Class III β-Tubulin Overexpression Is a Marker of Poor Clinical Outcome in Advanced Ovarian Cancer Patients

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Abstract

Purpose: Overexpression of β III tubulin has been involved in paclitaxel resistance in several experimental models. We investigated the role of β III tubulin as predictor of clinical outcome in ovarian cancer patients given platinum/paclitaxel treatment. We also investigated whether β III tubulin expression could be modified after the selective pressure represented by chemotherapy *in vivo*.

Experimental Design: The study was designed to include a series of consecutive ovarian cancer patients with unresectable disease at time of first surgery, who underwent interval debulking surgery with pathologic assessment of response to treatment with platinum/paclitaxel chemotherapy. Immunostaining was done on formalin-fixed, paraffin-embedded tissue sections from pretreatment and posttreatment tissue biopsies by using the polyclonal rabbit anti–class III β-tubulin antibody.

Results: β III Tubulin immunoreaction was observed in 51 of 62 (82.2%) cases. β III Tubulin positivity was neither associated with clinicopathologic variables nor with pathologic response to chemotherapy. Significantly lower percentages of β III tubulin positivity were observed in posttreatment (range, 5-80%; median, 20%) versus pretreatment (range 10-100%; median, 40%) tissue biopsies ($P = 0.0011$). Cases with high β III tubulin expression showed a worse overall survival with respect to cases with low β III tubulin expression (median overall survival, 25 versus 46 months; $P = 0.002$). Multivariate analysis showed that high content of β III tubulin remains independently associated with a worse prognosis.

Conclusions: Assessment of β III tubulin could be useful to identify poor prognosis ovarian cancer patients candidates to more aggressive and/or targeted therapy.

Ovarian cancer represents the fifth leading cause of death for cancer in women (1). More than 70% of cases present with advanced stage of disease at diagnosis and despite the advances in cytoreductive surgery and the establishment of carboplatin/paclitaxel combination as the standard chemotherapy regimen, intrinsic or acquired tumor chemoresistance remains the major determinant of chemotherapy failure and unfavorable clinical outcome (1, 2).

Among the molecular alterations proposed to support tumor resistance to platinum agents, such as mutation of p53, and alterations of DNA mismatch repair system (3), much attention has recently been focused on the role of tubulin alterations in supporting resistance to taxanes (4); in particular, point mutations of tubulin genes have been associated with taxane resistance in *in vitro* models (5, 6) although no univocal data have been documented (7–9). Moreover, overexpression of selective β-tubulin isotypes (i.e., class III β-tubulin) has been advocated as an additional mechanism involved in paclitaxel resistance (10–12).

In particular, β III tubulin has been hypothesized to counteract suppression of microtubule dynamicity by paclitaxel through the enhancement of microtubule instability or, alternatively, the reduction of microtubule polymerization rate (13, 14). Few data are currently available about the clinical role of class III β-tubulin status in human tumors; expression of tubulin isotypes has been reported to predict clinical outcome in breast cancer patients given taxanes (15, 16) and high expression of class III β-tubulin has been associated with poor chance of response to taxane/vinorelbine–containing regimens and poor prognosis in non–small-cell lung cancer patients (17–19). We also recently suggested that class III β-tubulin overexpression might represent a prominent mechanism of resistance to paclitaxel-platinum treatment in ovarian cancer (20).

To our knowledge, no data have been reported until now on the clinical role of the expression of class III β-tubulin in predicting clinical outcome in ovarian carcinoma. Moreover, no data are currently available about the possible modulation

