The miR-200 family controls β-tubulin III expression and is associated with paclitaxel-based treatment response and progression-free survival in ovarian cancer patients

Susanna Leskelä[1], Luis J Leandro-García[1]*, Marta Mendiola[2,3], Jorge Barriuso[4], Lucía Inglada-Pérez[1,5], Iván Muñoz[5,6], Beatriz Martínez-Delgado[5], Andrés Redondo[4], Javier de Santiago[7], Mercedes Robledo[1,5], David Hardisson[2] and Cristina Rodríguez-Antona[1,5]*

[1]Hereditary Endocrine Cancer Group, Human Cancer Genetics Programme, Spanish National Cancer Research Center, Melchor Fernández Almagro 3, 28029 Madrid, Spain
[2]Department of Pathology, Hospital Universitario La Paz, Universidad Autónoma de Madrid, IdiPAZ, 28046 Madrid, Spain
[3]Laboratory of Pathology and Oncology, Research Unit, Fundación para la Investigación de Hospital Universitario La Paz (FIBHULP), IdiPAZ, 28046 Madrid, Spain
[4]Department of Medical Oncology, Hospital Universitario La Paz, Universidad Autónoma de Madrid, IdiPAZ, 28046 Madrid, Spain
[5]Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER), Madrid, Spain
[6]Human Genetics Group, Human Cancer Genetics Programme, Spanish National Cancer Research Center, 28029 Madrid, Spain
[7]Department of Obstetrics and Gynecology, Hospital Universitario La Paz, Universidad Autónoma de Madrid, IdiPAZ, 28046 Madrid, Spain

(Correspondence should be addressed to C Rodríguez-Antona; Email: cristina.rodriguez-antona@cnio.es; D Hardisson; Email: dhardisson.hulp@salud.madrid.org)

*(S Leskelä and L J Leandro-García contributed equally to this work)

Abstract

Ovarian cancer remains one of the leading causes of cancer deaths. Thus, new biomarkers predictive of response to the standard paclitaxel–carboplatin treatment are needed to improve chemotherapy strategies. MicroRNAs have the potential to modify drug outcomes. Based on this, we have demonstrated in this study that patients with a high expression of the miR-200 family show low levels of β-tubulin class III in ovarian carcinoma. In addition, we have established the clinical relevance of these microRNAs for ovarian cancer patients' treatment response and survival. In a well-characterized series of 72 ovarian carcinomas, the expressions of miR-141, miR-200a, miR-200b, miR-200c, and miR-429 were quantified by quantitative reverse transcription-PCR, and the protein content of β-tubulin isotypes I, II, and III was determined by immunohistochemistry. The relationship between these microRNAs, β-tubulin expression, response to paclitaxel-based treatment, progression-free survival (PFS) and overall survival was determined. While isotype I had constant high levels, protein expression of β-tubulins II and III was mutually exclusive. Low tumoral miR-200 expression was significantly associated with high β-tubulin III protein content (P values range, 0.047–$<$0.0001), and patients without complete response (CR) had lower miR-200c levels than patients with CR (hazard ratio (HR)$=$1.43, 95% confidence interval (CI)$=$1.02–1.99, $P=0.037$, multivariate analysis). Additionally, low miR-200 family expression had a trend toward poor PFS (HR$>$2.0, P values 0.051, 0.054, and 0.079 for miR-200c, miR-141, and miR-429 respectively, multivariate analysis). In conclusion, miR-200 family members affect the final β-tubulin III protein content of ovarian carcinomas. Furthermore, these microRNAs might constitute the biomarkers of response to paclitaxel-based treatments and relapse/progression of advanced stage ovarian carcinoma patients.

Endocrine-Related Cancer (2011) **18** 85–95

1351–0088/11/018–085 © 2011 Society for Endocrinology *Printed in Great Britain*
DOI: 10.1677/ERC-10-0148
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Introduction

Ovarian cancer is the leading cause of death for gynecologic malignancies (Jemal *et al*. 2009). More than 70% of patients are diagnosed at late stages of the disease and, although the current standard treatment combining surgery with chemotherapy (mainly based on paclitaxel–carboplatin regimens) is efficient in almost 80% of cases, the 5-year survival rate is low due to the high incidence of recurrence and to the ultimate resistance to taxanes and/or platinum-based drugs (Ozols *et al*. 2003, Heintz *et al*. 2006). New biomarkers predictive of treatment response urgently need to be identified in order to improve chemotherapy strategies.

