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Expression of VEGF, VEGFR, EGFR, COX-2 and MVD in cervical carcinoma, in relation with the response to radio-chemotherapy

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Abstract

Introduction: Despite the improvement in the treatment results due to modern irradiation techniques and to the association of chemo-radiotherapy, cervical cancer remains an unsolved problem of oncology both due to the increased rate of local failures and of the distant metastasis. Efforts to implement new therapeutic strategies in order to obtain better results in patients with cervical cancer appear justified. Neovascularization is an important step in the tumor progression and the therapeutic targeting of the tumor blood vessels appears to be a good strategy to follow in the anti-cancer treatment. Thus, even in an incipient phase of the clinical research process, the combination between the anti-angiogenic aimed therapies and the current radio-chemotherapy seems to represent a new, feasible and promising approach. The aim of the present study was to determine the prognostic and/or predictive value of some biological markers of tumor angiogenesis and of their implication in increasing the efficacy of current treatments for this cancer. *Materials and Methods*: So far, 54 women were included in a prospective trial: 44 having an advanced cervical carcinoma and 10 healthy women, as controls. A tumor biopsy and a blood sample were obtained from each patient before the start of therapy. The density of microvascularization was assessed using CD34 monoclonal antibody (hot spot technique), the expression of angiogenic factors VEGFR, EGFR and COX-2 were determined in tumor biopsies by specific immunohistochemistry techniques, using primary antibodies anti-EGFR, anti-VEGF and anti-COX-2 respectively. The quantitative polymerase chain reaction (Real Time PCR) was employed for assessing the expression level of the genes involved. Serum VEGF was determined by quantitative ELISA technique. *Results*: Among the studied clinical and molecular factors, we found to be predictive for the type of response the following factors: tumor size at diagnosis (p=0.01), VEGFR2 expression (p=0.02) and a tendency to significance for patients' age (p=0.06). From the large panel of studied markers it was observed correlation between MVD expression with stromal COX-2 (p=0.01) and a tendency with epithelial COX-2 (p=0.06). Stromal COX-2 has higher correlation with VEGFR2 (p=0.01) and MVD (p=0.01) and also has a lower correlation with tumor size (p=0.08). *Conclusions*: Univariate analysis demonstrates that the response to radio-chemotherapy in cervical cancer is related to a set of clinical and molecular factors as: the tumor size, the expression of VEGFR2 as mRNA level and the patients' age. Unfortunately, the multivariate analysis by logistic model selects only VEGFR2 expression for prediction of tumor response. The interrelations between the different biomarkers demonstrate the complexity of the tumor progression process and the necessity of further studies to identify new therapeutic targets.

Keywords: cervix cancer, angiogenesis, biomarkers, prediction.

Introduction

Cervical cancer remains one of the greatest killers of women worldwide. According to Globocan 2008, it is estimated that in 2008 the number of patients diagnosed with and those who died from this disease was 529 828 and 275 128, respectively [1]. Romania ranks first in Europe regarding the incidence and mortality caused by cervical carcinoma, with 3402 new cases and 2005 deceased in 2008 [1], seventy percent of the newly diagnosed cases are already in advanced stages. In these advanced cases classic treatment approaches (surgery, radiotherapy, and, lately, chemotherapy) yield only inconspicuous results: 5-year survival rate is 70% for stage IIB, 50% for stage IIIA, 40–30% for stage IIIB and 10% for stage IVA [2].

The classic therapeutic approach for locally advanced cervical cancer is surgery (S) and radiotherapy (RT). Because of the research carried out in the last decade, concomitant cisplatin-based radio-chemotherapy (RTCT) has become the "gold standard" for the advanced stages and for early high-risk stages, as it has proved to be superior to radiotherapy alone, leading to a 16% significant increase of the overall survival rate. Nevertheless, approximately 50% of these patients present local relapse or distant metastases within the first two years after the initial treatment [3]. The efficacy of the classic treatments have reached a plateau, there is a need for the enhancement of the current treatments based on new prognostic factors and on the implementation of new treatment strategies as the target therapies are.