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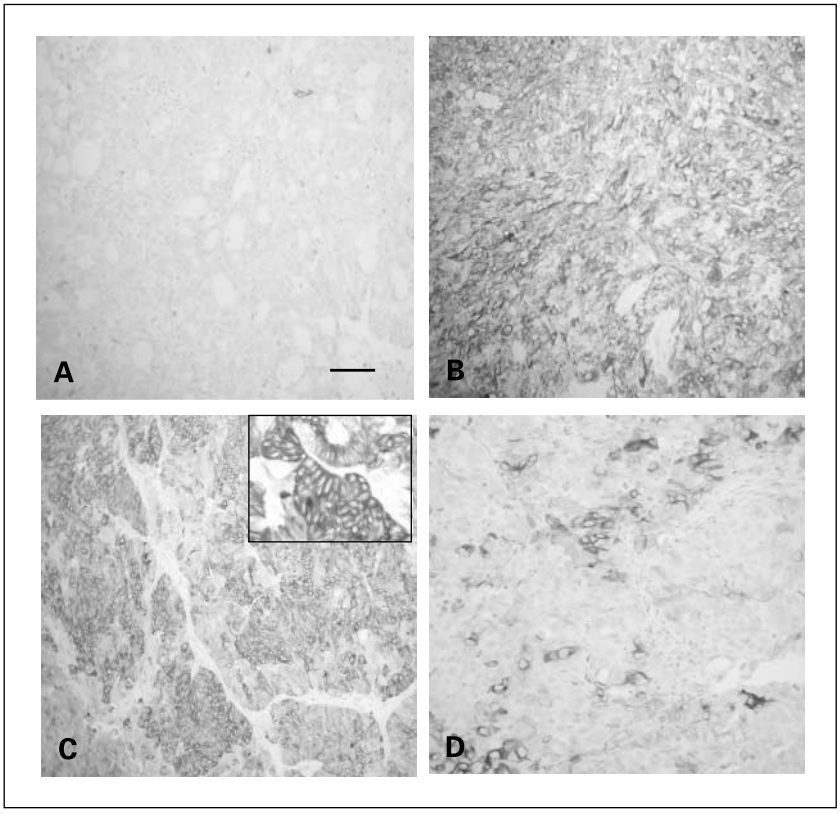

Fig. 1. β III Tubulin immunoreaction in primary ovarian cancer. Negative (*A*) and positive (*B*) controls (human brain tissue specimen) for β III tubulin staining. Representative examples of high (*C*) and low (*D*) β III tubulin expression. *A* to *D*, magnification ×20. Inset, magnification ×40. Bar, 100 μm.

of β III tubulin expression after the selective pressure represented by platinum/paclitaxel–containing chemotherapy *in vivo*.

The aim of the study was to investigate the clinical role of β III tubulin as predictor of survival and response to first-line platinum/paclitaxel treatment in a single institutional series of advanced unresectable ovarian cancer patients. We were also prompted at investigating whether carboplatin/paclitaxel regimen could modulate, and to what extent if any, the expression of class III β-tubulin protein in ovarian cancer tissues.

Patients and Methods

Patients. The study included 62 ovarian cancer patients admitted to the Gynecologic Oncology Unit, Catholic University of Rome, between January 1998 and December 2004. To make the analysis of the association of β III tubulin status with response to chemotherapy the most reliable, the study was designed to include a series of consecutive advanced ovarian cancer patients with unresectable disease at time of first surgery, who underwent interval debulking surgery with pathologic assessment of response to treatment after platinum/paclitaxel chemotherapy.

Both primary and secondary surgery were done by the same gynecologic oncology team. All cases were submitted to exploratory laparotomy and were judged unresectable because of diffuse abdominal carcinomatosis and/or infiltration of the upper gastrointestinal tract and/or the major vessels (21). Only multiple biopsies were done to obtain the pathologic diagnosis. Staging was done according to Fédération Internationale des Gynecologistes et Obstetristes classification.

All patients underwent three or four cycles of platinum (75-100 mg/m^2 or area under the curve = 5 for cisplatin or carboplatin, per cycle, respectively) and paclitaxel–containing (175 mg/m^2 for each cycle) chemotherapy. A direct assessment of the extent of response to chemotherapy was carried out at time of second laparotomy, and interval debulking surgery included surgical removal of tumor masses along with total abdominal hysterectomy, adnexectomy, radical omentectomy, and appendectomy plus additional surgery (diaphragm stripping, intestinal resection, posterior pelvic exenteration, and low rectal anastomosis) if necessary. Residual tumor at interval debulking surgery was also recorded.

Immunohistochemistry. Pretreatment tumor tissues biopsies were obtained at first surgery in all cases whereas posttreatment tumor tissue biopsies were obtained at interval debulking surgery after three or four cycles of chemotherapy. Tissue specimens were fixed in 10% formalin and paraffin embedded according to standard procedures. Immunostaining was done on 3-μm tissue sections mounted on poly-L-lysine–coated slides and dried at 37°C overnight. After the slides were deparaffinized in xylene and rehydrated conventionally, the endogenous peroxidase activity was blocked with 3% H_2O_2 in TBS for 5 minutes. Antigen retrieval procedure was done by microwave oven heating in 1 mmol/L EDTA (pH 8). Sections were incubated with 20% normal goat serum 20% for 30 minutes at room temperature to reduce nonspecific binding, then with the polyclonal rabbit anti–class III β-tubulin antibody (diluted 1:200; Covance, Princeton, NJ) in 1% bovine serum albumin-PBS (20).