First-line treatment for advanced ovarian cancer consists of a combination of paclitaxel and carboplatin (Ozols *et al*. 2003, Omura 2008). Carboplatin is equally efficient but less toxic and easier to administer than cisplatin, thus, it replaced cisplatin as the standard treatment for ovarian cancer (Ozols *et al*. 2003, Omura 2008). Paclitaxel is an antimitotic drug, which alters the dynamics of the cellular microtubules, which maintain cellular structure and are essential for diverse cellular functions such as cell cycle, cell signaling, and intracellular trafficking (Seve & Dumontet 2008). Paclitaxel binds to β-tubulin, leading to cellular microtubules stabilization, mitotic arrest, and finally to cell death. In humans, there are at least eight different isotypes of β-tubulin, which exhibit an altered expression pattern in tumoral tissue (Leandro-Garcia *et al*. 2010). In ovarian carcinoma, high protein levels of classes I and IV, intermediate levels of class III, and low levels of class II β-tubulin have been reported (Ohishi *et al*. 2007). High tumoral β-tubulin III expression has been associated with worse survival in non-small cell lung cancer (Rosell *et al*. 2003, Seve *et al*. 2005), breast (Seve & Dumontet 2008), head and neck (Koh *et al*. 2009), and ovarian cancer (Ferrandina *et al*. 2006), although Aoki *et al*. (2009) reported a better survival for patients with ovarian clear cell adenocarcinoma positive for class III expression. In ovarian cancer, β-tubulin III has also been associated with worse treatment response (Kavallaris *et al*. 1997, Mozzetti *et al*. 2005, Umezu *et al*. 2008). On the other hand, absence of class II β-tubulin expression has been associated with advanced stage and short progression-free survival (PFS) in ovarian tumors (Ohishi *et al*. 2007).

The molecular mechanisms leading to the upregulation of class III β-tubulin in tumors remain largely unknown. Hypoxia-inducible factor 1 seems to play a role in ovarian carcinomas (Raspaglio *et al*. 2008), and epigenetic modifications have been suggested to be a contributing factor in ovarian tumors (Izutsu *et al*. 2008) and melanoma cells (Akasaka *et al*. 2009). Recent studies suggest an important role of microRNAs, specifically, Cochrane *et al*. (2009, 2010) demonstrated that miR-200c had a binding site in the 3′ untranslated region (UTR) of class III β-tubulin and that overexpression of miR-200c in cell lines decreased β-tubulin III protein content and restored sensitivity to microtubule-targeting agents. Interestingly, the miR-200 family, formed by five microRNAs (miR-141, miR-200a, miR-200b, miR-200c, and miR-429) and located in two clusters in the genome, is involved in the epithelial to mesenchymal transition (EMT) through regulation of E-cadherin expression via suppression of ZEB1 and ZEB2 (Gregory *et al*. 2008, Korpal *et al*. 2008, Park *et al*. 2008). In ovarian cancer, a recent study with 55 advanced tumor samples showed that a high expression of miR-200a, miR-200b, and miR-429 was associated with improved survival (Hu *et al*. 2009). Nevertheless, another study including 20 serous carcinomas found that high expression of the miR-200 family members significantly correlated with poor prognosis (Nam *et al*. 2008). Thus, although there is evidence suggesting that the miR-200 family might play a key role in the response to microtubule-binding drugs and ovarian cancer survival, the relevance of these microRNAs as clinical markers for patients is largely unknown.

In this study, we quantified the expression of the miR-200 family in a well-characterized series of 72 epithelial ovarian tumors and examined their contribution to the protein expression of β-tubulin isotypes I, II, and III. We also investigated the impact of these microRNAs on the patients' response to and survival following paclitaxel-based therapy. The data provided in this study improved our comprehension of treatment failures in ovarian carcinoma and indicated the miR-200 family as a potential novel target for improved treatment strategies.

Materials and methods

Patient selection

This study included 72 formalin-fixed and paraffin-embedded ovarian carcinoma samples from patients treated at the Hospital Universitario La Paz (HULP), Madrid, Spain. All patients underwent a baseline computed tomography (CT) scan and exploratory laparotomy for diagnosis, staging, and debulking when feasible. All patients received a platinum/taxane-based chemotherapy for at least six cycles. Patients were divided into stages according to the International

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Federation of Gynecology and Obstetrics (FIGO) classification. Optimal debulking was defined as ≤ 1 cm (diameter) residual disease. A complete response (CR) was defined as absence of all clinical/radiographic evidence of disease. In addition, a second-look laparotomy (SLL) was performed on most of the patients who achieved a CR after planned treatment and all of them who were optimally debulked. In patients who achieved a CR after the planned treatment and did not accept an SLL or for whom this procedure was not feasible, and in patients with a partial response (PR), a second CT scan was performed 1 month after the first evaluation to confirm the response. Follow-up data were obtained by retrospective chart review. PFS was defined as the time interval between the start of the treatment and the first confirmed sign of disease recurrence or progression. Overall survival (OS) was defined as the time interval between the start of the treatment and the date of death or end of follow-up. Approval for the study was obtained from the local ethics committee. Relevant clinicopathological data of the patients are shown in Table 1.

Treatment response was studied in a homogeneous subgroup of 57 patients with both advanced tumor stage (FIGO stages III and IV) and serous carcinoma histology. In this subgroup of patients, 84 and 16% of the tumors corresponded to III and IV stages respectively. Regarding tumor grade in this subset of tumors, 61, 32, and 7% of the tumors were grades 3, 2, and 1 respectively. The clinical response to the treatment, debulking status after surgery, and survival data in this subgroup of patients are presented in Table 2.

