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Pretreatment serum hemoglobin level and a preliminary investigation of intratumoral microvessel density in advanced ovarian cancer

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Abstract

Objective. The primary aim of this study was to evaluate the prognostic and predictive value of pretreatment serum hemoglobin level (Hb) in advanced ovarian cancer; second aim was to perform a preliminary investigation of intratumoral microvessel density (IMD).

Methods. The influence on survival and response to treatment of several clinico-pathological features, including Hb, was analyzed in 72 patients with advanced ovarian cancer. IMD was assessed in tumor specimens of 25 of the 72 patients to compare three different endothelial markers: anti-FactorVIII, anti-CD31 and anti-CD34. In this subgroup of patients, a preliminary analysis of the prognostic and predictive value of IMD, and its relationship with Hb and other clinico-pathological features, was performed.

Results. Hb ≥ 12 g/dl was significantly associated with a better overall survival in univariate analysis ($P = 0.0181$) and was the only independent prognostic variable in multivariate analysis ($P = 0.0160$). Hb was directly related to progression-free survival ($P = 0.0240$) and complete response to treatment ($P = 0.016$). In the preliminary investigation of IMD, mean microvessel count did not show any significant difference among the three endothelial markers used, but anti-CD34 revealed a more consistent staining reaction. The relationship between IMD and complete response to treatment was found near to statistical significance ($P = 0.05$); Hb and IMD were inversely related ($r = -0.47$; $P = 0.045$).

Conclusions. Hb has a prognostic and predictive value in advanced ovarian cancer. In our preliminary study, which was performed on a limited number of patients, we found anti-CD34 to be an optimal marker for IMD determination, IMD to be a possible predictive factor of complete response to treatment, and IMD and Hb to be inversely related.

Keywords: Hemoglobin; Angiogenesis; Ovarian cancer

Introduction

Ovarian carcinoma has the highest mortality rate among gynecological malignancies and the majority (almost 70%) of ovarian cancer patients are diagnosed at an advanced stage of the disease [1]. Cytoreductive surgery combined with chemotherapy is the standard treatment of advanced ovarian cancer [2]; carboplatin and paclitaxel association is, by now, the standard first-line chemotherapy regimen [3,4].

The identification of predictive factors of response to a specific treatment, among already known prognostic factors, should open a new way through a tailored therapy for each patient [5,6].

Tumor anemia is a common symptom in cancer patients. Several reports have documented the prognostic role of

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pretreatment serum hemoglobin level (Hb) in patients with a variety of malignancies, but only two authors showed its influence on survival in ovarian cancer [7–9]. Hb was also reported to influence the response rates to radiotherapy and to radio-chemotherapy in cervical cancer and other non-gynecological tumors [10].

Angiogenesis plays a critical role in tumor growth. It is the result of predominantly proangiogenic inputs; there is strong evidence of a pivotal role of the hypoxic starting point and VEGF [11]. Many studies analyzed the prognostic role of intratumoral microvessel density (IMD) in ovarian cancer: the results are not homogeneous and the endothelial markers used not the same. While some authors found that IMD influenced survival, others did not. The most frequently used endothelial markers were anti-FactorVIII, anti-CD31, and anti-CD34 [12–21]. Gasparini et al. [22] showed a statistically significant inverse association between IMD and response to chemotherapy, while Gadducci et al. [23] demonstrated a positive association between IMD and response to chemotherapy, mainly represented by a paclitaxel–platinum regimen.

Primary aim of this study was to evaluate the influence of Hb on survival and on response to treatment in advanced ovarian cancer. Second aim was to perform a preliminary investigation of IMD.

Materials and methods

Patients selections

Criteria for eligibility to the study were: histologically confirmed epithelial ovarian cancer; advanced stages (III–IV); no previous treatment; no previous or concurrent malignancy; clinical, pathological and laboratory data availability; follow-up informations.