Angiogenesis represents an important stage of tumor growth; for this reason, it is extensively studied by investigating certain biomarkers. However, the results of the studies published so far are contradictory and somehow incomplete. Nevertheless, aside from their prognostic value, inhibiting those biomarkers may

yield a therapeutic effect in several circumstances [4]. Neovascularization is an important step in the tumor progression from the *in situ* lesions to the disease in its extended and metastatic phase; for this reason, based on pre-clinic models, the therapeutic targeting of the tumor blood vessels appears to be a good strategy to follow in the anti-cancer treatment.

Microvessel density (MVD) has been extensively evaluated as a measure of angiogenesis. Obviously, MVD is not generally accepted as an established and new prognostic factor, although in some studies MVD has been found to negatively affect survival in carcinoma of the cervix [5].

The vascular endothelial growth factor (VEGF) is a cytokine having a central role in angiogenesis. VEGF is currently considered one of the most important angiogenic factors, which is released by the tumor cells and induces the formation of the capillary vessel network by receptor tyrosine kinases (RTKs) mediated signaling. VEGF signals through three specific receptors: VEGFR1, also known as Flt-1, VEGFR2 or Flk-1 and VEGFR3 [6]. VEGF binding to VEGFR2 is considered the most important signaling pathway in angiogenesis and blocking its activity potentially enhances the therapeutic response [7]. In numerous studies, it has been pointed out as an independent prognostic element for the survival rate, while its relevance in this respect has not been proved in other studies [8]. Several investigators reported a correlation between the expression of the VEGF with the tumor invasion or the lymph node metastasis [9, 10]. Targeting VEGF/VEGFR2 signaling pathway as a new therapeutic strategy is largely explored in several tumors.

In certain studies, the expression of the receptor for the epidermal growth factor (EGFR – epidermal growth factor receptor) proved to be an independent predictor of poor prognosis for the cervical cancer decreasing local tumor control after RT [11, 12], while other studies did not confirm this result [13, 14]. Inhibiting EGFR during radiotherapy may be a promising therapeutic strategy in certain squamous cell carcinomas.

A correlation was observed in cervical cancer between the expression of the cyclooxygenase-2 (COX-2) and the lymph nodes and parametrial invasion [15]. The increased expression of COX-2 in patients with cervical cancer seems to be predictive for a weak response to treatment, as well as for an increased mortality rate [16, 17]. Inhibiting COX-2 may increase the sensitivity of the tumor tissues to radiotherapy without significantly affect the response of the normal tissues to radiation [18].

Although extensive research has been carried out, currently there are only few studies, which investigated multiple biomarkers on the homogenous population who underwent the some treatment strategies. As the results have been contradictory and failed to lead to a unitary view regarding the prognostic and therapeutic value of these biomarkers in any of the tumor localizations, further research is needed in this domain.

The purpose of our study was to evaluate the intra-tumoral and serum levels of different biomarkers and the MVD in patients with advanced cervix cancer and search correlation between them and with different already known clinico-pathological prognostic factors. The final aim is to reach a more exact understanding of the molecular mechanisms and of the natural course of the disease and to identify and validate new prognostic factors in cervix carcinoma – which can indicate the use of new therapeutic methods.

Materials and Methods

Between November 2009–November 2010, 54 women were included in this prospective study: 44 with locally advanced squamous cell carcinoma (18 in stage IIB, 15 in IIIA and 11 in stage IIIB) and 10 women with normal cervix, as controls. The median tumor size was 4.5 cm (2–8 cm) and the patients' age was between 28 and 73 years (median: 50.5 years).

Before the beginning of treatment, a tumor biopsy and a blood sample were taken from each patient and stored at -8^{0}C until processing.

The patients with stage IIB–IIIB cervical cancer included in the study, were treated according to the same therapeutic protocol: concomitant radiochemo-therapy (RTCT) associated or not with surgery (S). The therapeutic schedule consisted of external beam radiotherapy to the pelvic region delivered with 15 MV X-rays and cervical boost by HDR intracavitary brachytherapy. Concomitant cisplatin was administered as radiosensitizer. The patients were reevaluated at 46 Gy/pelvis and, according to tumor response, the continuation of RTCT or surgery (after a 4–6 week interval) was decided. The tumor response for patients with RTCT was evaluated clinically at the end of the treatment and for the operated patients the evaluation of the response was based on pathological examination of the surgical specimen and defined as complete response (CR) and non-complete response (NCR) (partial response, stable disease) (Table 1).

