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ORIGINAL ARTICLE

Expression of Nestin and CD133 in serous ovarian carcinoma

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Summary

Pupose: *Nestin and CD133 are regarded as putative markers of cancer stem cells (CSCs) and related to poor prognosis in various cancer sites. Since few studies have focused on their role in ovarian cancer, we aimed to investigate their predictive value and association with neoangiogenesis.*

Methods: *Immunohistochemical analysis for nestin and CD133 was performed on 85 serous ovarian carcinoma tumor samples using tissue microarray technique. Nestin immunoreactivity was detected in both tumor and endothelial cells, whilst CD133 was only identified in tumor cells. CD34 endothelial expression was used to assess intratumor microvessel density (MVD).*

Results: *Of the tissue samples 49.4% were nestin-positive and 24.7% were positive for CD133. In both univariate and multivariate analysis nestin or CD133 expressions in tumor cells were not significantly associated with clinicopathological parameters (age, serum CA125, peritoneal carcinomatosis, malignant ascites, tumor grade). However, in multivariate analysis nestin expression in tumor cells proved to be an independent prognostic factor, associated with poorer survival and time to progression (p=0.025 and p=0.05, respectively). This has not been achieved for CD133. Furthermore, a significant concordance between nestin endothelial expression (nestin-determined MVD) and CD34-determined MVD was achieved.*

Conclusion: *In addition to the well-known clinicopathological characteristics, tumor expression of nestin might be a valuable prognostic factor for survival in patients with advanced ovarian cancer. With regard to its endothelial expression, nestin might be as reliable as CD34 for quantifying tumor angiogenesis. Further investigation is justified in order to better clarify the role of these biomarkers.*

Key words: *cancer stem cells, CD133, nestin, ovarian cancer, prognosis*

Introduction

Ovarian cancer represents a major women's health problem, being reported as the deadliest gynecologic malignancy (3.8/100,000 mortality rate, worldwide in 2012) [1,2]. Despite advances in surgical procedures and systemic treatment, it is still characterized by delayed diagnosis until advanced-stages (75% of the cases) [2] due to non-specific symptoms and the lack of more reliable screening procedures and high recurrence rates, largely due to its heterogeneity regarding development, progression and response to treatment [3,4]. Hence, novel prognostic biomarkers and therapeutic targets are being required.

A more recent hypothesis that aims to explain the conspicuous heterogeneous phenotype of ovarian cancer is CSC theory [3,5-7]. It reflects

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a small subpopulation of undifferentiated tumor cells with "stem-like" capacities such as self-renewal, proliferation and migration, being able to nourish processes like tumorigenesis and tumor progression [8-10]. Extended from hematological malignancies, this hypothesis has been further tested in various solid tumors, including ovarian carcinoma [9-11].

Nestin is a class VI intermediate filament protein (IF) taking part in cellular processes such as adhesion and migration through modulation of the cytoskeleton [12,13]; CD133 is a 5-membrane cell-surface glycoprotein, also known as prominin-1, showing a polarized apical membrane distribution in epithelial and other cells [14]. Both nestin and CD133 have been originally used to depict neuroepithelial or CD34+ hematopoietic stem cells [13-15]. Progressively, they attracted attention as putative markers of CSCs, being related to poor prognosis and therapeutic resistance in various cancer sites [15-22]. Furthermore, nestin has been also focused on as a putative marker for tumor neoangiogenesis due to its patterns of endothelial expression [13]. There are several studies that have investigated the predictive value of nestin and CD133 in serous ovarian carcinoma [23-26], and yet their clinical impact is marked by uncertainty.

Methods

Patients

The study included 85 patients diagnosed with advanced (FIGO stage III and IV) serous ovarian carcinoma between January 2006 and November 2011, who underwent primary debulking surgery followed by platinum-based adjuvant chemotherapy at the "Prof. Dr. Ion Chiricuta" Institute of Oncology from Cluj-Napoca. This study was approved by the Ethics Committee of the Institute.

Exclusion criteria were as follows: different histology (other than serous histotype), suboptimal surgery with >1cm residual tumor, neoadjuvant chemotherapy as primary treatment, absence of follow-up within the Institute, and other synchronous or metachronous malignancies. Clinicopathological features of patients, including demographic and clinical characteristics such as diagnosis, FIGO stage, tumor differentiation grade, the presence of ascites and peritoneal carcinomatosis, cancer antigen 125 (CA125) serum level, chemotherapy protocols, time of relapse, were retrospectively collected by reviewing the patient files. Preoperative CA125 levels were considered high when they exceeded 10 times the upper limit of normal (>35 U/ml). Response to chemotherapy was assessed and patients were classified as follows: responders/platinum sensitive when had a progression-free interval (PFI) of more than 12 months; partially platinum sensitive when progression occurred within 6 to 12 months after treatment; and non-responders/platinum resistant when disease progression was reported during/within 6 months after chemotherapy.

