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The Role of NF κ B, HIF-1 α and Neuropeptide (ET-1) - Peptidase (CD10, NEP) Axis in Ovarian Cancer: An Immunochemistry Approach

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Abstract

Introduction: Ovarian cancer is considered to be the leading cause of death due to gynecological malignancies. Because of lack of effective screening methods, most patients are diagnosed at an advanced stage. Therefore, there is a mandatory need to develop new markers for early detection and prognostication as well as for response to treatment and detection of disease recurrence after definitive therapy.

Aim: Our study was designed to analyse immunohistochemically the expression of transcription factors NFκB and HIF-1α, in addition to biomarkers of endothelin axis (CD-10, ETAR and ETBR) in patients with ovarian cancer and relate their expression with overall survival and response to therapy.

Methods: Ninety four samples from paraffin-embedded tissues from patients with ovarian cancer were included. The final analysis of the samples compared two cohort groups that were dichotomized to positive and negative according to histoscore (either nucleoscore or cytoscore) of each patient.

Results: Patients expressing high NFκB nuclear score, high HIF-1 α nuclear score, low CD-10 score and high ETAR and ETBR scores had worse overall survival. Moreover, platinum sensitive patients expressed lower nuclear NFκB, higher cytoplasmic HIF-1α, higher CD-10 and lower ETAR expression. NFκB cytoplasmic score and ETAR score were correlated with disease of early stage, while CD-10 histoscore was associated with advanced stages. More importantly, a combination of specific biomarkers was correlated with OS of patients.

Conclusion: Our study confirms the prognostic value of CD10 in ovarian carcinoma through its association with endothelin axis, while the last one (ET axis), mediated mainly through ETAR, may also have clinical and therapeutical impact. Finally, the nuclear expression of known transcriptional factors such as HIF-1α and NFκB could be prognostic biomarkers to assess clinical outcome and possibly predict resistance to platinum-based chemotherapy.

Keywords: Breast cancer; Immunohistochemical; Ovarian cancer; NFκB; HIF-1α

Introduction

Ovarian cancer is the leading cause of gynecological cancer mortality worldwide. Approximately two thirds of the patients present with advanced stage disease (stage III or IV) at diagnosis [1]. As invasion and metastasis at the time of diagnosis significantly worsen the prognosis of survival of patients (<50% with a 5-year survival rate) [1], efforts to detect biomarkers that may be clinically relevant to cancer progression and response to current therapies are warranted.

One of the major transcription factors which has been investigated in various diseases including cancer is NF-κB (nuclear factor-kappa B). The central role of NF-κB expression in normal conditions is focused on the control of biochemical pathways promoting growth, apoptosis and differentiation. NF-κB seems to be activated in various types of cancers. In addition NF-κB can stimulate transcription of a big number of regulating genes involving in angiogenesis, metastasis and other biological responses [2]. There is evidence that NF-κB is implicated in oncogenesis in many types of cancers [3] promoting cell survival, angiogenesis and metastasis leading to cancer progression and resistance to chemotherapy in various solid tumours [4].

In addition, oxidative stress has been implicated in the pathogenesis of tumour progression and vascularization [5]. Hypoxia-inducible factors (HIF) are master regulators of the cellular response to hypoxia. Hypoxia-inducible factor 1 (HIF-1), a heterodimer composed of HIF-1aα and HIF-1b subunits, is the major key molecule which can help the hypoxic cells to cope with hypoxia and plays a critical role in tumorigenesis, angiogenesis and apoptosis [6].

Endothelin (ET-1) and its receptors have also been implicated in cancer development and progression through both autocrine and paracrine pathway. ET-1 is produced mainly in endothelial cells and secondary in vascular cells and acts through two distinct subtypes of receptors, ETAR and ETBR [7]. Upon activation, the receptors mediate a variety of signalling involved in the control of cell proliferation,

survival, migration and invasion [8]. Regarding ovarian carcinoma, it has been demonstrated that expression of ET-1 is significantly increased in carcinomas compared to normal ovarian tissues [9]. ET-1 selectively acts mainly through the endothelin A receptor (ETAR), and is involved in cell proliferation, invasiveness, neovascularization and prevention of apoptosis, thus promoting carcinogenesis [10].