Table 1 – ***Characteristics of patients with locally advanced cervical carcinoma***

Squamous cell carcinoma: 44 patients	***Stage IIB***	***Stage IIIA***	***Stage IIIB***
	18 (41%)	*15 (34%)*	*11 (25%)*
Tumor size [cm]			
Min.	2	2	3
Max.	7	7	8
Median	2	4.5	5
All malignant patients			
Min.	2		
Max.	7		
Median	4.5		
Age [years]			
Min.	28	36	28
Max.	73	69	68
Median	47	53	56
All malignant patients			
Min.	28		
Max.	73		
Median	50.5		

The study is carried out according to the relevant national guidelines and was approved by the Ethical Commission of the "Iuliu Haţieganu" University of

Medicine and Pharmacy from Cluj-Napoca. Clinical research respects the Helsinki Declaration on humans, each patient being informed and asked to give written consent. All experimental activities were carried out according to Good Laboratory Practice Rules.

Tissue VEGF and VEGFR evaluation

Total RNA isolation

Total RNA was isolated with TriReagent (Sigma-Aldrich), purified with RNAeasy Mini Kit (Qiagen) and further analyzed for quantity and quality with ND-1000 and Agilent Lab-on-a-chip Bioanalyzer 2100 (Agilent Technology). The RNA integrity number (RIN) and the 28/18S ribosomal RNA ratio were used as the quality control. All samples included in this study had a RIN between 8 and 9.8 and a 28/18S ribosomal RNA ratio more than 1.7.

The quantitative polymerase chain reaction (Real Time PCR)

The mRNA expression level was quantified by qRT–PCR (Real time PCR). 1000 ng of the total RNA from each sample were used for cDNA synthesis by reverse transcription using *FirstStrand cDNA Synthesis Kit* (Roche). The cDNA was subsequently amplified with the LightCycler Taqman Master Kit (Roche) in a 98-well plate using the LightCycler 480 instrument (Roche) as follows: 10 minutes at 95^0C for enzyme activation followed by 40 cycles of 15 seconds at 95^0C, 20 seconds at 55^0C and one second at 72^0C for the amplification step and 30 seconds at 40^0C for cooling step. Changes in the expression of target genes (VEGF and VEGFR2) were measured relative to the mean critical threshold (CT) values of 18S housekeeping gene, by the $\Delta\Delta C_t$ method.

The primers and UPL probes (Roche) used in the qRT–PCR evaluation were specific for every gene. For all the genes, we used 1μM of both primers and 0.2 μM from UPL (Universal Probe Library). The primers structure for VEGF were: F primer (5'-CCAC TTCGTGATGATTCTGC) and R primer (5'-TACCT CCACCATGCCAAGT). The UPL probe for VEGF was #29 (Roche Catalogue). The primers structure for VEGFR2 were: F primer (5'-CGGAAGAACAATGTA GTCTTTGC) and R primer 5'-GAACATTTGGGAAA TCTCTTGC) and #18 UPL. For the 18 S housekeeping, we used the #48 UPL probe and F primer (5'-GCAA TTATTCCCCATGAACG) and R primer (5'-GGGAC TTAATCAACGCAAGC).

Serum VEGF evaluation

Soluble VEGF was determined quantitatively in patients' serum using Quantikine Human VEGF Immunoassay kit (R&D Systems). This assay employs the quantitative sandwich enzyme immunoassay technique. Briefly, a monoclonal antibody specific for VEGF has been pre-coated onto a microplate. Standards and samples were pipetted into the wells and any VEGF present was bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for VEGF was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells and color developed in proportion to the amount of VEGF bound in the initial step. The color development was stopped and the intensity of the color was measured.

