RESEARCH ARTICLE

Expression of Cytokines Interleukin-2, Interleukin-4, Interleukin-10 and Transforming Growth Factor β in Gastric Adenocarcinoma Biopsies Obtained from Mexican Patients

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Abstract

Objective: In this study, expression of Interleukin-2, Interleukin-4, Interleukin-10 and transforming growth factor beta in diffuse and intestinal type gastric cancers from Mexican patients was assessed for use as markers of malignancy. **Methods:** A total of 30 biopsies from gastric adenocarcinomas, 60% diffuse, 20% intestinal and 20% mixed in type, were studied by immunohistochemistry. **Results:** Regarding expression of cytokines, 23% were positive for IL-2, 26.7% for IL-4, 16.6% for IL-10 and none for TGF-β. There were found Significant statistically stage differences were noted.For example, for stages I-II 100% were IL-2 positive (p = 0.009), 87.5% were IL-4 positive (p = 0.005) and 100.0% IL-10 positive (p = 0.009). Young women were more likely to suffer gastric adenocarcinoma. In biopsies of male patients with gastric cancer, there was an increased expression of IL-2 and in biopsies from female patients in IL4. There was significantly greater detection of IL-4 and IL-10 expression in stages I and II than in stages III and IV. It was also found that IL-4, IL-10 had a higher positive expression in patients biopsies with low-level differentiations than patients with well differentiated gastric cancer in which cases were undetected. **Conclusions:** These results suggest that positive expression of IL-4 and IL-10 may be useful as a molecular marker to distinguish stage I and II diffuse gastric cancers which can be more readily controlled.

Keywords: Gastric adenocarcinoma- Cytokines- IL2- IL4- IL10- TGFβ

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Introduction

One of the most aggressive kinds of cancer in the digestive tract is gastric cancer, which is usually detected in advanced stages. Gastric cancer (GC) is the third type of cancer to its malignity and the second most common cause of death by cancer worldwide; nearly it is two thirds of new cases per year occur in the developing countries. It occurs more in men, than women (2:1), 95% of cases are adenocarcinomas which is the most common malignant tumor regardless of age, race or predisposing factors presented by a patient (Parkin et al., 2002; Tsugane et al., 2007; Ferlay et al., 2010). Tumor staging has been validated and established as the best prognosticator of patient survival. Gastric cancer has two well-known classification systems, the Lauren classification, subdivides intestinal gastric adenocarcinoma (well differentiated) and diffuse (poorly differentiated). The World Health Organization divides gastric cancer in: papillary, tubular, mucinous (colloid), and little cohesive carcinomas (Liu et al., 2010; Hu et al., 2012; Lauren, 1965).

It has been found that the immune microenvironment in tumor tissues is highly organized in a molecular and cellular level. It is compound of many different kinds of cells: such as endothelial cells, fibroblasts, lymphocytes and macrophages. It also contains numerous soluble molecules: such as growth factors, cytokines, chemokines which may have protumoral or anti-tumoral properties that depend on the context of the immune response (Fridman et al., 2012, 2013; Coussens et al., 2013; Bindea et al., 2013; Bhome et al., 2016). It has been shown that through cytokine production, may promote tumor angiogenesis, metastasis and induce to T cell differentiation and activation (Riabov et al.,2014; Zhang et al., 2009; Kuang et al., 2010, 2014; Wu et al., 2013; Zhao et al., 2015). In different tumors, a tendency is observed on the expression of anti-inflammatory cytokines and a decreased expression of proinflammatory cytokines; this