In addition, CD10 (also known as CALLA-common acute lymphoblastic leukemia antigen or NEP- neutral endopeptidase), a T-cell differentiation antigen has been reported to be involved in tumour progression in certain human malignancies including ovarian carcinoma. Specifically, neutral endopeptidase (NEP) is a cell surface peptidase with an ubiquitous expression involved in the catalytic degradation of a number of bioactive peptides including ET-1 [11].

NEP is involved in neoplastic transformation and tumor progression in certain human malignancies including lung, breast, prostate and ovarian carcinomas by inactivating ET-1 which is considered to be an autocrine growth factor for these tumors. As a result, loss or decrease in NEP expression allows the presence of its peptide substrates (including ET-1) on cell surface resulting in unopposed signalling through their cognate receptors and thus facilitating progression of neoplasia [12].

Until now, only few data exist in the literature on the impact of NF-κB, HIF-1a, CD10 and ETR expression on prognosis of ovarian cancer, whereas most data derive from *in vitro* studies in cancer cells cultures.

The aim of our study was to demonstrate *in vivo*, by using paraffin embedded tissue and an immunohistochemistry approach, if the expression of those markers has any prognostic or even predictive value in patients with ovarian cancer treated with platinum-based chemotherapy.

In the era of personalised medicine and targeted therapies, identification of target-drugable molecules could be of paramount importance in an effort to increase efficacy of the applied therapy while minimizing toxicity in large patient population. Because of our previous research experience in which we have shown that the NFκB/ETAR/NEP (CD10) pathway involved in prostate cancer, we sought to investigate the potential involvement of these interacting pathways in ovarian cancer, in order to define prognostic but more importantly predictive factors carrying the potential of drugability.

Materials and Methods

A total of 94 consecutive patients diagnosed with ovarian cancer at the Department of Medical Ongology at the University Hospital of Larissa were included in the study. The median age of patients at the time of surgery was 52.3 ± 12.1 years. The histological types were as follows: serous (n=54, 57.4%), mucinous (n=9, 9.6%), endometrioid (n=9, 9.6%), clear cell carcinoma (n=2, 2.1%), mixed cell type (n=15, 16%) and other uncommon types (n=5, 5.3%). Tumors were classified and staged after surgery (if surgery was performed) as I, II, III and IV in 23 (24.5%), 7 (7.4%) 59 (62.8%) and 5 (5.3%) of the cases, respectively. Stage and grade were determined according to the International Federation of Gynecology and Obstetrics standards. At the end of follow up period, 41/94 (43.6%) were deceased due to their disease.

Patients received postoperatively 6 cycles of paclitaxel 175 mg/m^2 and carboplatin after calculating the area under the concentration curve (AUC:6) every 3 weeks. Among 94 patients, 17 (18.1%) developed resistance to platinum based chemotherapy, while the remaining 77 (81.9%) were platinum-sensitive (defining as platinum sensitive the disease with progression or relapse at least 6 months after the end of adjuvant or 1st line platinum- based chemotherapy).

Response to chemotherapy was evaluated as follows: No evidence of disease was defined as the complete response of the disease for at least 4 weeks, (confirmed by physical examination, ultrasound or computed scan tomography), while progressive disease was defined as an increase of at least 25% in the size of the measurable lesion or the appearance of an unequivocal new lesion after beginning of chemotherapy. The clinical and demographic data of the patients summarized in Table 1.

	n=94	%
Stage		
I	23	24.5
II	7	7.4
III	59	62.8
IV	5	5.3
Type		
Serous	54	57.4
Mucinous	9	9.6
Endometrioid	9	9.6
Clear cell carcinoma	2	2.1
Mixed cell type	15	16
Uncommon types	5	5.3
Sensitive to platinum	77	81.9
Resistance to platinum	17	18.1
Status		
Dead	41	43.6
Alive	53	56.4

Table 1: Clinicopathological parameters of patients with Ovarian cancer included in the study.

Immunohistochemical staining of ovarian tumour tissue (paraffin blocks)

Tissue samples of ovarian cancer which were previously fixed in 10% buffered formalin, processed and embedded in paraffin routinely and stored where used for our study. Sections were cut at 3 μm by using a Leica microtome TP1020 and dried overnight at 60°C.

