EXPRESSION OF INDUCILE NITRIC OXIDE SYNTHASE AND CYCLOOXYGENASE-2 IN OVARIAN CANCER: CORRRELATION WITH

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Expression of inducible nitric oxide synthase and cyclooxygenase-2 in ovarian cancer: correlation with clinical outcome

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Abstract

Objectives. Cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) play a critical role in cancer development. We investigated iNOS and COX-2 expression in relation to clinical outcome in 78 International Federation of Gynecology and Obstetrics (FIGO) stage III ovarian serous carcinoma with a low grade of differentiation (G3).

Methods. Disease-free interval and cause-specific survival rates (Kaplan–Meier method) were compared using the log rank test. A multivariate analysis (Cox-proportional hazards models) was used to determine the independent effect of each variable on prognosis. Fisher's exact test was used to analyze the distribution of iNOS and COX-2 expression according to clinical complete response to chemotherapy and to the presence of a brief disease-free interval (≤12 months).

Results. Overall 60 and 125 months cause-specific survival rates were 32% and 11%, respectively. In univariate analysis, iNOS ($P = 0.005$ and $P = 0.003$, respectively), COX-2 ($P = 0.002$ and $P = 0.007$, respectively), residual disease after surgery ($P = 0.017$ and $P = 0.032$, respectively), and FIGO stage ($P = 0.008$ and $P = 0.025$, respectively) were associated with survival and a disease-free interval. In multivariate analysis (Cox proportional hazards models), the factors that were found to be significantly independent predictors of disease relapse as well as survival were iNOS ($P = 0.014$ and $P = 0.001$, respectively), COX-2 expression ($P = 0.007$ and $P = 0.029$, respectively), and FIGO stage ($P = 0.026$ and $P = 0.05$, respectively). iNOS and COX-2 expressions were correlated with a brief disease-free interval ($P = 0.001$) and clinical complete response to first-line chemotherapy ($P = 0.038$ and $P = 0.033$, respectively).

Conclusions. The evaluation of iNOS and COX-2 expression may give additional prognostic information concerning the clinical outcome of patients with ovarian carcinoma and may encourage them to select more tailored therapies.

Keywords: Cyclooxygenase-2; Inducible nitric oxide synthase; Ovarian carcinoma; Chemotherapy response; Prognosis

Introduction

Invasive ovarian cancer is the most common cause of death from gynecological cancers in the Western world. In particular, serous carcinoma, the most frequent malignant ovarian tumor, is generally diagnosed at later stages and is associated with low survival rates. In the majority of the cases, women with advanced ovarian cancer are treated with surgery followed by adjuvant therapy. The patients with unresectable disease were submitted to exploratory laparotomy with multiple biopsies and received three cycles of chemotherapy before performing a cytoreductive surgery.

The current recommendations for chemotherapy are based on sequential prospective randomized trials in ovarian cancer. These trials were based on the analysis of earlier results that platinum combinations were superior to non-platinum-based chemotherapy [1,2]. Despite improvements in median and overall survival with the earlier development of the platinums and the more recent addition of taxanes to

Abbreviations: iNOS, inducible nitric oxide synthase; NO, nitric oxide; COX-2, cyclooxygenase-2.

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the therapeutic combination, a significant fraction of patients with advanced ovarian cancer will not achieve a complete response to adjuvant therapy and long-term survival rates for patients with advanced epithelial ovarian carcinoma remain low.

The identification of additional prognostic and predictive factors would be very helpful to better tailor treatments for patients with ovarian carcinoma. A multimodality approach using a combination of cytoreductive surgery, chemotherapy, and assessment of biochemical factors more strictly related to individual tumor biology and intrinsic aggressiveness is the direction of the future and can improve the treatment of patients with ovarian carcinoma.

The cyclooxygenase enzymes COX-1 and COX-2 are responsible for the conversion of arachidonic acid to prostaglandins. Research over the last decade, primarily in studies focused on colorectal cancer, has established that nonsteroidal anti-inflammatory drugs are effective in both cancer prevention and as adjuvant therapy in the treatment of established tumors [3].