Immunohistochemistry

Construction of the tissue microarray (TMA) block

For a simultaneous assessment of tumor tissues in a more effective and rapid way, we have chosen the technique of tissue microarray (TMA). This permits the positioning of tens or hundreds of spots, from different tissue samples, on the same histological slide. For the construction of the composite TMA block, we used the manual TMA kit from 3DHISTEC (Hungary). Two mm diameter insular carrots were extracted from the paraffin blocks, corresponding to initial biopsies. The most representative part of each tumor was selected and positioned in the TMA block. This block was used for all the immunohistochemical colorations except for CD34. For this particular case, the whole section of the biopsy was preferred, for a better estimation of the micro vascular density. For similar reasons we used whole sections also for normal cervical tissues included in the study.

Immunohistochemical technique

The immunohistochemical technique was realized on tissular samples fixed in 10% buffered formalin, paraffin included, and sectioned at 3–4 μm. The antigen retrieval was not necessary for VEGFR and COX-2. For the EGFR the pre-treatment with trypsin was mandatory. The heat induced epitope retrieval at pH6 was used for CD34 reaction. Primary antibodies were incubated at room temperature as follows: CD34 (clone QBEna10 from DAKO) at a dilution of 1:350 for 30 minutes; *VEGFR* (clone KLT9 from NOVOCASTRA) at a dilution of 1:50 for 60 minutes; EGFR (clone E30, DAKO at a dilution of 1:25 for 30 minutes; *COX-2* (goat polyclonal antibody anti-human recombinant COX-2, aa18-604, R&D Systems) at a dilution of 1:25 for 60 minutes. The reactions were visualized with the kit Novo Link Max Polymer from NOVOCASTRA, using a solution of 3,3'-diaminobenzidine tetrahydro-chloride (DAB) as chromogen. For the goat anti-COX-2 antibody, we used the kit LSAB+ from DAKO, who includes a secondary anti-goat antibody. Finally, the sections were counterstained with Hematoxylin, dehydrated and mounted.

Immunohistochemical analysis

The micro vascular density (MVD) was assessed on CD34 stained slides using the well-known "hot spot" method. Vascular profiles were identified and counted on three microscopic fields at ×200 magnification, selecting the first three most vascular regions of each tumor and avoiding necrotic or sclerotic areas. The images were captured with a high definition video camera (Mega Video IP Camera, de la Arecont Vision, USA), mounted on an Olympus CX31 microscope. With this technical setting, the area of the microscopic field was 0.0542 square-mm.

The immunoassays for VEGFR, EGFR and COX-2 were semi quantitatively assessed, separately for the epithelial and for stromal component. Using the intensity of the cytoplasmic staining and the proportion of positive cells, each case was arbitrarily classified in four categories: score "3" – for intense staining of more than 30% of cells; score "2" – for moderate staining of more than 30% of cells; score "1" – for weak staining of more than 30% of cells; score "0" – for absence of staining or for less than 30% of cells staining.

Statistical methods

Results connected with categorial variables were evaluated with chi-square test and if appropriate Yates corrections was used. For continuous variable as tumor VEGF, VEGFR2, MDV, serum VEGF, tumor size and age, due to lack of normality and consensus on the cut-off values, we used the non-parametric test of Kruskal–Wallis. At last, for the significant variables versus complete remission at the end of treatment we did a ROC curve analysis on VEGFR2, tumor size and age. Correlations were established generally by Pearson method, and for COX-2 and other ordinal variables, we used Spearman correlation. All statistics were considered significant if *p*-values were under 0.05.

Results

From the 44 patients with cervical cancer, at the date of analysis the clinical response could be evaluated in 34, 10 of them still being in the course of treatment. From the 34 evaluated patients 12 (27%) were operated after RTCT. Due to RTCT, complete response was obtained at 19 (55.8%) patients: 8/14 (57%) in stage IIB and 11/19 (55%) in stage IIIA–IIIB (Table 2) without statistically significant differences between the stages.

