

Research Article

Current Opinion in Gynecology and Obstetrics C-ERB-2, P53, Ki67 Proteins and Receptors of Estrogen and Progesterone on the Prognosis of Epithelial Ovarian Cancer

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Abstract

Ovarian cancer is the seventh most common cancer diagnosed in women worldwide. To date, many studies in epithelial ovarian cancer (EOC) have reported on the association HER-2/neu, p53 proteins and steroid hormones and their respective receptors with prognosis and/or the carcinogenesis process, but no definitive conclusion has been reached.

Objectives: To assess the proteins c-erbB-2, p53, Ki67 and receptors of estrogen (ER) and progesterone (PR) of EOC, with regard to clinical stage findings and its effect on survival.

Methods: 125 patients with a diagnosis of EOC treated by primary surgery and chemotherapy have participated. A surgical stage was noted and analyzed the correlation with c-erbB-2, p53, Ki67, ER and PR. Immunohistochemical analysis, using the anti-c-erbB-2, p53, Ki67 monoclonal antibodies, the antibody cod PR clone PgR and code ER-6-F11 Anti human estrogen. The c-erbB-2 study was complemented by genetic amplification and was reported univariate and multivariate analysis.

Results: Age 55.7 ± 16 ; 50.2% with residual disease (< 2 cm); initial (54.6%) and advanced (45.4%) stage. Univariate analysis showed positive staining for c-erbB-2, p-53, Ki67, PR and ER. The patients with negative receptors had a significantly shortened survival time ($p = 0.01$) than patients with positive receptors. Multivariable analysis revealed only clinical FIGO stage as an independent prognostic of overall survival ($p = 0.002$). Other variables like c-erbB-2, p53, Ki67, and ER were not significantly related to survival.

Conclusions: We concluded that patients with negative PR had a significantly shortened survival time than patients with positive receptors. The overexpression of markers c-erbB-2, p53, Ki67, and ER, were not significantly related to survival in EOC. Only the FIGO stage was achieved to be an independent predictor of overall survival. They should be evaluated together with the patient's clinical status and other prognostic factors.

Keywords: Epithelial ovarian carcinoma, Immunohistochemistry and CISH, Proteins: c-erbB-2, p53, Ki67, Steroid receptors, Prognosis

Introduction

Ovarian cancer is the seventh most common cancer diagnosed in women worldwide [1]. Approximately one-fourth of all gynecologic malignancies are of ovarian origin, and 47% of all gynecologic cancer-related deaths are due to ovarian cancer. Ovarian cancer carries the highest mortality among all gynecological malignancies. The high mortality is due mostly to the fact that the tumor is frequently diagnosed late, in advanced stages (III, or even IV), because the early stages are often asymptomatic, and no effective screening methods are available [2]. The average 5-year survival of patients in all stages of the disease is only 40%, in patients with the advanced disease only 10-20% [3]. Some studies reported a decreasing incidence in women, attributed to the use of an oral contraceptive pill [4,5].

It seems that there has been no significant decrease in incidence or mortality from ovarian cancer since the early 1980s, although the imagiologic exams had permitted diagnosed more and more the disease in early stages. In the absence of preventable etiologic factors or effective tools for screening, the only possible means of improving survival currently lies with the optimal management of patients after initial diagnosis. The prognosis of epithelial ovarian cancer can be correlated with biological (age), social (performance status) and clinical factors (tumor stage, histological grade, histological type, presence or absence of ascites; size and number of residual lesions after primary cytoreduction surgery, and chemotherapy) [6-8]. Identification of new prognostic factors might be useful in directing the therapy and intensifying the follow-up of a selected group of patients. A variety of prognostic factors have been reported, but their independent prognostic significance remains unclear. Immunohistochemistry has been widely used in the biomarkers search.

The oncoprotein c-erbB-2 (HER-2/neu; Neu), encodes a transmembrane glycoprotein that is member of the class I receptor tyrosine kinase family, which includes the epidermal growth factor, HER-2/neu, HER-3 and HER-4 [9]. Proto-oncogene HER-2/neu is located on chromosome 17 and is not activated by a point mutation but through amplification and overexpression of the wild-type gene. Amplification of the HER-2/neu oncogene may be observed in 20-30% of cases in a wide spectrum of neoplastic disorders (e.g., breast, lung carcinomas, others), and HER-2/neu overexpression has been associated with a poor prognosis of patients with cancer arising from other primary sites, but studies of ovarian cancer have

produced conflicting results [10-12]. Patients with breast carcinomas with amplified or overexpressed HER-2/neu can benefit from anthracycline-based regimens as well as Trastuzumab (Herceptin), a recombinant humanized monoclonal antibody against the Her-2/neu protein [13-15]. HER-2/neu is generally assessed as protein overexpression by using immunohistochemistry (IHC), and patients with tumors that either have 2+ or 3+ results with this method become good candidates for treatment with Trastuzumab.