Experimental data show that colorectal cancer cell growth is primarily through the inhibition of cyclooxygenase-2 (COX-2), and also that selective COX-2 inhibitors have potent antineoplastic effect in vivo in preclinical models of several solid malignancies.

Nitric oxide (NO) that comes from L-arginine by the inducible nitric oxide synthase enzyme (iNOS) is a molecule involved in several biological activities, such as vasodilatation, neurotransmission, and cellular immune system.

Stimulation of inducible nitric oxide synthase and release of nitric oxide by tumor cells play a critical role in cancer development; in fact, the inducible nitric oxide synthase enzyme has been implicated in tumor angiogenesis and colon cancer progression [4] and has been reported in human gynecological cancer [5].

Experimental evidence indicates that nitric oxide mediates diverse aspects of tumor biology, such as host's immune suppression accompanying tumor growth [6], and is advantageous to tumor growth and metastasis [7–9].

In a recent study, Klimp et al. [10] showed that not only malignant, but also borderline and benign ovarian tumors can exhibit increased levels of COX-2 and iNOS expression. In their analysis, the correlation of COX-2 and iNOS status with clinical outcome is lacking.

Nose et al. [11] have suggested that enhanced expression of both COX-2 and iNOS may have important roles in the processes underlying thyroid tumorigenesis.

In ovarian carcinoma, COX-2 positivity has been recently correlated with the clinicopathological outcome of patients [12–14]; while to our knowledge, no data have been reported to the present about the expression of iNOS and its possible clinical significance in ovarian cancer.

The adaption of COX-2 and iNOS as prognostic factors for survival may add information to the well-accepted clinicopathological parameters and enhance research towards more tailored therapies. Whether any additional benefit will result from the inclusion of selective COX-2 inhibitors and iNOS inhibitors in the therapeutic plan of patients whose tumors express them is an interesting hypothesis which needs to be explored.

Thus, the aim of the present study is to investigate the possible correlations between iNOS and COX-2 expression in primary untreated ovarian carcinoma and the clinical outcome of patients to give additional data to modulate therapeutic tools for the patient.

Materials and methods

Case selection

The files of the Department of Human Pathology and Oncology of the University of Florence were searched from 1985 to 1999 for the diagnosis of ovarian carcinoma. Out of these 494 cases, we selected a very homogeneous series of International Federation of Gynecology and Obstetrics (FIGO) stage III ovarian serous carcinoma with low grade of differentiation (G3). The specimens come from 78 patients, with a known follow-up, who had undergone surgical and adjuvant therapy at the Department of Gynecology, Perinatology and Reproductive Medicine of the University of Florence.

The mean age of our patients was 58 years and the median was 60 years (range: 33–79 years). The age of 40 women (51%) was higher than 60. The patients underwent laparotomy for optimal debulking of the gross neoplastic masses with abdominal hysterectomy, bilateral salpingo-oophorectomy, appendectomy, and omentectomy with careful examination of all serosal surface and biopsies of any suspected lesions. All the patients presented residual disease after surgery: 50 women (64%) had minimal residual disease (<2 cm) and 28 (35%) had bulk residual disease (≥ 2 cm) on completion of the initial surgery before chemotherapy as described by the surgeon or by the histological examinations. All patients were staged retrospectively according to a modified staging system of the International Federation of Gynecology and Obstetrics (FIGO) for malignant surface epithelial–stromal tumors. Ten cases were FIGO stage IIIA, 8 were IIIB, and 60 cases were FIGO stage IIIC [15].

A postoperative treatment was performed in all patients independently of the presence of minimal residual disease or bulk residual disease and consisted of combined chemotherapy regimes with six cycles of cisplatin-based chemotherapy, unless they showed disease progression during adjuvant treatment.

Until 1995, chemotherapy was based on combined regimens with cisplatin and cyclophosphamide. After 1995, the chemotherapeutic treatment was based on combined regimens with carboplatin (AUC 5) and either taxol or cyclophosphamide.