All cases were histopathologically revised; disease was staged according to FIGO staging system and tumor grade was defined according to WHO classification 2014 (high grade vs low-grade serous ovarian carcinoma).

Immunohistochemistry

Immunostaining for nestin, CD133 and CD34 was performed on formalin-fixed paraffin-embedded tissue samples achieved through tissue microarray (TMA) technique [27]. Cores were obtained from the most representative/non-necrotic areas of the primary tumors. Sections of 4 μm were mounted on silane-coated slides and incubated overnight at 37˚C, were de-paraffinized in xylene, then were rehydrated with graded ethanol and washed. Antigen retrieval was performed by heating the slides in 10 mM citrate buffer (pH 6.0) for 20 min. After cooling at room temperature and rinsing in tris-buffered saline (TBS), endogenous peroxidase activity was blocked with 3% H_2O_2 for 5 min. The slides were next incubated for 30 min with the primary mouse monoclonal antibody to human CD133 (clone AC133, MACS Miltenyi Biotec, Germany) and monoclonal mouse antibody to human nestin (10c2, SC:23927, Santa Cruz Biotechnology, Dallas, USA) at 1:150 and 1:50 dilutions, respectively. After washing in TBS, the slides were incubated with Post Primary Block Leica Biosystems, Nusslook, Germany) for 30 min and with the secondary antibody to mouse/rabbit IgG-Poly-HRP (Novolink Polymer Novocastra, Leica Biosystems, Nusslock, Germany. Peroxidase activity was developed with diaminobenzidine (DAB) working solution (Leica Biosystems, Nusslock, Germany). Sections were counterstained with Mayer's hematoxylin, rinsed in saturated solution of lithium carbonate, dehydrated, cleared in xylene and mounted in Faramount Mounting Medium (S3025;Dako, Glostrup, Denmark). Omitting primary antibodies served as negative controls. Positive control for CD133 expression was achieved through immunostaining of normal human fetal brain tissue [28] which showed a strong apical cytoplasmic signal for the ependymal cells of the choroid plexus. Positive control for nestin showed cytoplasmic staining of glomerular endothelial and fibroblastic cells in human kidney [29].

Immunohistochemical analysis for nestin and CD133 was performed focusing on the percentage of positive tumor cells, staining intensity as well as endothelial expression. Nestin immunoreactivity was detected in both tumor cells and endothelial cells. In tumor cells, a diffuse cytoplasmic pattern of expression has been observed in most of the cases. CD133 positivity was identified only in tumor cells, showing a pre-

dominant distribution at the apical membrane level. Based on staining intensity and the percentage of positive tumor cells, nestin expression was considered positive when more than 30% of tumor cells showed moderate to strong intensity of staining, whilst for CD133 when more than 10% of tumor cells were moderately/strongly stained. Otherwise, cases fitted into the negative expression category.

Given the endothelial expression of nestin, MVD was quantified on TMA sections stained with monoclonal antibodies for nestin and CD34, through identification of the most vascular areas and counting of the positive microvessels at high magnification (400x, field size 3.14 mm^2). The presence of a vascular lumen was not mandatory; hence, immature vessels identified through stained endothelial cells or clusters of cells were also considered [27].

Objectives

In the present study we aimed to investigate the existing associations between nestin and CD133 expressions in tumor cells and the above mentioned clincopathological parameters, as well as their influence on overall (OS) and progression-free survival (PFS) of patients with advanced serous ovarian cancer. We also intended to propose nestin as a new endothelial marker for tumor angiogenesis estimation by assessing the concordance with CD34-positive vessels.

Statistics

In the present research, the quantitative variables were summarized as mean±standard deviation, while qualitative variables were expressed as absolute and relative frequencies (%).

In order to test the bivariate association between various qualitative nominal variables Fisher's Exact test or Chi-square test were used. The effect size of the analyzed relationships was described by the odds ratio (OR) and 95% CI for OR. A p value <0.05 was considered as statistically significant.

