

Preoperative Anti-Class III β -Tubulin Antibodies As Relevant Clinical Biomarkers in Ovarian Cancer



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Abstract

Class III β -tubulin (TUBB3) overexpression in ovarian cancer (OC) associates with poor prognosis. We investigated whether TUBB3 overexpression elicited anti-TUBB3 antibody production in OC patients and whether these antibodies may have diagnostic and prognostic impact. The presence of serum anti-TUBB3 antibodies was investigated in 49 untreated OC patients and 44 healthy individuals by an in-house developed ELISA that used recombinant TUBB3 as the antigen. Receiver operating characteristic (ROC) curves were generated to assess the diagnostic accuracy of the assay. Anti-TUBB3 antibodies discriminated OC patients and healthy individuals with excellent sensitivity and specificity (91.8% and 90.9%, respectively). In multivariate analysis, anti-TUBB3 antibody level emerged as an independent prognostic factor for progression free and overall survival. The ELISA was then optimized using a biotin-labeled TUBB3 C-terminal peptide₄₂₄₋₄₅₀ instead of recombinant TUBB3 as the antigen and streptavidin-coated plates. The diagnostic role of the anti-TUBB3 antibodies was studied in an independent series of 99 OC patients and 80 gynecological benign disease patients. ROC-curve analysis showed a valuable diagnostic potential for serum anti-TUBB3 antibodies to identify OC patients with higher sensitivity and specificity (95.3% and 97.6%, respectively). Overall, our results provide evidence that preoperative anti-TUBB3 antibody level is a promising diagnostic and prognostic biomarker for the management of OC patients.

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Introduction

Ovarian cancer (OC) is the most lethal among gynecological malignancies and represents the fourth most common cause of cancer-related death in women in the western countries [1]. Since OC patients are asymptomatic or moderately symptomatic in the earlier stages of the disease, the majority of OC patients are diagnosed after the primary tumor has already metastasized and despite the initial response to surgical debulking and first-line therapy, most tumors eventually develop drug resistance, with a 5-year survival generally around 30–40% [2]. Although the past decade has seen significant changes in the available therapeutic agents and strategies, taxane–platinum regimens remain the mainstay of treatment for ovarian cancer.

Taxanes act as microtubule-stabilizing agents by binding to β -tubulins, cytoskeleton proteins that belong to one of two core protein families (alpha and beta tubulins) and that heterodimerize to form

microtubules [3]. These drugs exert their growth-inhibitory effects by arresting the growth of tumor cells at the G2-M phase. Others and we have proposed that selective overexpression of class III β -tubulin (TUBB3) by OC cells is associated with resistance to taxanes and poor prognosis [4–9]. Collectively, these early studies point to

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TUBB3 overexpression by OC cells as both potential predictive biomarker for chemotherapy chemosensitivity and negative prognostic indicator in OC patients. However, the assessment of TUBB3 by IHC in the context of OC has some intrinsic limitations mostly related to the method of pathologist semi-quantification, which is costly and inherently subjective, and error-ridden, producing ordinal rather than continuous variable data. Additionally, TUBB3 expressing OC cells might be located in sites that are not available to the pathologist, e.g. distant metastases, as it has been demonstrated to be the case in different tumor settings [10,11]. All these factors have limited the clinical utility of TUBB3 as a biomarker in OC.

It is well known that, besides being present in OC, TUBB3 is largely expressed in normal neurons [12]. A study in patients affected by cerebral malaria demonstrated that TUBB3 elicits antibodies as a consequence of neuron damage induced by *Plasmodium falciparum* infection [13]. Given this background, we hypothesized that in OC TUBB3 might become immunogenic and elicit antibody production as a consequence of tumor cell damage and release of the intracellular components into the tumor microenvironment. We reasoned that if TUBB3 overexpressed by OC cells elicited antibody production, these antibodies could be revealed in the serum of OC patients, in analogy with a number of antibodies to tumor-associated proteins in a variety of tumor settings [Reviewed in 14], and could be used as a more precise indicator of TUBB3 expression by OC cells. To this end, we generated recombinant TUBB3 and developed an ELISA system using the recombinant TUBB3 as the antigen to detect the presence of anti-TUBB3 antibodies in the serum of OC patients. To improve sensitivity and specificity, we optimized the assay by substituting recombinant TUBB3 with a biotin-labeled TUBB3 C-terminal peptide₄₂₄₋₄₅₀.

Results demonstrate for the first time that anti-TUBB3 antibodies are present in the vast majority of OC patients irrespective of histotype and disease stage and could serve as both a diagnostic and prognostic biomarker.

Patients and Methods

Samples Studied by the ELISA System Using the Recombinant TUBB3 as the Antigen

The study included 49 stage I-IV OC patients, (median age 57 years, range: 25-81), consecutively admitted to the Department of Woman and Child Health, Fondazione Policlinico Universitario A.

