miR-203 is an independent molecular predictor of prognosis and treatment outcome in ovarian cancer: a multi-institutional study

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Abstract

Ovarian cancer (OC) accounts for the most gynecological cancer-related deaths in developed countries. Unfortunately, the lack of both evident early symptoms and effective asymptomatic population screening results in late diagnosis and inevitably poor prognosis. Hence, it is urgent to identify novel molecular markers to support personalized prognosis. In the present study, we have analyzed the clinical significance of miR-203 in OC using two institutionally-independent cohorts. miR-203 levels were quantified in a screening (n=125) and a validation cohort (n=100, OVCAD multicenter study). Survival analysis was performed using progression and death as clinical endpoint events. Internal validation was conducted by bootstrap analysis, and decision curve analysis was used to evaluate the clinical benefit. Increased miR-203 levels in OC patients were correlated with unfavorable prognosis and higher risk for disease progression, independently of FIGO stage, tumor grade, residual tumor after surgery, chemotherapy response and age. The analysis of the institutionally-independent validation cohort (OVCAD study) clearly confirmed the shorter survival outcome of the patients overexpressing miR-203. Additionally, integration of miR-203 levels with the established disease prognostic markers led to a superior stratification of OC patients that can ameliorate prognosis and benefit patient clinical management. In this regard, miR-203 expression constitutes a novel independent molecular marker to improve patients' prognosis in OC.

Summary

miR-203 overexpression in ovarian tumors is associated with disease progression and worse patient's survival, independently of FIGO stage, grade, residual tumor, chemotherapy response and age. Interestingly, miR-203-incorporating multivariate models benefit disease prognosis and clinical management.

Introduction

Ovarian cancer (OC) represents the most lethal and the second most frequently diagnosed gynecological cancer in developed countries (1). Histologically, more than 90% of ovarian tumors are of epithelial cell origin, including high-grade serous (70%), endometrioid (10%), clear cell (10%), low-grade serous (<5%), mucinous (3%), and undifferentiated carcinomas (2,3). Disease incidence and mortality have been declining in recent years, possibly attributed to the uptake of oral contraceptives, as well as to advances in disease treatment, respectively (4-6). However, OC patients' 5-year survival is the poorest of all gynecological cancers, with only 38% in Europe (7) and 47% in USA (6), due to the absence of early-symptoms and thus late-stage diagnosis.

Although early diagnosis of OC could effectively improve patients' survival outcome and quality-of-life, PLCO and UKCTOCS large-scale randomized clinical studies for the use of transvaginal ultrasound (TVU) and CA125 levels in asymptomatic population screening and early diagnosis of OC, have not identified a significant mortality benefit in average-risk women after 15 years of follow-up (8,9). Unfortunately, the majority (~70%) of patients with epithelial OC (EOC) are still diagnosed at advanced disease stages (FIGO III/IV), highlighting the urgent clinical need of personalized prognosis and treatment decisions to improve disease management.

The gold standard treatment for newly diagnosed OC patients is radical surgical debulking and platinum-based first-line chemotherapy, usually, with addition of a taxane. However, despite active treatment, disease progression occurs in most patients at a median of 15-20 months from diagnosis (3,10). Disease prognosis is mainly performed by FIGO staging, success of surgical debulking (presence of residual tumor after surgery) and the clinical response to first-line chemotherapy (3,11). In this regard, the heterogeneity of ovarian tumors (12), with respect to their cellular and molecular background, could be exploited for the

identification of novel molecular markers to improve disease prognosis and to support precision medicine in the modern era.

MicroRNAs (miRNAs) are small single-stranded (18-25 nt) non-coding RNA molecules, which extensively regulate gene expression at the post-transcriptional level, mainly by binding through imperfect base pairing to the 3′ UTR of their target mRNAs (13,14). Their function is linked to many cellular processes and, consequently, their abnormal levels are highly correlated with initiation and progression of the majority of human malignancies, exerting an oncogenic or tumor suppressive role (15-17). Concerning OC, recent studies have clearly documented that miRNAs are actively involved in hallmarks of the disease establishment, representing ideal candidates for novel molecular indicators of disease course (18,19).

MicroRNA-203 (miR-203) is transcribed by the *MIR203A* gene, located at the chromosomal locus 14q32.33, and is deregulated in various human malignancies (20,21). With respect to OC, Iorio *et al.* (22) first confirmed overexpression of miR-203 in ovarian tumors due to *MIR203A* gene demethylation, while Xiaohong *et al.* (23) have clearly demonstrated an oncogenic role for miR-203 through the enhancement of glycolysis *via* PDHB (pyruvate dehydrogenase B) targeting. On the contrary, a tumor-suppressive role has also been proposed, as miR-203 upregulation has been demonstrated *in vitro* to attenuate epithelial-to-mesenchymal transition (EMT) and to downregulate SNAI2, vimentin and β-catenin, all representing mesenchymal markers, as well as to inhibit OC growth and metastasis *in vivo* (24,25).

Considering the lack of a comprehensive clinical evaluation of miR-203 in OC, we have studied, for the first time, the prognostic value of miR-203 expression in improving prediction of treatment outcome and providing superior risk-stratification of the patients, using two institutionally-independent cohorts of EOC patients.