In order to prepare sections for immunohistochemistry, after a first step of deparaffinization using xylene, the sections were rehydrated in decreasing ethanol solutions. After that, dilutions of 0.3% hydrogen peroxidase for 10 min were used to block endogenous peroxidase activity. Then, optimal antigen retrieval was achieved by microwaving tissue sections in 0.01 M citrate buffer solution (pH 6) for 20 min. Finally, sections cooled and washed in Tris Buffer Saline (TBST) for three times.

Tissue sections were incubated with each antibody at room temperature. Immunostaining procedure was performed with the antibodies that listed in Table 2. Immunostaining was performed in all patients (n=94) for NFκB, HIF-1a and CD-10 detection while for ETAR and ETBR detection was performed in only 70 samples. The incubation period was 30 min for NFκB, HIF-1a and CD-10, and 60 min for ETAR and ETBR. After the immunostaining, slides were washed in TBST and a sensitive detection fluid was added (a novel system of non-biotin polymeric technology containing 2 major components: Super Enhancer and a Poly-HRP reagent in order to eliminate problems associated with endogenous biotin) (Biogenex), followed by incubation for 50 min. The antibodies that were bound, were visualized by using 0.05% 3,3'-diaminobenzidine solution (DAB solution, DAKO). Finally, the enzyme horseradish peroxidase (HRP) catalyzes the conversion of chromogenic substrates (e.g., DAB, AEC) into coloured products facilitating in this way tissue staining. At a final step, sections were counterstained with hematoxylin.

Antibody	Optimal dilution	Company
NFkB p65, clone F-6	1:500 for 30 min	Santa Cruz
HIF-1A, clone H1alpha	1:25 for 30 min	Novus
CD10, clone 56C6	1:30 for 30 min	Dako
ETAR, clone N-15	1:100 for 60 min	Santa Cruz
ETBR, clone A-20	1:100 for 60 min	Santa Cruz

Table 2: Antibodies used in this study.

All slides were reviewed blindly and independently by two pathologists. The median values of results were used for all further calculations. If differences of more than 30% between observers occurred, these slides were re-assessed by both investigators and a final consensus score was obtained for further statistical analysis.

Determination of NFκB/p65 and HIF-1a was performed in both cytoplasmic and nuclear compartment. ETAR and ETBR detection was membranous while CD10 immunostaining gave a diffuse cytoplasmic or membranous staining pattern.

Intensity of immunoreactivity for each immunostaining marker was graded as follows: 0 – none (no detectable immunostaining on tumor cells), 1-weak (weak staining in the majority of tumor cells), 2-moderate (medium staining intensity of tumor cells), and 3-strong (high staining intensity of tumor). The prevalence of neoplastic cells with cytoplasmic staining was approximately determined in the entire tumour area, while the percentage of nuclear staining was determined by counting positive tumour nuclei in 500 tumour cells. The overall immunohistochemical score (histoscore) was expressed as the percentage of positive tumour cells (100%) multiplied by their staining intensity (0=negative, 1=weak, 2=moderate, 3=strong). Therefore, the total histoscore for each marker ranged from 0 to 300.

Statistical Analysis

All numerical values are expressed as mean ± SD or median according to their distribution (normally and non-normally respectively). Data were analyzed by t-test, $\chi 2$ test (two-by-two with Yates' correction) and Fisher's exact test where applicable. Each p-value below 0.050 was considered statistically significant.

Overall survival (OS) was defined as the length of time from either the date of diagnosis or the date of surgery, that patients diagnosed with ovarian cancer are still alive, while disease-free survival (DFS) was defined as the period until the patient survives without any signs of reappearance of the disease (symptoms or new tomography findings). These two parameters were used to check the probability of survival using the Kaplan-Mayer method. All parameters that were significant by Kaplan-Mayer analysis (univariate analysis, $p<0.050$) where used in a multivariate Cox regression model in order to identify possible independent risk factors. Statistical analyses were performed using SPSS version 17.0 (IBM, Armonk, New York, USA).

Results

Mean ± SD histoscore expression of each protein was as follow; NFκB cytoscore: 219.4 ± 63.9, NF-κB nucleoscore: 32.7 ± 46, HIF-1a cytoscore: 119.6 ± 81.5, HIF-1a nucleoscore: 108.9 ± 84.3, CD-10 score: 30.8 ± 58.2, ETAR score: 84.1 ± 112.1, ETBR score: 48.1 ± 70.3. The final results for analysis were performed after the patients were dichotomized in two parts based on histoscore of each marker (according to the median value of each marker). Overall immunohistochemical staining results are summarized in Table 3.