Table 1. Clinicopathological Features of OC Patients Evaluated for the Anti-TUBB3 Antibodies by the ELISA System that Uses the Recombinant TUBB3 as the Antigen

Characteristics	No. of Patients (%)
FIGO stage	
I-II	10 (20.4)
III-IV	39 (79.6)
Carcinomatosis	
Yes	26 (53.1)
No	23 (46.9)
Tumor Histotype	
Serous	39 (79.6)
Endometrioid/Clear Cell	10 (20.4)
Tumor Grade	
G1	3 (6.1)
G2-G3	46 (93.9)
Residual tumor at 1 st surgery	
≤1cm	26 (53.1)
>1cm	23 (46.9)

Gemelli, Rome, Italy between January 2005 and December 2006. The clinicopathological characteristics are summarized in Table 1. Follow-up data including progression-free survival (PFS) and overall survival (OS) were available for all patients.

As a control, normal serum samples (n = 44) were collected from female blood donors who volunteered to participate in the study. Serum samples of OC patients were obtained before surgery and any medical treatment. Patients' and normal serum samples were stored at -80°C until assay.

Samples Studied by the ELISA System Using the Biotin-Labeled TUBB3 C-Terminal Peptide₄₂₄₋₄₅₀

The study included an independent cohort of 99 OC patients consecutively admitted to our Department between February and December 2016. The clinicopathological characteristics are summarized in Supplementary Data. As a control, serum samples were collected from 80 patients suffering from benign gynecological diseases (including uterine myoma and ovarian cysts), admitted at our department during the same period. Serum samples were obtained before surgery and any medical treatment and were stored at -80°C until assay.

Patients' Evaluation and Informed Consent

For all OC patients, pretreatment evaluation consisted of a history and physical examination, biopsy and gynecologic examination under general anesthesia, abdominal pelvic MRI, pelvic ultrasonography and chest X-ray. Serum from the OC patients was collected before surgery and any medical treatment; likewise, tumor tissue samples for the tissue-based analysis were obtained at the first surgery. Standard taxanes-including chemotherapy followed surgery in all cases. This study was approved by our institutional review board, and written informed consent was obtained from all patients for collection of their clinical data, as well as tissue materials for research purposes. Clinical information was obtained from the existing medical records in accord with institutional guidelines. All data were managed using anonymous numerical codes.

Production of Recombinant TUBB3 in Sf9 Cells

Human TUBB3 gene open reading frame (NCBI reference: NM_006086) was PCR amplified and cloned into pFastHTA. The cloned DNA sequence was confirmed by DNA sequencing. Bacmids carrying TUBB3 were prepared using a Bac-to-Bac® Baculovirus Expression System (Invitrogen, Carlsbad, CA, USA) and transfected into Sf9 cells to amplify the recombinant baculovirus using Cellfectin (Invitrogen). The Sf9 cells were infected with the viruses, with a multiplicity of infection of approximately 0.5 at 28°C. The Sf9 cells were harvested and used for protein preparation after a 72-h infection. The procedure of TUBB3 expression in Sf9 cells and purification were performed according to manufacturer's instructions. Quality of the purified protein was tested by silver staining of a SDS page gel using the Proteo Silver silver stain kit from Sigma Aldrich.

Detecting the Anti-TUBB3 Antibodies Using Recombinant TUBB3 as the Antigen

The ELISA was performed using flat-bottom, 96-well microtiter plates (Sigma-Aldrich Inc.). Wells were coated with 100 µl phosphate-buffered saline at pH 7.4 (PBS) containing 0.25 µg/µl recombinant TUBB3 at 4°C overnight. Wells were carefully washed with PBS-0.1% Tween-20 and then blocked with PBS-1% gelatin at 37°C for 1 h. Wells were washed 3 times with PBS and incubated

with 100 μ l of test serum (1:100 dilution in PBS-0.1% tween-20) at 37°C for 1 h. A rabbit polyclonal anti-TUBB3 antibody (Covance, Princeton, NJ) served as positive control. As a background control, the recombinant TUBB3 was omitted in some wells. After incubation, the plates were extensively washed with PBS 0.1% tween-20 before adding horseradish peroxidase-conjugated goat anti-human IgG (final dilution 1: 20,000) or anti-rabbit IgG (final dilution 1: 2,000) both from Santa Cruz Biotechnology (Santa Cruz, CA). The plates were incubated 37°C for 1h and then extensively washed with PBS-0.1% tween-20. 3,3',5,5'-tetramethylbenzidine solution (eBioscience, San Diego, CA) was added to the wells and incubated at room temperature for 10 min. The reaction was stopped by adding 0.5 M H₂SO₄. The optical density (OD) was read at 450 nm by a plate reader (EnSpire, Perkin Elmer). All samples were examined in triplicate and the median value was used for analysis. Results were expressed as OD after subtraction of background value.