Materials & Methods

Patients and tissue samples

Screening cohort

The screening cohort of the study (n=103) consisted of patients diagnosed with primary EOC, including 67 patients with high grade serous, 14 with low grade serous, 8 with endometrioid, 8 with mucinous (among which 2 with signet ring cells), 2 with clear cell and 4 with undifferentiated carcinomas. In addition, 22 patients with benign lesions of the ovary were analyzed. The EOC and benign samples were from the Department of Obstetrics and Gynecology, School of Medicine, Technical University of Munich, Munich, Germany. Ovarian tumor samples were obtained by radical cytoreductive surgery and were fresh-frozen at -80ºC until analysis. Following surgery, 102 patients received systemic adjuvant platinum-based therapy; while for 1 patient taxane (paclitaxel) monotherapy was administrated. Neoadjuvant therapy was applied in 14 patients. Patients post-treatment monitoring was performed *via* follow-up visits, as archived in patients' files or from the Tumorregister Munich. Disease progression was diagnosed by imaging (CT or PET-CT). Evaluation of patients' response-to-chemotherapy was assessed using tumor markers or imaging. The study was conducted according to ethical standards of the 1975 Declaration of Helsinki, as revised in 2008, and approved by the Ethics Committee of the Faculty of Medicine, Technical University Munich (491/17). Informed consent was obtained by all the participating patients.

Validation cohort

A homogenous cohort of 100 primary advanced serous OC (SOC) patients, from the OVCAD multicenter consortium (26), was used as institutionally-independent validation cohort. All patients underwent standard stage-related primary radical debulking surgery and tissue specimens were fresh-frozen and stored in liquid nitrogen until analysis. Following

tumor resection, patients received adjuvant treatment according to consensus recommendations, while neoadjuvant therapy prior to surgery was administrated in 14 patients. Response to treatment and diagnosis of progression was determined according to RECIST criteria or according to CA125 variations (GCIG-criteria) during follow-up (27,28). Patients with progression during primary therapy or within 6 months after primary therapy were defined as non-responders (29). Progression-free survival (PFS) was calculated as time from end of platinum based first-line chemotherapy to progression of disease, and overall survival (OS) as time from initial diagnosis to death or loss to follow-up. The study was conducted according to ethical standards of the 1975 Declaration of Helsinki, as revised in 2008. The study protocol was approved by the local ethics committees of the participating OVCAD partners (EK207/2003, ML2524, HEK190504, EK366, and EK260). Written informed consent was obtained from all participated patients.

Expression analysis

The handling of the tissue samples for the evaluation of miR-203 expression and analysis of the clinical value was conducted as previously reported (30,31) and is described in detail below.

Extraction of total RNA

40-150 mg of fresh-frozen samples were pulverized and total RNA was isolated using TRI-Reagent (Molecular Research Center, Cincinnati, OH, USA) according to the manufacturer's instructions. The RNA was dissolved in RNA Storage Solution (Ambion) and its concentration and purity were determined spectrophotometrically at 260 and 280 nm. Agarose gel electrophoresis confirmed RNA's integrity.

Polyadenylation of total RNA and cDNA synthesis

1 µg of total RNA was polyadenylated at the 3'-end in a 10 µl reaction containing 800 µM ATP and 1 U of *E. coli* poly (A) polymerase (New England Biolabs, Inc., Ipswich, MA, USA) at 37°C for 60 minutes. Thereafter, the enzyme was inactivated at 65°C for 15 min. Following polyadenylation, total RNA was reverse transcribed in a 20 µl reaction containing 50 U MMLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA), 40 U recombinant ribonuclease inhibitor (Invitrogen) and 0.25 µM oligo (dT) adapter 5′-GCGAGCACAGAATTAATACGACTCACTATAGGTTTTTTTTTTTTVN-3′ (V = G, A, C and N = G, A, T, C), at 37ºC for 60 minutes. Enzyme inactivation was accomplished at 70°C for 15 min.

Quantitative real-time PCR

For the quantification of miR-203 expression levels, a SYBR-Green fluorescent-based quantitative real-time PCR (qPCR) assay was optimized. Specific primers for the small nucleolar RNA C/D box 48 (SNORD48), also known as RNU48, (F: 5'-TGATGATGACCCCAGGTAACTCT-3') and miR-203 (F: 5'-GGTGAAATGTTTAGGACCACTAGAA-3') were designed with the use of published RNA sequences (NCBI: NR_002745.1 for SNORD48 and NR_029620.1 for miR-203) and *in silico* analysis. The specific forward primers and the universal reverse primer (5'-GCGAGCACAGAATTAATACGAC-3') were used for the amplification of 105 bp SNORD48-specific and 67 bp miR-203-specific amplicons.

The qPCR assays were performed in the 7500 Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA). The 10 µl reaction consisted of Kapa SYBR Fast Universal 2X qPCR MasterMix (Kapa Biosystems, Inc., Woburn, MA), 200 nM of each qPCR primer and 2 ng of each cDNA tissue sample. The thermal protocol consisted of polymerase activation step

at 95 °C for 3 minutes, followed by 40 cycles of denaturation at 95 °C for 3 seconds and lastly the annealing and elongation step at 60 °C for 30 seconds. Following amplification, melting curve analysis and agarose gel electrophoresis were performed to discriminate specific amplicons from non-specific PCR products or primer dimers.

Each reaction was performed in duplicate and the threshold cycle (C_T) mean was calculated for the quantification analysis. The $2^{-\Delta\Delta CT}$ relative quantification (RQ) method was used to evaluate miR-203 levels. SKOV3 OC cell line was used as our assay calibrator and RNU48 as endogenous reference control to normalize the values.

Statistical analysis

IBM SPSS Statistics 20 software (IBM Corp., Armonk, New York, USA) was used for the statistical analysis of our data. Sapiro-Wilk and Kolmogorov-Smirnov tests were used for the evaluation of the normal distribution of the data. Due to absence of normal distribution, non-parametric tests were performed to analyze miR-203 expression between ovarian tumors and benign lesions, as well as between patient clinicopathological features (FIGO stage, tumor grade, residual tumors, chemotherapy response etc.). Kaplan-Meier curves, using log-rank test, and Cox proportional regression analysis were performed for the survival analysis of the patients. X-tile algorithm (32) was assessed for the adoption of miR-203 levels optimal cut-off values. Internal validation was performed by bootstrap Cox proportional regression analysis based on 1000 bootstrap samples. Finally, the clinical net benefit of multivariate prediction models on patients survival outcome following treatment was evaluated by decision curve analysis (DCA) according to Vickers *et al.* (33).