Table 2 – ***Tumor response vs. stage and treatment***

Stages/Treatment	Complete remission	Non-complete remission	Total
IIB	8 (57%)	6 (43%)	14
RTCT	3		3
RTCT+S	5 (45.5%)	6 (54.5%)	11
IIIA–IIIB	11 (55%)	8 (45%)	19
RTCT	10 (52 %)	9 (48%)	19
RTCT+S	1		1
Total	19 (55.8%)	15 (44.2%)	34

It was analyzed the relation between the different clinical prognostic factors, biomarkers and tumor response.

Analyzing the correlation between the different clinical prognostic factors and biomarkers and the tumor response, statistically significant values were obtained for tumor size at the diagnosis (p=0.01) and VEGFR2 expression (p=0.02). A tendency to significance for the patients' age (p=0.06) was also observed.

The tumors with complete remission had an average size of 3.92 cm and those with non-complete remission 5.17 cm (Figure 1).

The ROC curve (p=0.01) suggests the value of ≥5 cm as a cut-off for the poor prognostic of the patients (Figure 2).

The age was also important for the tumor response, better response being found for older patients (Figure 3).

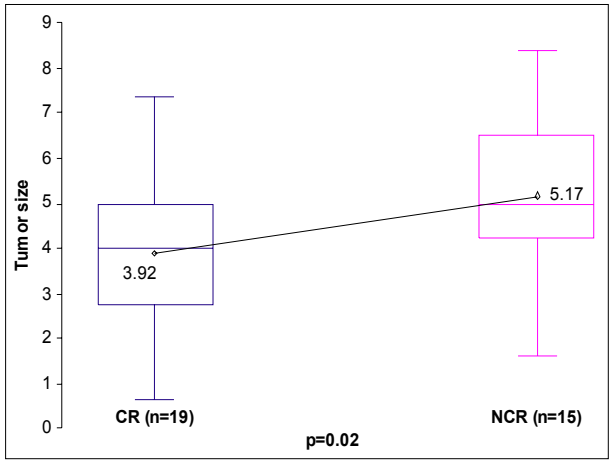

Figure 1 – ***Complete response (CR) and non-complete response (NCR) vs. tumor size.***

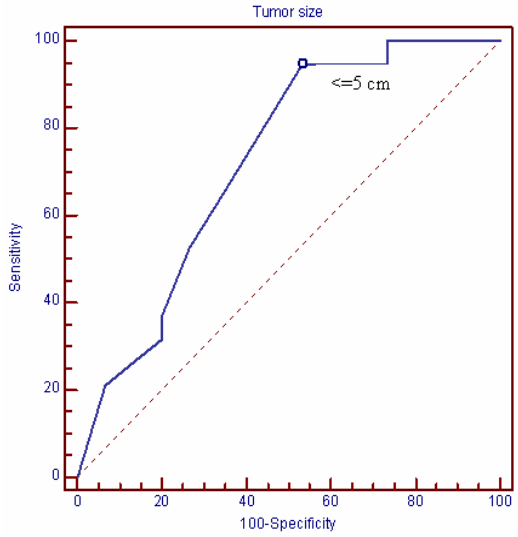

Figure 2 – ***ROC curve for tumor size; AUC=0.73, p-value =0.01; criterion: tumor size ≤5 cm.***

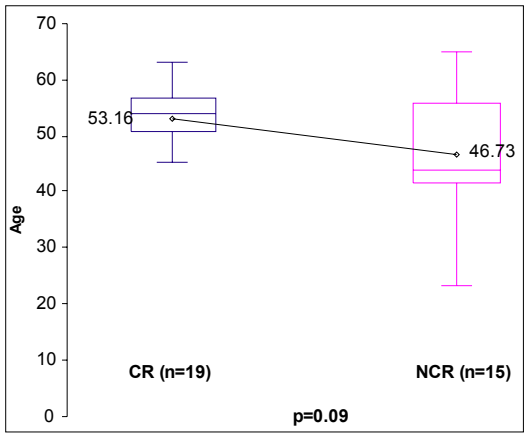

Figure 3 – ***Complete response vs. patients' age.***

The ROC curve supports this result showing that the age above 44 years is a criterion for favorable prognostic of the patients (p=0.06) (Figure 4).