β III Tubulin detection was evaluated by a labeled polymer. The EnVision-rabbit+ System-HRP System (DAKO, Carpinteria, CA) was used. Diaminobenzidine was used as a chromogen (DAB Substrate System, DAKO). Negative controls were done by omitting the primary antibody. Positive control for β III tubulin was represented by sections taken from the brain (20). Results were expressed as the proportion of immunostained tumor cells.

The analysis of all tissue sections was done without any prior knowledge of the clinical variables by two authors (G.F.Z. and E.M.) by means of light microscopy. The proportion of immunostained tumor cells was scored at low magnification (5× objective lens) by evaluating the entire tumor area. In case of disagreement (n = 5, 8.1%), sections were submitted to a rejoint evaluation.

The value of 30% immunostained tumor cells (corresponding to the median value) was arbitrarily chosen as cutoff value to distinguish cases with high versus low β III tubulin content without any prior knowledge of the clinicopathologic variables and patient clinical outcome.

Statistical analysis. Fisher's exact test (or χ^2 test for proportion) was used to analyze the distribution of class III β-tubulin positivity according to clinicopathologic features and response to treatment. Wilcoxon signed rank sum test for paired samples was used to analyze the expression levels of class III β-tubulin in pretreatment versus posttreatment tumor tissue samples.

Time to progression and overall survival were calculated from the date of diagnosis to the date of progression/death or date last seen. Medians and life tables were computed using the product-limit estimate by the Kaplan and Meier method (22) and the log-rank test was employed to assess the statistical significance (23). Statistical analysis was carried out using SOLO (BMDP Statistical Software, Los Angeles, CA). Multivariate analysis assessing the clinical role of βIII tubulin expression matched with other clinicopathologic characteristics was done by Cox proportional hazards model (24).

Results

Figure 1 shows representative examples of high versus low β III tubulin immunoreaction in primary ovarian cancer.

Table 1 summarizes the clinicopathologic characteristics of cases in the whole series. Median age was 58 years (range, 39-77 years). Fifty-two (83.9%) cases were stage III and 10 (16.1%) cases were stage IV disease.

After chemotherapy, all cases underwent exploratory laparotomy; cytoreduction to <0.5 cm residual tumor was achieved in 30 cases whereas residual tumor between 0.5 and 2 cm and >2 cm was left in 19 and 13 cases, respectively.

At pathologic examination, tumor was represented by only microscopic foci in 11 cases, which were considered as responsive cases. Partial response or no change of disease was documented in 41 and 10 cases, respectively, whereas we could not detect any case showing progression of disease.

β III Tubulin immunoreaction was observed in 51 of 62 (82.2%) cases and the percentage of positively stained cells showed a wide range of variability (range, 0-100%; median, 30%). In Table 1, the percentage of cases with high β III tubulin expression is summarized according to clinicopathologic features. β III Tubulin positivity was found not to be associated with any of the clinicopathologic variables examined. No association with pathologic response to chemotherapy was found. Similar results were found when analyzing the percentage of β III tubulin immunostained cells as a continuous variable (data not shown). We could not find any statistically significant difference in the percentage of cells expressing β III tubulin in pretreatment versus posttreatment samples; in tumor tissues obtained at interval debulking surgery after chemotherapy exposure, the percentages of β III tubulin positive cells ranged from 0% to 80% (median, 20%) with respect to baseline expression (range 0-100%; median, 30%; $P = 0.09$; Table 2). There was no difference in pretreatment versus posttreatment β III tubulin content samples in patients considered chemotherapy sensitive (pathologically assessed complete response or persistence of only microscopic foci) or chemotherapy resistant (pathologically assessed partial response or no change of disease; data not shown). To avoid the possibility that cases absolutely lacking β III tubulin at baseline could be a priori not susceptible to treatment modulation, we tried

Table 1. Clinicopathologic characteristics of the overall series and β III tubulin expression

Characteristics	No. patients	β III tubulin positive tumors, no. (%)	P*
All cases	62	26 (41.9)	
Age (y)			
<65	44	18 (40.9)	
>65	18	8 (44.4)	0.9
Fédération Internationale des Gynecologistes et Obstetristes stage			
III	52	22 (42.3)	
IV	10	4 (40.0)	0.9
Ascites			
No	8	4 (50.0)	
Yes	54	21 (38.9)	0.7
Histotype			
Serous	50	22 (44.4)	
Other	12	3 (25.0)	0.3
Pathologic response to chemotherapy			
Microscopic foci	11	4 (36.4)	
Partial/no change	51	22 (43.1)	0.7
Residual tumor at interval debulking surgery (cm)			
<0.5	30	14 (46.7)	
0.5-2	19	5 (26.3)	
>2	13	5 (38.5)	0.4

*Calculated by Fisher's exact or χ^2 test for proportion.