Clinical records of patients treated at the Department of Gynecologic Oncology, University of Turin, from 1997 to 2000 were collected. Seventy-two consecutive patients were selected.

All the eligible patients underwent primary debulking surgery followed by platinum-based chemotherapy. Forty of the 72 patients received a regimen without paclitaxel: 24 carboplatin alone; 4 cisplatin + cyclophosphamide; 4 cisplatin + doxorubicin + cyclophosphamide. Thirty-two of the 72 patients received a regimen with paclitaxel: 25 carboplatin + paclitaxel; 7 cisplatin + paclitaxel. The mean number of cycles administered was 6.

One month after the last course of chemotherapy, response to treatment was assessed by clinical examination, Ca125 evaluation and CT scan. Patients with clinical complete response (CR) underwent second look surgery, if response had to be confirmed.

At the end of the complete treatment program, Ca125 serum level evaluation and a clinical and gynecological examination were performed every 3–4 months for the first 2 years; patients underwent instrumental evaluation according to clinical status.

Clinico-pathological features analyzed

The following clinico-pathological features were analyzed: age, performance status (on the basis of the World Health Organization), ascites at diagnosis, FIGO stage, histologic type, tumor grade, peritoneal cytology, post-operative residual disease, and pretreatment serum hemoglobin level (Hb).

Intratumoral microvessel density determination

In 25 of the 72 patients, intratumoral microvessel density (IMD) was assessed. IMD determination was performed on formalin-fixed paraffin-embedded specimens with the methodology described by Weidner [24] and by the International Consensus in 1996 [25].

All histologic slides of hematoxylin and eosin stained sections from the primary tumor were examined at low magnification (10×) to select the most representative tissue block of the invasive carcinoma for each patient. Five-μ-thick sections of the selected blocks were obtained. Sections were deparaffinized in xylol and rehydrated in alcohol, then exposed to 3% H_2O_2 for 15 min to block endogenous peroxide activity. After pretreatment in a microwave for 10 min to unmask the antigens, the microvessels were highlighted by immunostaining the sections with three different endothelial markers using a standard immunoperoxidase technique. The three markers were anti-FactorVIII, anti-CD31, and anti-CD34 (Biogenex).

The immunostained sections were scanned at low magnification (40× to 100×) to select those areas of invasive carcinoma with the greatest numbers of distinctly highlighted microvessels (i.e., neovascular "hotspot"). All the vessels within a 0.74 mm^2 area of this neovascular "hotspot" were counted at high power (200×). Microvessel counts were performed without knowledge of the clinical outcome.

Outcome measures and statistical analysis

The overall survival and the response to treatment have been evaluated and have been compared in patients treated with regimens containing paclitaxel or not.

In all the 72 patients, the influence on survival and response to treatment of the selected clinico-pathological features was analyzed. The mean microvessel counts with the three different endothelial markers were compared. In the 25 patients assessed for IMD, a preliminary analysis to evaluate the relationship between IMD and overall survival rate, response to treatment, and the other clinico-pathological variables was performed.

The overall survival (OS) was considered as the period of time from diagnosis until the time of death or to the date of

the last available follow-up. The progression-free survival (PFS) was considered as the period from surgery to the first documented relapse.

Responses to treatment were defined as CR, partial response (PR), stable disease (SD) or progressive disease (PD) according to standard criteria.

All clinical data were revised and recorded in a database. Statistical analyses were performed with SPSS package (SPSS Advanced Statistics for Window version 9).

OS and PFS curves were obtained using the Kaplan–Meyer method. Log Rank, Breslow and Tarone–Ware statistics were used to test the difference between two groups.

Responses were correlated to continue variables with the Analysis of Variance and independent-samples Student's t test in a parametric setting. χ^2, Mann–Whitney and Kruskal–Wallis statistics were adopted for non-parametric distributions. To correlate continuous variables the parametric Pearson's coefficient and the non-parametric Spearman test were used.