Analyzing the results for angiogenesis factors, we found that a complete response was obtained in patients with higher level of VEGFR2 (Figure 5), the cut-off value on ROC curve being 0.54 units (p=0.02) (Figure 6).

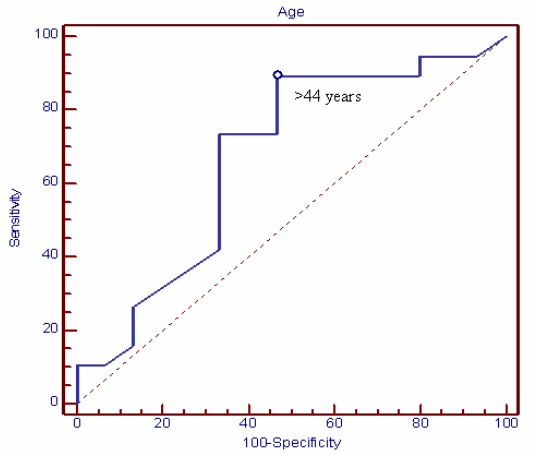

Figure 4 – *ROC curve for age; area under curve (AUC) =0.67, p-value =0.06; criterion: age >44 years.*

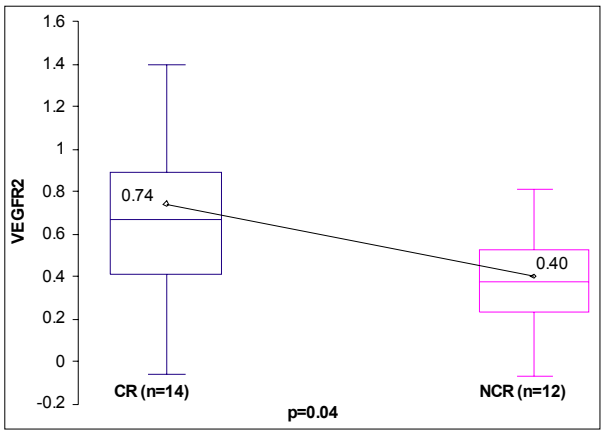

Figure 5 – *Tumor response vs. VEGFR2.*

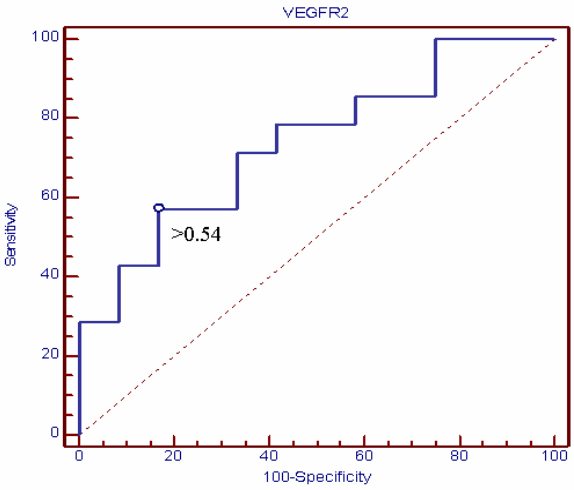

Figure 6 – *ROC curve for VEGFR2; AUC=0.74 and p-value =0.02; criterion: VEGFR2>0.54.*

The other variables: tissue VEGF, MVD, serum VEGF, epithelial VEGFR, stromal VEGFR, EGFR, epithelial COX-2 and stromal COX-2 did not comply with the chosen significance level.

Micro vascular density (MVD) was determined using "hot spots" method both in tumors from patients with cervical cancer and in normal cervical tissue from control group. As we expected, MVD was higher in tumor tissue in comparison with normal tissue (Figure 7).