After completion of treatment, the patients were followed-up with a pelvic examination, the measurement of

tumor markers (CA125, CEA, CA19.9), and pelvic and abdominal ultrasonography (every 3 months for 2 years, afterwards every 6 months), computed tomography of the pelvis and the abdomen (once a year).

The follow-up period for each patient was until death or at least 5 years after surgery, the median follow up was 32 months, and the mean value was 47 months, with observed values ranging between 3 and 204 months following surgery and first-line adjuvant therapy.

We have evaluated the clinical response to first-line chemotherapy treatment according to computed tomography of the pelvis and the abdomen with WHO methods [16]: complete response (CR), partial response (PR), stable disease (S), and progression (P).

A brief disease-free interval is defined as when recurrence or metastasis comes no later than 12 months after surgery [17,18]. A disease-free interval was defined as the interval time from primary treatment to recurrence or metastasis. Cause-specific survival was defined as the survival time from primary treatment to death due to the disease.

Immunohistochemical staining

The specimens were obtained by surgical resection in all cases and fixed in 10% formalin before being processed in paraffin. Hematoxylin–eosin-stained sections from each histological specimen were reviewed by two pathologists to confirm the histological diagnosis. A representative section for each case was selected for immunohistochemical analysis.

The immunohistochemical study was performed by the streptavidin–biotin–peroxidase method (UltraVision kit, LAB VISION; Fremont, CA) with diaminobenzidine (DAB) as chromogen and Mayer's hematoxylin as nuclear counterstain with two different antibodies.

We studied the inducible nitric oxide synthase enzyme with anti-iNOS polyclonal antibody (Biomol Laboratories, Plymouty, PA; 1:600 dilution for 5 h, at 4 °C), with biotinylated goat anti-polyvalent secondary antibody (UltraVision, Lab Vision Corporation, Fremont, CA), and with antigen rescue in the microwave with citrate buffer, pH 6 for 10′.

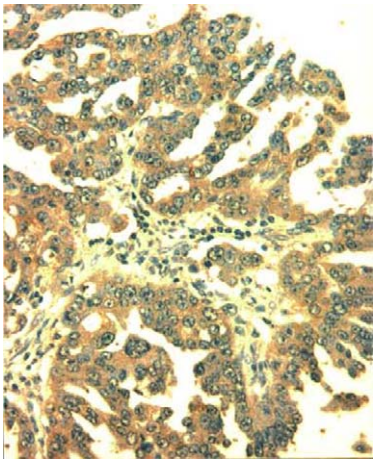

Fig. 1. Immunohistochemical positive staining of anti-iNOS polyclonal antibody of the cell membrane and cytoplasm.

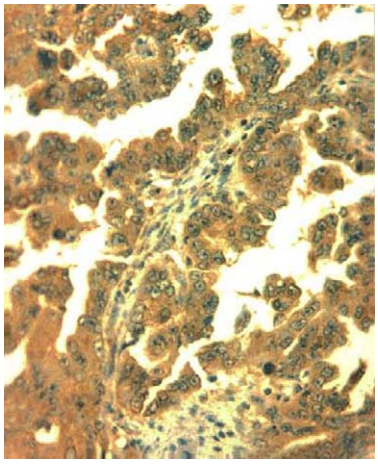

Fig. 2. Immunohistochemical positive staining of anti-COX-2 polyclonal antibody of the cell membrane and cytoplasm.

The second enzyme analyzed was cyclooxygenase-2 with goat polyclonal antibody anti-COX-2 (Santa Cruz Biotechnology, Santa Cruz, CA; 1:50 dilution overnight, at 4 °C), with biotinylated rabbit anti-goat secondary antibody (Dako, Carpenteria, CA; 1:100 dilution overnight, at

Table 1
Clinical characteristics and outcome of 78 patients with serous ovarian carcinoma