Histoscore	Sample (n=94)	%
NFkB cytoscore Mean ± SD	219.4 ± 63.9	-
SCORE <200/>200	39/55	41.5/58.5
NFkB nucleoscore Mean ± SD	32.7 ± 46	-
SCORE <25/>25	32/62	34/66
HIF-1a cytoscore Mean ± SD	119.6 ± 81.5	-
SCORE <75/>75	35/59	37.2/62.8
HIF-1a nucleoscore Mean ± SD	108.9 ± 84.3	-
SCORE <75/>75	31/63	33/67
CD-10 score Mean ± SD	30.8 ± 58.2	-
SCORE <25/>25	69/25	73.4/26/6
ETAR score Mean ± SD	84.1 ± 112.1	-
SCORE <75>75	44/26	62.9/37.1
ETBR score Mean ± SD	48.1 ± 70.3	-
SCORE <75/>75	47/23	67.1/32.9

Table 3: NFκB and HIF-1α cytoplasmic and nuclear histoscore, CD-10, ETAR and ETBR histoscore value.

Correlation between overall survival (OS) and histoscore of each biomarker

Despite using different cut-off points of histoscore for positivity, cytoscore of NFκB was not associated with any gain considering OS of patients. On the contrary, nuclear NFκB expression was inversely correlated with OS (low NFκB nucleoscore was associated with increased OS) regardless of stage. In particular, patients with NFκB nucleoscore less than 25 had an OS 113.5 ± 9.1 months compared to 88.1 ± 7.4 months of patients with nucleoscore >25, (p=0.048) (Figure 1).

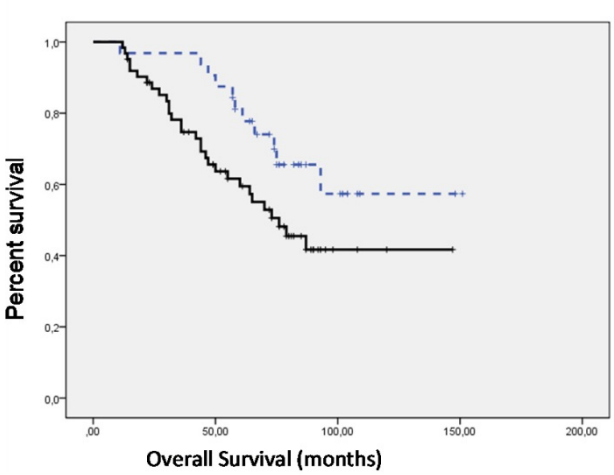

Figure 1: High NFκB nuclear expression correlates with a clinically aggressive tumor phenotype. The Kaplan-Meier survival curves show that patients with high histoscore (black solid-line) had a reduced overall survival compared to patients with low NFκB nucleoscore (blue dot-line) (88.1 ± 7.4 vs. 113.5 ± 9.1 months, p=0.048).

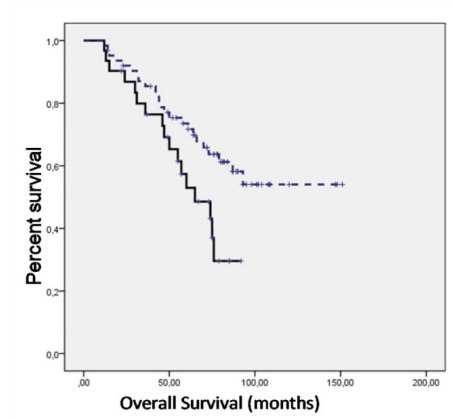

Figure 2: High HIF-1α nuclear expression correlates with a clinically aggressive tumor phenotype. The Kaplan-Meier survival curves show that patients with high histoscore (black solid-line) had worse overall survival compared to patients with low HIF-1α nucleoscore (blue dot-line) (62.2 ± 5 vs. 105.6 ± 7.2 months, p=0.029).

Cytoplasmic HIF-1a expression was also not associated with OS of patients in any comparison (using different cut-off points of histoscore for positivity), while nuclear expression was inversely associated with OS (low HIF-1a nucleoscore was associated with high overall survival). Specifically, patients with HIF-1a nucleoscore less than 75 had an OS 105.6 ± 7.2 months compared to 62.2 ± 5 months of patients with nucleoscore >75 (p=0.029) (Figure 2).