Results

Baseline clinical and experimental data

The REMARK diagram of our study is presented in Fig. 1. The median age of the patients enrolled in our screening and validation cohorts was 62.0 (range: 25-83) and 61.0 (range: 26-83), respectively. The majority of the patients were diagnosed at advanced (III/IV) FIGO stages (screening cohort: 84.4%, validation cohort: 100%) and G3/Undifferentiated tumor grades (screening cohort: 74.5%, validation cohort: 71.7%). Additionally, 66 and 92 patients were optimally debulked (residual tumor ≤1cm), whereas 30 and 8 had a residual tumor larger than 1 cm in the screening and validation cohort, respectively.

Regarding patients' chemotherapy response, 51.6% and 26% of women experienced progressive disease (PD-progressive disease) in the screening and validation cohort, respectively. The median follow-up time (reverse Kaplan-Meier method) of our screening cohort was 91 months (95% CI: 87.08 – 94.92), and successful follow-up was achieved for 103 patients, with a median OS of 55.00 months (95% CI: 42.74 – 67.26; n=103) and median PFS (2 patients were excluded due to unclear monitoring data) of 22.00 (95% CI: 16.71 – 27.29; n=101). Regarding the validation cohort, during a median follow-up time (reverse Kaplan–Meier method) of 75.56 months (95% CI: 71.76 - 79.36), 100 patients were successfully followed-up with median OS of 45.40 months (95% CI: 34.73 - 56.06; n=100) and median PFS (2 patients were excluded due to unclear monitoring data) of 12.24 (95% CI: 9.37 – 15.11, n=98). Finally, 1 and 3 patients were excluded from the survival analysis of the screening and validation cohort, respectively, due to low quality of qPCR data. The clinicopathological characteristics of the two cohorts are presented in Table 1.

Up-regulated miR-203 tumor levels are associated with significantly shorter overall survival outcome and higher risk for disease progression

Expression analysis (Supplementary Figure 1) of the screening cohort did not highlight any statistically significant correlation of miR-203 tumor levels with the established clinical markers of OC, including tumor grade (p=0.098), response to chemotherapy (p=0.309) and the presence of residual tumor after surgery (p=0.335). Similarly, the upregulated miR-203 levels in ovarian tumors compared to benign ovarian lesions were not proven to be statistically significant (p=0.162)

Survival analysis of our screening and validation cohorts was conducted using patients' death and disease progression as clinical endpoint events for the OS and PFS, respectively. Regarding our screening cohort, using the X-tile algorithm, the 50th percentile (median) of miR-203 was adopted as the optimal cut-off value. Kaplan-Meier curves (Figure 2) illustrated the significantly shorter OS and PFS expectancy of the EOC (p=0.026; Fig. 2A and p=0.020; Fig. 2B) and SOC patients (p=0.024; Fig. 2C and p=0.020; Fig. 2D) overexpressing miR-203. Univariate Cox proportional regression analysis (Figure 3) confirmed the significantly higher risk for death (HR: 1.741; 95% CI: 1.059-2.862; p=0.029) and disease progression (HR: 1.721; 95% CI: 1.075-2.754; p=0.024) of EOC patients with up-regulated miR-203 levels. Additionally, multivariate Cox models established that miR-203 can effectively predict worse survival outcome (HR: 2.812; 95% CI: 1.590-4.973; p<0.001) and disease progression (HR: 2.327; 95% CI: 1.340-4.041; p=0.003), independently of FIGO stage, tumor grade, residual tumor after surgery, response to chemotherapy and age, which was affirmed with Bootstrap analysis (BCa 95% CI: 1.507-7.142; p=0.002 for OS and BCa 95% CI: 1.288-4.540; p=0.007 for PFS).

The OVCAD multicenter validation cohort clearly confirmed the unfavorable nature of miR-203 overexpression for EOC prognosis. Using the X-tile algorithm the optimal cut-off value was set to 40th percentile. More precisely, SOC patients overexpressing miR-203 were highlighted to be in significantly higher risk for worse survival outcome as highlighted by

Kaplan-Meier survival curves (p=0.042; Fig. 2E) and Cox regression analysis (HR: 1.644; 95% CI: 1.013-2.668; p=0.044; BCa 95% CI: 1.019-2.729; p=0.034). The correlation of miR-203 expression with the PFS in our validation cohort was not statistically significant (p=0.226; Fig. 2F).

The evaluation of miR-203 expression ameliorates the clinical value of the established and clinically used disease prognostic markers.

The abovementioned vigorous and independent prognostic significance of miR-203 for EOC motivated us to study miR-203 ability to improve the clinical value of the established prognostic markers of the disease. Residual tumor and response to chemotherapy represent the generally used clinical markers of EOC progression. Integration of miR-203 levels with these markers clearly improved the prediction of patients' treatment outcome and achieved a better risk-stratification (Figure 4). Concerning our screening cohort, miR-203 overexpression could effectively predict the patients with increased risk for disease progression and worse survival expectancy within residual tumor <2 cm patient group (OS: p<0.001; Fig. 4A, PFS: p<0.001; Fig. 4B), and responding to first-line platinum-based chemotherapy (CR/PR/SD) group (OS: p<0.001; Fig. 4C, PFS: p<0.001; Fig. 4D). Likewise, the analysis of the validation cohort, clearly confirmed the significantly shorter survival outcome of the completely debulked patients (p=0.006; Fig. 4E) or CR/PR/SD patients (p<0.001; Fig. 4E) with increased miR-203 levels compared to those underexpressing miR-203.