	No. of cases
Age	
<60 years	38
≥60 years	40
FIGO stage	
IIIA	10
IIIB	8
IIIC	60
Residual disease	
<2 cm	50
≥2 cm	28
Clinical responsiveness to chemotherapy	
Complete response	55
Partial response	7
Progression after treatment	16
Disease-free interval ≤12 months	
Present	52
Absent	26
Relapse	
Present	69
Absent	9
Status	
Deceased	65
Living (no evident disease)	13
iNOS immunohistochemistry	
Positive	50
Negative	28
COX-2 immunohistochemistry	
Positive	54
Negative	24

Table 2
Prognostic factors by univariate analysis (Cox proportional hazards model)

Variable	Relapse				Death			
	Score	Hazard ratio	95% CI	*P*	Score	Hazard ratio	95% CI	*P*
iNOS	0				0			
	1	2.09	1.24–3.51	0.005	1	2.27	1.33–3.89	0.003
COX-2	0				0			
	1	2.41	1.37–4.23	0.002	1	2.19	1.24–3.87	0.007
Residual disease	0				0			
	1	1.82	1.11–2.98	0.017	1	1.73	1.04–2.87	0.032
FIGO stage								
IIIA	0				0			
IIIB	1	2.91	0.96–8.74	0.057	1	1.36	0.41–4.51	0.607
IIIC	2	3.16	1.34–7.41	0.008	2	2.69	1.13–6.42	0.025
Age ≥60 years	0				0			
	1	0.97	0.60–1.57	0.92	1	0.9	0.55–1.48	0.7

CI, confidence interval.

4 °C), and with antigen rescue in the microwave with TEC buffer (Tris-EDTA-citrate), pH 8 for 20′.

Negative control was performed by substituting the primary antibody with nonimmune sera. Appropriate positive and negative controls were run simultaneously.

The immunohistochemically stained sections were evaluated without previous knowledge of the clinical outcome of each patient.

Evaluation of inducible nitric oxide synthase and cyclooxygenase-2 expression

The tumor sections showing brown staining of the antibodies specific iNOS and COX-2 of cytoplasm were scored as positive. The proportion of immunostained cells was scored at low magnification (5× objective lens) by evaluating the entire tumor area. When the tumor area with positive immunostaining was >10% of the total tumor area, the case was scored as positive. The intensity of staining was also evaluated subjectively using a range from 0 (none) to 1 (feint) to 2 (strong). Cases in which the intensity of staining was scored <2 were considered negative; as previously described for cyclooxygenase-2 expression [12].

Statistical analyses

A disease-free interval and cause-specific survival rates were calculated according to the Kaplan–Meier method [19] and differences were evaluated using the log rank test.

A univariate analysis, with Cox proportional hazards models, was used to determine which variables had an effect on clinical outcome. A P value ≤0.05 was considered to be statistically significant. In the multivariate analysis, we had analyzed the variables which in the univariate analysis had a P statistically significant. A multivariate analysis (Cox proportional hazards model) was used to determine which variables had an independent effect on clinical outcome [20]. A P value ≤0.05 was considered to be statistically significant.

The optimized cut-out points were calculated as those corresponding to the lowest values that by multivariate analysis were significantly and independently associated with both disease-free interval and cause-specific survival.

Fisher's exact test was used to analyze the distribution of iNOS and COX-2-positive cases according to clinical response to chemotherapy and to the presence of recurrence of

Table 3
Significant prognostic factors by multivariate analysis (Cox proportional hazards model)

Variable	Relapse				Death			
	Score	Hazard ratio	95% CI	*P*	Score	Hazard ratio	95% CI	*P*
iNOS	0				0			
	1	1.95	1.14–3.34	0.014	1	2.46	1.41–4.29	0.001
COX-2	0				0			
	1	2.19	1.23–3.88	0.007	1	1.9	1.06–3.4	0.029
Residual disease	0				0			
	1	1.56	0.92–2.66	0.097	1	1.4	0.82–2.41	0.21
FIGO stage								
IIIA	0				0			
IIIB	1	2.69	0.88–8.21	0.082	1	1.01	0.3–3.36	0.98
IIIC	2	2.71	1.12–6.51	0.026	2	2.43	0.99–5.95	0.05

CI, confidence interval.

the disease that comes no later than 12 months after surgical treatment.