The concordance between quantitative MVD determinations was highlighted by Bland-Altman graph or difference plot which was used to show the absence of any systematic bias between MVD measurements. The average of the MVD measurements was represented against the difference between these and 95% of the estimated differences expected to lie within the limits of agreement (mean ± 1.96 SD).

Univariate analysis of possible predictors of OS and PFS was performed by Cox regression method, while the survival curves were created by the Kaplan-Maier method and compared with the log rank test. OS was defined as the time period between diagnosis confirmation and the time of death or the last follow-up within the Institute. PFS was considered as the time period between diagnosis confirmation and the date of the objective relapse.

For multivariate analysis, the Cox regression was performed in order to identify the independent predictors of survival, the proportionality assumption being verified using the Schoenfeld residuals or test of significance. The multivariate model of regression was composed using all independent variables whose estimated significance level in univariate analysis was p< 0.25. The final model was obtained by comparing multiple nested models using Deviance (Likelihood Ratio) test. All covariates whose exclusion would produce a change in the coefficients of the remaining variables above 20% were included in the final model. Adjusted OR, with associated 95% CI, were also calculated to quantify the importance of significant predictors in OS and PFS respectively.

The significance level (α) for all two-sided tests was equal to 0.05 and the statistical significance was accomplished when the estimated significance level p was inferior to 0.05.

The statistical analysis was performed with the IBM SPSS v.19 (Armonk, NY: IBM Corp), Statistica, v.6.0. (StatSoft) and R version 3.2.2 (R Foundation for Statistical Computing, Vienna, Austria).

Results

The age of the 85 patients at diagnosis ranged from 35 to 79 years, with a mean age of 56±9 years. Fifty four (63.5%) patients were less than 60 years old. Advanced stage disease was classified as follows: 75 (88.2%) cases were stage III and 10 (11.8%) cases were stage IV disease. Preoperative CA125 levels were higher than 350 U/ml in 51 (60.0%) cases.

Primary optimal surgical debulking was achieved in 55 (64.7%) cases. Suboptimal cytoreduction with less than 1cm residual tumor had been performed in the rest of the cases (35.3%). Peritoneal carcinomatosis was detected intraoperatively in 74 (87.1%) patients, as well as malignant ascites in 66 (77.6%) patients. High-grade ovarian serous carcinoma was determined in 79 (92%) patients.

Taking into consideration both staining intensity and the percentage of positive tumor cells, nestin and CD133 positivity was observed in 49.4% (42/85) and 24.7% (21/85) of the ovarian cancer TMA, respectively (Figure 1A, 1B). CD34 expression in endothelial cells was used for the assessment of MVD (Figure 1C). The mean CD34-determined MVD was 47.72±22.58. The endothelium of tumor microvessels also showed reactivity for nestin (Figure 1D). The mean value of nestin positive tumor vessels was 42.59±19.36.

Expression of nestin or CD133 in tumor cells was not significantly associated with clinicopath-

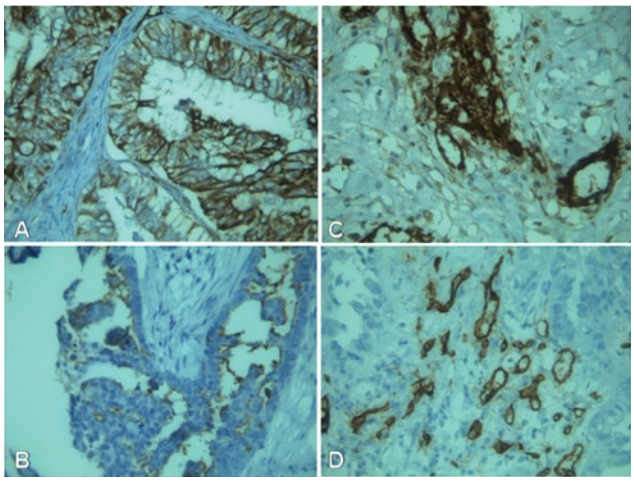

Figure 1. Immunostained ovarian cancer tissue samples: **A)** strong expression for nestin, **B)** strong apical CD133 expression, **C)** high nestin-positive microvessel density (MVD), D) high CD34-positive MVD.

