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Prognostic factors for response to chemotherapy in advanced tumors of the uterine cervix: the role of neoangiogenesis.

Fatores prognósticos de resposta à quimioterapia em tumores avançados do colo uterino: o papel da neoangiogênese.

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A B S T R A C T

Objective: to analyze the expression of Vascular Endothelial Growth Factor (VEGF), its receptor (VEGFR-2), age and histological type of advanced cervical carcinomas with respect to the clinical response to neoadjuvant chemotherapy. **Methods:** we studied 40 patients with cervical carcinoma (IB2 and IVA) diagnosed by biopsies prior to treatment. All patients underwent neoadjuvant chemotherapy and evaluation for clinical response and expression of VEGF. We considered a tumor regression greater than 50% as a good clinical response. **Results:** eighteen patients (45%) had good response to chemotherapy, and 22 (55%), poor response. VEGF expression was positive in 16 patients and negative in 24. When analyzed separately for response to chemotherapy, only the positive expression of VEGF was associated with good clinical response (p=0.0157). **Conclusion:** VEGF expression alone was an important marker of good response to neoadjuvant chemotherapy in patients with advanced carcinoma of the cervix.

Keywords: Cervix Uteri. Uterine Cervical Neoplasms. Neovascularization. Pathologic.

INTRODUCTION

Cervical cancer is the third most common in the female population, surpassed only by breast and bowel cancers. According to absolute data on cancer incidence and mortality from the Brazilian National Cancer Institute (INCA), cervical cancer was responsible for the death of 5430 women in Brazil in 2015[1]. In the world, it is the second cause of cancer death in women[2]. The most effective approach for the control of cervical carcinoma is the screening and treatment of pre-neoplastic lesions. The introduction of the Pap smear as a screening test significantly reduced the incidence of advanced cases and their mortality[3]. However, diagnosis is still common in advanced stages. The five-year survival rate for patients with initial disease (stages 0-IIA) remains around 80%, and for patients with more advanced disease (stages IIIB-IVA) it is only 20% to 42%[3].

For the treatment of uterine cervix carcinoma in stages IB, IIA and some selected cases of stage IIB, surgery or radiotherapy are used[4]. In the United States, patients with stage IIB tumors were treated primarily with radiotherapy, while, in Europe, radical hysterectomy was the preferred approach[5]. For stages III and IVA, the treatment of choice was radical radiotherapy comprising external pelvic irradiation associated with brachytherapy, when possible[4]. With radiotherapy, survival rates remain around 65% for patients with tumors in stage IIB and 40% for those in IIIB, but with increased morbidity[6].

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The search for better results has led several centers throughout the world to introduce, for advanced cervical cancer, treatments employed for solid tumors from other locations, i.e., the combination of chemotherapy and radiotherapy with or without surgery[7]. Cisplatin is reported as the most active chemotherapy drug in cervical cancer and has been included as the basic component of its primary treatment[3]. Duration of treatment is critical and the ideal would be to anticipate which patients would present good response to chemotherapy, avoiding indicating it to those without prospects of good response, gaining time on the prescription of another therapeutic modality: radiotherapy alone or chemosensitization. On the other hand, patients who presented good response could undergo surgery, dispensing with radiotherapy and thus avoiding complications due to the association of treatments[3,4].

Angiogenesis is a critical factor in the progression of solid tumors, including cancer of he cervix. Several studies have shown correlation of angiogenesis with worse prognosis in solid tumors such as breast, prostate, colon[8,9]. The mechanisms responsible for angiogenesis in the cervical neoplasia, however, are not well defined[10]. The increased expression of Vascular Endothelial Growth Factor (VEGF) in primary tumors correlates with a more aggressive biological behavior (increased vascular invasion index, metastatic lymph nodes and liver metastases) and with a worse prognosis compared with cases in which its expression is decreased[11]. The presence of VEGF and VEGFR-2 in a uterine cervix tumor can be determined by immunohistochemistry, and its subtypes, by Western blot, ELISA and PCR[12].

There are few studies that correlate the expression of VEGF, or its VEGFR-2 receptor, with the response to chemotherapy. The objective of this study was to evaluate the expression of tumor VEGF in patients submitted to neoadjuvant chemotherapy for locally advanced uterine cervix cancer.