Data analysis was performed using the Statacorp.2001. Stata Statistical Software: Release 7.0. College Station, TX: Stata Corporation [21].

Results

iNOS and COX-2 immunostaining

Intense iNOS immunostaining was observed in the cytoplasm of tumor cells in 50 cases (64%), and high staining intensity for COX-2 was observed in 54 cases (69%). Figs. 1 and 2 show representative examples of an ovarian carcinoma with intense iNOS and COX-2 immunostaining, respectively. The tumor cells presented a positive immunostaining for both the two antibodies in 37 cases (47%).

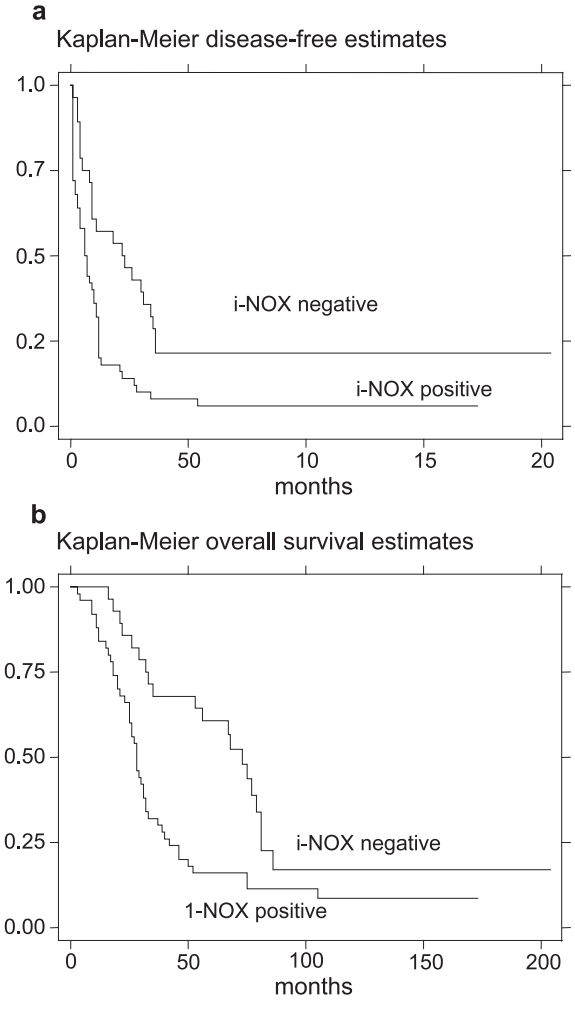

Fig. 3. Probability of disease-free survival (a) and cause-specific survival (b) according to the presence versus the absence of iNOS expression (P = 0.014 and P = 0.001, respectively).