The evaluation of miR-203 levels results in superior clinical benefit in OC prognosis

Ultimately, we have performed decision curve analysis (Figure 5), according to Vickers *et al.* (33), in order to evaluate the clinical net benefit of the multivariate prognosis prediction model integrating miR-203 along with established and clinically used markers of the disease

for the survival outcome of the OC patients. The analysis revealed that the multivariate model integrating miR-203 with FIGO stage, tumor grade, residual tumor and chemotherapy response offers higher clinical net benefit for OS prognosis, compared to the model of the clinical prognostic markers only (Fig. 5). Consequently, the incorporation of miR-203 evaluation supports personalized treatment decision to achieve a superior clinical benefit for disease management.

Discussion

OC is characterized by high heterogeneity on both the molecular and the clinical level, incorporating a group of malignancies that vary in etiology, molecular and genetic driver events, and, subsequently, treatment outcome (2,3,34,35). Despite the reduction of disease-specific mortality, the lack of accurate markers for asymptomatic population screening still results in late stage disease diagnosis (FIGO III/IV) and, thus, in poor prognosis. Consequently, the identification of adequate molecular markers (36-38) is a novel approach that would strongly support personalized treatment and monitoring decisions, and ameliorate patients' quality-of-life.

The ENCODE project revealed that >75% of the genome is actively transcribed into non-coding RNAs (ncRNAs) (39), while miRNAs are gaining an ever-growing attention with respect to their function and clinical impact in human malignancies (40,41). It has been established that numerous miRNAs exert oncogenic or tumor-suppressive roles in OC tumorigenesis and progression, thus representing promising molecular markers to support precision medicine in OC (18,42,43). The aim of the present study was the evaluation of the clinical utility of miR-203 expression in improving prediction of treatment outcome and promoting personalized patients' prognosis.

The survival analysis of our screening cohort revealed an independent and unfavorable

significance of increased miR-203 expression concerning treatment outcome of OC patients. In particular, miR-203 overexpression was significantly associated with worse survival outcome of EOC and SOC patients following tumor resection and first-line platinum-based chemotherapy compared to patients underexpressing miR-203. Moreover, this association with higher risk for disease progression and shorter survival expectancy of the patients overexpressing miR-203 was independent of FIGO stage, tumor grade, residual tumor after surgery, chemotherapy response and age. To confirm our findings, a cohort of 100 advanced SOC patients from the OVCAD multicenter study (26) was used as an institutionally-independent validation cohort. The analysis strongly confirmed the unfavorable prognostic value of miR-203 overexpression in SOC prognosis.

The major limitation of our study is the relatively small number of OC patients that have been included in our screening and validation cohorts. Besides, different optimal cut-off values have been adopted for the two institutionally-independent cohorts, which could be attributed to the small sample size and to the distinct clinicopathological features of the cohorts. More specifically, the cohorts include mainly advanced EOC patients, however, there is a significant variation in residual tumor size and patients' response-to-chemotherapy that could impact the optimal cut-off values of miR-203 levels. Future multi-institutional large-scale studies could identify the appropriate cut-off value of miR-203 to be exploited in clinical disease prognosis.

Poor outcome of patients displaying upregulated miR-203 levels in tumor tissue has also been observed in breast (44), colorectal (45), prostate (46) and pancreatic (47,48) cancers. In agreement with our findings, Wang *et al.* (20) and Azizmohammadi *et al.* (49) have recently reported an association of miR-203 overexpression in ovarian tumors with unfavorable outcome of the patients. Regarding the mode of miR-203 overexpression in OC, Iorio *et al.* (22) demonstrated that miR-203 levels are regulated by methylation *in vitro*, suggesting that

DNA hypomethylation could represent an epigenetic mechanism responsible for the *in vivo* upregulation of miR-203. Concerning the molecular function of miR-203, it has been established *in vitro* that miR-203 and p53 are inversely correlated and, upon p53 overexpression, miR-203 expression is attenuated (23). In addition, miR-203 transfection in OC cells enhances cell growth and migration as well as glycose uptake and lactate formation *via* PDHB-targeting, supporting the oncogenic role of miR-203 in OC progression.

Likewise, oncogenic function of miR-203 has been revealed also in pancreatic cancer cells, where the miR-203-mediated downregulation of tumor suppressor SIK1 led to enhanced cell proliferation and migration (50). Moreover, miR-203 has been documented contributing to oxaliplatin resistance in colorectal cancer cells. In this regard, exogenous overexpression of miR-203 resulted in oxaliplatin resistance of chemo-naïve tumor cells, through suppressing the expression of the DNA damage response regulator, ATM. The sensitization of chemoresistance cells to oxaliplatin either by miR-203 knockdown or mutation of ATM mRNA 3'-UTR biding site of miR-203 highlights the active role of miR-203/ATM regulatory axis in inducing chemoresistance in colorectal cancer (51).

In our study design, the analysis of institutionally-independent multicenter cohorts, for the first time, strengthens the unfavorable clinical value of miR-203 overexpression in EOC treatment response and patients' survival outcome. Additionally, taking advantage of miR-203 powerful and independent prognostic significance, we have highlighted the additional impact of miR-203 evaluation in improving the prognostic performance of established disease markers. Indeed, multivariate models incorporating miR-203 overexpression with the widely-used prognostic disease markers resulted in superior positive prediction of the patients at higher risk for poor treatment outcome, as well as in improved patients' risk-stratification and clinical benefit of OC prognosis.