METHODS

We conducted a case-series, prospective study, correlating the clinical response to chemotherapy with the degree of VEGF expression in tumor specimens collected prior to treatment initiation. The study was approved by the Ethics in Research Committee of the Hospital das Clínicas of the Medical School of Ribeirão Preto (FMRP-USP), according to proceeding nº 2952/00.

We included 40 patients enrolled in the protocol established for the research project, assisted in the Gynecological Oncology and Mastology Sector of the Department of Gynecology and Obstetrics of FMRP-USP. Recruitment of patients was the usual of the outpatient clinic of the Hospital das Clínicas of FMRP-USP, having been referred by the Unified Health System (SUS - primary care) to the Gynecological Oncology Outpatient Clinic (tertiary care).

All patients included had locally advanced uterine cervix cancer, i.e. stages IB2 (tumor limited to the cervix, but volume >4cm) through IVA (tumor extension with involvement of the rectum or the bladder mucosa). Patients should meet the following criteria: performance status of 0 or 1, corresponding to the Karnofsky indices between 70 and 100; number of leukocytes greater than 4000/ml; and number of platelets greater than 100,000/mm^3.

Urinalysis should not show signs of infection. We excluded patients who had positive serology for the human immunodeficiency virus and those who had not received at least two cycles of primary chemotherapy after initial inclusion.

We prospectively divided the patients into two groups of good and poor clinical response to neoadjuvant chemotherapy according to the WHO criteria.

The care procedures used in this research were the same as those used in the care protocol of the Gynecological Oncology Service of the FMRP-USP, with no exception, plus the collection of an additional fragment of the tumor and the collection of 10ml of blood. Two observers clinically assessed the volume of the primary tumor by specular examination and vaginal exam, and parametrial invasion, by rectal examination. Image exams, such as transrectal ultrasonography, computed tomography or magnetic resonance imaging were occasionally used, but the evaluations considered for analysis were those obtained by clinical examination. Staging was completed with cystoscopy, rectosigmoidoscopy, excretory urography, and chest X-ray.

We performed the biopsies in areas without necrosis with a Baliú drill. Part of the material obtained in the biopsy was subjected to formalin fixation and inclusion in paraffin and the remaining part stored in liquid nitrogen. The paraffin material was cut for the slides to the histopathological diagnosis necessary for the beginning of treatment and the sections destined to the research of the expression of the vascular endothelial growth factor (VEGF) and its receptor VEGFR-2, carried out by the Service of the Surgical Pathology of FMRP-USP.

The planned treatment is standardized, is part of the routine care of the Service, and includes neoadjuvant chemotherapy followed or not by radiotherapy and, depending on the regression of neoplasia, especially in the parametrium, is completed with radical hysterectomy. All patients are referred for pelvic lymphadenectomy (laparoscopic or open) with intraoperative evaluation of the lymph nodes. In the presence of compromised pelvic lymph nodes, the staging is completed with para-aortic lymphadenectomy. We assessed the preoperative clinical conditions of each patient, and contraindicated the surgical procedure for those who did not present acceptable clinical conditions, those which for some reason largely postponed the onset of chemotherapy, and those who did not accept their inclusion in the protocol. Therefore, not all patients included in this study underwent lymphadenectomy.

The chemotherapy protocol includes the administration of three cycles of neoadjuvant chemotherapy is the USAN cisplatin and 5-fluorouracil secu ing the following schedule: Day 1- Platiram 50mg/m^2 IV + Fluoracil 500mg IV t.i.d.; Day 2- Platiram 50mg/m^2 IV + Fluoracil 500mg IV t.i.d.; Days 3/4/5- Fluoracil - 500mg IV t.i.d.

This scheme was repeated three with 28-day intervals. After each cycle, we assessed the patients' response. The cases that showed good response continued with the above-mentioned scheme. Patients who did not respond with a significant decrease in tumor volume after the second cycle of this regimen and those who had not enough tumor reduction to allow radical surgery were considered resistant to chemotherapy and classified as cases with poor response.

The same observers examined the patients three or four weeks after the end of each cycle of chemotherapy. According to WHO criteria, we considered a complete response the disappearance of the entire lesion; a partial response, when there was an estimated decrease in tumor size of 50% or more; stable disease, when there was an estimated decrease of less than 50% or an estimated increase of less than 25%; and progression of the disease, when a new unidentified lesion appeared or there was an estimated increase of 25% or more of the existing lesion[13]. We considered a good response to chemotherapy when there was a complete and/or partial response, and a poor response when there was stable disease and/or tumor progression.