Detecting the Anti-TUBB3 Antibodies Using the Biotin-Labeled TUBB3 C-Terminal Peptide₄₂₄₋₄₅₀ as the Antigen

The ELISA was performed using the biotin-labeled TUBB3 C-terminal peptide₄₂₄₋₄₅₀ (QYQDATAEEEGEMYEDDEEE SEAQGPK, Primm Biotech, Milano, Italy) and streptavidin-coated microtiter plates (Pierce Thermo Scientific Waltham, MA). Wells were coated with 100 μ l PBS containing 1 μ M biotin-labeled TUBB3 C-terminal peptide₄₂₄₋₄₅₀ at 4°C overnight. All the remaining washing and staining steps were as described in the 2.5 section. All samples were examined in triplicate and the median value was used for analysis. Results were expressed as OD after subtraction of background value.

TUBB3 Immunohistochemical Analysis

For each specimen, five to seven paraffin embedded sections were randomly selected. Immunostaining was performed on 3 μ m paraffin tissue section mounted poly-l-lysine-coated slides as detailed earlier [4]. Briefly, TUBB3 expressing OC cells were identified using the monoclonal anti-human TUBB3 antibody (clone TUJ1; 1:300; Covance). Binding of the monoclonal anti-human TUBB3 antibody was revealed by the EnVision-rabbit+ System-HRP System (Dako, Carpinteria, CA). Diaminobenzidine was the chromogen (DAB Substrate System, DAKO). Negative controls were done by omitting the primary antibody. Microscopic enumeration of TUBB3 expressing tumor cells was done blinded without any prior knowledge of clinical parameters by two authors (GFZ and EM). The proportion of immunostained OC cells was scored at high magnification (40x objective lens) in ten randomly selected tumor areas.

Statistical Analysis

Receiver operating characteristic (ROC) curves were generated to assess the diagnostic accuracy of the assays, and the Youden's Index was used to determine the best OD cutoff point to define OC patients [15]. The χ^2 test or Mann-Whitney test was employed to assess statistical significance of differences in parameter distributions, as indicated (STATA statistical software). Survival probabilities were estimated according to the method of Kaplan and Meier and compared by the log rank test. Cox's regression model with stepwise variable selection was used to analyze the role of clinicopathological parameters as prognostic factors for progression free survival (PFS) and overall survival (OS). Only variables with $P < .10$ in the univariate analysis were included in the multivariate analysis.

Pearson's correlation was used to verify linear relationship between two variables. Statistical analysis was carried out using SOLO (BMDP Statistical Software, Los Angeles, CA), Statview survival (Abacus Concepts Inc, Berkeley, CA) and Graphpad Prism 5.0 software packages (Graphpad Software Inc., CA).

Results

The Anti-TUBB3 Antibody Level is a Prognostic Factor in OC Patients

By the ELISA system that used the recombinant TUBB3 as the antigen, the median level of anti-TUBB3 antibodies (expressed in OD) in 49 OC patients and 44 healthy individuals was 0.31 (range 0.04-0.92) and 0.07 (range 0.00-0.32), respectively. The ROC curve analysis and the Youden's index identified 0.15 OD as optimal cutoff point to discriminate OC patients from healthy subjects (Figure 1). Using this cutoff, sensitivity was 91.8% and specificity 90.9% (Figure 1). Only 4 OC patients out of 49 had OD values below the cutoff and only 4 healthy individuals out of 44 had OD values above the cutoff.

Tumor tissue samples from 34 OC patients were available for the immunohistochemical determination of TUBB3 overexpression. Representative examples of TUBB3 immunoreaction in primary OC samples are shown in Figure 2A. TUBB3 was detectable in most cases, the percentage of positively stained cells ranging from 0 to 90% (median = 20%). Subgrouping OC patients according to the median value of the anti-TUBB3 antibody level showed that OC patients with higher anti-TUBB3 antibody level had significantly higher ($P < .001$) percentage of positively stained cells (Figure 2B). However, two OC patients that scored positive for anti-TUBB3 antibodies (OD above the cutoff) did not show positively stained cells in tumor tissue by IHC and two of the 4 OC patients with OD below the cutoff showed the presence of TUBB3 expressing cells (percentage of positively stained cells 5% and 7%).