CD-10 expression was strongly correlated with OS (cut off value: 25 histoscore). Specifically, patients with low CD-10 expression (histoscore<25) had a worse OS 86.8 ± 6.8 compared to 128.1 ± 9 months of patients with high CD-10 expression (histoscore>25) (p=0.011) (Figure 3) regardless of the stage.

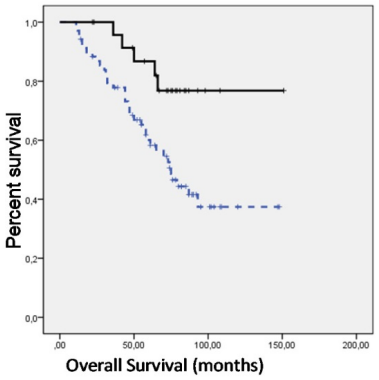

Figure 3: Low CD-10 expression correlates with a clinically aggressive tumor phenotype. The Kaplan-Meier survival curves show that patients with high histoscore (black solid-line) had better overall survival compared to patients with low histoscore (blue dot-line) (cut off value: 25).

Finally, ETAR expression was strongly correlated with OS. Patients with low ETAR histoscore had better OS than patients with high ETAR histoscore (cut off value: 75 histoscore) 113.5 ± 7.7 vs. 66.4 ± 6.9 months p=0.026 (Figure 4).

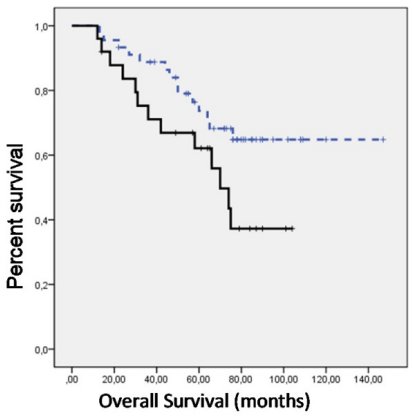

Figure 4: High ETAR expression correlates with a clinically aggressive tumor phenotype. The Kaplan-Meier survival curves show that patients with low histoscore (blue dot-line) had better overall survival compared to patients with high histoscore (black solid-line) (cut off value: 75).

On the contrary, ETBR expression was not associated with OS of patients with ovarian cancer. However, there was a trend for better OS for those patients with ETBR histoscore<75 vs. patients with histoscore >75 (110.3 ± 7.7 vs. 69 ± 7.1 months, p=0.076) (Figure 5).

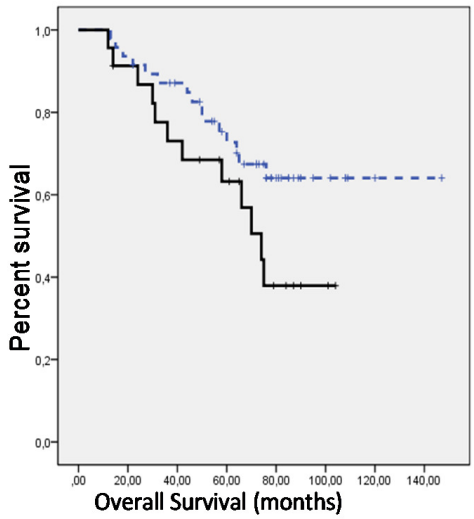

Figure 5: Expression of ETBR and OS. The Kaplan-Meier survival curves show that patients with high histoscore (black solid-line) had a trend to be associated statistically with reduced overall survival compared to patients with low score (blue dot-line) (110.3 ± 7.7 vs. 69 ± 7.1 months, p=0.076).

In conclusion, our statistical analysis shows that low NFκB, HIF-1a, ETAR and ETBR expression and high CD-10 expression (implying lower levels of ET-1) correlated with longer overall survival of patients with ovarian cancer.

Expression of biomarkers and correlation with platinum-based chemotherapy response

Histoscore		Chemotherapy Sensitivity: Resistant	Sensitive	p value
NFkB nucleoscore	<25	2	30	0.046
	>25	15	47	-
HIF-1a cytoscore	<75	10	25	0.042
	>75	7	52	-
CD-10 score	<25	17	52	0.004
	>25	0	25	-
ETAR score	<75	6	38	0.063
	>75	8	18	-

Table 4: Correlations between the histoscore of various biomarkers and response to chemotherapy.