The samples collected were fixed in formalin and included in paraffin. Histological preparations were submitted to immunohistochemical reaction using mouse monoclonal primary antibodies produced by clone C1, anti-VEGF (reference SC-7269, Santa Cruz, CA, USA). For its VEGFR-2 receptor, we used as the primary antibodies the FLK-1 mouse monoclonal, produced by clone A3 (reference SC-6251, Santa Cruz, Ca, USA).

For the immunohistochemical study for VEGF and VEGFR-2 we used the Quik Novostain Universal Kit (reference NCL-RTU-Qu, Novocastra Laboratories, Newcastle, UK), which includes normal horse serum to block non-specific antigens; pan-specific secondary biotinylated antibody to bind to the primary antibody; and the extreptavidin-peroxidase complex for detection of the reaction. The preparations were developed with 3'3' diaminobenzidine tetrahydrochloride, using Liquid DAB (3'3' Diaminobenzidine Tetrahydrocloride) Substrate Kit (reference NCL-L-DAB; Novocastra, UK) for tissues embedded in paraffin and counter-stained with Mayer's hematoxylin.

We performed the statistical analysis with the GraphPad Prisma software 2.01. For the analysis of the contingency tables (2x2) and comparison between VEGF and VEGFR-2 expression and clinical response to chemotherapy alone, we used the Fisher's exact test (univariate analysis).

To verify independence (age less than or equal to 60 years *versus* greater than 60, histological type, expression of VEGF and VEGFR-2) and how it would affect the dependent variable (response to chemotherapy), we used logistic regression (multivariate analysis)[14].

RESULTS

Regarding the epidemiological aspects, the 40 patients included in this study were aged between 28 and 76 years, the mean age being 53.2 years. As for clinical staging, five patients had stage IB2, two IIA, 23 IIB, seven IIIB, and three had IVA. We observed that the mean age showed a tendency to increase with the more advanced stages (Table 1).

Table 1. *Clinical stage.*

Stage	n	%	Average age (years)
IB2	5	12.5	42.6
IIA	2	5.0	35.5
IIB	23	57.5	53.4
IIIB	7	17.5	60.0
IVA	3	7.5	51.0

Thirty-six patients underwent pelvic and/or para-aortic lymphadenectomy (surgical staging) and in 15 cases there was staging alteration, of which ten were re-staged for a higher stage, and in five, for less. When considering surgical staging, there was the following distribution: Seven had stage IB2, three IIA, 18 IIB, one IIIA, six IIIB, and five had stage IVA.

As for histological type, 33 individuals had squamous cell carcinomas, five had adenocarcinomas, and two, adenosquamous carcinoma. Good response rates were similar between cases of squamous cell carcinoma (45.4%) and adenocarcinomas (42.8%) (Table 2).

Table 2. *Distribution of patients according to histological type.*

Histological type	n	%
Spinal cell carcinoma	33	82.5
Adenocarcinoma	5	12.5
Adenosquamous carcinoma	2	5.0
Total	40	100

Regarding the clinical response to chemotherapy, after the first two cycles, 18 patients showed good response and 22 presented with poor response (Table 3). Twenty-six patients became operable after initial treatment with neoadjuvant chemotherapy, and underwent surgery.

As for VEGF, 16 patients presented positive expression and 24 patients were considered negative due to low expression. The positive expression of VEGF was more frequent in adenocarcinomas (5 in 7 - 71.4%) than in squamous cell carcinomas (11 in 33 - 33.3%), but the results did not show significant differences (OR=5.0, 95% CI 0.83-30.03, p=0.0942) (Table 4).

Regarding the VEGF receptor (VEGFR-2), 11 patients were considered positive, and 29, negative. The positive expression of VEGFR-2 was more frequent in cases of adenocarcinomas (4 in 7 - 57.1%) than in squamous cell carcinomas (7 in 33 - 21.2%), though without significant difference (OR=4.952, 95% CI 0.89-27.50, p=0.0755).

When compared separately with response to chemotherapy, only VEGF expression was positively associated with good clinical response (OR=5.34, 95% CI 1.35-21.15, p=0.0157) (Table 5).