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change in expression could facilitate tumor progression by subversion of the mechanisms of cell immunosurveillance (Lee et al., 2014). Interleukin 2 (IL2) is produced in an immune response Th1 cytokine, and interleukin 4 and 10 (IL4, IL10) are an immune response Th2 cytokines. These cytokines are critical mediators of the Th1/ Th2 balance and they are involved in the process of inflammation-mediated carcinogenesis in human organs, including the gastrointestinal tract. Interleukin-10 (IL-10) is a pleiotropic cytokine produced by macrophages, T-helper 2 (Th2) cells, and B lymphocytes (CD5subset) and both can stimulate and suppress the immune response. (Howard et al., 1992). Another cytokine that mediates suppression of immune response as a strong inhibition of epithelial-cell growth involves the transforming growth factor-β (TGF-β). It can regulate multiple cellular functions, including both inhibition and stimulation of proliferation, apoptosis and differentiation. TGF-ß it is also an inducer of extracellular matrix (ECM) protein synthesis and has-been Implicated as the key mediator of fibrogenesis in several tissues (Border et al., 1994; Nishimura et al., 1998; Ihn et al., 2002). It has been reported that tumor cells can secrete inhibitory cytokines to avoid immune surveillance (Mantovani et al 2010). The expression of cytokines IL-4, IL-10 and TGF-β1 is reached in correlation with disease severity (Pisa et al.,1992).

There are many reports of molecular and clinical studies on the immune context on tumors; however they had not been related to the immune response associated with this type of malignancy. The aim of the present study was to analyze the clinic-pathological characteristics and describe the immune response T helper 1 (Th1), T helper 2 (Th2) and T helper 3 (Th3) by the cytokines presence in biopsies from a population of Mexican patients with gastric adenocarcinoma in different stages of malignity to be used as a marker of malignancy and for implementation of targeted immunotherapies for the treatment of this disease.

Materials and Methods

Patients and biopsies

30 biopsies from patients of both genders were studied, with in an age range of 14-80 years with a histological test confirming gastric adenocarcinoma were underwent a total gastrectomy, obtained by the Pathology Department of the Instituto Mexicano del Seguro Social, Unidad Médica de Alta Especialidad, Hospital de Especialidades, Centro Medico Nacional General de Division "Manuel Ávila Camacho", Puebla, Mexico.

Paraffin inclusion

Tissue samples were fixed with 10% formalin immediately after surgical resection and embedded in paraffin. 6 cuts 4um, one paraffin block for each patient, then were collected on glass slides coated with Poly-L-lysine Solution (P 8920, Sigma-Aldrich) and processed according to technical considerations Haines were obtained (Haines et al., 1991). All cuts were deparaffinized and rehydrated in decreasing concentrations of ethanol baths. In a cut of each biopsy of each patient, they underwent the hematoxylin-eosin staining to determine their histological difference; with the remaining cuts one immunoperoxidase histological test was performed to determine the following cytokines IL-2, IL-4, IL 10 and TGFβ.

Cytokines

The cytokines detection was performed by indirect immunoperoxidase staining of paraffin-embedded tissue sections in biopsies of patients with gastric adenocarcinoma, following the technical indications of Kashani (Kashani et al., 2013). The fixed paraffin-embedded tissue was sectioned at 4-um thickness. Representative tissue sections were stained with hematoxylin-eosin to confirm the diagnosis prior to immunoperoxidase staining. The hydrated tissue sections were treated with 10% H2O2 in methanol for 10 minutes to Block endogenous peroxidase and incubated with Target Retrieval Solution pH 9 (Dako Cytomation DenmarkA / S, Denmark) for 40 min at 95° C in a water bath for antigen retrieval. After each treatment, the sections were washed 3 times in PBS for 5 minutes. The sections were incubated with a panel with specific monoclonal antibodies for each cytokine in dilutions of 1: 100 to 1: 1,000. Sections were incubated with monoclonal antibody specific IL-2 (Anti-Human IL-2 Purified, and Biocience, California), IL-4 (Anti-Human IL-4 Purified, and Biocience, California), IL-10 (Anti-Human IL-10 Purified, and Biocience, California) and TGFβ (Mouse Anti-Human Beta TFG, AbD. Serotec, UK), All sections were incubated overnight in a humid chamber at 4 °C. Subsequently, the slides were washed 3 times in PBS for 5 minutes. As a secondary antibody, was used an anti-mouse IgG coupled to peroxidase (Polyclonal goat Anti-Mouse Immunoglobulins DakoCytomation A/S, Denmark.), at a dilution 1: 1000 for an hour, at room temperature and washed again three times with PBS. Peroxidase activity was revealed by 3, 3'-Diaminobenzidine tetrahydrochloride (Sigma) (DAB)-H2o2 reaction. The slides were incubated with DAB (0.5 mg/ml PBS) containing 0.005% H2O2 for 10 minutes at room temperature, and then washed with distilled water. Finally, slides were counterstained with hematoxylin for 30 seconds and dehydrated in increasing concentrations of ethanol baths, clarified with xylene and mounted with resin (Entellan, Merck, Germany). All slides were observed and analyzed in a light microscope (Nikon, Eclipse E600) and different images were captured.