In summary, miR-203 constitutes a powerful molecular indicator of patients' prognosis

in ovarian carcinoma. More precisely, miR-203 overexpression is an unfavorable and independent marker of patients' treatment outcome, and is strongly associated with higher risk for disease progression and shorter survival expectancy of the patients. The analysis of the institutionally-independent multicenter validation cohort further confirmed the clinical value of elevated miR-203 in disease prognosis. Finally, multivariate prognosis prediction models incorporating miR-203 overexpression with clinically used markers, established the superior stratification specificity and improved positive prediction of OC patients' poor treatment outcome, offering a significantly higher clinical benefit for patients' prognosis and monitoring compared to models of the established and clinically used prognostic markers alone.

Supplementary material

Supplementary Figure 1 can be found at http://carcin.oxfordjournals.org/

Conflict of Interest

The authors declare no conflicts of interest.

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Table 1. Clinicopathological characteristics of the screening and validation cohorts.

Variable	*Screening cohort* No. of patients (n=103)	*Validation cohort* No. of patients (n=100)

Age		
<65	**61** (59.2%)	**62** (62%)
≥65	**42** (40.8%)	**38** (38%)
FIGO		
I/II	**16** (15.5%)	
III	**61** (59.2%)	**87** (87%)
IV	**26** (25.2%)	**13** (13%)
Tumor		
T1	**15** (14.7%)	**2** (2%)
T2	**6** (5.9%)	**3** (3%)
T3	**81** (79.4%)	**95** (95%)
Excluded (no data)	**1**	
Nodes		
N0	**34** (42.5%)	**17** (23.9%)
N1	**46** (57.5%)	**54** (76.1%)
Excluded (no data)	**23**	**29**
Metastasis		
M0	**66** (71%)	**53** (82.8%)
M1	**27** (29%)	**11** (17.2%)
Excluded (no data)	**10**	**36**
Grade		
G1	**16** (15.7%)	**6** (6.1%)
G2	**10** (9.8%)	**22** (22.2%)
G3	**71** (69.6%)	**71** (71.7%)
Undifferentiated	**5** (4.9%)	
Excluded (no data)	**1**	**1**
Neoadjuvant		
Yes	**14** (13.7%)	**14** (14%)
No	**88** (86.3%)	**86** (86%)
Excluded (no data)	**1**	
Ascites		
none	**28** (30.4%)	**17** (18.3%)
<500ml	**21** (22.8%)	**31** (33.3%)
≥500ml	**43** (46.7%)	**45** (48.4%)
Excluded (no data)	**11**	**7**
Residual tumor		
No tumor	**46** (46.5%)	**68** (68%)
≤1cm	**20** (20.2%)	**24** (24%)
1 - 2cm	**18** (18.2%)	**1** (1%)
>2cm	**12** (12.1%)	**7** (7%)
Inoperable	**3** (3%)	
Excluded (no data)	**4**	

Figure legends

Response to chemotherapy		
Progressive disease (PD)	**47** (51.6%)	**24** (26%)
Stable Disease (SD)	**1** (1.1%)	**2** (2.2%)
Complete Response (CR)	**36** (39.6%)	**62** (67.4%)
Partial Response (PR)	**7** (7.7%)	**4** (4.4%)
Excluded (no data)	**12**	**8**
Overall survival		
Follow-up patients	**103**	**100**
Alive	**38** (36.9%)	**29** (29%)
Dead	**65** (63.1%)	**71** (71%)
Disease Progression		
Follow-up patients	**101**	**98**
Progression	**74** (73.3%)	**87** (88.8%)
Event-free survival	**27** (26.7%)	**11** (11.2%)
Excluded from follow-up	**2**	**2**

Figure 1. REMARK diagram of the study.

Figure 2. Up-regulated miR-203 levels are associated with worse overall survival and disease progression. Kaplan-Meier survival curves were plotted based on miR-203 expression for overall survival (OS) and progression-free survival (PFS) of the epithelial ovarian cancer (EOC) patients (A, B) and serous ovarian cancer (SOC) patients (C, D) of the screening cohort; Kaplan-Meier survival curves for overall survival (OS) and progression-free survival (PFS) of the serous ovarian cancer (SOC) patients (E, F) of the validation cohort in relation to miR-203 levels. *p*-values calculated by log-rank test.

Figure 3. miR-203 overexpression is an independent prognostic predictor of poor survival of EOC patients and progression following treatment. Forest plots of the univariate (A, C) and multivariate (B, D) Cox regression analysis are depicted. Multivariate analysis was adjusted for miR-203 expression, FIGO stage, tumor grade, residual tumor size after surgery, response to chemotherapy and age. Internal validation was performed by bootstrap Cox proportional regression analysis based on 1000 bootstrap samples. HR: Hazard Ratio, 95% CI:

95% confidence interval of the estimated HR, BCa 95% CI: bootstrap bias-corrected and accelerated 95% confidence interval of the estimated HR based on 1000 bootstrap samples.

Figure 4. Evaluation of miR-203 levels improves prediction of EOC prognosis. Kaplan-Meier survival curves of miR-203 levels complied with residual tumor size and response to chemotherapy for the overall survival (OS) (A,C) and progression-free survival (PFS) (B,D) of the screening cohort and for the overall survival (OS) (E,F) of the validation cohort patients are shown. *p*-values were calculated by log-rank test. R: residual tumor size, CR: complete response, PR: partial response, SD: stable disease, PD: progressive disease

Figure 5. Decision curve analysis demonstrates the superior clinical net benefit of the model that integrates miR-203 up-regulation with the clinically established prognostic markers (FIGO stage, tumor grade, residual tumor size, chemotherapy response) for prediction of overall survival (OS) of OC patients. Net benefit is plotted against various ranges of threshold probabilities.