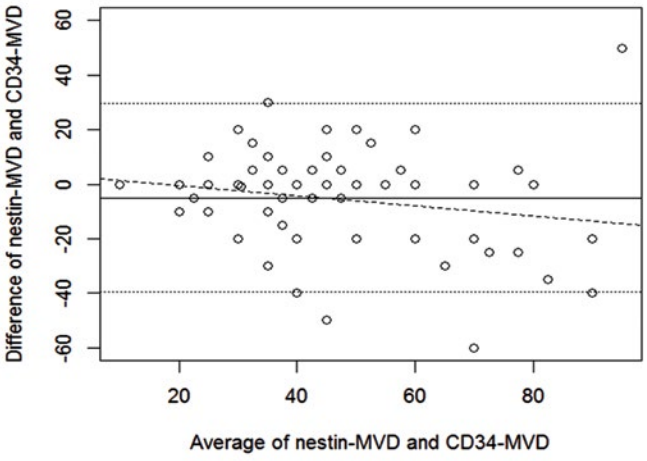

Figure 2. The agreement between nestin-determined microvessel density (MVD) and CD34-determined MVD. The continuous line= the reference line (zero bias line); the outer parallel lines =95% CI for difference; the oblique line = regression line.

Table 1. Univariate analysis results

Dependent variables	*Progression free survival (months)*		*Overall survivall (months)*	
Characteristics	x^2	*p*	x^2	*p*
Age (≥ vs < 60 years)	1.48	0.225	2.38	0.123
Preoperative CA125 (≥ vs < 35 U/L)	5.15	0.023	5.15	0.023
Surgery – residual tumor (0 mm vs 0-10 mm)	0.62	0.430	5.14	0.023
High vs low grade tumors	0.03	0.857	0.720	0.396
Peritoneal carcinomatosis (present vs absent)	0.23	0.630	0.116	0.734
Ascites (present vs absent)	0.66	0.417	3.65	0.056
Response to chemotherapy (PFS <6 vs 6-12 vs >12 months)	155.55	<0.001	29.10	<0.001
Nestin expression (positive vs negative)	3.96	0.05	5.04	0.025
CD133 expression (positive vs negative)	0.20	0.653	0.13	0.719

ological parameters such as age, serum CA125, peritoneal carcinomatosis, malignant ascites, and tumor grade (p>0.05).

Moreover, neither nestin-positivity in tumor vessels nor CD34-determined MVD were significantly associated with nestin or CD133 expression in tumor cells (data not shown).

The agreement between nestin-determined MVD and CD34-determined MVD is illustrated in Figure 2. Each data point of Bland-Altman graph represents the differences between the nestin-determined MVD and CD34-determined MVD measurements for each patient. The differences were distributed symmetrically around the zero bias line and according to the regression line that is situated within the limits of agreement, there was no systematic bias.

The median follow-up period was 54 months (range 24-97). During the study period, 70 (82.35%) patients relapsed after first-line treatment (53 pelvic and/or abdominal recurrences, 6 distant metastases, 11 loco-regional and distant relapse). Nineteen (23.17%) patients were considered non-responders/platinum-resistant, 16 (19.51%) patients were partially sensitive, while the majority (47 cases-57.31%) of patients was considered platinum-sensitive.

The median (25th,75th percentile) PFS time in the study group was 18.84 months (range 11.77-28.67). Fifty two (61.17%) patients were deceased at the end of the study. Hence, the median (25th,75th percentile) OS time was 43.8 months (range 31.16-67.72).

Kaplan-Meier analysis demonstrated that high preoperative CA125 levels (>35 U/ml), suboptimal surgery (macroscopic residual tumor less