Table 1 Clinicopathological data of the ovarian cancer patients[a]

	All cases ($n=72$)		Serous III/IV ($n=57$)[b]	
	n	**%**	***n***	**%**
Age (years)				
Median	57.0		54.0	
(minimum–maximum)	(35–85)		(35–85)	
Histological subtype				
Serous carcinoma	57	80	57	100
Clear cell carcinoma	6	8	–	–
Endometrioid carcinoma	4	6	–	–
Mucinous carcinoma	3	4	–	–
Mixed endometrioid–clear cell carcinoma	1	1	–	–
Mixed endometrioid–serous carcinoma	1	1	–	–
FIGO stage				
I	3	4	–	–
II	5	7	–	–
III	54	75	48	84
IV	10	14	9	16
Tumor grade				
Well differentiated (grade 1)	8	11	4	7
Moderately differentiated (grade 2)	24	33	18	32
Poorly differentiated (grade 3)	39	54	35	61
Unknown	1	1	–	–

FIGO, International Federation of Gynecology and Obstetrics.
[a]All patients included in the study were Caucasian females.
[b]Clinicopathologial characteristics of the subset of 57 patients with FIGO stages III and IV and serous ovarian carcinoma.

Tissue microarray construction

Representative areas of the tumors were selected on hematoxylin and eosin-stained sections and marked on individual paraffin blocks. Two tissue cores (1 mm in diameter) were obtained from each specimen. The tissue cores were arrayed into a receptor paraffin block using a tissue microarray workstation (Beecher Instruments, Silver Spring, MD, USA) as previously described (Hardisson *et al*. 2003). A hematoxylin and eosin-stained section of the array was reviewed to confirm the presence of morphologically representative areas of the original lesions.

Immunohistochemistry

Immunohistochemistry was performed on 4 μm sections of formalin-fixed, paraffin-embedded tissues. Briefly, the tissue sections were deparaffinized and rehydrated in water, after which antigen retrieval was carried out by incubation in EDTA solution, pH 8.2 at 50 °C for 45 min in an autoclave. Endogenous peroxidase and nonspecific antibody reactivity were blocked with peroxidase-blocking reagent (Dako, Glostrup, Denmark) at room temperature for 15 min. The sections were then incubated for 60–90 min at 4 °C with the following antibodies: class I β-tubulin (clone SAP.4G5, Sigma–Aldrich, dilution 1:100), class II β-tubulin that recognizes classes IIa and IIb β-tubulins, which differ in one single amino acid, (clone 7B9, Covance, Emeryville, CA, USA, dilution 1:100), and class III β-tubulin (clone TUJ-1, Santa Cruz Biotechnology, Heidelberg, Germany, dilution 1:200). Detection was performed with Envision Plus Detection System (Dako).

Immunohistochemical results were evaluated and scored by one pathologist (D H) blinded to the clinical data of the patients. Immunoreactivity was scored by estimating the percentage of tumor cells with cytoplasmic immunostaining, regardless of intensity. The intensity of the immunostaining was not considered

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Table 2 Clinical characteristics of patients with International Federation of Gynecology and Obstetrics (FIGO) stages III and IV and serous ovarian carcinoma

	n	%
Response to treatment		
Complete response	38	67
Partial response	11	19
Stable disease	3	5
Progressive disease	4	7
Unknown	1	2
Pathological response		
Complete response	14	25
Stable disease	10	17
Unknown	33	58
Debulking status		
Optimal (<1 cm)	24	42
Suboptimal (>1 cm)	12	21
Unknown	21	37
Relapse/progression		
Yes	47	83
No	8	14
Unknown	2	3
Progression-free survival (months)		
Median	16.0	
(minimum–maximum)	(1–128)	
Deceased		
Yes	35	61
No	19	33
Unknown	3	5
Overall survival (months)		
Median	35.1	
(minimum–maximum)	(1–128)	

since in our study the intensity of the immunostaining in the positive tumors was predominantly high, precluding discrimination between the samples. Class I β-tubulin expression was not categorized since the expression levels of all samples were consistently high. In the tissue sections, negative controls were used with only the secondary, replacing the primary antibody.

RNA isolation and real-time quantitative reverse transcription-PCR

Tissue sections previously stained with hematoxylin and eosin were reviewed by an experienced pathologist. Eligible samples included at least 80% of tumor cells and no large necrotic areas. Four to eight 4 μm sections were used for RNA isolation with the Masterpure RNA Purification kit (EPICENTRE Biotechnologies, Madison, WI, USA) according to the manufacturer's instructions. For microRNA quantitative reverse transcription (qRT)-PCR, 25 ng total RNA were reverse transcribed using the miRCURY LNA First-Strand cDNA kit (Exiqon A/S, Vedbaek, Denmark) and the miRCURY LNA microRNA Primer Sets (Exiqon) corresponding to hsa-miR-141, hsa-miR-200a, hsa-miR-200b, hsa-miR-200c, hsa-miR-429, and the control primer set 5S rRNA, according to the manufacturer's instructions. Negative controls with reaction mix without reverse transcriptase were included for the different microRNAs studied. Real-time qPCR was performed with the Sequence Detection System 7900HT (Applied Biosystems, Carlsbad, CA, USA) using the miRCURY LNA SYBR Green Master Mix (Exiqon), following the manufacturer's instructions. The amplification conditions consisted of an initial step at 95 °C for 10 min, followed by 50 cycles of 20 s at 95 °C and 1 min at 60 °C. Negative controls were included in all PCRs, and all assays were performed in triplicate. The $\Delta\Delta C_t$ method was used for the calculation of the different amounts of mRNA (Livak & Schmittgen 2001). Normalization was carried out with the endogenous control 5S ribosomic RNA. The relative abundance of different miR-200 family members cannot be precisely estimated because the hybridization characteristics of the different qRT-PCR probes could vary, however, approximate conclusions can be drawn due to the large differences found (Supplementary Figure 3, see section on supplementary data given at the end of this article).