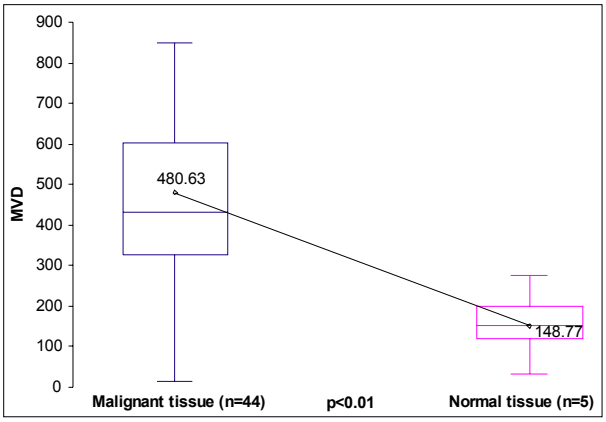

Figure 7 – *MVD vs. type of tissue.*

Analyzing the relation between variables (VEGF, epithelial VEGFR, stromal VEGFR, VEGFR2, EGFR, stromal COX-2, epithelial COX-2, MVD, tumor size and patients age), a strong variation is induced in VEGFR2 by stromal COX-2 (p=0.03) (Figure 8), respectively in MVD by stromal COX-2 (p=0.02) (Figure 9).

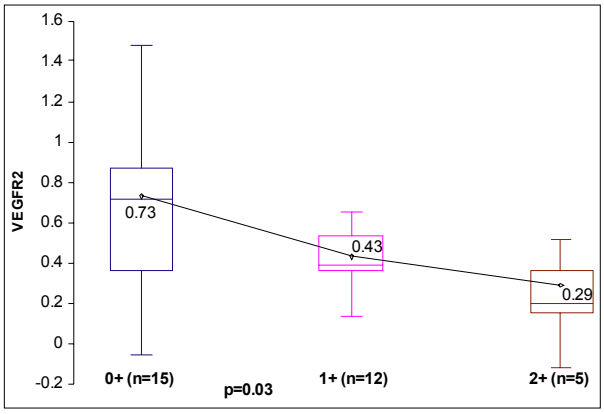

Figure 8 – *Expression of VEGFR2 vs. stromal COX-2 levels.*

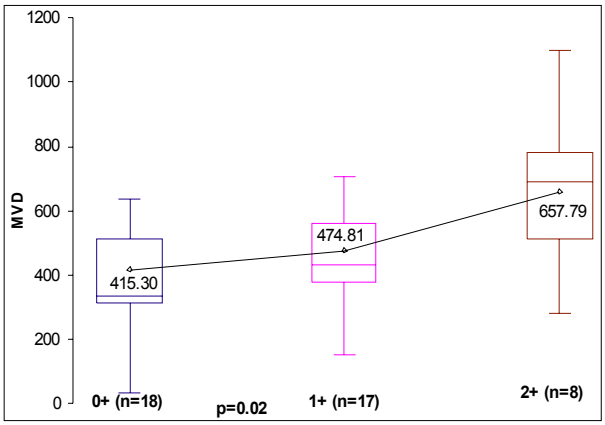

Figure 9 – *Stromal COX-2 vs. MVD.*

It has to be mentioned a tendency in variation of MVD by epithelial COX-2 (p=0.06), and in variation of tumor size by stromal COX-2 (p=0.08).

With the three factors with level of significance ≥0.94 related to the treatment response: tumor size, patients' age and VEGFR2 we try a multivariate analysis by logistic model. Unfortunately, tumor size and patients' age were rejected and only VEGFR2 was accepted.

Discussion

Efforts to implement new therapeutic strategies in order to obtain better results in patients with cervical cancer continue to be made in our Institution. One of these domains of interest is represented by targeted therapies. Thus, even in an incipient phase of the clinical research process, the combination between the anti-angiogenic aimed therapies and the current radio-chemotherapy seems to represent a new, feasible and promising approach in several types of cancer, including the cervical cancer. The indication of the target therapy has to be based on some predictive factors of the response to this treatment. The different new biomarkers can be prognostic factors and predictive ones, as well. They can be used to indicate the association of target therapies to standard RTCT.

Currently, the prognostic value of these biologic factors and their involvement in the treatment of cervical cancer is not confirmed; on the contrary, it is still controversial and, therefore, intensively investigated.