However, studies indicate that HER-2/neu determined as gene amplification provides better prognostic information and is associated with a better response to Trastuzumab. HER-2/neu gene amplification is primarily detected by *in situ* hybridization and uses fluorescence (FISH) to detect the signals [16]. This method is expensive, it requires a fluorescence microscope, appropriate filters, and a sophisticated camera; so it is not practical as a screening tool. Chromogenic *in situ* hybridization (CISH) is a recently introduced method, and although it makes use of the *in situ* hybridization technology of FISH, it also takes advantage of the chromogenic signal detection of IHC that can be detected with the ordinary light microscope and has fewer costs [17,18]. CISH is potentially able to detect HER-2/neu gene amplification and to minimize, if not eliminate, the false positive fraction with the IHC procedure.

To date, many studies in epithelial ovarian cancer have reported on the association between HER-2/neu expression and outcome; some earlier studies reported that HER-2/neu overexpression was a poor prognostic factor, but later studies reported that HER-2/neu expression had no relationship with prognosis. Thus, no definitive conclusion has been reached as to the relationship between HER-2/neu expression and prognosis [14,19]. Just like of studies in breast also for the ovary, more studies should be conducted for studies in order to enhance the HER2 prognostic value and advantage therapy with monoclonal antibodies (Trastuzumab or other) [9,20].

P53 is a tumor suppressor gene (inhibit cell division and/or promote cell death/apoptosis) located on the short arm of chromosome 17 [21]. It suppresses cell growth by controlling entry into the S-phase of the cell cycle. Mutation or deletion of the p53 gene is believed to result in uncontrolled cell proliferation. Most p53 gene mutations result in stabilization of the protein. In contrast to the short half-life of the wild type p53 protein,

increased the stability of the mutant forms allows their detection by immunohistochemical techniques. Mutations of the p53 gene are the most common genetic abnormalities described in human cancers and have been implicated in the pathogenesis of several human tumors [22]. Mutations of the p53 have been found in approximately 40-80% of epithelial ovarian cancer cases. Studies have demonstrated an association between p53 protein overexpression and poor prognosis in patients with several tumor types. In epithelial ovarian carcinoma, the role of p53 protein is contentious, and there are a number of studies with contradictory results [23]. There are several studies that identified the p53 protein as an adverse prognostic factor for survival in ovarian cancer; others studies suggested that alterations in p53 expression in ovarian cancer can to affect the sensibility to chemotherapy [24]. In contrast, there are a number of studies that suggest that p53 expression has no prognostic value in epithelial ovarian cancer [25].

The proliferation activity of the tumor cell can be determined using a variety of methods, but many of these methods have significant technical limitations (DNA flow cytometry, DNA image cytometry, immunohistochemistry and others)[26]. Immunohistochemistry allows evaluating the Ki67 a nuclear non-histone protein expressed in cells in G1, S, G2, and M cell cycle phases, but absented from quiescent cells in G0. High cellular proliferative activity was associated with poor outcome. On the other hand, other studies did not confirm the relationship between proliferation activity and epithelial ovarian cancer prognosis [27].

Steroid hormones (estrogen and progesterone) are important hormones secreted by the ovary and acting through specific receptors. The interaction between steroid hormones and their respective receptors (estrogen receptor (ER) and progesterone receptor (PR)) are thought to play an important role in the process of carcinogenesis in gynecologic cancers as well as other primary tumors. Since ER and PR were first recognized as prognostic factors for breast cancer, much interest has been focused on steroid receptors in tumors thought to be related to gonadal hormones (endometrium, prostatic, ovarian cancer). ER and PR have been found in about 50% of ovarian tumors. Although the significance of their presence in the pathogenesis of epithelial ovarian tumors has not yet been defined [28], a role similar to that in breast cancer has been claimed, in that their presence seems to be inversely related to tumor differentiation but has not yet been confirmed the relationship. Tumor

expression of ER and/or PR, as well as their pattern of combinations (ER+/PR+, ER+/PR-, ER-/PR-), has been identified as predictive factors for response to endocrine treatment. Some studies found expression of PR to be an independent indicator of favorable prognosis in epithelial ovarian cancer [29,30]. However, other studies did not confirm these results.