A multivariate analysis was performed according to Cox proportional hazard model for statistical significant variables at the univariate level.

Results

Patient characteristics

The clinico-pathological characteristics of the 72 patients are reported in Table 1. The numbers of patients treated with or without paclitaxel are not statistically different (P = 0.239).

Forty-three (59.7%) patients had a complete response to treatment (CR), while the remaining 29 (40.3%) had a partial response or a stable disease or a progressive disease (PR + SD + PD). No statistically significant difference in the response to treatment between patients treated with or without paclitaxel were found: CR were 21 in patients treated with paclitaxel and 22 in patients treated without paclitaxel (P = 0.454); PR + SD + PD were 11 in patients who received paclitaxel and 18 in patients who did not receive paclitaxel (P = 0.639). Median progression-free survival and overall survival were similar in these two subgroups of patients.

Table 1 shows also the characteristics of the 25 patients evaluated for IMD. Twelve (48%) of these patients had a complete response to treatment, while the remaining 13 (52%) had a partial response or a stable disease or a progressive disease.

Prognostic factors analysis

In our series age at diagnosis, performance status, FIGO stage, histological type, and tumor grade did not influence survival (data not shown).

A statistically significant association between overall survival and ascites at diagnosis, postoperative residual disease, and pretreatment serum hemoglobin level was found (Table 2).

In Table 3 multivariate analysis for variables influencing overall survival is reported: Hb was the only independent prognostic variable (P = 0.0160). Hb was statistically associated with progression-free survival too.

Fig. 1 shows the Kaplan–Meier curve for overall survival in patients with pretreatment serum hemoglobin levels ≥12 and <12 g/dl; Fig. 2 shows the Kaplan–Meier curve for PFS in the same patients.

Predictive factors analysis

As reported in Table 4, a statistically significant association between complete response to treatment and ascites at diagnosis, postoperative residual disease, and pretreatment serum hemoglobin level was found.

IMD determination and preliminary analyses

Microvessel counts were similar with the three endothelial markers. The mean microvessel count was anti-CD34 = 17.48; anti-CD31 = 17.31; anti-FactorVIII = 18.23.

Table 1
Clinico-pathological characteristics

Characteristics	All the patients		Patients evaluated for IMD	
	Number (mean)	% (range)	Number (mean)	% (range)
Number	72		25	
Age	(58.4)	(25–80)	(58)	(33–79)
Performance status WHO				
0	39	54.2	10	40
1	25	34.7	9	36
2	8	11.1	6	24
Ascites				
Absent	28	38.9	5	20
Present	44	61.1	20	80
FIGO stage				
IIIA	13	18	2	8
IIIB	6	8.3	4	16
IIIC	48	66.7	19	76
IV	5	7	0	0
Histologic type				
Serous	44	61.1	15	60
Mucinous	9	12.5	4	16
Endometrioid	7	9.7	0	0
Clear cells	1	1.4	0	0
Undifferentiated	11	15.3	6	24
Tumor grade				
1	3	4.2	1	4
2	24	33.4	6	24
3	45	62.4	18	72
Peritoneal cytology				
Negative	8	11.1	2	8
Positive	64	88.9	23	92
Postoperative residual disease				
<2 cm	33	45.8	12	48
≥2 cm	38	52.8	13	52

Table 2
Clinico-pathological features influencing cumulative overall survival (univariate analysis)

Clinico-pathological features	% survival	*P* value
Ascites		
Absent (n = 28)	71.43	0.0123
Present (n = 44)	50.00	
Postoperative residual disease		
<2 cm (n = 33)	75.76	0.0008
≥2 cm (n = 38)	44.74	
Pretreatment serum hemoglobin level		
≥12 g/dl (n = 42)	69.05	0.0181
<12 g/dl (n = 30)	43.33	

Anti-CD34 revealed a more consistent staining reaction. Data presented below are from anti-CD34 staining.