Figure 1

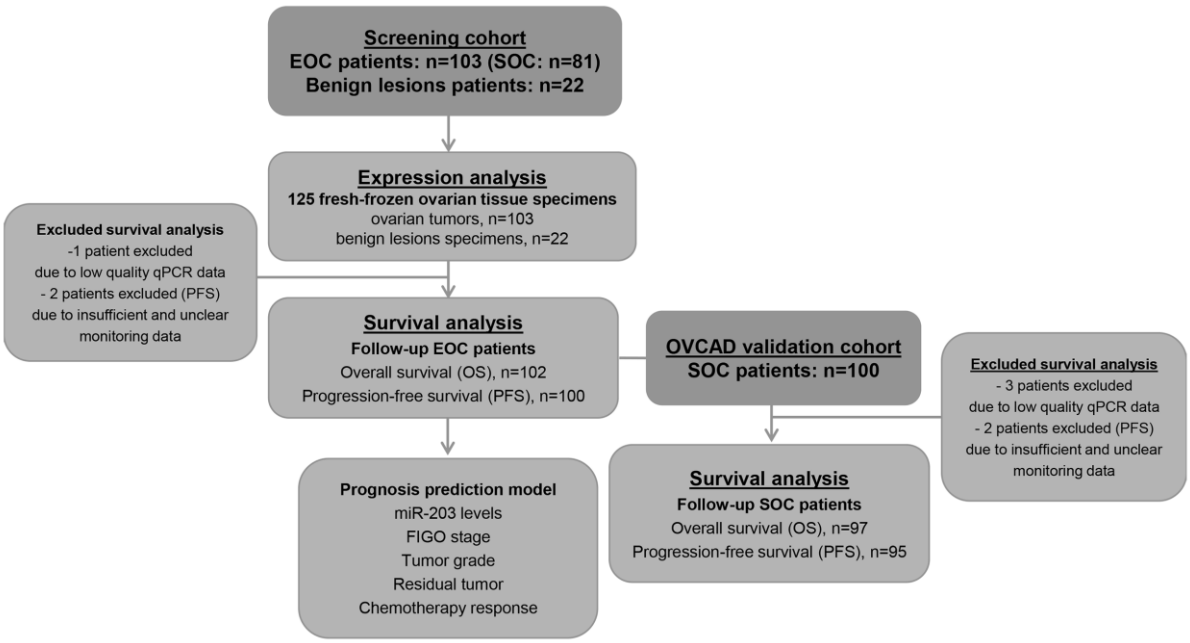


Figure 2

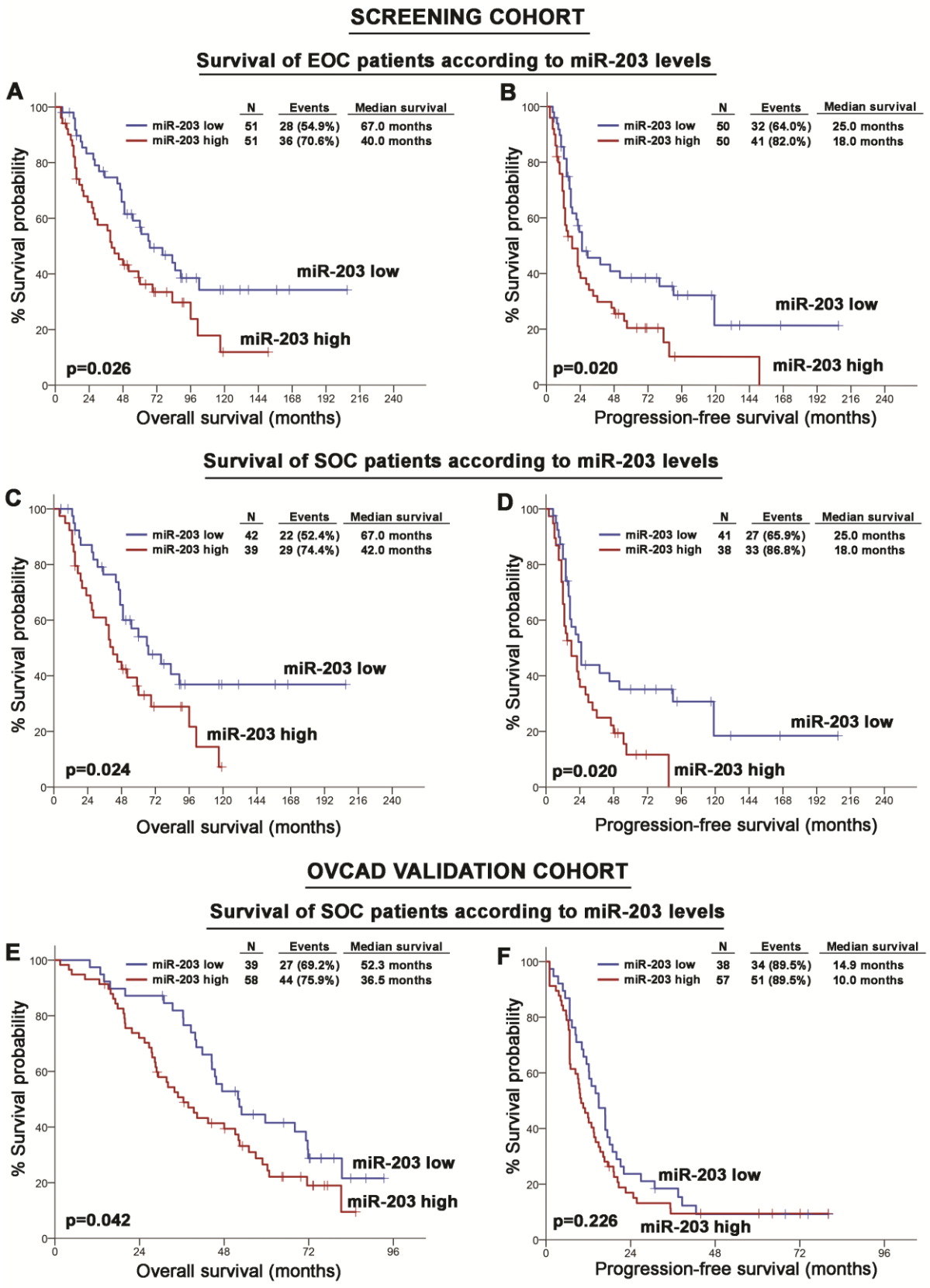


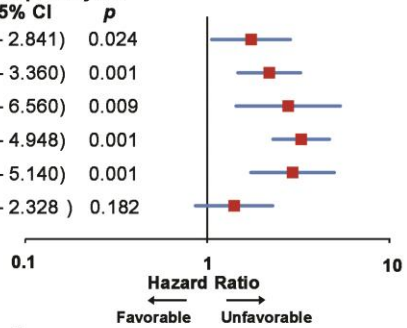
Figure 3

Overall survival (OS)

A

Univariate Cox regression analysis

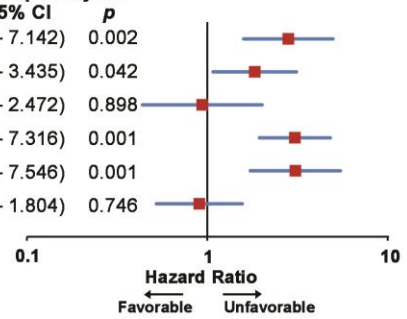
Covariants: tested *vs.* control (HR=1)	HR	95% CI	*p*	Bootstrap analysis BCa 95% CI	*p*
miR-203 levels: High *vs.* Low	1.741	(1.059 – 2.862)	0.029	(1.091 – 2.841)	0.024
FIGO Stage: IV *vs.* III *vs.* I/II	2.191	(1.475 – 3.255)	<0.001	(1.522 – 3.360)	0.001
Tumor Grade: G3/Undif *vs.* G1/G2	2.783	(1.440 – 5.379)	0.002	(1.409 – 6.560)	0.009
Residual tumor: >2cm *vs.* <2cm *vs.* no	3.281	(2.297 – 4.688)	<0.001	(2.418 – 4.948)	0.001
Response to chemo: PD *vs.*CR/PR/SD	2.942	(1.737 – 4.985)	<0.001	(1.781 – 5.140)	0.001
Age: ≥65y *vs.* <65y	1.405	(0.862 – 2.289)	0.173	(0.880 – 2.328)	0.182



B

Multivariate Cox regression analysis

Covariants: tested *vs.* control (HR=1)	HR	95% CI	*p*	Bootstrap analysis BCa 95% CI	*p*
miR-203 levels: High *vs.* Low	2.812	(1.590 – 4.973)	<0.001	(1.507 – 7.142)	0.002