Statistical analysis

All statistical analyses were carried out using SPSS version 17.0 statistical software (SPSS Inc., Chicago, IL, USA). The association between the expression levels of the five members of the miR-200 family and continuous demographic variables (such as age) was determined by the Pearson coefficient. β-Tubulins II and III protein expression, treatment response, pathological response, mortality, relapse, and tumoral characteristics (histology subtype, differentiation grade, and tumor stage) were analyzed as categorical variables. Similar to previous publications, protein expression of β-tubulins II and III was used as a binary variable (low expression versus high expression) using 75% of positive cells as the cutoff (Ohishi *et al*. 2007). The response to treatment was divided into two categories: patients with CR and those with PR, stable disease, and disease progression grouped together. Associations between the response to treatment and clinical variables (histology subtype, differentiation grade, tumor stage, and patient's age) were evaluated with χ^2 test, Fisher's exact test, and Student's *t*-test, when appropriate. P values <0.05 were considered statistically significant and all of them are two-sided. To analyze the associations between miR-200 expression and β-tubulin content and treatment

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response, since the tumoral miR-200 family content followed a normal distribution (Kolmogorov–Smirnov test), Student's *t*-tests were used, applying the Welch correction when the S.D.s differed significantly between the groups. To further analyze miR-200 expression and response to treatment, logistic regression was applied and the hazard ratio (HR) was estimated, adjusting for relevant clinicopathological variables. To analyze miR-200 expression and PFS and OS, the univariate analysis was carried out by using the Kaplan–Meier plots coupled to log-rank test and univariate Cox regression model was applied for the HR estimation. The multivariate Cox proportional hazards regression model was used to evaluate the prognostic significance of the microRNA adjusted by clinicopathological variables.

Results

Predicted binding sites of miR-200b/200c/429 in the β-tubulin 3′ UTR and immunohistochemical determination of β-tubulin isotypes I, IIa, and III expression

Recently, *in vitro* studies have shown that the 3′ UTR region of class III β-tubulin has a miR-200c-binding site. The important role that this microRNA plays in metastasis and the high degree of conservation among β-tubulin isotype functions and genetic structure led us to further explore in tumor samples the correlation between the expression of the tubulin isoforms and the miR-200 family. By means of an *in silico* analysis (http://www.targetscan.org/), miR-200b/200c/429-binding sites were predicted in the 3′ UTR not only of class III β-tubulin but also of classes I and IIa, while no binding sites were predicted in the rest of the human β-tubulin isotypes. The miR-200b/200c/429 microRNA-binding site was broadly conserved among vertebrates for classes I and III β-tubulins, but poorly conserved for class IIa. The *in silico* analysis did not predict miR-141 and miR-200a binding, due to one nucleotide difference in the seed sequence (Supplementary Figure 1, see section on supplementary data given at the end of this article), but there is evidence for miR-200 family common targets.

We then determined the protein expression of classes I, II, and III β-tubulins in 72 ovarian cancer samples by immunohistochemical analysis (Fig. 1). The protein staining showed substantial differences for the different isotypes, while class I protein expression showed no variation among the cases, β-tubulins II and III exhibited substantial intersample differences. All samples exhibited a very strong staining for class I β-tubulin; class II protein was absent in 46 (69%) tumors, while 15 cases had low and 6 cases had a high protein expression (22 and 9% of the tumors respectively). Class III protein was absent in 34 (48%) cases, while 29 (41%) had low and 8 (11%) had a high protein expression (Supplementary Figure 2A, see section on supplementary data given at the end of this article). With regards to the correlation between the different isotypes, classes II and III β-tubulin expression proved to be mutually exclusive events, with samples exhibiting a high β-tubulin class III content lacking isotype II expression and vice versa (Supplementary Figure 2B, see section on supplementary data given at the end of this article).