Thus, we studied c-erbB-2 (ERBB2, HER-2/neu, neu), p53, Ki67 and steroid receptors (ER and PR) tumor expression and their possible prognostic value in epithelial ovarian cancer.

Objective

The objective was to evaluate the value of proteins c-erbB-2, p53, Ki67 and steroids receptors (ER and PR) in predicting long-term survival of patients with epithelial ovarian cancer.

Material and Methods

This retrospective study comprised one hundred and twenty-five patients with an epithelial ovarian cancer diagnosis and treated, at the Gynecologic Oncology Centre, Hospital Geral Santo Antonio, Porto, Portugal.

All patients were treated by multidisciplinary medical-surgical teams and by international protocols. All patients were staged according to the criteria of the International Federation of Gynecology and Obstetrics (FIGO) staging system I-IV, and after surgery received six courses of chemotherapy basis platinum.

In this series, all were invasive tumors. All histological sections were reviewed by reference pathologist and histological classifications were performed using the criteria defined by the World Health Organization (WHO). The tumors were graded according to the WHO histologic grading system as grade 1, 2 or 3. Clinical information was available for all patients (age, date of initial diagnosis, surgical stage, histological type, tumor grade, initial tumor volume, residual tumor volume, treatment, follow-up), and the date of death confirmed.

Immunohistochemical staining

The expression of markers was detected using the three-step streptavidin-biotin immunoperoxidase method. The monoclonal antibodies to human c-erbB-2 (1:100, CB11, Zymed), p53 (1:30, D07, Novocastra), Ki67 (1:20, MM1, Novocastra), ER (1:20, ER-6-F11, Novocastra), and PR (1:20, PGR, Novocastra) were used to identify the immune staining. Tissue sections from paraffin-

embedded tissue were deparaffinized in xylene, rehydrated through a downgraded alcohol series and washed with phosphate-buffered saline (PBS).

To improve antigen detection, the sections were pre-treated in a microwave oven (900 W) for 20 min in a 10 mM citrate buffer pH 6.0 or EDTA buffer pH 8.0, for the deferent's markers. After cooling, the sections were immersed in 3% hydrogen peroxide (H_2O_2) and distilled water for 30 min to block endogenous peroxidase activity. Nonspecific staining was eliminated by 60 min incubation. Excess normal serum was removed and replaced by primary antibodies used and incubated overnight (4°C) in a humidified chamber. After washing the slides, the sections were incubated in streptavidin-biotin-complex (HRP, Labvision Corporation) for 20 min at room temperature. Subsequently, the color was developed with 3, 3 diaminobenzidine tetrahydrochloride with H_2O_2 in PBS buffer for 5min. Slides were counterstained with Gill's hematoxylin, were dehydrated and mounted. Primary antibodies and biotinylated secondary antibodies were diluted in PBS. Negative controls were carried out by replacing the primary antibody with PBS. Paraffin sections from ovarian cancer with known immunoreactivity to c-erbB-2, p53, Ki67, ER, and PR antigens were used as positive controls. For each case, positively-stained tumor cells within five microscopic fields with the highest immunoreactivity ("hot spot" areas) were counted at high magnification (400x) using a 10×10 grid.

For the determination of c-erbB-2 protein overexpression, only the membrane staining intensity was evaluated. In samples in which CB11 were used as a primary antibody, intensity was graded as 0 (staining not greater than the negative control), 1+ (light staining - < 10%), 2+ (moderate staining -10 to 30%), or 3+ (intense staining - > 30%). The samples were considered to present c-erbB-2 overexpression when staining was intense (3+).