FIGO Stage: IV *vs.* III *vs.* I/II	1.832	(1.076 – 3.120)	0.026	(0.946 – 3.435)	0.042
Tumor Grade: G3/Undif *vs.* G1/G2	0.938	(0.437 – 2.013)	0.870	(0.441 – 2.472)	0.898
Residual tumor: >2cm *vs.* <2cm *vs.* no	3.058	(1.946 – 4.807)	<0.001	(2.000 – 7.316)	0.001
Response to chemo: PD *vs.*CR/PR/SD	3.079	(1.732 – 5.473)	<0.001	(1.887 – 7.546)	0.001
Age: ≥65y *vs.* <65y	0.903	(0.522 – 1.562)	0.714	(0.439 – 1.804)	0.746

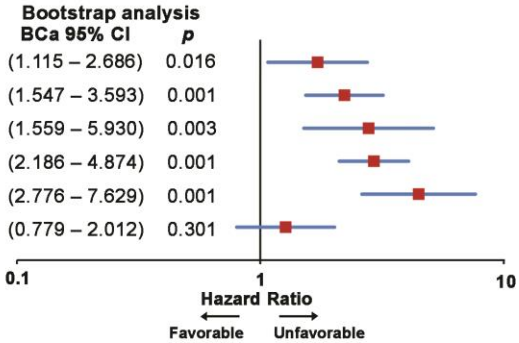


Progression-free survival (PFS)

C

Univariate Cox regression analysis

Covariants: tested *vs.* control (HR=1)	HR	95% CI	*p*	Bootstrap analysis BCa 95% CI	*p*
miR-203 levels: High *vs.* Low	1.721	(1.075 – 2.754)	0.024	(1.115 – 2.686)	0.016
FIGO Stage: IV *vs.* III *vs.* I/II	2.217	(1.538 – 3.196)	<0.001	(1.547 – 3.593)	0.001
Tumor Grade: G3/Undif *vs.* G1/G2	2.787	(1.510 – 5.145)	0.001	(1.559 – 5.930)	0.003
Residual tumor: >2cm *vs.* <2cm *vs.* no	2.931	(2.111 – 4.069)	<0.001	(2.186 – 4.874)	0.001
Response to chemo: PD *vs.*CR/PR/SD .	4.475	(2.616 – 7.654)	<0.001	(2.776 – 7.629)	0.001
Age: ≥65y *vs.* <65y	1.272	(0.800 – 2.020)	0.309	(0.779 – 2.012)	0.301