We then compared the clinicopathological characteristics of the OC patients according to the anti-TUBB3 antibody levels, using a

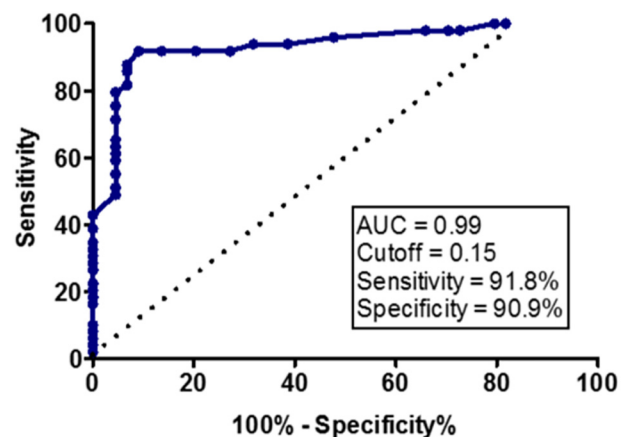


Figure 1. Anti-TUBB3 antibody level discriminates ovarian cancer (OC) patients from healthy individuals. Receiver operating characteristic (ROC) curve analysis of anti-TUBB3 antibody level to discriminate OC patients from healthy individuals is shown. The area under the ROC curve (AUC) corresponding to the comparisons between the two groups, cutoff value, sensitivity and specificity are indicated.

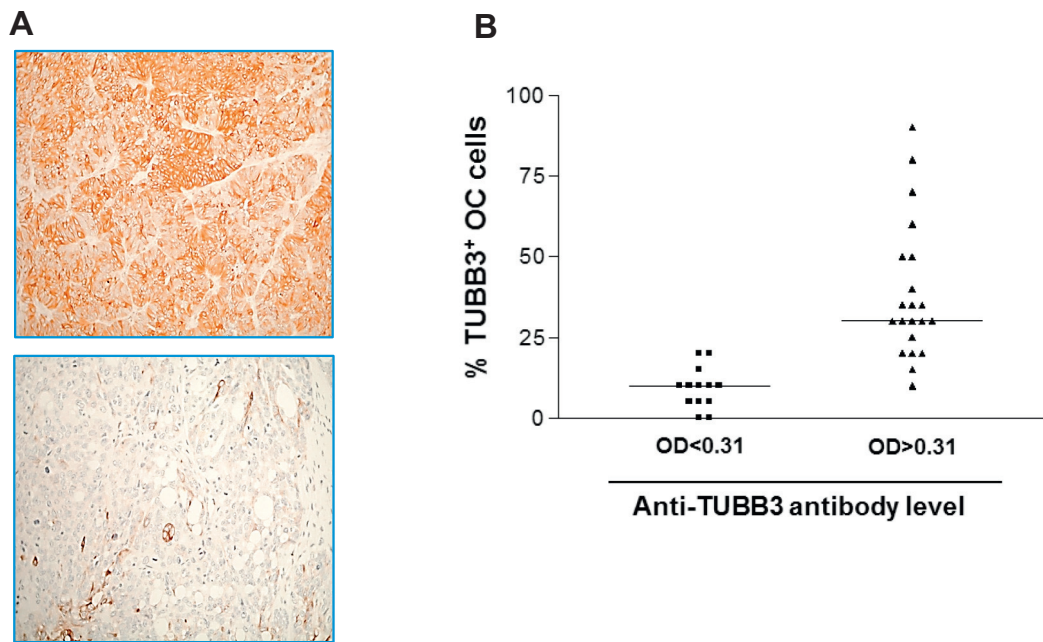


Figure 2. TUBB3 positive ovarian cancer (OC) cells in tumor samples according to anti-TUBB3 antibody level. (A) Representative immunohistochemistry for TUBB3 in OC samples. Examples of high (upper panel) versus low (lower panel) TUBB3 expression in tumor sample are shown. Specific immunostaining for TUBB3 was confined into the cytoplasm. Original magnification 10x. (B) OC patients with anti-TUBB3 antibody level below median (OD 0.31) show a lower percentage of TUBB3 expressing cells in tumor samples by IHC analysis ($P < .0001$, Mann Whitney two-tailed test). Horizontal bars denote median values.

cutoff based on the median value of the OC patient population (OD 0.31). No clinicopathological characteristics associated with anti-TUBB3 antibody levels (Table 2). Conversely, a significant association between high anti-TUBB3 antibody level and lower PFS and OS was found (Figure 3, A and B, respectively). The 5-year cumulative PFS was 55% and 30% for OC patients with low and high anti-TUBB3 antibody level ($P = .023$), respectively (Figure 3A). The 5-year cumulative OS was 79% and 46% for OC patients with low and high anti-TUBB3 antibody level ($P = .006$), respectively (Figure 3B). In multivariate analysis anti-TUBB3 antibody level emerged as independent predictor of PFS ($P = .039$), together with FIGO stage ($P = .047$) and residual tumor at first surgery ($P = .042$)

(Table 3). Likewise, anti-TUBB3 antibody levels emerged as independent predictor of OS ($P = .045$), together with FIGO stage ($P = .042$) and PFS ($P = .001$) (Table 4). Finally, in the univariate analysis, the percentage of TUBB3 expressing cells in tumor tissue did not predict either PFS (Table 3) or OS (Table 4).