A statistically significant association between IMD and overall survival or disease-free survival has not been found.

A correlation near to the statistical significance (P = 0.05) has been evidenced between IMD and complete response to chemotherapy. Table 5 shows the mean microvessel counts for patients with CR or PR+SD+PD.

There was no statistically significant association between IMD and the other clinico-pathologic features. Table 6 reports the mean microvessel count according to different histological type.

An inverse correlation has been demonstrated between IMD and Hb (r = −0.47; P = 0.045).

Discussion

Primary aim of this study was to evaluate the prognostic and predictive value of pretreatment serum hemoglobin level (Hb) with other clinico-pathological features in advanced ovarian cancer. Nowadays, paclitaxel plus platinum-based chemotherapy is the standard treatment in these patients [3,4]. In our experience, the paclitaxel addition to platinum schedules increased neither response rates nor survival. Because of our small number of patients, we do not want to draw any conclusion about the effectiveness of paclitaxel addition to platinum-containing regimens, but our results allow us to analyze together both groups of patients.

Table 3
Clinico-pathological features influencing cumulative overall survival (multivariate analysis)

Variable	Wald χ^2	RR	95% CI lower	95% CI upper	*P* value
Ascites	0.1553	1.2943	0.3588	4.6686	0.6935
Postoperative residual disease	1.6415	2.7284	0.5876	12.6687	0.2001
Pretreatment serum hemoglobin	4.4605	2.9588	1.0812	8.0969	0.0347
Multivariate analysis (final model)					
Pretreatment serum hemoglobin	5.8011	3.2792	1.2475	8.6192	0.0160

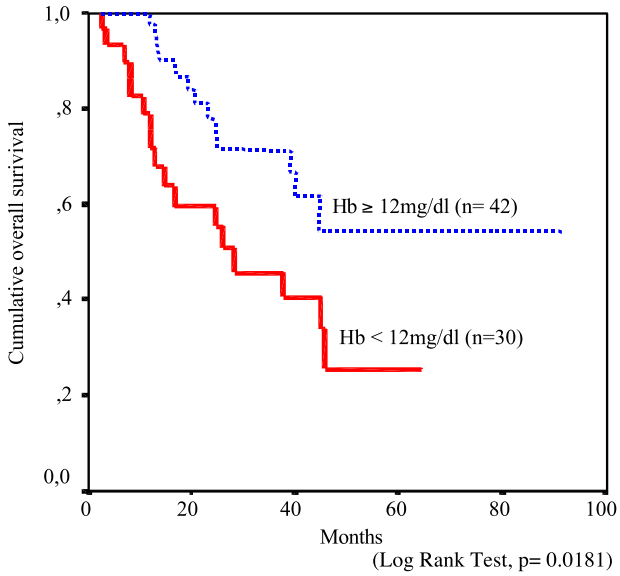

Fig. 1. Overall survival by pretreatment serum hemoglobin level (Hb).

In agreement with existing literature [5,6], in our study, the postoperative residual disease was a prognostic factor which influences survival in a positive way if it's smaller than 2 cm. Ascites at diagnosis resulted as an index of worse outcome.

The prognostic role of pretreatment serum hemoglobin level in patients affected by different neoplasms has already been demonstrated. In ovarian cancer, Obermair et al. [7,8] found the relationship of hemoglobin levels before surgery and survival, especially in early stages of the disease; hemoglobin levels prior and during chemotherapy were identified as a prognostic factor for overall survival by