Table 2. β III tubulin expression in ovarian cancer tissue samples before and after platinum/paclitaxel treatment

Ovarian cancer tissue specimens	% β III tubulin positive cells, median (range)	P*	% β III tubulin positive cells,† median (range)	P*
Pretreatment	30 (0-100)	0.09	40 (10-100)	0.0011
Posttreatment	20 (0-80)		20 (5-80)	

*Calculated by Wilcoxon signed rank sum test.
†Only cases with detectable β III tubulin expression (n = 51).

analyzing the data after excluding from the analysis the cases showing no evidence of any positively immunostained tumor cells in pretreatment tissue samples: significantly lower percentages of β III tubulin positivity were observed in posttreatment (range 5-80%; median, 20%) versus pretreatment (range 10-100%; median, 40%) tissue biopsies (P = 0.0011). The decrease in β III tubulin positivity in posttreatment tissue samples was observed both in the groups of chemosensitive and chemoresistant cases (data not shown).

Survival analysis. Follow-up data were available for all patients. After a median follow-up of 27 months (range, 7-84 months), progression and death of disease were observed in 48 (77.4%) and 36 (58.1%) cases. Figure 2 shows the time to progression and overall survival curves in the whole series. There was no statistically significant difference in terms of time to progression in cases with high versus low β III tubulin expression (median time to progression, 15 versus 15 months, respectively; P = 0.5). On the other hand, cases with high β III tubulin expression showed a worse overall survival with respect to cases with low β III tubulin expression (median overall survival, 25 versus 46 months; P = 0.002).

Multivariate analysis showed that a large extent of residual tumor at interval debulking surgery and high content of β III tubulin were the most important variables independently associated with an unfavorable overall survival (Table 3).

Discussion

This is the first study analyzing the association between the expression of β III tubulin protein and clinical outcome in advanced unresectable ovarian cancer patients submitted to neoadjuvant platinum/paclitaxel–containing chemotherapy.

We showed that patients whose tumors expressed high levels of β III tubulin experienced a shorter overall survival with respect to cases with low β III tubulin content. This finding has been confirmed in multivariate analysis documenting that the extent of residual tumor at interval debulking surgery as well as the status of β III tubulin maintained their independent correlation with worse survival. These findings seem even more relevant from a clinical point of view when considering that the prognosis of patients judged unresectable at primary surgery is so unfavorable that any other factor, besides residual tumor, is expected to become of minor, if any, relevance in terms of prognostic discrimination.

The association between β III tubulin overexpression and poor prognosis has been reported in several human tumors including lung and breast tumors (15–19) but often related to poor chance of response to antitubulin agents (16–18), so that it is difficult in this clinical setting to discriminate whether the prognostic effect of the molecular factor is due to its value either as a predictor of response or as a marker of intrinsic tumor aggressiveness (pure prognostic factor).

Interestingly enough, we could not detect in our series any difference in the distribution of β III tubulin expression according to response to treatment or time to progression, the latter being strictly associated with treatment susceptibility, thus suggesting that overexpression of β III tubulin might more likely indicate tumor intrinsic biological aggressiveness rather than represent only a marker of chemotherapy resistance.

In this context, it is worth noting that the promoter of β-tubulin gene contains transcription factors involved in the hypoxic response (25–27), which translates into the transcription of genes relevant for tumor cell adaptation and survival, but is also associated with resistance to chemo/radiotherapy and clinical outcome (28–30). Therefore, it is conceivable that