Our research indicates that histoscores of various biomarkers was significantly correlated with chemotherapy responses. In general, platinum- sensitive patients had lower nuclear NFκB, higher cytoplasmic HIF-1a, higher CD-10 and lower ETAR expression (Table 4). Histoscores of cytoplasmic NFκB, nuclear HIF-1a and ETBR were not correlated with response to chemotherapy (data not shown).

Correlation between histoscore and stage of disease

NFκB cytoscore (<100 vs. >100) was positively correlated with early stage of disease (I vs II-IV, p=0.058). Furthermore, high CD-10 histoscore (<50 vs. >50) was associated with advance stage of disease (I vs. II-IV, p=0.045). In addition, lower ETAR histoscore (<100 vs. >100) was more often found in early stage (I vs. II-IV, p=0.042). All other comparisons between histoscore and stage of the disease did not reveal any significant correlation.

Multivariate analysis

For the multivariate analysis Cox proportional hazards model was used. NF-κB, HIF-1a, CD-10 and ETAR expression, stage of the disease and platinum sensitivity were entered into Cox regression. For all tests, p<0.05 was considered as significant. In multivariate analysis, strong HIF-1α and ETAR expression remained independent prognostic factors (p<0.0001), as well as tumour stage and platinum resistance. NF-κB, ETAR expression and stage had the biggest effect on the OS (Higher Hazard ratio-relative risk) (Table 5).

	Univariate analysis	Multivariate analysis		
	p	p	Hazard Ratio	95% CiI
NF-kB	0.048	**0.003**	**5.608**	1.83-17.19
HIF-1a	0.029	0.077	0.403	0.14-1.10
CD10	0.011	0.787	1.119	0.357-3.89
ETAR	0.026	**0.006**	**3.975**	1.47-10.72
STAGE	0.012	**0.014**	**3.607**	1.29-10.08
Platinum-Resistance	<0.001	**<0.001**	**9.055**	2.097-18.199
Serus Type	0.049	0.054	**2.78**	0.98-7.90
Strong NFκB and ETAR expression as well as tumor stage and resistance to chemotherapy remained independent prognostic factors.				

Table 5: Multivariate cox regression analysis.

Interaction of biomarkers with OS

Given the impact of the above studied biomarkers on OS, we decided to study the interaction of some of these biomarkers with overall survival. Specifically, patients with high NFκB and HIF-1a histoscores (both with nucleus pattern) (n=23) had worse OS compared to those with low histoscores (n=22, p=0.003). Patients with low CD10 and high ETAR expression (n=18) had worse OS compared to those with high CD10 and low ETAR expression (n=41, p=0.022). In addition, patients with high HIF-1α and low CD10 expression (n=24) had also worse OS compared to those with low HIF-1α and high CD10 expression (n=18, p=0.002). Finally, patients with a histoprofile of NFκB high/HIF-1α high/CD10 low/ETAR high (n=9) gave the worst overall survival (30.3 months). The results are summarized in Table 6.

	Mean			
			95% Confidence Interval	
	Estimate	Std. Error	Lower Bound	Upper Bound
CD10 low/ETAR high	59,881	8,372	43,471	76,290
CD10 high/ETAR low	1,13,916	7,933	98,368	1,29,465
NFkB low/HIF-1a low	1,17,995	10,424	97,565	1,38,426
NFkB high/HIF-1a high	55,945	6,358	43,483	68,406
HIF-1a low/CD10 high	1,20,131	11,568	97,458	1,42,803
NFkB high /CD10 low	81,189	8,111	65,291	97,088
NFkB low/CD10 high	1,33,926	10,954	1,12,454	1,55,395
HIF-1a high/CD10 low	54,511	5,295	44,134	64,889
NFkB low/HIF-1a low/CD10 high/ETAR low	1,39,345	11,453	1,00,234	1,62,458
NFkB high/HIF-1a high/CD10 low/ETAR high	30,32	4,23	22.67	39.45

Table 6: Interaction of biomarkers with OS.

Discussion

Although, factors with traditionally prognostic significance have been mainly derived from the clinical and pathological staging, it has been suggested that the knowledge of molecular behavior of a tumor may represent a fundamental step to identify high-risk categories of patients and predict clinical outcome. According to this assumption, in recent years, targeted agents have become an integral part of treatment in an effort to inhibit specific cancer pathways. In this study we investigated the expression pattern of NF-κB, HIF-1a, CD-10, ETAR and ETBR in ovarian carcinomas.