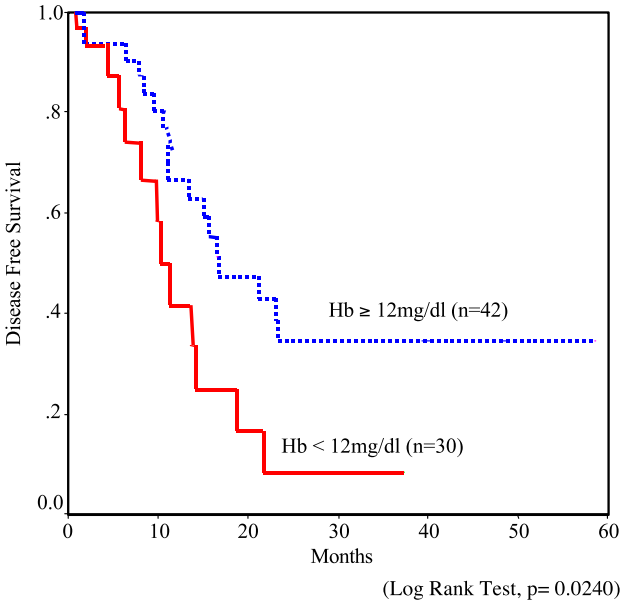

Fig. 2. Progression-free survival by pretreatment serum hemoglobin level (Hb).

Table 4
Clinico-pathological features predictive of complete response to treatment

Clinico-pathological features	CR (%)	PR + SD + PD (%)	*P* value
Ascites			
Absent (n = 28)	22 (78.6)	6 (21.4)	0.0092
Present (n = 44)	21 (47.7)	23 (52.3)	
Postoperative residual disease			
<2 cm (n = 33)	28 (84.8)	5 (15.2)	0.00009
≥2 cm (n = 38)	15 (39.5)	23 (60.5)	
Pretreatment serum hemoglobin level			
≥12 g/dl (n = 42)	30 (71.4)	12 (28.6)	0.016
<12 g/dl (n = 30)	13 (43.3)	17 (56.7)	

Munstedt et al. [9]. Like these two authors, in our series, pretreatment serum hemoglobin levels ≥12 g/dl were significantly associated with a better overall survival (P = 0.0181) and retained significance in multivariate analysis (P = 0.0160). The reasons of these results are presently unexplained. Obermair hypothesized a paraneoplastic phenomenon. Tumor-released cytokines play a major role in the development of tumor-associated anemia by hemolysis, suppression of erythropoiesis, and impairment of erythropoietin response on erythroid progenitor cells; marked tumor anemia might indicate the presence of biologically aggressive tumor cell clones. Another possible explanation is that hypoxia, due to low levels of circulating hemoglobin, causes a worse response to treatment [7,10].

In our study, three factors were linked to a better response to treatment: the postoperative residual disease smaller than 2 cm, the absence of ascites at diagnosis and pretreatment serum hemoglobin levels ≥12 g/dl. The predictive value of Hb is a datum that still needs further study. A considerable number of studies report radiotherapy and chemotherapy to be more effective under well-oxygenated conditions. Anemia might contribute to reduce tumor oxygenation via reduced oxygen delivery to the tumor [26]. In vitro and animal models have shown that cellular hypoxia, the consequence of anemia, may provide a selection pressure for tumor cells with higher rate of mutation, which may ultimately result in increased metastatic potential, increased cellular growth, therapy resistance, and decreased apoptotic potential [27].

In literature different endothelial markers have been used to highlight microvessels with immunohistochemistry. Hollingsworth et al. [13] initially performed counts with CD34, Ulex and von Willebrand Factor (vWF, also designated Factor VIII) antibodies. Subsequently, it was noted that variability in staining was occurring with both Ulex and vWF with misleading of low vessel counts and understaining of endothelium in approximately 50% of the slides. CD34 antibody stained consistently and reproducibly and did no exhibit such problems. Alvarez et al. [21] evaluated IMD with antibodies to von Willebrand Factor (vWF) and CD31: vessel counts for vWF and CD31 were highly correlated and comparable results for outcome were seen for the two factors. In our study, three different endothelial markers for IMD determination were used: anti-Factor VIII, anti-CD31, and anti-CD34. The comparison did not show any difference in terms of mean microvessel count, but anti-CD34 revealed a more consistent staining reaction. In agreement with Heiburg et al. [18,19], we consider CD34 as an optimal marker for IMD determination in tumoral neoangiogenesis and have used anti-CD34 staining for our analyses.