D

Multivariate Cox regression analysis

Covariants: tested *vs.* control (HR=1)	HR	95% CI	*p*	Bootstrap analysis BCa 95% CI	*p*
miR-203 levels: High *vs.* Low	2.327	(1.340 – 4.041)	0.003	(1.288 – 4.540)	0.007
FIGO Stage: IV *vs.* III *vs.* I/II	2.167	(1.292 – 3.635)	0.003	(1.255 – 4.088)	0.005
Tumor Grade: G3/Undif *vs.* G1/G2	1.124	(0.554 – 2.279)	0.747	(0.527 – 2.654)	0.766
Residual tumor: >2cm *vs.* <2cm *vs.* no	2.372	(1.581 – 3.558)	<0.001	(1.619 – 5.425)	0.005
Response to chemo: PD *vs.*CR/PR/SD	4.573	(2.503 – 8.353)	<0.001	(3.016 – 9.384)	0.001
Age: ≥65y *vs.* <65y	0.869	(0.521 – 1.451)	0.592	(0.432 – 1.614)	0.618

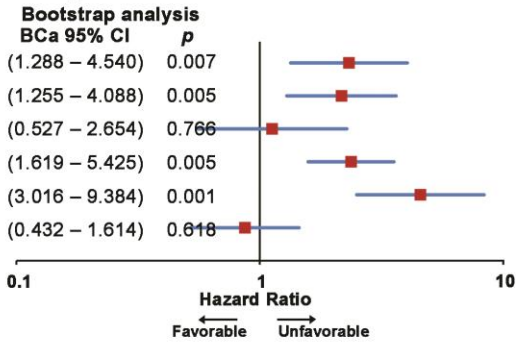


Figure 4

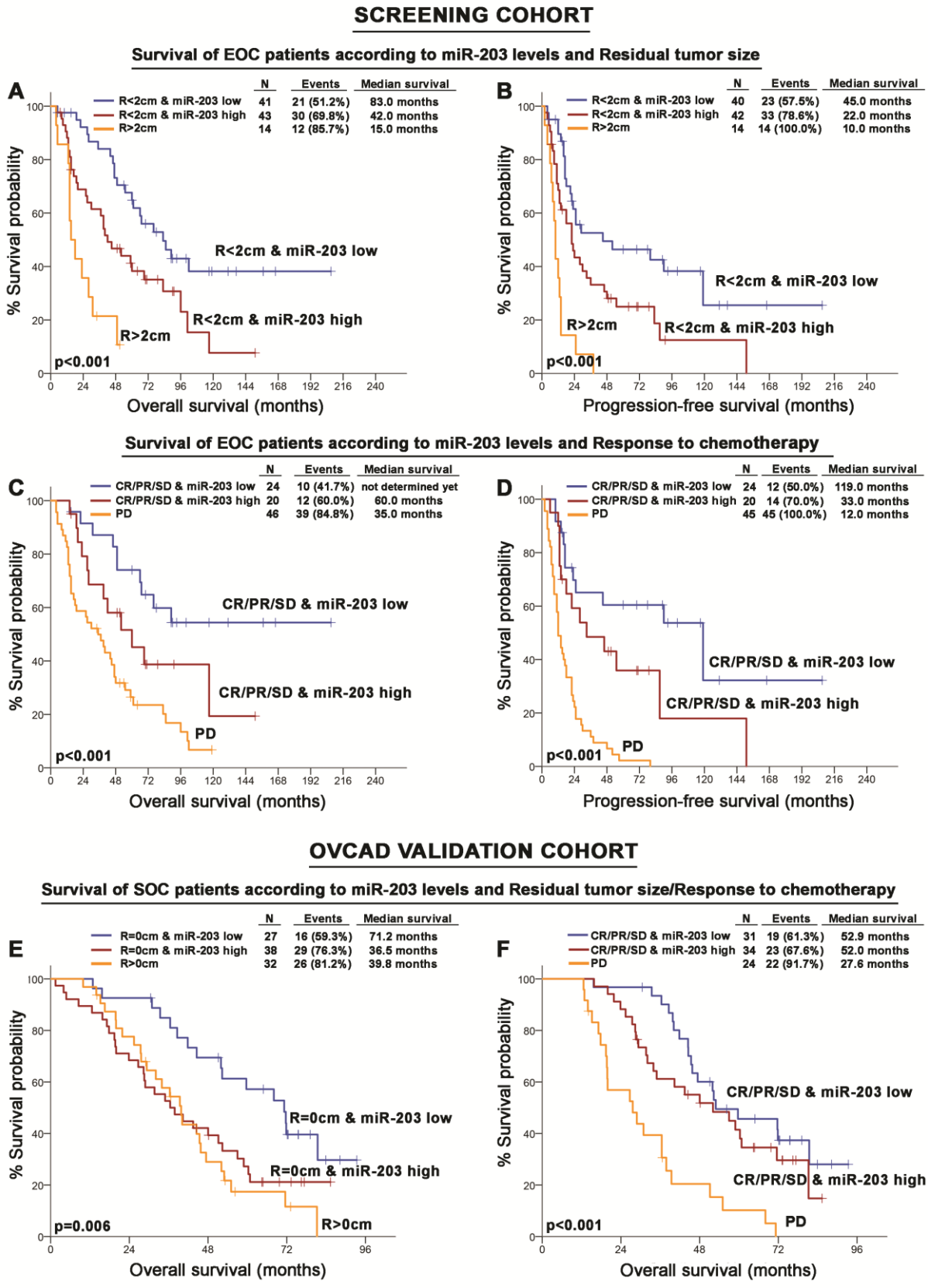


Figure 5