Measuring the Anti-TUBB3 Antibody Level to Diagnose OC

Having shown that the anti-TUBB3 antibody level is a powerful diagnostic and prognostic factor in OC patients we decided to improve the sensitivity and specificity of the ELISA test. To this end we modified the assay by substituting recombinant TUBB3 with a biotin-labeled TUBB3 C-terminal peptide₄₂₄₋₄₅₀. By this ELISA

Table 2. Distribution of OC Patients' Clinicopathological Characteristics According to the Low (OD <0.31) and High (OD >0.31) Levels of Anti-TUBB3 Antibodies (Evaluated by the ELISA System that Uses the Recombinant TUBB3 as the Antigen)

Characteristics	Low Anti-TUBB3 Antibody Level No. (%)	High Anti-TUBB3 Antibody Level No. (%)	P^a
FIGO stage			
I-II	4 (16.0)	6 (25.0)	.335
III-IV	21 (84.0)	18 (75.0)	
Carcinomatosis			
Yes	12 (48.0)	14 (58.3)	.469
No	13 (52.0)	10 (41.7)	
Tumor histotype			
Serous	21 (84.0)	18 (75.0)	.335
Endometrioid/clear cell	4 (16.0)	6 (25.0)	
Tumor grade			
G1	3 (12.0)	0 (0.0)	.080
G2-G3	22 (88.0)	24 (100.0)	
Residual tumor at first surgery			
≤1cm	14 (56.0)	12 (50.0)	.447
>1cm	11 (44.0)	12 (50.0)	

^a Calculated by χ^2 test.

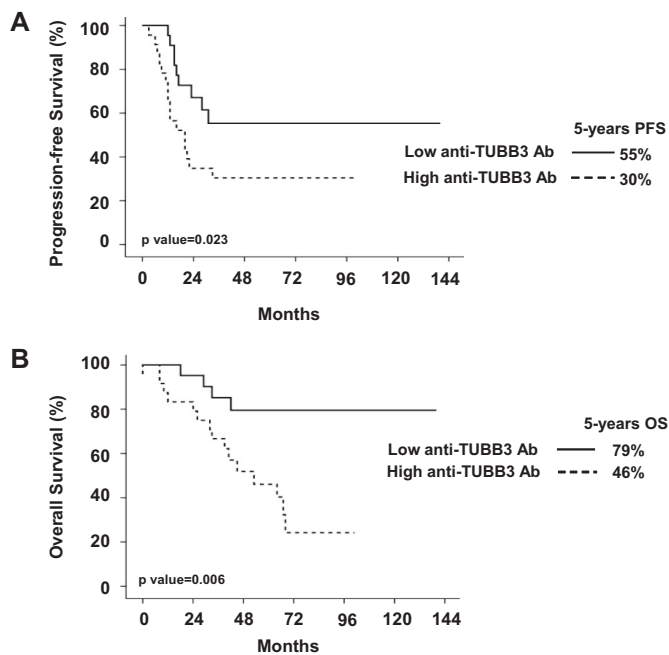


Figure 3. Kaplan-Meier survival analysis according to anti-TUBB3 antibody level. Kaplan-Meier estimates showing significant association between high anti-TUBB3 antibody level and shorter A) progression-free survival (PFS) and B) overall survival (OS). Solid line: low anti-TUBB3 antibody level; dotted line: high anti-TUBB3 antibody level.

system, we examined the presence of serum anti-TUBB3 antibodies in an independent set of samples of 99 OC patients and 80 benign gynecological diseases patients. The median level of anti-TUBB3 antibodies (expressed in OD) was 0.15 (range 0.05-0.44) and 0.00 (range 0.00-0.11) in OC patients and benign gynecological disease patients, respectively. The area under the ROC curve comparing OC cases against benign disease was 0.99 (95% CI: 0.9865-1.001) (Figure 4A). ROC curve analysis and the Youden's index identified 0.075 OD as the optimal cutoff point to discriminate OC patients from benign gynecologic disease patients. Using this cutoff, sensitivity was 95.3% and specificity 97.6% (Figure 4A). Thus, the ELISA system that uses the biotin-labeled TUBB3 C-terminal peptide₄₂₄₋₄₅₀ resulted much more specific and sensitive than the ELISA system that uses the recombinant TUBB3 as the antigen. The analytical performance of the assay included evaluation of intra- and inter-assay reproducibility (Figure 4B). Intra-assay coefficient of variability percentage (CV%) was calculated by testing at least five technical replicates of four serum samples containing decreasing concentrations of the marker. The range of CV% was 6.4% to 19% and was deemed acceptable. Inter-assay CV% was calculated after testing a high and a low serum sample in triplicate on 3 different plates. The inter-assay CV% was 15% and 14% for samples of high and low anti-TUBB3 antibodies, respectively and was deemed acceptable.