The study of c-erbB-2 was complemented by genetic amplification through the chromogenic *in situ* hybridization technique (CISH), with the following procedure: tissues 4-5 μ m thick were mounted on Histogrip-treated microscope slides, dried at 37°C, and baked for 2-4 hours at 60°C. The slides were deparaffinized for 15 min three times in xylene at room temperature (22-27°C) and washed for 2 min three times in 100% ethanol at room temperature. The slides were microwaved in SPOT-Light Tissue Heat Pretreatment Buffer for 10 min at 92°C and washed for 3 min twice in PBS. They were covered with 100 μ L SPOT-Light Tissue Pretreatment

Enzyme for 10 min at 37°C and washed for 2 min three times in PBS at room temperature. The slides were then dehydrated in 70%, 85%, 95%, and 100% ethanol for 2 min each, then air-dried. Denatured probe (15 μ L) was added to the center of each sample and covered with a 24 mm × 32 mm coverslip, the edges of which were sealed with a thin layer of rubber cement to prevent the evaporation of probe solution during incubation. The slides were denatured at 94°C for 3 min and placed in a dark humidity box for 16-24 hours at 37°C. After removal of the rubber cement and coverslip, the slides were immersed in 0.5 × SCC buffer in a Coplin jar for 5 min at 75°C. They were then washed for 2 min three times in PBS-Tween 20 buffer at RT. The slides were submerged in peroxidase quenching solution and then washed for 2 min three times with PBS, after which endogenous biotin blocking was performed with Reagent A (100 μ L of CAS Block). Using Zymed's SPOT-Light Detection Kit, 100 μ L each of fluorescein isothiocyanate-labeled sheep anti-digoxigenin, horseradish peroxidase-labeled goat anti-fluorescein isothiocyanate, and diaminobenzidine chromogen were sequentially added to the slides, with three 2 min rinses with PBS-Tween between the additions of reagents. The slides were counterstained with 150 μ L of Gill-2 hematoxylin and incubated for 3 min. They were then dehydrated with a graded series of alcohol, cleared in xylene, and mounted with a coverslip. The results of amplification were recorded as follows: two to four signs - lack of amplification; four to six signs - result misunderstanding; more than six signs - the presence of amplification. In all cases where there was amplified, the minimum number of signs was always clearly exceeding 6. The signs were or clearly distinct from each other or confluent, among themselves, in the stain.

Positive staining for p53 was nuclear. The reaction for p53 was considered positive when more than 25% of the tumors cells exhibited strong diffuse immunostaining. The Ki67 labeling index (LI) was calculated as the percentage of positive nuclei divided by the total number of cells examined. At least 1000 cells per tumor were examined. Staining in more than 10% (LI) of the tumor cells was considered positive. For the ER and PR, the percentage of tumor cells that exhibited nuclear staining for a particular receptor regardless of intensity was considered positive when more than 10% of cells showed stained.

Statistical analysis

The statistical analysis was done using the SPSS statistical package for Windows, version 22 (IBM SPSS, Chicago, IL). For univariate analysis, survival time was

analyzed by the Kaplan-Meier method, and the log-rank test was used to assess differences among groups. For multivariate analysis, Cox proportional hazard regression model was used to examine all factors found to be predictive of survival in univariate analysis simultaneously. Associations between tested parameters were studied by Spearman rank correlation. Differences were considered statistically significant at $p = 0.20$.

Results

The mean age of 86 patients was 55.7 ± 16 years (range 23-85 years). The median follow-up of patients was 70.5 months. In this series, the FIGO's stage I and II were 47 cases and stage III and IV were 39 cases. Concerning the survival rate, the group I and II were grouped and groups III and IV as well. Overall survival was defined as the time from diagnosis until death or the date of the last follow-up.

The immunohistochemical results (N and %) for Ki67, p53, PR, and ER are presented in table 1.

The study of HER-2/neu in our series was some difficulties arising largely because the older cases have had histologic, stages processing more or less time-consuming Bouin fixing, which is an excellent fixative for certain tissues (e.g., for lymphomas), but it has the disadvantage of hamper studies immunocytochemistry, as occurs with the HER-2/neu. This limitation is felt in studies of immunocytochemistry and much more obvious becomes when we perform molecular genetic studies, such as CISH. Therefore, only 42 cases to obtain a material with an acceptable quality of fixing to perform immunocytochemistry and CISH. The results are shown in table 2, which underline the following data: of the 42 cases included 7 had intensity of positivity 2; 8 with the intensity was 3. In the other, the intensity 1 was 22 cases and 0 presented 5 cases.

In all cases, where the intensity of reaction was 0 or 1, gene amplification was through CISH. In 7 cases in which the intensity of positivity was 2, there were 4 amplification and absence in the other 3. In 8 cases, where the intensity was 3, all gene amplification showed to CISH. So, the concordance between 3+ IHC and CISH-amplified cases was 100%, denoting all gene amplified cases to be overexpressing the HER-2/neu protein.