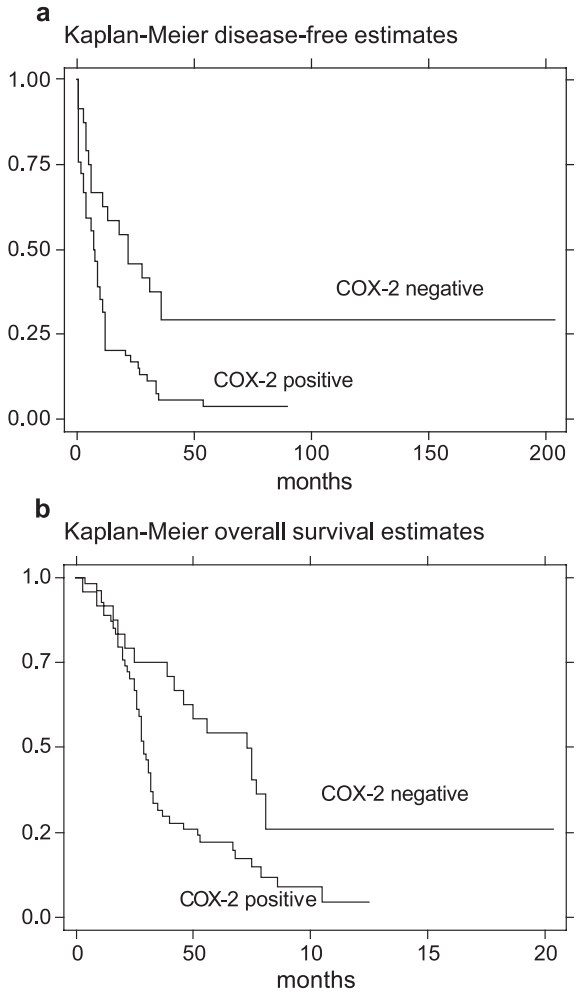

Fig. 4. Probability of disease-free survival (a) and cause-specific survival (b) according to the presence versus the absence of COX-2 expression (P = 0.007 and P = 0.029, respectively).

Correlation of iNOS and COX-2 expression with clinicopathologic parameters

Clinical characteristics and outcome of 78 patients with serous ovarian carcinoma are shown in Table 1.

Fifty-five patients (70%) presented a complete clinical response to first-line chemotherapy after surgical treatment, evaluated according to the WHO methods [16]; seven

Table 4
iNOS and COX-2 expression in correlation with the disease-free interval ≤12 months (P = 0.001. Fisher's exact test)

	Disease-free interval ≤12 months	Disease-free interval >12 months	P value
iNOS expression			
Positive	40 cases (80%)	10 cases (20%)	0.001
Negative	12 cases (42%)	16 cases (57%)	
COX-2 expression			
Positive	43 cases (79%)	11 cases (20%)	0.001
Negative	9 cases (37%)	15 cases (62%)	

patients (8%) presented a partial response, and in 16 patients (20%) the disease was in progression after surgery and adjuvant treatment. None of the patients were with stable disease.

The overall 60 and 125 months cause-specific survival rates were 32% and 11%, respectively. Fifty-two patients (66%) showed a brief disease-free interval, and 69 patients (88%) showed recurrence of disease.

Using univariate analysis, several parameters, such as iNOS ($P = 0.005$ and $P = 0.003$, respectively) and COX-2 expression ($P = 0.002$ and $P = 0.007$, respectively), the residual disease after surgery ($P = 0.017$ and $P = 0.032$, respectively), and the FIGO stage IIIC even more than FIGO stage IIIA ($P = 0.008$ and $P = 0.025$, respectively), were found significantly associated with the risk of recurrence or metastases and death from disease (Table 2).

Using multivariate analysis (Cox proportional hazards models), the only factors that were found to be significant independent predictors of disease relapse as well as survival were iNOS ($P = 0.014$ and $P = 0.001$, respectively) and COX-2 expression ($P = 0.007$ and $P = 0.029$, respectively), and FIGO stage IIIC even more than FIGO stage IIIA ($P = 0.026$ and $P = 0.05$, respectively) (Table 3).

Kaplan–Meier estimates of a disease-free interval and cause-specific survival by iNOS and COX-2 expression are shown in Figs. 3 and 4, respectively.

iNOS and COX-2 expressions are significantly correlated with the disease-free interval of ≤12 months (Fisher's exact test, $P = 0.001$) (Table 4).

iNOS and COX-2 negative ovarian carcinomas are significantly correlated with clinical complete response to first-line chemotherapy. In fact, in 85% of the iNOS-negative cases, the response to first-line chemotherapy was complete; while 38% of the patients with iNOS-positive ovarian carcinoma presented partial response or progression of the disease after the first-line chemotherapy (Fisher's exact test, $P = 0.038$). We can observe the same trend of poorer prognosis of iNOS-positive cases also for COX-2-positive cases. In fact, in 87% of the COX-2 negative cases, the response to first-line chemotherapy was complete, while 37% of the patients with COX-2-positive ovarian carcinoma presented partial response or progression of disease after the first-line chemotherapy (Fisher's exact test, $P = 0.033$) (Table 5).