For these reasons, our study proposes the examination of a set of angiogenetic biomarkers in the cervical cancer, with the following purposes: a more exact understanding of the molecular mechanisms and of the natural course of the disease; identification and validation of new prognostic factors; validation of certain prediction factors for the response to radiochemo-therapy; introduction of new therapeutic strategies having a curing potential superior to that of the classic ones.

The microvascular density is one of the most studied histopathologic factors of prognostic, considered a measure of the tumor angiogenesis and a significant prognostic factor in many types of tumors. However, it is not unanimously accepted as a stable prognostic factor. In a study published by Obermair A *et al.*, MVD did not correlate with the patients' age, histology type, tumor size, vascular space invasion and lymph node invasion, but it influenced disease free survival (DFS) and overall survival (OS) [19]. In this study, the 5-years OS probability was 80.7% (±0.03%) in patients whose tumors had an MVD≤ to 20/field and 63.0% (±0.06) in patients whose primary tumors had an MVD >20/field (*log*rank, $p<0.0001$). In our study, MVD proved to have an increased level in tumor tissues, but the correlation with the tumor response was not observed. This, however, does not mean that it could not influence the long-term results (DFS or OS).

VEGFR2 receptors were originally thought to be present only in endothelial cells but recent studies showed that VEGFR2 is widely distributed in human tissues and tumors [20] suggesting an autocrine signaling VEGF/VEGFR2 loop [20–22]. To our knowledge, only one study reports VEGFR2 expression in cervical adenosquamous carcinomas and its overexpression has been associated with lack of metastases, but no association was found between VEGFR2 and overall survival and disease recurrence [23]. The authors suggest that other alternative molecules can drive the metastatic spread in these types of tumors. However, another study investigated the soluble level of VEGFR2 in patients' serum plasma and found it to be higher in recurrent compared to pre-invasive and primary invasive carcinoma, in node-positive disease and in patients with distant metastases [24]. We found that VEGFR2 is higher in patients with clinical complete response at the end of treatment; therefore, the pre-therapeutical level of VEGFR2 could be used as a predictive factor for clinical response in cervical cancer.

Amongst the newly studied prognostic factors for the cervical carcinoma is the over-expression of cyclo-oxygenase-2 (COX-2), considered to be an unfavorable prognostic factor. However, at present there are only few studies (and they are pre-clinical studies) regarding this factor [25]. COX-2 is induced in the presence of an inflammatory stimulus and has been shown to be involved in cell-cycle regulation, apoptosis, and pathological angiogenesis by promoting carcinogenesis, tumor proliferation, and tumor spreading [26, 27]. Several studies have shown that COX-2 is up-regulated in many cancers, including uterine cervical cancer [15, 28]. COX-2 expression has been determined to be significantly greater in younger patients than in older ones, which suggest that COX-2 expression is related to the poor prognosis of young women with advanced uterine cervical cancer [29]. We also found that COX-2 values are higher in younger patient, in concordance with the literature. Cyclooxygenase-2 is known to be related to angiogenesis, its expression being associated with neovasculature and tumor-related angiogenesis [27]. We measured the COX-2 expression in epithelial cells as well as in stromal cells of tumors. Interestingly, at least for now, only COX-2 expression in tumor stroma was linked with micro vessel density (in a direct correlation) and also with the level of VEGFR2 but in an inverse relationship (high level of COX-2 associated with lower expression of VEGFR2).

Conclusions

Our study is a prospective one, including multiple biomarkers evaluated in a homogenous group of patients with locally advanced cervical carcinoma, treated by the same therapeutic protocol.

The results demonstrate that the response to radio-chemotherapy in cervical cancer is related to a set of clinical and molecular factors as: the patients' age, the tumor size, the expression of VEGFR2 as mRNA level. Unfortunately, the multivariate analysis by logistic model selects only VEGFR2 expression for prediction of tumor response. The interrelations between the different biomarkers demonstrate the complexity of the tumor progression process and the importance of using a battery of molecular assays to predict the clinical response to radio-chemotherapy.

The long-term follow-up of a bigger number of patients should validate the biomarkers with prognostic value for survival. Their use for the therapeutic decision would identify the indications of newly targeted therapies and, in that way, could help in treatment individualization.

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