The entire cases with gene amplification and thus positive, for HER-2/neu, was 12 in 42, that is 28.57% of cases. We have not found an association between

overexpression/amplification (IHC/CISH) and prognosis. We noted that IHC/CISH-positive cases, as well as CISH-positive-only cases, had the same prognosis regarding survival, whereas IHC-positive-only cases had a prognosis similar to that of IHC/CISH-negative tumors.

Table 1: Immunohistochemical: Ki67, p53, PR and ER.

	N	%
Ki67		
Negative	68	54.4
Positive	30	24
Unknown	27	21.6
N=28		
(overall±standard deviation)	23.92 ± 15.71	
Median		20
Min; Max	10; 60	
Variation Interquart.		30
P53		
Negative	67	53.6
Positive	52	41.6
Unknown	6	4.8
N=52		
(overall±standard deviation)	62.07 ± 21.84	
Median		70
Min; Max	10.0; 90.0	
Variation Interquart.		35
PR		
Negative	98	78.4
Positive	24	19.2
Unknown	3	2.4
N=24		
(overall±standard deviation)	38.96 ± 24.75	
Median		37.5
Min; Max	10.0; 90.0	
Variation Interquart.		47.5
ER		
Negative	99	80
Positive	26	20
N=26		
(overall±standard deviation)	36.73 ± 22.71	
Median		30
Min; Max	10.0; 90.0	
Variation Interquart.		42.5

The amplification evidenced by CISH was the second two patterns: or present themselves as signals and points individually and independently; or existing between

themselves, presenting itself as a stain (Figures 1 and 2).

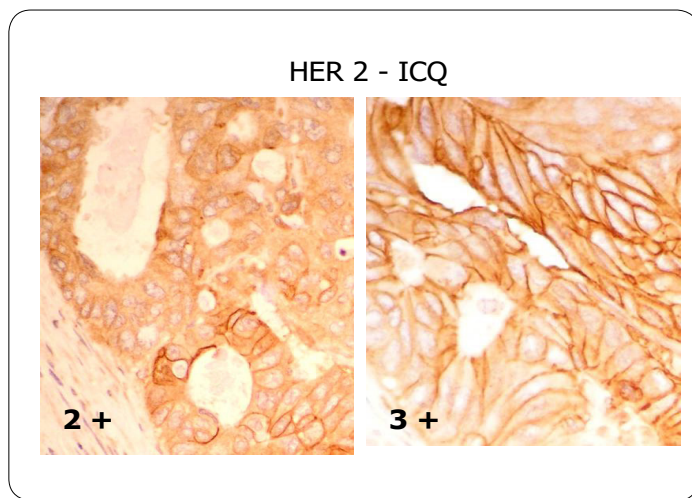


Figure 1: HER-2/neu - Immunocytochemistry (IHC)- (400x).

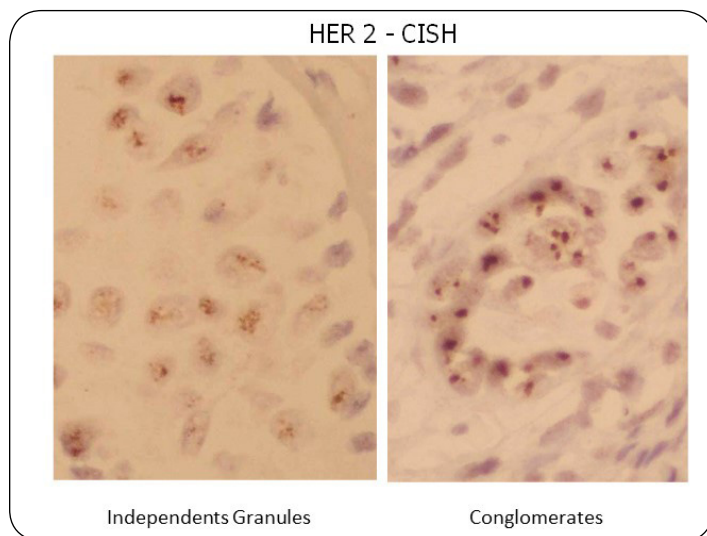


Figure 2: HER-2/neu- Chromogenic *in situ* hybridization (CISH).

Results for FIGO stage showed that this variable correlated significantly with survival ($P = 0.002$) (Table 3 and Figure 3).

In univariate analysis, the survival time was longer in patients with stage I-II disease than in those with stages III-IV disease (Cox $p = 0.002$). In the first group (I-II) the overall survival was 179.7 months (IC = 143.2-216.2) and in the second group (III-IV), the overall survival was 73.4 months (IC = 39.4-107.0).

