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Correlation of cytokines and inducible nitric oxide synthase expression with prognostic factors in ovarian cancer

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ABSTRACT

The study related the immunohistochemical staining of cytokines (IL2, IL5, IL6, IL8, IL10, and TNF-alpha), and iNOS staining with clinical and pathological parameters of patients with primary ovarian malignancy. We prospectively evaluated 40 patients who underwent surgical treatment in accordance with pre-established criteria and later confirmed diagnosis of ovarian cancer. Immunohistochemistry study for cytokines (IL2, IL5, IL6, IL8, IL10, TNF-alpha) and iNOS was performed. The evaluation of prognostic factors was performed using the Fisher's exact test. The significance level was less than 0.05. Histological grade 1 was significantly correlated with strong intensity for TNF-α ($p = 0.0028$). In addition, early stages showed strong expression intensity of TNF-α, but this was at the limit of significance ($p = 0.0525$). Strong staining immunohistochemical IL5 was related to disease-free survival less than or equal to 24 months, suggesting that a factor of poor prognosis, but there was no statistical significance ($p = 0.1771$). There was no statistical significance in relation at other cytokines studied. Therefore, immunohistochemical staining in strong intensity for TNF-α was related to histological grade 1 and early stages of ovarian cancer in our sample of patients.

1. Introduction

Despite significant clinical and laboratory advances and the increasing availability of chemotherapeutic agents, ovarian cancer remains the most dangerous gynecological malignancy and a major cause of morbidity [1].

The sensitivity of gynecological examinations for screening ovarian neoplasms is not exact [2]. Ultrasound may facilitate the diagnosis of ovarian tumors and help differentiate between neoplastic and non-neoplastic, and benign and malignant tumors. It also delivers important information regarding tumor size and content [3]. In addition, a transvaginal ultrasound image associated with color Doppler can increase ultrasound specificity, enabling an assessment of vascular tumor and blood flow conditions [4]. Notwithstanding these advantages, early diagnosis of ovarian cancer by ultrasound is uncommon due to their low prevalence in the general population.

Cytokines are glycoproteins and peptides of low molecular weight (less than 80 kD) that can be secreted and/or expressed in cell membranes, or stored in the extracellular matrix. They can be produced by any cell in the body, except red blood cells. They are important in mediating immune response events, by triggering and coordinating an inflammatory response [5], thus playing key roles in host defense [6].

Cytokines are molecular messengers that allow the immune cells to communicate with each other to generate a coordinated response to a target antigen. The secretion of cytokines allows rapid propagation of immune signaling in an efficient manner. Cytokines directly stimulate immune effector cells and stromal cells at the tumor site and increase tumor cell recognition by cytotoxic effector cells [5], thus playing key roles in host defense [6].

Cytokines also play fundamental roles in the regulation of biological processes such as growth and cell activation, chemotaxis, inflammation, immunity and tissue repair. The immune

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response can be assessed according to the profile of the cytokines secreted by CD4+ lymphocytes. The immune response of TH1 cytokines involves IL-2, IL-8, TNF-α, and IFN-γ production; while TH2 responses involve IL-3, IL-4, IL-5, IL-6, and IL-10 production. TH1 cells participate in cytotoxicity reactions and inflammation, and are involved in the host's defense against viral, microbial, and neoplastic infection. TH2 cells primarily act in the humoral immune response, producing antibodies in atopic reactions and regulating host defenses against parasitic diseases [7].

Cytokine production has been recently linked to cancer progression, as they can stimulate cell growth and contribute to metastasis. If permanently synthesized, cytokines can be used as markers of immune system activation. This involvement has demonstrated roles in oncogenesis (alone or in combination with other cytokines) and in the immunomodulatory activities of the immune response against neoplasia, such as the signaling between inflammatory cells and invasive tumor tissue [8,9].

Several reports have revealed the expression of inducible nitric oxide synthase (iNOS) by malignant cells or tumor microenvironment, both at mRNA and protein levels [10], with immunohistochemical labeling found in malignant and not benign tumors [11]. In human carcinogenesis, iNOS is related to immune system regulation, cell apoptosis and angiogenesis [12]. Nitric oxide (NO) has been implicated in the initial steps of carcinogenesis, which combined with other factors, may lead to uncontrolled cell differentiation and cytostasis. High concentrations of NO may also induce apoptosis; however, low concentrations stimulate tumor growth via the induction of angiogenesis [13]. NO is therefore an attractive target for novel strategies for diagnosis and prognosis of cancer onset and subsequent therapy.

The aims of the present study were to relate the immunohistochemical staining of cytokines (IL2, IL5, IL6, IL8, IL10, and TNF-alpha), and iNOS staining with clinical and pathological parameters of patients with primary ovarian malignancy.