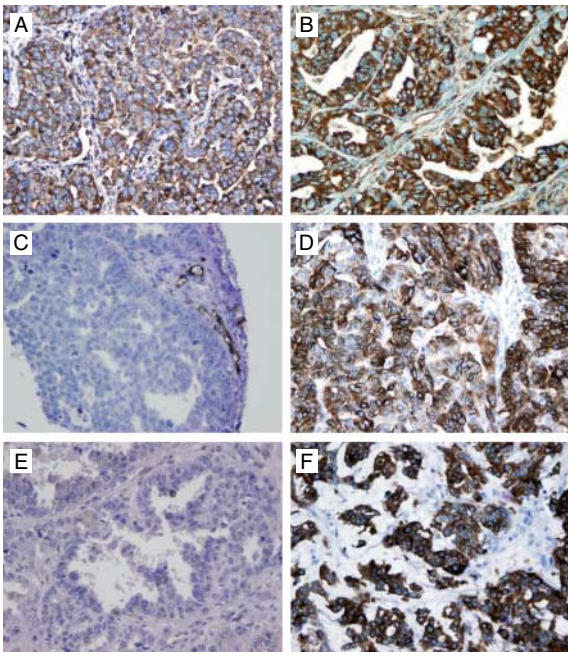

Figure 1 Protein expression of β-tubulin isotypes I, II, and III in ovarian carcinomas. Isotype I exhibited a high expression in all cases analyzed with minimal differences among samples (A) and (B). Illustrative cases with low (C) and high (D) β-tubulin isotype II expression. Illustrative cases with low (E) and high (F) β-tubulin isotype III expression. All cases shown correspond to serous carcinomas.

Expression of the miR-200 family is associated with tumoral β-tubulin III protein expression

To investigate whether the miR-200 family could regulate β-tubulin isotypes I, II, and III, we measured the expression of these microRNAs in ovarian cancer samples. The expression levels found were variable, with miR-200c expressed at the highest level, then, miR-200b, miR-200a, and finally miR-429 and miR-141, which were expressed at similarly low-expression levels (Supplementary Figure 3, see section

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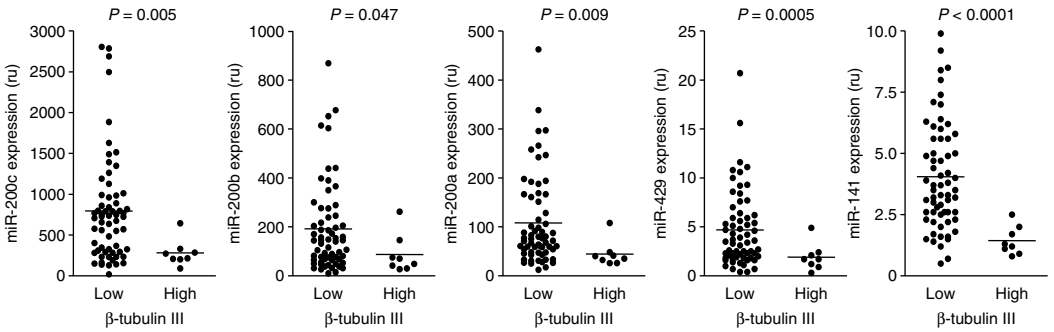

Figure 2 Tumors with high levels of β-tubulin III protein have significantly decreased miR-200 expression. Samples with high isotype III expression (more than 75% positive cells) showed a significantly lower miR-200c, $P=0.005$; miR-200b, $P=0.047$; miR-200a, $P=0.009$; miR-429, $P=0.0005$; and miR-141, $P<0.0001$ expression compared with samples with low isotype III expression. MicroRNAs are shown in the figure according to the expression levels. To express the microRNAs content as whole numbers, their expression was multiplied by 100 and expressed as relative units (ru).

on supplementary data given at the end of this article). As expected, correlations were found among the miR-200 family members, with miR-141/miR-200a and miR-200a/miR-200b showing the highest correlation and miR-429 the lowest (Supplementary Table 1, see section on supplementary data given at the end of this article).

We then determined whether the miR-200 family could regulate the protein expression of β-tubulin isotypes I, II, and III. We found a statistically significant association between class III β-tubulin protein expression and the tumoral content of all miR-200 members (Fig. 2). According to this, the ovarian tumors with low miR-200 expression exhibited high levels of class III protein, suggesting that the absence of these microRNAs in the tumor results in lack of class III β-tubulin degradation and accumulation of high levels of the protein. The strongest associations corresponded to miR-141, miR-429, and miR-200c ($P<0.006$) among which miR-200c showed the highest expression. No association was found between protein levels of β-tubulins I and II with miR-200 family expression (data not shown).

miR-200c expression determines the response to paclitaxel-based chemotherapy in serous ovarian carcinoma patients

Owing to the importance of tumor type and stage, we selected a homogenous subgroup of patients with serous carcinomas and advanced tumor stage (FIGO stages III and IV) to study whether the miR-200 family could influence clinical response to treatment: fifty-seven patients met these inclusion criteria. In these samples, we did not find statistically significant associations between the expression of classes I, II, or III β-tubulin content and response to treatment, relapse, or survival of the patients. Notwithstanding this, we found a statistically significant association between miR-200c expression and response to treatment ($P=0.0027$ with t-test; HR = 1.43, 95% CI = 1.02–1.99, $P=0.037$ with logistic regression multivariate analysis, Table 3). The patients who did not achieve a complete clinical response had lower miR-200c levels than those patients with CRs. A significant association was also found between miR-200c and pathological response using the t-test ($P=0.045$), although it did not reach significance in a logistic regression multivariate analysis (HR = 1.45, 95% CI = 0.94–2.25, $P=0.094$). With respect to the number of recurrence and mortality events, low expression of miR-200c was associated with recurrence (odds ratio (OR) = 1.17, 95% CI = 1.01–1.34, $P=0.030$), while no significant association was observed for mortality (OR = 1.11, 95% CI = 0.97–1.28, $P=0.128$; Table 3). miR-200c expression did not show any association with other clinicopathological characteristics. No association with treatment response was found for any of the other miR-200 family members.