To begin with, the results of our study demonstrate that a significant proportion of patients with ovarian cancer had detectable nuclear NF-κB expression which was correlated with more aggressive disease (due to association with worse OS and PFS). We studied both cytoplasmic and nuclear expression of NF-κB. When NF-κB is located in the cytoplasma it is considered to be in resting state, whereas when it translocates to the nucleus, following TLR (toll like receptor)-mediated signaling, it is considered to be activated [13]. NF-κB possesses a role in the regulation of the expression of targeted genes involved in normal procedures such as immune response, cell growth and survival [13]. According to this observation, the detection of cytoplasmic expression of NF-κB has minor importance since it represents the inactive form of this anti-apoptotic transcription factor, while the nuclear expression represents the activated state of this factor, reflecting to worse OS. Our finding is in agreement with previous studies, the majority of which assert that patients with a nuclear NF-κB expressing cancer have a shorter overall survival.

In addition, our study also demonstrates that the expression of HIF-1a in our cohort was associated with poor prognosis and also with resistance to chemotherapy. It is well established that hypoxia (low O2 levels) is common aspect in carcinogenesis and many types of hypoxic tumor cells activate several survival pathways to carry out their essential biological processes. Recent studies highlighted the HIF-1α pathway as a crucial pathway for which novel strategies of therapy could be developed [14]. Although the results of various studies are conflicting, the more prevalent belief is that HIF-1a nuclear expression is associated with poor prognosis and worse survival of patients with ovarian cancer, in agreement with our results [15-18]. In a previous study the authors found that ovarian clear cell carcinoma had the highest HIF-1a expression compared to other histological types [19] while other studies failed to see any correlation to histological type [17]. Furthermore, in accordance with our results, studies show that HIF-1a is overexpressed in the majority of hypoxic metastatic tumors and its expression is associated with chemoresistance [17,18,20-22]. In cell lines, expression of HIF-1a reduced ciplatin-induced apoptosis in sensitive cells whereas genetic knockdown of HIF-1a enhanced response to cisplatin in both cisplatin sensitive and resistant ovarian cancer cells [23].

We also showed that CD-10 expression in patients with ovarian cancer is a good marker of prognosis and sensitivity to chemotherapy. High expression of CD-10 was correlated with better OS and DFS in patients and also with good response to chemotherapy. In addition, CD10 expression decreased significantly in high grade tumors in ovarian and other types of cancer [24-26]. All these findings imply that high CD-10 expression can reduce progression of disease [24] and prolong survival through chemotherapy. The precise role of CD-10 in cancer evolution is controversial. Some studies report CD10 as tumor suppressor molecule in certain tumors included ovarian carcinoma [27]. In addition, reports have shown that CD10 plays a key role in the neoplastic progression through degradation or re-modulation of specific substrates including endothelin-1 and growth factors in various types of cancer [28-30].

Our findings are in agreement with previous studies. CD10 seems to be used as a sensitive marker to identify normal and atypical endometrial stromal cells [31]. Others studies reported that most of myoinvasive endometrial carcinomas expressed CD10 and this marker can distinguish atypical polypoid adenomyomas from endometrial carcinomas [32-34]. In ovarian carcinoma CD10 was expressed in both tumor cells and stromal cells although the intensity of the staining

varied among tissues. In another study CD10 expression was lower in patients with ovarian cancer with high grade tumors, similar to our results [35]. We also found that CD10 expression is correlated with good response to chemotherapy, in agreement with other studies where the expression of CD10 enhanced susceptibility to paclitaxel, [24,31] perhaps through the inhibition of FAK phosphorylation necessary procedure for the cell migration through formation of microtubular skeleton [36,37]. This study also showed that there was a significant decrease of cell proliferation and invasiveness in CD10-transfected ovarian carcinoma cells and so the overexpression of CD10 *in vivo* can reduce tumorigenesis in ovarian cancer cell lines [24]. Whether CD10 is more than just a marker of prognosis of OS and response to therapy or could be used as a potential target of therapy in patients with ovarian cancer requires further studies.