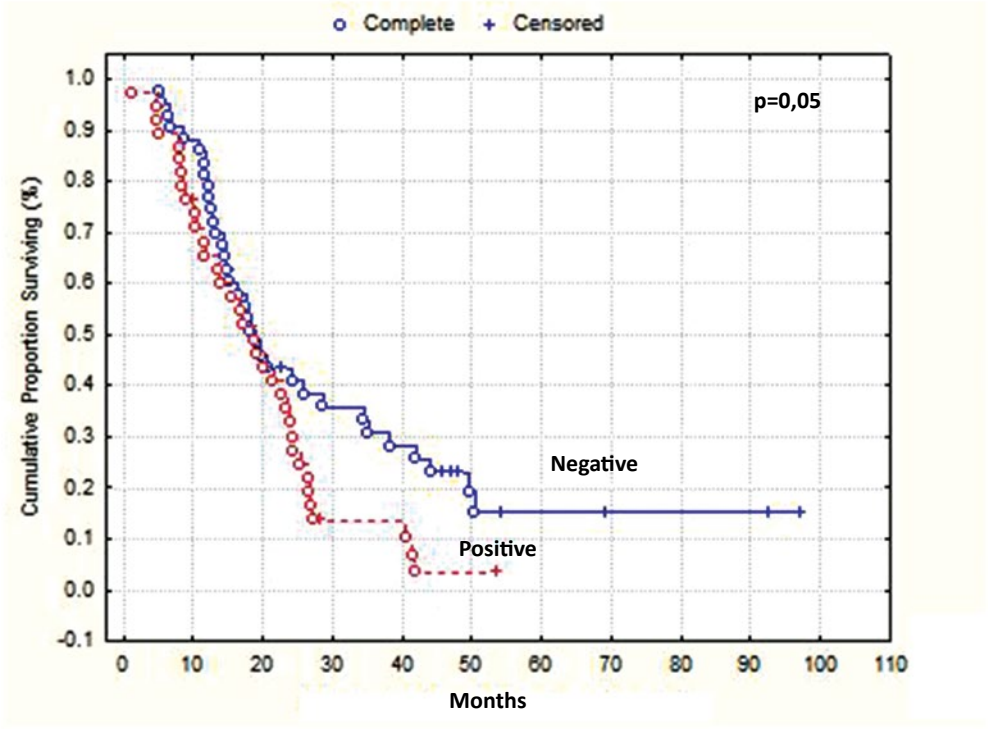

Figure 3. Kaplan-Meier progression-free survival of patients with serous ovarian cancer according to the expression of nestin in tumor cells.

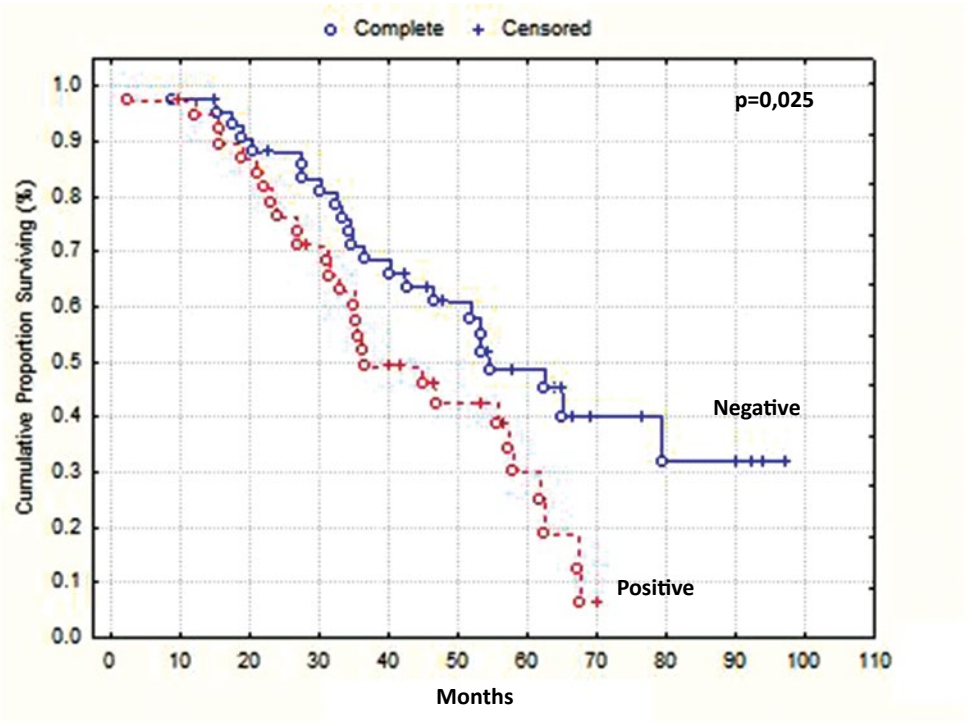

Figure 4. Kaplan-Meier overall survival of patients with serous ovarian cancer according to the expression of nestin in tumor cells.

than 1cm) and resistance to chemotherapy were significantly associated with worse OS (p=0.023, p=0.023, and p<0.001, respectively). There was no difference regarding time to progression or survival according to age, tumor grade, presence of peritoneal carcinomatosis and CD133 expression in ovarian cancer cells (Table 1). A trend toward a shorter survival was also observed when malignant ascites was present (p=0.056).