miR-200 family expression is associated with prognosis in serous ovarian carcinoma patients

Of the miR-200 family, only miR-429 expression showed a statistically significant association with the recurrence-free survival and OS of the patients (HR = 2.01, 95% CI = 1.11–3.66, $P=0.021$ and HR = 2.08, 95% CI = 1.03–4.20, $P=0.041$; Fig. 3). The PFS rate at 12 months post treatment was 85% in the group of high miR-429 expression and 48% for those with low expression. For miR-200c, the PFS rate at 12 months was 73 and 54% for high and low expressors respectively. After the multivariable analysis, adjusting for relevant clinicopathological

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Table 3 miR-200c expression and response to paclitaxel–carboplatin chemotherapy

	Number of samples	miR-200c		Difference of means	Logistic regression	
		Mean	95% CI (ru)	*P* value[a]	HR (95% CI)	*P* value[b]
Response						
CR	37	9.1	(6.8–11.3)	**0.0027**[c]	**1.43 (1.02–1.99)**	**0.037**
No CR (PR, SD, PD)	17	4.8	(3.1–6.5)			
Pathological response						
CR	14	8.0	(5.6–10.3)	**0.045**	1.45 (0.94–2.25)	0.094
No CR	10	4.6	(2.0–7.1)			
Recurrence						
No	8	11.8	(3.1–20.6)	0.243[c]	**1.17 (1.01–1.34)**	**0.030**
Yes	45	7.0	(5.5–8.5)			
Deceased						
No	19	9.3	(5.4–13.1)	0.249[c]	1.11 (0.97–1.28)	0.128
Yes	35	6.9	(5.0–8.7)			

CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease. Values in bold are statistically significant.
[a]Univariate analysis using unpaired *t*-test.
[b]Multivariate analysis using logistic regression with debulking status, tumor grade, and FIGO stage as covariates.
[c]The Welch correction was applied when the S.D. differed significantly between the groups.

variables (debulking status, tumor stage, and histological grade), a tendency was observed for miR-429 expression to associate with recurrence-free survival (HR = 2.10, 95% CI = 0.92–4.79, $P = 0.079$). Similarly, miR-200c and miR-141 also showed this trend (HR = 2.24, 95% CI = 1.00–5.03, $P = 0.051$ and HR = 2.35, 95% CI = 0.98–5.59, $P = 0.054$ respectively) (Supplementary Table 2, see section on supplementary data given at the end of this article). When analyzing OS in the multivariate analysis, the association was not statistically significant for miR-429 or the other microRNAs.

Discussion

Ovarian cancer remains one of the leading causes of cancer death. Most ovarian cancers are detected at advanced stages and, although substantial progress has been made in the treatment of this tumor, lack of response and relapse due to intrinsic or acquired resistance greatly reduce survival rates. Thus, there is a need to improve patient care through the identification of biomarkers predictive of treatment response. This study focuses on the new field of microRNAs, because of their potential to provide novel drug response markers (Yang *et al*. 2008*a*,*b*, Adam *et al*. 2009, Li *et al*. 2009), and gives insight into the role of the miR-200 family in paclitaxel–carboplatin response and survival.

The miR-200 family has been shown to maintain the cellular epithelial phenotype via repression of ZEB1 and ZEB2 and to play an important role in tumor progression (Gregory *et al*. 2008). Interestingly, using *in silico* tools we have found that miR-200b/200c/429 had putative binding sites in the 3′ UTR of the β-tubulin isotypes I, IIa, and III (Supplementary Figure 1, see section on supplementary data given at the end of this article). Since β-tubulin is the therapeutic target of paclitaxel, we speculated that these microRNAs might influence the response of ovarian cancer to paclitaxel-based treatments through the downregulation of these isotypes in the tumoral cells. The miR-200 family of microRNAs has seed sequences differing by one nucleotide (Supplementary Figure 1, see section on supplementary data given at the end of this article) and, although target prediction algorithms assume significant differences in the genes targeted by miR-200b/200c/429 and miR-200a/141, there is evidence indicating a high degree of overlap in target genes (Park *et al*. 2008). This data suggests that multiple members of the miR-200 family may target a large common subset of genes to enhance the efficiency of genetic regulation.