Notably, the level of anti-TUBB3 antibodies in OC patients was not affected by disease stage, median values being 0.156 (range 0.075-0.442, n = 25) and 0.147 (range 0.045-0.420, n = 74) in stage I/II and stage III/IV, respectively. Likewise, there were no relevant differences in the anti-TUBB3 antibody levels among different histotypes, median values being 0.161 (range 0.075-0.31), 0.252 (range 0.132-0.419), 0.210 (range 0.106-0.35) and 0.143 (range

Table 3. Univariate and Multivariate Analysis of Clinicopathological Parameters of OC Patients (Evaluated for the Anti-TUBB3 Antibodies by the ELISA System that Uses the Recombinant TUBB3 as the Antigen), and Treatment Details as Predictor of Progression-Free Survival

Variable	Univariate Analysis		Multivariate Analysis	
	χ^2	P	χ^2	P
Age ^a	3.605	.058	-	-
FIGO stage				
I-II	4.391	.036	2.233	.047
III-IV				
Carcinomatosis				
Yes	8.363	.004	4.530	.145
No				
Tumor histotype				
Serous	1.107	.293	-	-
Endometrioid/clear cell				
Residual tumor at first surgery				
≤1 cm	11.441	.001	4.674	.042
>1 cm				
Anti-TUBB3 antibody level				
Low	4.114	.023	3.893	.039
High				
TUBB3 ^b in tumor sample				
Low	0.239	.625	-	-
High				

^a Considered as continuous variable.

^b By immunohistochemistry.

0.045-0.442) in clear cells (n = 8), endometrioid (n = 4), mucinous (n = 7) and serous OC (n = 80), respectively. The anti-TUBB3 antibody level was not related to grade of tumor differentiation, median values being 0.145 (range 0.079-0.21) and 0.154 (range 0.045-0.442) in grade 1 (n = 7) and 2/3 (n = 92), respectively.

A comparison of anti-TUBB3 antibody levels assessed by the two ELISA systems is reported in Supplementary Results.

Combined Detection of Serum Anti-TUBB3 Antibodies and CA125 Improves OC Diagnosis

CA125 is routinely used in the assessment of patients presenting with a pelvic mass and in OC patients monitoring after treatment. Elevated CA125 serum levels are present in up to 80% of patients presenting with a pelvic mass [16]. However, CA125 is not sufficiently sensitive and specific to make diagnosis [16]. CA125 levels at admission were available for 90 out of the 99 OC patients and 33 out of the 80 benign gynecological diseases patients that were tested for serum anti-TUBB3 antibodies by the ELISA system that uses the biotin-labeled TUBB3 C-terminal peptide₄₂₄₋₄₅₀. At the used cutoff of CA125 (35 U/ml), we found a sensitivity of 88% and a specificity of 64% in our cohort of patients, in keeping with literature data [17]. The anti-TUBB3 antibody ELISA test showed that 11 out of the 12 OC patients with CA125 <35U/ml tested positive for anti-TUBB3 antibodies (Figure 4C) and only 4 out of the 78 OC patients with CA125 >35U/ml tested negative for anti-TUBB3 antibodies (Figure 4C). Thus, combining anti-TUBB3 antibody with CA125 assessment may contribute to OC diagnosis. In addition, 5 out of the 6 CA125 false positive (CA125 >35U/mL) benign gynecological disease patients could be correctly diagnosed by the anti-TUBB3 antibody ELISA test (not shown).

Discussion

Cancer progression is often associated with high levels of circulating tumor-specific antibodies, and their detection may provide a reliable serum biomarker for cancer diagnosis and/or prognosis in a variety of

Table 4. Univariate and Multivariate Analysis of Clinicopathological Parameters of OC Patients (Evaluated for the Anti-TUBB3 Antibodies by the ELISA System that Uses the Recombinant TUBB3 as the Antigen), and Treatment Details as Predictor of Overall Survival

Variable	Univariate Analysis		Multivariate Analysis	
	χ^2	P	χ^2	P
Age ^a	2.606	.102	-	-
FIGO stage				
I-II	3.741	.035	2.865	.042
III-IV				
Carcinomatosis				
Yes	5.051	.025	1.230	.883
No				
Tumor histotype				
Serous	0.601	.614	-	-
Endometrioid/clear cell				
Residual tumor at first surgery				
≤1 cm	8.958	.001	2.462	.177
>1 cm				
Progression-free survival ^a	13.210	.001	9.249	.001
Anti-TUBB3 antibody level				
Low	6.170	.006	4.213	.045
High				
TUBB3 ^b in tumor sample				
Low	0.662	.416	-	-
High				

^a Considered as continuous variable.