The univariate analysis did not show an association of HER-2/neu protein expression with overall survival. Also, in the multivariate analysis the overexpression/ amplification protein was not proven as an independent prognostic indicator.

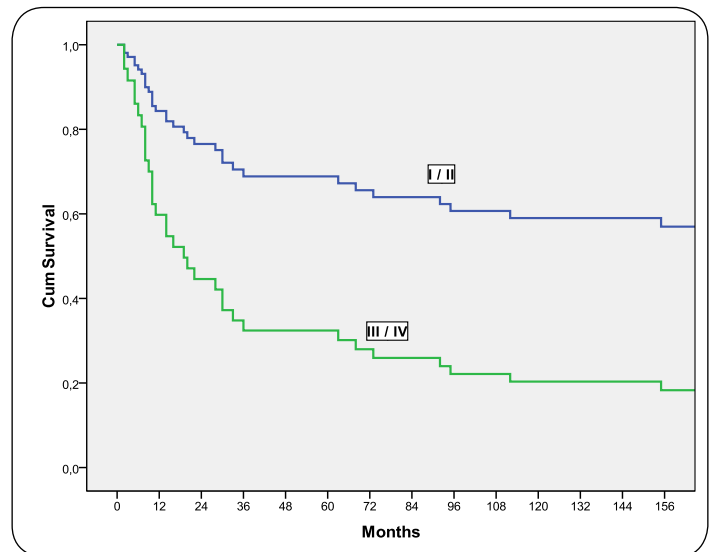


Figure 3: Overall survival of patients with epithelial ovarian carcinoma related to the FIGO stage.

The survival in relation to markers HER-2/neu, p53, Ki67, and steroids receptors (ER and PR), the univariate analysis of clinical follow-up data revealed that patients with negative PR, had a significantly shortened survival time ($p = 0.01$) than patients with positive (Table 3 and Figure 4).

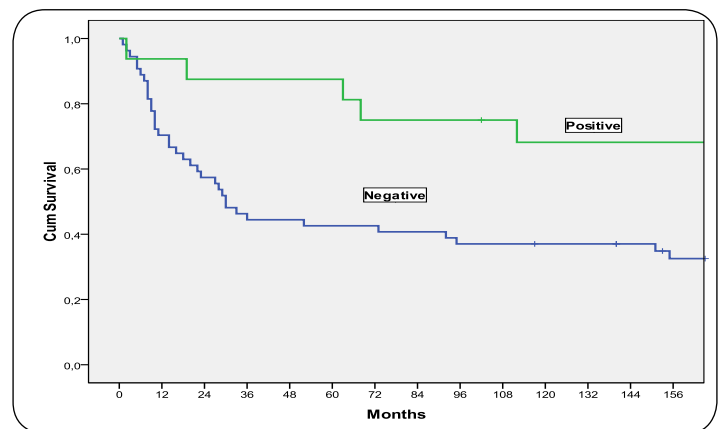


Figure 4: Overall survivals of patients with epithelial ovarian carcinoma relative to progesterone receptor.

Table 2: HER-2/neu: Immunohistochemistry and CISH.

N° of cases	HER-2/neu (ICQ)				CISH (AMPLIFICATION)				
	0	1+	2+	3+	0	1+	2+	3+	Total
42	5	22	7	8	0	0	4	8	12

Other variables like HER-2/neu, P53, Ki67 and ER were not significantly related to survival (Table 3).

Table 3: Univariate survival analysis (Kaplan-Meier)

Variables	Unadjusted relative risk	P value
	(95% CI)	
FIGO		
I+II	1	0.002
III+IV	3.02 (1.50-6.06)	
HER-2/neu		
Negative	1	0.46
Positive	1.55 (0.48-5.02)	
P53		
Negative	1	0.17
Positive	1.52 (0.83-2.79)	
Ki67		
Negative	1	0.45
Positive	1.28 (0.67-2.47)	
PR		
Positive	1	0.01
Negative	3.20 (1.25-8.16)	
ER		
Positive	1	0.58
Negative	1.26 (0.56-2.83)	

In the multivariable statistical analysis only FIGO stage ($P = 0.002$) was achieved to be independent predictor of overall survival (Table 4). None of the other variables showed any independent predictive value for patient prognosis.