When we measured the expression of the five miR-200 family members in 72 epithelial ovarian cancer samples (Supplementary Figure 3, see section on supplementary data given at the end of this article), we confirmed a high degree of variation (Park *et al*. 2008, Hu *et al*. 2009). The protein expression of classes I, II, and III β-tubulin (Supplementary Figure 2, see section on supplementary data given at the end of this article) showed isotype-specific differences, similar to those reported in a previous study (Ohishi *et al*. 2007). Interestingly, we found classes II and III β-tubulin

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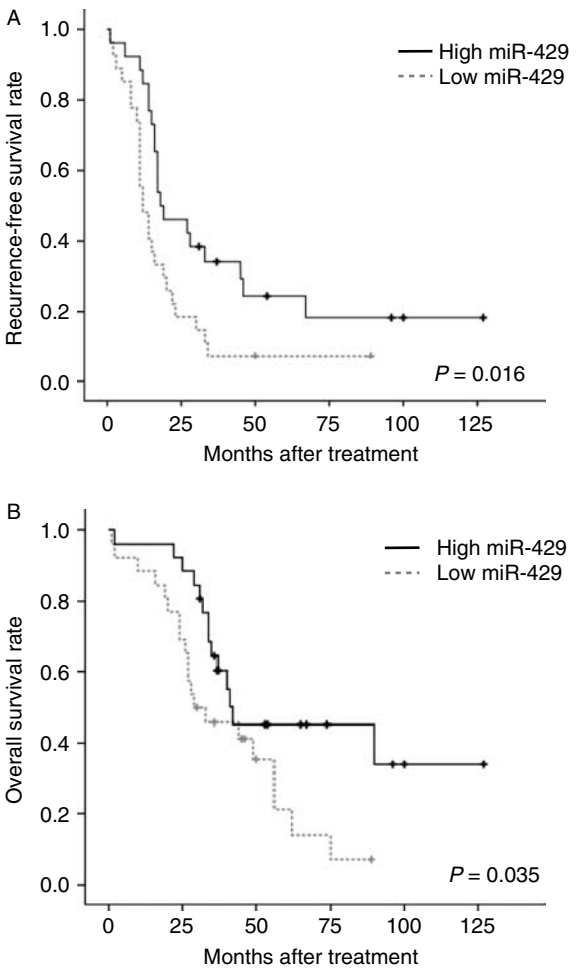

Figure 3 miR-429 is associated with recurrence-free survival and overall survival. The Kaplan–Meier survival analysis evaluating the effect of miR-429 expression on disease outcome for (A) recurrence-free survival and (B) overall survival. The miR-429 median expression was used as cutoff value to divide the patients into low and high miR-429 expressors. The median progression-free survival was 12 months (95% CI 8.2–15.8) for the low and 18 months (95% CI 8.8–27.2) for the high miR-429 expressors. *P* values shown correspond to the log-rank test.

expression to be mutually exclusive, suggesting a complex regulatory mechanism. In other tumor types, isotype III has been detected in 36% of gastric (Urano *et al*. 2006), 40% of head and neck (Koh *et al*. 2009), and 84% of breast (Paradiso *et al*. 2005) cancer samples respectively. β-tubulin III tumoral overexpression has been associated with poor prognosis in a variety of cancer types (Seve *et al*. 2005, Seve & Dumontet 2008, Koh *et al*. 2009), including ovarian carcinomas (Ferrandina *et al*. 2006), although there is one contradictory report (Aoki *et al*. 2009) in clear cell ovarian cancer patients. Concerning response to taxanes, which is closely related to survival rates, high β-tubulin III protein expression was reported to be associated with lack of response in breast (Hasegawa *et al*. 2003, Paradiso *et al*. 2005), lung (Rosell *et al*. 2003, Seve *et al*. 2005), and ovarian (Umezu *et al*. 2008) cancers. In our study, we were not able to detect a significant association between β-tubulin III expression and treatment response, probably due to a small number of high level class III samples. Altogether, these findings seem to reflect an increased resistance of class III to the effect of microtubule-binding drugs (Cochrane *et al*. 2009). In contrast, lack of isotype II expression has been associated with advanced stage and short PFS in ovarian cancer (Ohishi *et al*. 2007). These findings are in agreement with the mutual exclusivity we found for β-tubulins II and III expression.

Interestingly, we found that low levels of miR-200 were associated with high levels of class III protein, implying that β-tubulin III expression could be regulated by this family of microRNAs in clinical samples. In support of this finding, it has been recently shown that the reinstatement of miR-200c in cell lines decreases class III β-tubulin expression and increases sensitivity to microtubule-targeting agents (Cochrane *et al*. 2009) through direct targeting of β-tubulin III (Cochrane *et al*. 2010). However, it should be noted that samples with low levels of miR-200 did not always exhibit high levels of β-tubulin III (Fig. 2). This suggests that in addition to microRNA depletion, other mechanisms, such as epigenetic modifications, are required for β-tubulin III upregulation. We did not find an association between miR-200 and β-tubulins I and II, suggesting that the predicted binding sites for miR-200 in β-tubulins I and IIa genes are either nonfunctional or alternative mechanisms are crucial for the regulation of the eventual protein expression. Mutations on the miRNA-binding sites and alternative cleavage or polyadenylation of the 3′ UTRs are frequent in cancer and might be the mechanism underlying this observation (Blenkiron & Miska 2007, Mayr & Bartel 2009).