Table 3. *Correlation between histological type and response to chemotherapy.*

Histological type	Good response		Poor response		Total
	n	%	n	%	
Adenocarcinoma	3	42.8	4	57.2	7
Squamous cell carcinoma	15	45.4	18	54.6	33
Total	18		22		40

Table 4. *Distribution of VEGF expression in relation to histological type.*

VEGF	Histological type		Total
	Adenocarcinoma	Squamous cell carcinoma	
Positive	5	11	16
Negative	2	22	24
Total	7	33	40

Fisher's exact test: p=0.0942; OR:5.0 (95% CI, 0.83 to 30.03).

Table 5. *Distribution of VEGF expression, positive or negative, regarding the clinical response to chemotherapy.*

Response	VEGF		Total
	+	-	
Good	11	7	18
Poor	5	17	22
Total	16	24	40

Fisher's exact test: p=0.0157, OR:5.4343 (95% CI,1.35-21.15).

The multivariate analysis included the variables positive VEGF, positive VEGFR-2, age less than or equal to 60 years and histological type. There was no association between variables and only the positive expression of VEGF was significantly associated with good clinical response to chemotherapy alone, increasing by 6.3 times the chance of a satisfactory response (*odds ratio*=6.34, 95% CI 1.21-33.18; p=0.0286) (Table 6).

DISCUSSION

Neoadjuvant chemotherapy for uterine cervix tumors has been incorporated into the conventional treatment in order to reduce tumor volume and extension, so that radiotherapy can be instituted with better local conditions or transform clinically inoperable cases in operable ones[15]. Another beneficial effect would be the possibility of treating micrometastases followed by surgery or irradiation, depending on the primary tumor response. Chemotherapy is also used simultaneously with radiotherapy, being called chemosensitization. In theory, they would have a synergistic effect, since chemotherapy can increase the tumor's sensitivity to radiation by synchronizing the cells to a radio-sensitive phase of the cell cycle[16]. However, its side effects also add up, increasing morbidity and mortality[8]. Tumor size and presence of metastasis are risk factors that affect the final result and are associated with worse prognosis and greater resistance to therapy[16,17]. Thus, it would be of great importance to determine the predictive factors of response to chemotherapy through biological markers.

The role of angiogenesis in tumor growth has been clearly established. For progression of solid tumors, a sufficient supply of blood vessels is necessary[18]. The tumor induces angiogenic mechanisms, directly and indirectly leading to the growth of microvessels, providing access for tumor cells to the vascular system for its metastatic spread. Among the various angiogenic factors, VEGF and its receptors play an important role in tumor neoangiogenesis. VEGF is widely distributed in squamous cell carcinomas of the head and neck, regardless of stage or histological grade[19].

Several clinical studies have shown that VEGF expression and angiogenesis play an important prognostic role in advanced squamous cell carcinoma, being associated in most cases with poor prognosis and decreased survival[20-24]. In cervical neoplasms, the correlation between VEGF expression and dysplasia progression has been demonstrated.

Table 6. *Multivariate analysis of positive VEGF, positive VEGFR-2, age less than or equal to 60 years and histological type versus clinical response to neoadjuvant chemotherapy (good or poor).*

	Response				OR	95% CI	p
	Good		Poor				
	n	%	n	%			
VEGF +	11	27.5	5	12.5	6,34	1.21-33.18	0.028
VEGFR-2 +	7	17.5	4	10.0	1.62	0.30-8.69	0.570
Age <60 years	15	37.5	16	40.0	2.32	0.35-14.02	0.356
Histological Type (AC+ASC)*	3	42.8	4	57.2	0.28	0.03-2.30	0.239

** AC+ASC: Adenocarcinoma + Adenosquamous carcinoma.*

However, the association between VEGF expression and the clinical-pathological features of the disease needs further studies[9,25]. In the present study, increased expression of VEGF and VEGFR-2 was more frequent in adenocarcinomas. Recently, patient's age, clinical stage, tumor differentiation, presence of lymph node metastases or lymphovascular space involvement were all considered significant prognostic indicators in patients with invasive colon cancer[21]. We did not observe significant differences between these variables. However, one study showed a significant association only between VEGF expression and tumor the histological type. This finding supports the idea that the mechanisms of angiogenesis may be different, depending on the organ involved and the histological type of the tumor.