In this study, the p53 suppressor gene showed a positive percentage (41.6%) and the univariate analysis did not show an association of p53 overexpression and prognosis (with overall survival) (Table 1). Also, the Multivariate analysis not showed that the p53 overexpression protein was not proven as an independent prognostic indicator.

Other marker studied by immunohistochemical, the proliferative marker Ki67 (with positive percentage, 24.0%) did not show association Ki67 expression with overall survival by the univariate analysis, as well as in multivariate analysis, the Ki67 was not proven as an independent prognostic indicator (Table 1).

The percentage of PR positive was 19.2% and ER positive 20.0%. We found that PR was associated with a better prognosis. Univariate analysis of clinical follow-up data revealed that patients with negative PR, had a significantly shortened survival time ($p = 0.01$) than Cur Op Gyn Obs, 2(1): 169-179 (2019)

patients with positive (Table 3 and Figure 4). The same was not observed with the ER.

Table 4: Multivariable analysis of survival time using cox's regression proportional hazards model for identification of independent prognostic factors for patients with ovarian carcinoma.

Variables	Adjusted Relative risk	P value
	(95% CI)	
FIGO		
I+II	1	0.02
III+IV	2.52 (1.13-5.61)	
PR		
Positive	1	0.16
Negative	2.02 (0.75-5.44)	
P53		
Negative	1	0.94
Positive	1.03 (0.48-2.20)	

Discussion

Wide substantial progress has been made and although more and more patients are living longer with their disease, the majority of patients with advanced ovarian cancer are not cured. The prognosis of ovarian cancer is discouraging compared to other malignancies of the female genital tract. Despite aggressive surgery and intensified chemotherapy, the outcome of patients with stage III and IV is poor. The importance of staging with regard to survival also stems from the influence it has on subsequent patient management. The prognostic value of stage according to the FIGO has been well established [8]. In a surgical staging procedure, there is some controversy over some aspects, especially in the early stages (I-II), regarding the status of retroperitoneal lymph nodes. When is presumed FIGO I and II stage, the extensive surgical staging showed that tumors were a more advanced stage, in general stage III. The "aggressive surgical staging" cannot significantly change the survival of the patients, but it can increase the operation risk [3,31]. In our study, unlike the great majority of the series, the percentage of early stages (I-II) cancers was higher compared to the advanced stage cancers. This is due to many of these cases been referred to the hospital. Most of the patients were diagnosed in routine gynecologic exams or by other specialties after the realization of imagiologic exams, for different clinical indications. In fact, nowadays, it seems like patients with ovarian cancer may be diagnosed at early stages, as a result of better primary care by general practitioners. Nevertheless, we still diagnose a great number of advanced stage cancers,

although this number may be decreasing.

In this study, only the FIGO stage was achieved to be an independent predictor of overall survival by the multivariable statistical analysis. None of the other variables showed any independent predictive value for patient prognosis. The stage is reorganized as the most important prognostic factor in epithelial ovarian cancer [8,32,33].

Valid prognostic factors are necessary to estimate the course of the disease and to define biologically similar subgroups for the analysis of therapeutic efficacy [32,34]. Many studies have been devoted to finding "Prognostic Factors", and numerous features have been described that can help predict the prognosis of early and advanced ovarian cancer with varying degrees of accuracy and may provide a better understanding of the biological behavior of ovarian tumors. Immunohistochemistry has been widely used in the search of such markers [35-37].

The HER-2/neu expression can be determined through IHC, FISH, CISH, and ELISA among other tests. This oncogene has been studied mainly in breast cancer by IHC and FISH where it has a prognostic, predictive and therapeutic target value. The HER-2/neu expression in epithelial ovarian cancer has been less studied and we studied the overexpression by IHC and the amplification by CISH, a promising practical alternative to FISH. After the first CISH study by Tanner and colleagues [38] other reports favorably validated CISH results and the concordance between CISH and FISH [17,39-41]. Some reports noted the advantages of CISH over FISH; because CISH is a specific, sensitive, and easily applicable method for the detection of HER-2/neu gene amplification, and it can be used together with IHC for the evaluation of patients with breast carcinoma [17,18]. In this study, different series reported positivity frequencies of overexpression, it may be attributable to the type of material analyzed (fresh or embedded in paraffin) and to differences in specificity among antibodies used. Enzyme and microwave treatment of the tissue during the staining process may greatly affect the staining results, and tissue fixation procedures may also influence immunostaining. Different scoring methods and subjective interpretation of immunohistochemical analysis may also be reasons for different results obtained by different studies.