Additionally, for class IIa the antibody available for immunohistochemistry detected both IIa and IIb isotypes, which differ in a single nucleotide, and the predicted binding site in IIa was only conserved among mammals. However, for class I the predicted binding site was broadly conserved among vertebrates, similar to that of isotype III.

We then explored a possible role for miR-200 expression as a marker of response to paclitaxel–carboplatin regimen in ovarian carcinomas. Owing to the impact of the cancer stage and histology on response to treatment, we analyzed a homogenous

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series of serous adenocarcinomas with FIGO stages III and IV. We found a significant association between miR-200c expression and treatment response: women lacking CR had tumors with significantly lower miR-200c levels than the ones who had achieved CR (HR = 1.43, 95% CI = 1.02–1.99, $P = 0.037$; Table 3); in addition, higher expression of miR-200c was associated with lower relapse/progression rates (HR = 1.17, 95% CI = 1.01–1.34, $P = 0.030$; Table 3). These data seem to indicate that a low miR-200c expression results in high β-tubulin III expression and, thus, increased resistance to paclitaxel-based therapies. *In vitro* studies further support this connection (Cochrane *et al*. 2009, 2010). Regarding prognosis, we found that low tumoral miR-429 was associated with poor PFS and OS (Fig. 3). Multivariate analysis adjusted to relevant clinicopathologic variables revealed a trend for miR-429, miR-200c, and miR-141 with PFS (Supplementary Table 2, see section on supplementary data given at the end of this article), while the association with OS was lost. Hu *et al*. (2009) found a statistically significant association of miR-200a expression with OS and PFS of cancer patients, but Nam *et al*. (2008) described opposite results for the miR-200 family. The discrepancy found by Nam *et al*. could be caused by the small number of samples included in the study (20 serous ovarian carcinoma samples).

Since low tumoral expression of the miR-200 family has been associated with tumor progression and metastasis (Gregory *et al*. 2008, Park *et al*. 2008, Baffa *et al*. 2009), this could lead to a lower OS, independent of treatment response. Our results suggest a possible role for the miR-200 family members as predictive factors for paclitaxel-based response, especially miR-200c, and as prognostic factors in ovarian carcinoma. Because all miR-200 family members share similar targets, but there are differences in the recognition site, we propose that specific members of the family might be more important for prognosis and others for treatment response. In addition, the relative expression levels in the tumor cells could be playing a role in the final regulation of target genes. Thus, the effect of low tumoral miR-200 family expression could be twofold: a decreased response to microtubule-binding drugs and an increased metastasis risk through increased EMT.

Whether the relationship between the expression of miR-200 and first-line treatment response is due to paclitaxel alone or due to combined therapy is unknown, however, this could only be studied using single-agent paclitaxel. Even so, in the context of the new regimens with better response rates (such as combination with antiangiogenic compounds (Burger *et al*. 2007, Cannistra *et al*. 2007, Penson *et al*. 2010)), markers which are able to identify patients who efficiently respond to carboplatin/paclitaxel treatment are relevant. This subset of patients might not benefit from newly targeted drugs, especially if their risk of serious adverse reactions (e.g. bowel perforation) is increased.

In conclusion, we suggest that miR-200 down-regulates β-tubulin III in ovarian tumors. Furthermore, our results suggest a possible role for the miR-200 family both as a prognostic factor and a marker of treatment failure in ovarian carcinoma. Thus, miR-200 might constitute an important biomarker for ovarian cancer patients and could provide the basis for future therapies restoring miR-200 expression in tumor cells. Nevertheless, these data should be further validated in independent cohorts and prospective trials.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1677/ERC-10-0148.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This article was supported in part by projects from the Fundación Ramón Areces, the Spanish Ministry of Science and Innovation (SAF 2006-01139 and 2009-08307), the 'Ramon y Cajal' program, the Fondo de Investigación Sanitaria (FIS), Instituto de Investigación Carlos III, Ministerio de Ciencia e Innovación, Spain (Grant PI07/0651), and the Fundación de Investigación Médica Mutua Madrileña. S Leskelä has a fellowship from the Spanish Ministry of Education and Science (AP2005-4514). Dr M Mendiola is supported by a postdoctoral research contract (FIS, Program 'Sara Borrell', Instituto de Salud Carlos III), Ministerio de Ciencia e Innovación, Spain.

Author contribution statement

C Rodríguez-Antona, D Hardisson, M Robledo, and B Martínez-Delgado contributed to the study design; D Hardisson, M Mendiola, J Barriuso, A Redondo, and J de Santiago conceived data collection; S Leskelä, L J Leandro-García, M Mendiola, I Muñoz, and J Barriuso carried out the experiments; S Leskelä, L J Leandro-García, L Inglada, J Barriuso, and C Rodríguez-Antona analyzed the data. All authors were involved in writing the article and had final approval of the submitted and published versions.

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Acknowledgements

We thank María Miguel-Martín, Rocío López-Vacas, and Esther Díaz-López for their excellent technical assistance and Carol Box for her help with the English of this paper.

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