^b By immunohistochemistry.

malignancies [14]. In OC, a number of studies [Reviewed in 18–20] have shown that various anti-tumor antibodies can be found in the sera of patients and have suggested their possible relevance for diagnostic and prognostic purposes. Prompted by the assumption that TUBB3 released from OC cells should elicit anti-TUBB3 antibodies, in analogy with the TUBB3 released from damaged neurons in cerebral malaria [13], we developed an ELISA system that used recombinant TUBB3 as the antigen. By this assay, we show here the presence of previously unreported antibodies against TUBB3 in the serum of OC patients. The assay identified the presence of anti-TUBB3 antibodies in OC patients with a sensitivity of 91.8% and a specificity of 90.9%. The multivariate analysis showed that the anti-TUBB3 antibody level was an independent marker of outcome, a high level predicting poor survival.

How high levels of anti-TUBB3 antibodies predict dismal prognosis is still speculative. It is likely that high anti-TUBB3 antibody levels correspond to high amounts of tumor cells. This would make anti-TUBB3 antibody level a reliable indicator of tumor burden, an obvious negative prognostic factor. In addition, high anti-TUBB3 antibody levels may correspond to an abundance of TUBB3 overexpressing OC cells, a negative predictive factor for response rate to taxane-based chemotherapy [4–9]. However, despite we found an overall good association between the anti-TUBB3 antibody level and the TUBB3 expressing tumor cell amounts in tissue sections, the IHC analysis of TUBB3 expression was non-informative of prognosis in our series. The lack of predictive power of IHC analysis is likely related to the inherent subjectivity and limited reproducibility of the IHC-based assay in accurately quantifying the TUBB3 expressing OC cells in a given patient. Adding to the uncertainty, the intrinsic tumor heterogeneity may induce to score an OC patient as low positive/negative merely because of sampling bias. Consistent with this view, the occurrence of TUBB3 expressing tumor cells in sites other than primary tumor may explain

why the few patients of the present series scored negative for TUBB3 expression by IHC yet showed anti-TUBB3 antibodies.

Although the sensitivity (91.8%) and specificity (90.9%) of our ELISA system largely exceeded the highest sensitivity and specificity so far described using an ELISA system for the detection of antibodies to various OC antigens (62.5% and 90.2%, respectively) [21], we decided to improve the overall performance of the assay and substituted the recombinant TUBB3 with the TUBB3 C-terminal peptide₄₂₄₋₄₅₀ as the antigen. In an independent set of serum samples that included 99 OC patients and 80 gynecological benign disease patients, this optimized assay proved to be much more specific (97.6%) and sensitive (95.3%). To date, no single biomarker displays high sensitivity and specificity to detect early OC. CA125, although routinely used for monitoring OC treatment and recurrence [22], is of scarce utility in OC diagnosis as it displays a low specificity, by rising in a variety of non-ovarian malignancies, benign diseases affecting pleura, pericardium and peritoneum and in several pelvic diseases including endometriosis, ovarian cysts, pelvic inflammatory diseases, myomas of the uterus and salpingitis [23]. Here we show that the vast majority of the OC patients that tested negative for CA125 scored positive for anti-TUBB3 antibodies, thereby indicating that the combined detection of serum anti-TUBB3 antibodies and CA125 would help to improve the diagnosis of OC.

Our series of OC patients was comprised of subjects at the first diagnosis and prior to patients being treated with surgery and/or chemotherapy. This implies that the anti-TUBB3 antibodies are induced by the antigenic stimulation due to spontaneous tumor cell death, a phenomenon that occurs since the early phases of tumorigenesis. Thus, we anticipate that our highly sensitive ELISA system that employs TUBB3 C-terminal peptide₄₂₄₋₄₅₀ as the antigen may detect anti-TUBB3 antibodies in asymptomatic or moderately symptomatic OC patients and contribute to early diagnosis. Significantly, if confirmed by *ad hoc* prospective studies, the present ELISA for anti-TUBB3 antibodies would allow predicting the chance of response to standard treatment for OC, thus enabling patient allocation to personalized treatment procedures with significant benefits to both patients and healthcare system.

It is widely recognized that the best prospects for further improvement in OC survival reside in the discovery of robust clinical biomarkers enabling early tumor detection as well as patient allocation to personalized and targeted treatment strategies. We show here for the first time the feasibility of using an ELISA system to detect the occurrence of previously unreported anti-TUBB3 antibodies in OC patients. We also demonstrate that preoperative anti-TUBB3 antibody levels are relevant clinical diagnostic and prognostic biomarkers in OC, independently of histotype and stage of disease.

Conflict of Interest

Enrica Martinelli, Andrea Fattorossi, Alessandra Battaglia, Marco Petrillo, Giuseppina Raspaglio, Mara Fanelli, Daniela Gallo and Giovanni Scambia filed a patent pending application on “New diagnostic and prognostic biomarkers in ovarian cancer” (International application number n. PCT/IB2017/052048).

Enrica Martinelli, Alessandra Battaglia, Marco Petrillo, Daniela Gallo and Giovanni Scambia filed a patent pending application on “Saggio e kit per la diagnosi del carcinoma ovarico” (Italian application number n. 102017000117860).

Gian Franco Zannoni has no conflict of interest.