In our study, the percentage positive of HER-2/neu has not found an association between overexpression/amplification (IHC/CISH) and it was not proven as an

independent prognostic indicator. Researchers have been trying to assess the real prognostic significance of HER-2/neu in ovarian cancer, but the results of their studies have been controversial. Some earlier studies reported that HER-2/neu overexpression was a poor prognostic factor [42,43], but later studies reported that HER-2/neu expression had not any relationship with prognosis [11,12,44,45]. Thus, no definitive conclusion has been reached as to the relationship between HER-2/neu expression and prognosis.

The same happens with the role of the p53 protein in epithelial ovarian cancer, several studies have identified the p53 protein as an adverse prognostic factor for survival in epithelial ovarian cancer [46]. However, other studies suggest that p53 expression has not prognostic value in epithelial ovarian cancer [47]. De Graeff and collaborators showed in meta-analysis that the outcome of analysis was influenced by FIGO stage, for example: in some studies the p53 overexpression was associated with shorter survival of patients in stage I and II, but not in advanced stage III and IV; in other studies, found contradictory results, with shorter survival in an advanced stage but not in the early stage of the tumors with p53 overexpression [47]. In our study, we don't find any association of the p53 overexpression with the predictive value of overall survival in early stage or advanced stage.

As for the other markers studied by immunohistochemical, we used the proliferative marker Ki67 that did not show association Ki67 expression with overall survival, as well as the Ki67 was not proven as an independent prognostic indicator. Studies on DNA content and proliferation in epithelial ovarian cancer have contradictory results regarding the prognostic significance of these parameters. Authors showed that ovarian cancer had a higher median percentage of Ki67 staining than borderline and benign tumors, and they found a significant relationship between the proliferative marker Ki67 and disease-free survival that was independent the others parameters as histologic type, histologic grade, and stage [48]. Others authors also observed that Ki67 is a marker that differs in expression significantly between the short and long-term survivors [14,48]. Poor outcome was associated with the high proliferative activity, however series of studies did not confirm the relationship between proliferative activity and prognosis in epithelial ovarian cancer.

In our study, the percentage of PR was associated with a better prognosis, the univariate analysis of clinical follow-

up data revealed that patients with negative PR, had a significantly shortened survival time than patients with positive. The same was not observed with ER. Therefore, some of the latest studies of the analysis of ER and PR status showed that steroids receptors have significant favorable prognostic value in ovarian cancer. Especially progesterone positive tumor receptor status is proved to be an independent prognostic variable of improved progression for free-survival among patients with ovarian carcinoma. This anti-tumor effect of PR (significant survival benefit) may be due to two hypotheses that have been proposed. Estrogen-responsive cells efficiently repair DNA and avoid apoptosis; progesterone promotes cell differentiation and apoptosis, and stimulation of PR inhibits DNA synthesis and cell division. What justified the theory, proposed to explain the causal mechanism of carcinogenesis, the hypothesis "incessant ovulation", that argues that repeated cycles of ovulation-induced trauma and repair of the ovarian surface epithelium (OSE) at the site of ovulation. According to with this hypothesis, the protective effect of progesterone will be: decreasing the exposure of the OSE to high levels of estrogens; antagonizing the growth-promoting effect of estrogens on OSE; inducing the apoptosis of tumor cells [49]; loss chromosome 11q23.3-24.3 decreasing PR expression and elevated risk for ovarian cancer and poorer prognosis. So, this may explain why patients with ER + and PR- tumors have the worst and patients with ER- and PR+ tumors the best prognosis. It is important to identify reliable prognostic markers, such as PR. So, maybe possible targets of therapy.

Conclusion

Although this study, with a relatively small number of cases, we conclude that patients with negative PR had a significantly shortened survival time ($p = 0.01$) than patients with positive. The overexpression of markers HER-2/neu, p53, Ki67 and ER, were not significantly related to survival in ovarian carcinoma. CISH is a promising, practical alternative to FISH that can be used in conjunction with IHC, which remains the first screening procedure of choice. IHC is easy to perform, relatively inexpensive, and able to detect a majority of ovarian cancer patients whose tumors have negative (0 or 1+) or positive (3+) HER-2/neu status, all three of which have complete concordance with CISH.

Only FIGO stage was achieved to be an independent predictor of overall survival. They should be evaluated together with the patient's clinical status and other prognostic factors.

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