2. Material and methods

2.1. Patients

We prospectively evaluated 40 patients in the Clinic Pelvic Mass, Discipline of Obstetrics and Gynecology/Oncology Research Institute (IPON), Federal University of Triângulo Mineiro (UFTM), who underwent surgical treatment in accordance with pre-established criteria, in from May 2009 to December 2011 and later confirmed diagnosis of malignancy ($n = 40$) of the ovary. The indication criteria for laparotomy were as follows; anechoic cysts with a maximum diameter smaller than 7.0 cm and persistence of change for more than 6 months and normal tumor markers; tumor markers changed; anechoic cysts with a maximum diameter greater than or equal to 7.0 cm; ovarian masses with solid content, presence of vegetation intracystic, thick septa, two or more thin septa; and a color Doppler resistance index less than or equal to 0.4 [2,14].

Data recorded included age, parity, race, smoking, history of hormone therapy, age at menarche and menopause, hormonal status, histological grade, stage [International Federation of Gynecology and Obstetrics (FIGO)], histological type, lymph node metastasis, response to chemotherapy (complete or partial), and a disease-free interval less than or equal to 24 months (present or absent).

In evaluating the histological grade, granulosa cell tumors and borderline tumors were considered in the same group as well differentiated tumors (grade 1). For other tumors, a review of the grade was conducted by a pathologist, and were subsequently divided into grade 1 (well differentiated), grade 2 (moderately differentiated), and grade 3 (poorly differentiated).

Inclusion criteria were diagnosis of postoperative primary ovarian malignancy by pathological paraffin. Exclusion criteria were the presence of adnexal torsion of the pedicle, the cyst ruptured during surgery, secondary ovarian malignancy (metastasis) chemotherapy treatment prior to surgery, and remission.

The study was reviewed and approved by the Ethics Committee of the Federal University of Triângulo Mineiro. Written informed consent was obtained from each patient or a family member.

2.2. Pathological Study

Pathological analysis was conducted by the Surgical Pathology Service, Federal University of Triângulo Mineiro. Cuts were embedded in paraffin, and the cases were reviewed by an experienced pathologist. The pathological evaluation and staging of the cases were performed according to the criteria of the International FIGO.

2.3. Immunohistochemistry

Paraffin blocks containing representative samples of tumors were selected from a review of the original slides. Specimens obtained from surgical resection were fixed in 10% formalin before being processed in paraffin. Hematoxylin–eosin-stained sections were reviewed by a pathologist and a representative section for each case was selected for immunohistochemical analysis.

Selected sections were deparaffinized, rehydrated, and heated in a microwave oven in 0.01 M citrate buffer (pH 6.0) for 30 min. Endogenous peroxidase activity was blocked by 3% hydrogen peroxide for 10 min, followed by a wash with PBS. The sections were incubated overnight at 4 °C with a primary antibody (anti-iNOS: rabbit polyclonal IgG 100 μg/ml, 1:200; anti-TNF: rabbit polyclonal IgG 100 μg/ml, 1:50; anti-IL-2: mouse monoclonal IgG 50 μg/0.5 ml, 1:200; anti-IL-5: rabbit polyclonal IgG 200 μg/ml, 1:200; anti-IL-6: mouse monoclonal IgG 100 μg/ml, 1:50; anti-IL-8: mouse monoclonal IgG 200 μg/ml, 1:50; anti-IL-10: rabbit polyclonal IgG 1 mg/ml, 1:600). Primary antibody was then detected with avidin-biotin peroxidase detection solution (*Dako Cytomation LSAB and System-HRP*) and the signal was visualized using diaminobenzidine (*Dako Cytomation Liquid DAB and substrate Chromogen System*). Slides were counterstained with Harris's haematoxylin, dehydrated, cleared, and mounted. Positive and negative controls were utilized. The cells were initially observed at a low magnification (100×) to assess the general distribution of primary antibody. The samples were then examined at a higher magnification (400×). The evaluation of cell staining was performed in tumor tissue. The tumor cells (showing gross and evident nucleolus, and irregular chromatin) were observed and counted at the higher magnification. Immunohistochemical staining was evaluated in the cytoplasm of tumor cells. Three observers evaluated the sections. The intensity of staining was evaluated subjectively using the following designations: 0–10% of cells stained: score 0; 11–25% of cells stained: score 1; 26–50% of cells stained: score 2; 51–100% of cells staining: score 3. When scores of multiple tissue stainings were combined, scores that were ≤1 were labeled "weak intensity" (Fig. 1 A/B), and scores ≥2 were labeled "strong intensity" (Fig. 1 C/D).

2.4. Statistical analysis

Data were analyzed using GraphPad Instat software. For immunohistochemical staining, the concordance between staining intensity scores was calculated according to the following classification: kappa <0.4: slight concordance; kappa ≥0.4 and <0.8: moderate concordance; kappa ≥0.8 and <1: strong concordance; and kappa = 1: perfect concordance. The first kappa inter-rater was between 0.8 and 1.0 of 1% (between strong and perfect