Moreover, in nestin-positive patients a poorer outcome in terms of PFS and OS was noted (p=0.05 and p=0.025, respectively) (Figures 3 and

Table 2. Results of multivariate Cox regression analysis using overall survival as dependent time variable in ovarian cancer patients

Factors	*Adjusted HR*	*95% CI*	*p value*
Preoperative CA125 (≥ vs < 35 U/ml)	1.25	0.65-2.41	0.509
Surgery – residual tumor (0-10 vs 0 mm)	1.72	0.96-3.12	0.073
Ascites (present vs absent)	1.37	0.62-3.02	0.437
Response to chemotherapy			<0.001
6-12 months vs >12 months*	3.81	1.77-8.20	<0.001
<6 months vs >12 months	5.06	2.49-10.30	<0.001
Nestin expression (positive vs negative)	1.92	1.07-3.45	0.030

*defined as progression free survival, HR: hazard ratio, CI: confidence interval

Table 3. Results of multivariate Cox regression analysis using PFS as dependent time variable in a sample of ovarian cancer patients

Factors	*Adjusted HR*	*95% CI*	*p value*
Preoperative CA125 (35 vs < 35 U/ml)	1.21	0.62-2.36	0.580
Surgery – residual tumor (0-10 vs 0 mm)	1.71	0.93-3.13	0.084
Ascites (present vs absent)	1.32	0.60-2.94	0.492
Response to chemotherapy			<0.001
6-12 months vs >12 months	3.65	1.66-8.00	<0.001
<6 months vs. >12 months	4.96	2.41-10.24	<0.001
Nestin expression (positive vs negative)	2.07	1.13-3.78	0.018

PFS: progression-free survival, HR: hazard ratio, CI: confidence interval

4). The differences in disease progression after 1st line treatment according to nestin expression, became significantly apparent after 20 months.

Multivariate Cox regression analysis demonstrated that shorter progression free interval and positive nestin expression were negatively associated with PFS and OS time and were independent risk factors for patients with ovarian cancer (Tables 2 and 3). Greater size of residual tumor was also negatively associated with OS (adjusted HR=1.72), with a trend toward statistical significance (p=0.07).

Discussion

The management of advanced stage serous ovarian carcinoma faces major challenges due to its heterogeneity in behavior, more often regarding disease progression and response to treatment [7,30]. Since the usual clinicopathological prognostic factors such as age, tumor grade, ascites, peritoneal carcinomatosis or CA125 level are not enough for predicting prognosis and response to chemotherapy, novel biomarkers need to be developed in order to decipher more profound and complex molecular processes [3,31].

Nestin and CD133 are two such markers, used to highlight a subpopulation of cells (CSCs) capable of initiating tumors through their self-renewal, proliferation and multi-lineage differentiation abilities [14,15]. Their prognostic significance and impact on survival have been validated for various cancer sites such as colorectal [21,32], hepatocellular [18,22], pancreatic [33], as well as brain tumors [16] and non-small cell lung carcinoma [19].

Researchers focusing on serous epithelial ovarian cancer have reported conflicting results so far. There are few studies that have tried to positively link overexpression of nestin and CD133 to clinicopathological characteristics, resistance to chemotherapy and poor prognosis [23,24]. Our results are partially in accordance with some studies [23,26] that have not supported the correlation

between nestin or CD133 expression in serous ovarian carcinoma tissues and clinicopathological parameters (age, tumor grade, peritoneal carcinomatosis, response to treatment). CD133 level of expression did not have a significant impact on patient outcome. Nevertheless, positive expression of nestin in tumor cells seems to negatively influence disease progression and OS, certifying its independent prognostic value and potentially explaining the more aggressive behavior of some cases of advanced ovarian cancer. Besides nestin, the size of residual tumor as well as response to chemotherapy have been able to predict outcome in terms of survival.

Additionally, some researchers have advocated nestin as a reliable marker for undifferentiated, activated endothelium of tumor neovasculature [13,15]. This is reflected in our results through the significant concordance between nestin-determined MVD and CD34-determined MVD, which states that nestin might be an endothelial marker as accurate as CD34 for quantifying tumor angiogenesis. Altogether, the putative involvement of nestin positive cells in serous ovarian cancer progression through the process of angiogenesis is envisaged.

Considering the still controversial results with regard to nestin and CD133 as prognostic biomarkers in serous ovarian carcinoma, further investigation in larger studies is justified in order to better clarify their prognostic significance with regard to tumor progression and angiogenesis and lay the pathway for more promising personalized therapies.

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Conflict of interests

The authors declare no confict of interests.

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