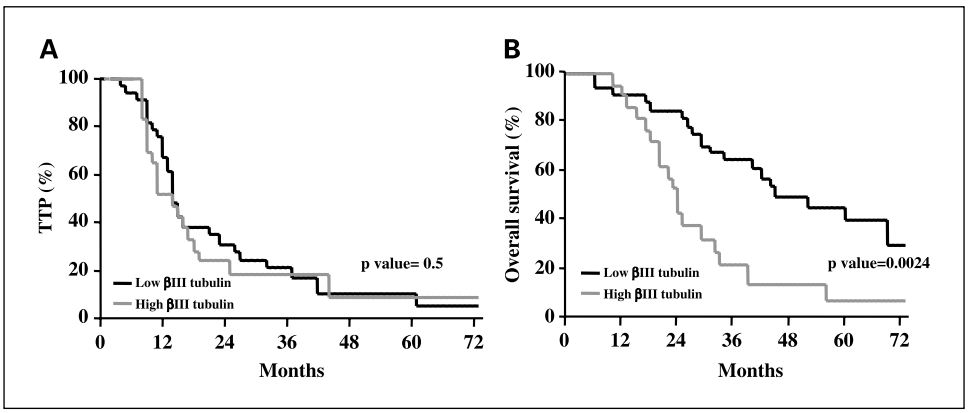

Fig. 2. Time to progression (*TTP*; *A*) and overall survival (*B*) curves according to low versus high β III tubulin expression in advanced ovarian cancer patients.

Table 3. Univariate and multivariate analyses of clinicopathologic variables and class III β-tubulin status as prognostic factors for overall survival in advanced ovarian cancer patients

Variable	Univariate			Multivariate*		
	RR1	χ^2	*P*	RR2	χ^2	*P*
Age (y)						
<65	1^0			1^0		
>65	2.4	6.0	0.014	2.3	3.0	0.08
Stage						
III	1^0					
IV	1.1	0.1	0.8	—	—	—
Ascites						
No	1^0					
Yes	1.4	0.6	0.3	—	—	—
Extent of residual tumor (cm) †						
<0.5	1^0			1^0		
>0.5	1.8	2.9	0.07	1.9	8.7	0.003
Response to treatment						
No	1^0					
Yes	1.8	1.4	0.2	—	—	—
β III tubulin expression						
Low	1^0			1^0		
High	3.3	11.1	0.0009	3.1	4.6	0.032

NOTE: RR1, unadjusted relative risk; RR2, relative risk after adjusting for all the factors listed; 1^0, reference category; χ^2 of the model = 14.3; $P = 0.0008$.
*Only variables with $P < 0.10$ in the univariate analysis were included in the multivariate model.
†Obtained at interval debulking surgery.

the overexpression of β III tubulin may represent a marker of the so-called "hypoxia lethal phenotype" (28), which is endowed by biological aggressiveness.

The lack of association between β III tubulin and response to chemotherapy does not confirm the results previously reported by our group (20) in a completely different clinical setting, which included patients submitted to primary cytoreduction and adjuvant chemotherapy before assessment of response by clinical or imaging procedures. Some issues have to be considered: (*a*) in the current series all patients were judged to be unresectable at primary surgery, a condition likely to heavily affect the overall response rate; (*b*) more importantly, response to treatment was pathologically assessed; (*c*) finally, we cannot exclude the possibility that ovarian cancer diagnosed with a disease so extensive to be unresectable might have intrinsic biological characteristics of aggressiveness (31), which could in turn affect tumor susceptibility to treatment, thus emphasizing the need to examine the biological correlations within a specific clinical setting.

It has been reported that selective *in vitro* pressure by antitubulin agents is able to force tumor cells to develop drug resistance by overexpressing β III tubulin isotype (10, 12, 32). Conversely, we observed a decrease in the percentage of β III tubulin positivity in posttreatment tissue samples regardless of response to chemotherapy.

It has been reported that the ability of taxanes to modulate β III tubulin gene transcription may be affected by the mutational status of p53 gene (33), of which the alterations are the most frequently observed in ovarian carcinoma (34). Therefore, the simultaneous assessment of β III tubulin and p53 status in a larger series of ovarian tumors has been planned in our institution. On the other side, it cannot be excluded that tissue specimens represent a different picture from *in vitro* models: the contribution of cellular components, such as flogistic and endothelial cells in the stroma, is likely to realize a regulatory microenvironment which might somehow hide the straight biochemical relationships found in cell culture with respect to *in vivo* models, as also reported by Nicoletti et al. (35).

In conclusion, although these findings need to be confirmed in a larger series, our study suggests that the assessment of class III β-tubulin status could be helpful to identify poor prognosis ovarian cancer patients who are potential candidates to more aggressive and/or targeted therapy. In this context, it is conceivable that cases expressing high β III tubulin expression could, in principle, benefit from the addition of novel taxanes able to selectively target β III tubulin. Indeed, the seco-taxane IDN5390, which has been recently reported to exhibit a strong synergistic antitumor activity when combined with paclitaxel in paclitaxel resistant β III tubulin overexpressing cells (32), could be regarded as the prototype of selective β III tubulin inhibitors.

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