Regarding the value of VEGF in predicting the response to chemotherapy, little is known so far. In this study, VEGF proved to be a good marker to predict response to chemotherapy. Due to the role of VEGF in tumor angiogenesis and vascular permeability, the greater distribution and release of drugs through the neoformed vessels could explain the relationship between the response to chemotherapy and the presence of VEGF. However, Foekens *et al*.[26] studied the role of VEGF as a predictor of chemotherapy response to recurrent breast cancer and found that elevated levels of tumor VEGF were associated with poor response to the chemotherapy used. The same occurred when the response to tamoxifen was analyzed. One explanation would be that VEGF, by inducing endothelial cell proliferation, would indirectly contribute to the formation of a drug resistant tumoral phenotype via expression of proteins associated with drug resistance, such as glutathione-S-transferase[26]. Another study, carried out by Shimada *et al*.[8], has shown that VEGF is an independent prognostic factor of response to chemo-radiotherapy in patients with esophageal squamous cell carcinoma (p=0.0147), though associated with a lower response to therapy and survival. The authors suggest that the expression of VEGF would contribute to the protection of tumor blood vessels from the effects of the cytotoxicity mediated by chemotherapy and, therefore, to greater resistance to treatment[8]. Unlike the cited authors, Boku *et al*.[27] studied the role of biological markers as predictors of response and prognosis in 39 unresectable gastric cancer patients treated with 5-fluoroacyl and cisplatin, and found that cases considered as VEGF positive had a higher response rate than those considered negative (11 out of 20 *versus* 2 out of 19 cases; p=0.0057). Therefore, positive VEGF cases were considered a favorable phenotype for response to chemotherapy. Takiuchi *et al*.[14] studied the immunohistochemical expression of VEGF in 30 patients with gastric adenocarcinoma and its relation with the response to 5-fluoracil and cisplatin. The response rate of positive VEGF and negative VEGF cases was 75% (12/16) and 16.7% (2/14), respectively, and positive VEGF cases had a significant treatment impact (p=0.0031). In the present study, VEGF-positive patients also had a better response to the use of neoadjuvant chemotherapy with 5-fluoracil and cisplatin. The correlation between the response to chemotherapy and the expression of VEGF might be explained by the greater release of drugs within tumor neoangiogenesis regions, greater vascular permeability and, consequently, better local performance.

The differences found between the studies in relation to VEGF expression and response to chemotherapy could be explained by the use of

different therapeutic schemes, the different tumor types involved, the different drug associations, the different mechanisms of action and varied dosages. In this way, a larger number of papers, with more cases and with similar therapeutic schemes are necessary to better understand the relationship between the clinical response to anti-neoplastic agents and the expression of biological markers.

We found no studies correlating the expression of VEGFR-2 with response to chemotherapy in solid tumors. In our study, the cases considered positive for VEGFR-2 showed no association with the clinical response to chemotherapy, nor when combined with positive VEGF expression.

The VEGF expression was the sole indicator of good response to neoadjuvant chemotherapy in patients with advanced cervical cancer. Age and tumor histological type showed no association with response to neoadjuvant chemotherapy.

R E S U M O

Objetivo: *analisar a expressão do Fator de Crescimento do Endotélio Vascular (VEGF), seu receptor (VEGFR-2), idade e tipo histológico de carcinomas avançados de colo uterino com relação à resposta clínica à quimioterapia neoadjuvante.* **Métodos:** *foram incluídas 40 pacientes com diagnóstico de carcinoma de colo uterino (IB2 e IVA), com biópsias prévias ao tratamento. Todas as pacientes foram submetidas à quimioterapia neoadjuvante e avaliadas quanto à resposta clínica e à expressão do VEGF. Considerou-se boa resposta clínica uma regressão tumoral total ou maior do que 50%.* **Resultados:** *em relação à resposta à quimioterapia, 18 pacientes (45%) apresentaram boa resposta e 22 (55%), má resposta. Quanto à expressão do VEGF, em 16 pacientes foi considerada positiva e em 24, negativa. Quando os casos foram analisados separadamente em relação à resposta à quimioterapia, somente a expressão positiva de VEGF foi associada à boa resposta clínica (p=0,0157).* **Conclusão:** *a expressão de VEGF mostrou ser isoladamente, um importante marcador de boa resposta ao tratamento quimioterápico neoadjuvante das pacientes com carcinoma avançado de colo uterino.*

Descritores: *Colo do Útero. Neoplasias do Colo do Útero. Neovascularização Patológica.*

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