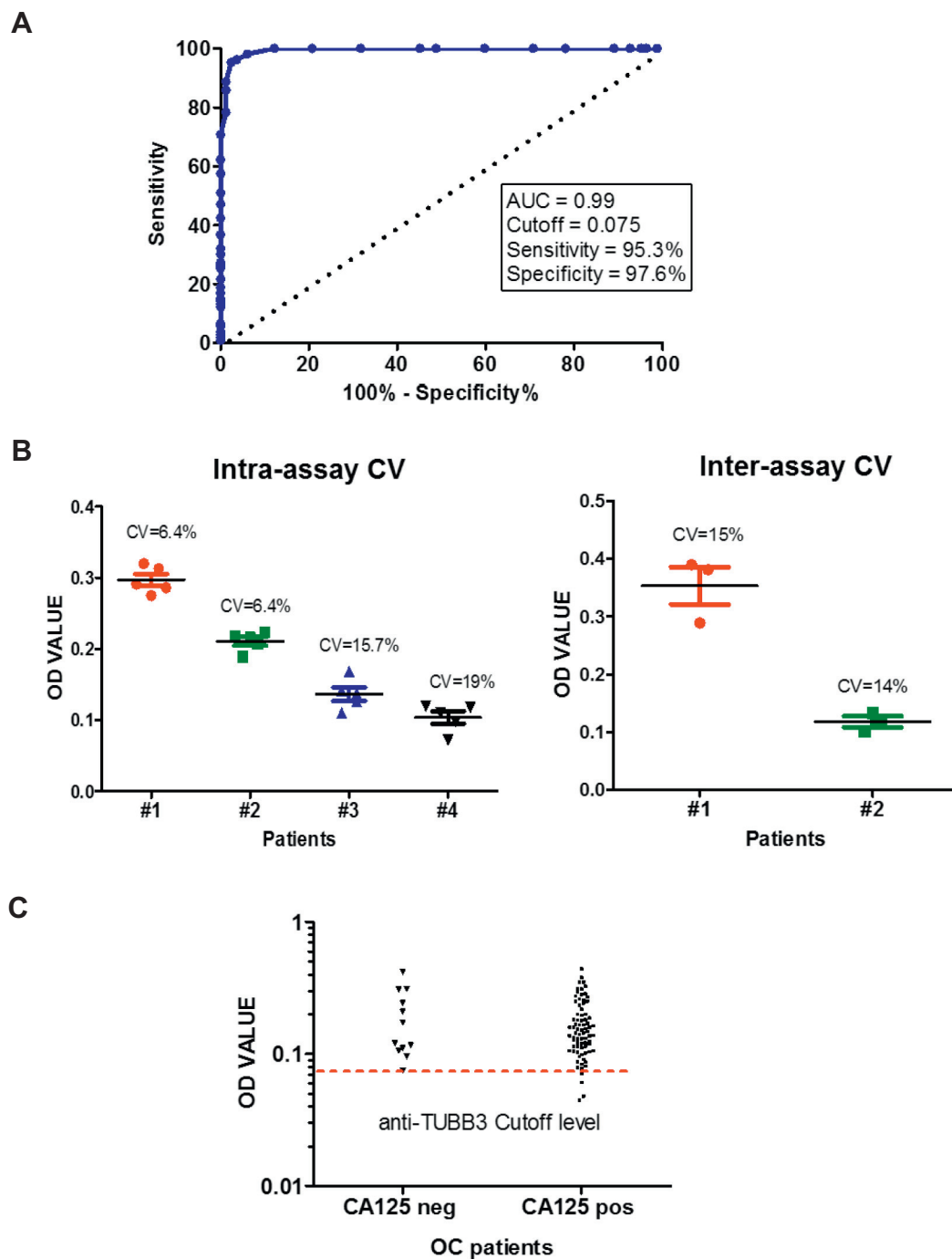


Figure 4. (A) Anti-TUBB3 antibody level discriminates ovarian cancer (OC) patients from benign gynecological disease patients. Receiver Operating Characteristic (ROC) curve analysis of anti-TUBB3 antibody levels to discriminate OC patients from benign disease controls is shown. The area under the ROC curve (AUC) corresponding to the comparisons between the two groups, cutoff value, sensitivity and specificity are indicated. (B) Intra- and Inter-assay reproducibility. Scatter plot with replicate values, mean and SEM, showing intra-assay (5 replicates per point) and inter-assay (3 experiments) coefficients of variation of samples with different anti-TUBB3 levels. OD: Optical Density. (C) Anti-TUBB3 antibody levels in OC patients stratified according to CA125 positivity. Scatter plot showing individual anti-TUBB3 levels in OC patients with negative and positive CA125 levels (<35 U/ml and >35 U/ml, respectively); dotted red line identifies the selected anti-TUBB3 cutoff level. OD: Optical Density.

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Appendix A. Supplementary data

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