Furthermore, our study has shown that expression of ETAR but not ETBR portends a worse prognosis and response to therapy in patients with ovarian cancer since high expression of ETAR correlated with worse OS, resistance to chemotherapy and presence of more advanced stage of the disease. Recent studies suggested that activation of receptor A of endothelin-1 can promote tumorigenesis and tumor progression by various mechanisms, mainly angiogenesis but also proliferation, invasion and inhibition of apoptosis, stromal reaction, epithelial mesenchymal transitions, metastases and drug resistance [38-41].

All ET-induced tumor effects in ovarian carcinoma selectively occur via ETAR receptors while overexpression of ETBR is not a unique finding and could be found only in melanoma, glioblastoma, multiple myeloma [39] and rarely in colon and breast cancer [38]. Thus, the above indicate that the expression of the receptor involved in the effect of ET-1 strictly depends on the cell type. Under this observation, our findings that expression of ETBR was lower than ETAR and was not correlated with survival or response to therapy in patients with ovarian cancers were in accordance with previous observations. Our data referring to ETAR are consistent with findings in ovarian cancers in which expression of endothelin and ETAR are significantly associated with neo-vascularization and VEGF expression [42-44]. This expression of VEGF in turn stimulates growth and angiogenesis by increasing the levels of HIF-1a in a dose dependent manner [44]. In addition, hypoxia can induce endothelin transcription in several tumor cell types [45,46]. There is also a functioning Hypoxia Response Element in the antisense strand of the promoter of ET-1 [47,48] and induction of endothelin expression by hypoxia is probably via HIF-1 [49]. A previous study also demonstrated that endothelin is present at high concentration in ascites is patients with ovarian cancer indicate that this molecule could participate in the progression and invasion of ovarian carcinoma [50]. All these data clearly demonstrate that expression of ETAR is associated with worse survival and rapid tumor progression.

We have also demonstrated that patients with high expression of ETAR had platinum resistance. This finding is in accordance with previous studies [51]. In one of those studies when authors targeted ETAR with antagonist (zibotentan) in combination to chemotherapy, they noticed that could sensitize tumor to classical chemotherapeutics [51]. This probably works by preventing EMT (epithelial-mesenchymal transition)-associated escape signaling [51]. In ovarian cancer specific ETAR antagonists inhibit *in vitro* cell proliferation and the ET-mediated cytoprotection against paclitaxel [38].

Conclusion

Our study was designed in order to investigate the possible role of CD-10/ endothelin axis as well as NFκB and HIF1a pathways in patients with ovarian cancer and to define if the expression of those biomarkers could be used as predictor factor of survival and resistance to chemotherapy. Actually, patients with ovarian cancer showed a markedly different expression map of biomarkers on tissue and these differences influence overall survival and thus can be used as prognostic markers. This current study demonstrates *in vivo*, according to real clinical data and long follow up, a possible prognostic and at the same time functional role of CD10 in ovarian carcinoma through its association with endothelin axis (ET-1/ETAR axis). Finally, the nuclear expression of known transcriptional factors such as HIF-1a and NFκB is demonstrated to be a potential prognostic biomarker associated with clinical outcome as well as a potential predictive one to platinum-based chemotherapy. Targeting the HIF-1α and NFκB pathway has been a promising progress in recent years.

It is obvious that our results need to be verified in larger and prospective studies, whereas the ways in which these three biochemical pathways (CD10/endothelin, HIF-1a and NFkB) are involved and affect each other should also be identified. If these results are confirmed, we could design studies testing drugs targeting the aforementioned pathways (taking into consideration the availability of ETAR antagonists or multiple agents targeting the antiangiogenic pathway to which HIF1a leads through the VEGF/ RAS/RAF/MAPK or VEGF/ PI3K/Akt pathways), especially in tumors with more aggressive biology, in the era of personal targeted medicine so as to enhance the therapeutic result.

Ethics Approval and Consent to Participate

The study was conducted after approval from ethical review board of University of Thessaly.

Availability of Data and Material

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request competing interests: "The authors declare that they have no competing interests"

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Authors' Contributions

C.P. conceived of the study, participated in its design and coordination, contributed to the acquisition, analysis and interpretation of data and drafted the manuscript. E.I.P. had the major contributor in writing the manuscript. C.L had the major contributor in writing the manuscript and performed the statistical analysis. D.N. helped with data interpretation. K.P and E.K. performed the histological examination. G.K participated in its design and coordination, contributed to the acquisition, analysis and interpretation of data and drafted the manuscript. All authors read and approved the final manuscript.

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