Table 5
Correlation between response to treatment and mean microvessel count (CD34)

Type of response	Mean count	DS	SE
CR (n = 12)	15.08	6.08	1.76
PR + SD + PD (n = 13)	19.68	5.71	1.58

Independent-samples Student's t test, P = 0.05.

Table 6
Mean microvessel count (CD34) according to histologic type

Histologic type	Mean count	DS	SE
Serous (n = 15)	17.60	7.70	1.99
Mucinous (n = 4)	16.50	5.07	2.53
Indifferenziated (n = 6)	17.83	1.94	0.79

(ANOVA, P = 0.944).

IMD was not a statistically significant prognostic factor for our patient sample. Surely, the small number of patients could be a cause, but the numbers of patients in some already published studies are only slightly higher than ours. Some, but not all, papers reported a statistically significant association between low IMD and an increased overall survival. However, in these studies, even patients at early stages were included and in those stages IMD is usually lower and the outcome is better [12].

The mean microvessel count in different histological types was almost the same in our study, and moreover we did not find an evident difference concerning mucinous histotype such as happened in Orre et al. [28] and Gasparini et al. [22] studies.

A correlation near to the statistical significance (P = 0.05) has been found between IMD and complete response to chemotherapy. The mean microvessel count was 15.08 for patients with CR and 19.69 for PR, SD, PD. Gasparini et al. [22] also showed a statistically significant inverse association between IMD and response to chemotherapy, while Gadducci et al. [23] found a positive association between IMD and response to chemotherapy, mainly represented by a paclitaxel–platinum regimen. Gadducci hypothesized that the antineoplastic activity of paclitaxel could be greater in highly vascularized tumors. Indeed paclitaxel, besides causing tumor cell death, displays antiangiogenic activity through organic and functional damage of endothelial cells.

An interesting evidence seems to be the finding of an inverse correlation between pretreatment serum hemoglo-

bin level and IMD. A low level of circulating hemoglobin should increase IMD because of hypoxia and at the end become a proangiogenic stimulation. The switch of tumors from the avascular to the vascular phase is a critical checkpoint in cancer progression. Folkman [29] proposed that neovascularization of a tumor was required to provide essential nutrients beyond the limit of simple diffusion and to allow a growth higher than 2 mm. At this time, tumor and host tissues produce a great variety of angiogenic factors that promote the development of a new capillary bed. The mechanism by which low hemoglobin levels could be involved in this process needs further elucidations by biochemical and molecular studies, but a hypothesis is that the decreased oxygen carrying capacity may lead to increased tumor hypoxia than to neoangiogenic stimulation and tumor growth. Neoangiogenesis may cause a misunderstanding in a positive way: the major perfusion granted by the new vessels may be seen as a new therapeutical option because a greater dose of drugs may arrive directly into the tumoral mass. Unfortunately, the first vascular phase is followed by a second phase during which there is a mechanical compression of new vessels due to the increased volume of the neoplasm mass.

In conclusion, our study confirms the prognostic role of pretreatment hemoglobin level, which has been demonstrated to be a predictive factor of response to treatment as well. We are continuing to evaluate some more patients to measure IMD in a larger number of cases to analyze the correlation among hemoglobin levels, IMD, and the response to treatment.

We are looking for a tailored treatment for selected ovarian cancer patients. Recently, new therapeutic approaches have been studied as a consequence of biological research development [30]: angiogenesis inhibitors could be the new frontier of treatment with genic therapies and vaccines. Paclitaxel may inhibit angiogenesis. The correction of anemia before the beginning of treatment could improve response rates and survival.

Acknowledgments

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