Table 5
iNOS and COX-2 expression, respectively, in correlation with the clinical complete response to chemotherapy versus clinical not complete response to chemotherapy ($P = 0.038$ and $P = 0.033$, respectively; Fisher's exact test)

	Clinical complete responsiveness to chemotherapy	Clinical not complete responsiveness to chemotherapy (partial response or progression)	*P* value
iNOS expression			
Positive	31 cases (62%)	19 cases (38%)	0.038
Negative	24 cases (85%)	4 cases (14%)	
COX-2 expression			
Positive	34 cases (62%)	20 cases (37%)	0.033
Negative	21 cases (87%)	3 cases (12%)	

Discussion

To the best of our knowledge, a study designed to determine the association between iNOS and COX-2 expression and reduced susceptibility to chemotherapy and prognosis in a series of primary advanced untreated ovarian serous carcinomas has never been performed.

Epidemiological data indicate that nonsteroidal anti-inflammatory drugs may be effective in the prevention of ovarian cancers [22,23]. Preclinical evaluation of these drugs as chemopreventive agents by Rodriguez-Burford et al. [24] provides in vitro evidence of direct growth inhibitory effects of these agents. In particular, the COX-2 inhibitors, across all the cell lines tested, call for additional studies for the use of nonsteroidal anti-inflammatory drugs in addition to the adjuvant therapy in ovarian cancer; in particular, in the cases that show COX-2 expression [25].

Recently, studies [12,13] have shown that increased cyclooxygenase-2 expression is associated with chemotherapy resistance and clinical outcome in a series of III and IV FIGO stage ovarian cancer patients who had undergone either primary debulking and subsequent chemotherapeutic treatment or exploratory laparotomy and chemotherapy. Denkert et al. [14] correlated COX-2 with the prognosis in ovarian carcinoma of different stages and histopathologic types and in low malignant potential ovarian tumors.

Our analysis consists of a series of III stage FIGO serous, G3, ovarian carcinoma patients who had undergone surgical treatment and following chemotherapy.

A preclinical study with regards to iNOS gene expression in ovarian carcinoma cell lines following incubation with different combinations of interferon-γ, interleukin-1β, *lipopolysaccharide,* and tumor necrosis factor-α demonstrated variations in nitric oxide production with interferon-γ and different patterns of nitric oxide release in response to inflammatory stimuli in ovarian carcinoma cell lines [26]. Saito et al. [27] previously showed that interferon-γ exerts antiproliferative effects on neoplastic cells, including ovarian cancer. Intraperitoneal treatment with interferon-γ has been shown to achieve documented surgical responses in the second-line therapy of ovarian cancer and also in the first-line [28].

Garman and Bonavida [29] demonstrated that induction of apoptotic cell death in the ovarian carcinoma cell line AD10 by interferon-γ induced iNOS gene expression. Rieder et al. [30] recently showed that nitric oxide produced by ovarian carcinomas is correlated to the intensity of tumor cell death by apoptosis; thus, it is conceivable that tumor cells generating large amounts of nitric oxide are susceptible to nitric oxide-mediated killing cell. Also, Kost et al. [31] found synergistic cytolytic effects of

interferon-γ and tumor necrosis factors-α in ovarian cancer cell lines.

The data suggest the utility of additional studies to better characterize the role of interferon-γ in ovarian cancer because it seems correlated with iNOS gene expression to hypothesize additional therapeutic strategies in ovarian cancer patients.

We detected iNOS and COX-2 expression in the majority of the ovarian cancer samples tested; in particular, they showed a correlation with clinical outcome. In our series of cases that included only serous ovarian cancer with low grade of differentiation, G3, FIGO stage III, which had undergone the same surgical and adjuvant treatment, we showed that iNOS and COX-2 immunohistochemical expression can give us prognostic information for clinical outcome of the patient. In our cases, both iNOS and COX-2 positivity are associated with a shorter survival period, with a relapse or metastases of disease and also with disease-free interval shorter than 12 months [17,18]. Moreover, our study proves that both iNOS and COX-2-negative ovarian carcinomas are statistically correlated with clinical complete response to first-line chemotherapy.

The above results indicate the necessity to further investigate the status of iNOS and COX-2 in ovarian cancer to develop additional treatment options as more studies and clinical trials are performed.

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