Statistical analysis

A statistical descriptive analysis was performed using SPSS 22 statistical software. Age comparisons were performed between negative and positive cytokines using Student's t preflight normal distribution with the Kolmogorov-Smirnov statistic; comparisons of proportions (age, gender, histological type, clinical stage, inflammatory reaction) were performed with Chi Square or the Fisher exact test case. Differences were considered significant at $p < 0.05$.

Results

Thirty biopsies of patients with gastric adenocarcinoma

Table 1. This Chart Shows the Percentage of Positive cytokines Studied, in the Biopsies of Patients with Gastric Cancer by Clinical Stagea

Cytokines	IL-2 n(%) p=0.009		IL-4 n(%) p=0.005		IL-10 n(%) p=0.009	
Clinica stage	Positive n=7	Negative n=23	Positive n=8	Negative n=22	Positive n=5	Negative n=25
I-II						
n=5 (I) and 8 (II)	7 (100.0)	6 (26.1)	7 (87.5)	6 (26.7)	5 (100.0)	8 (32.0)
III-IV						
n=9 (III) and 8 (IV)	0 (0.0)	17 (73.9)	1 (12.5)	16 (72.2)	0 (0.0)	17 (68.0)

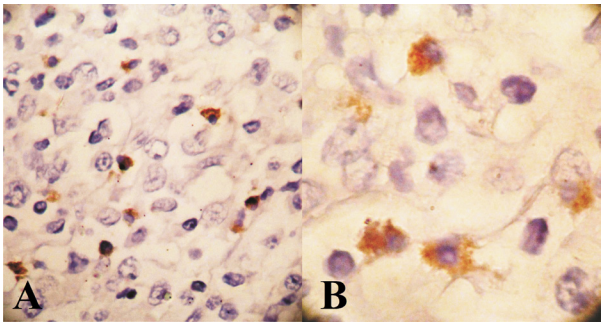

Figure 1. Photograph of Gastric Cancer Diffuse is Shown the Positive Expression of Interleukin -2 (IL-2) Detected by the Immunoperoxidase Technique in Section Paraffin of Biopsies of Patients with Gastric Adenocarcinoma. Typical Expression of IL-2 is Observed in the Tumor Cells Cytoplasm Using Anti-IL2 Moab and IL-2 Signals are Reveled in Brown Precipitate. A) 40X, B) 100x

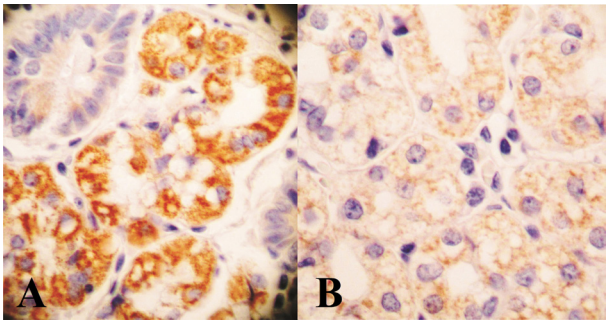

Figure 2. Photograph of Gastric Cancer Diffuse is Shown the IL-4 Cytokine Expression by Immunohistochemistry in Biopsies of Patients with Gastric Adenocarcinoma. Section Paraffin, were Stained with Anti- IL-4 Moab and IL-4 Signals are Revealed in Brown in the Glandular Epithelium (Brown Precipitate). A) 40X, B) 100X

Table 2. This Chart Shows the Percentage of Positive Cytokines Studied, in the Biopsies of Patients with Gastric Cancer by Histologic Classification

Cytokines	IL-2 n(%) p=0.07		IL-4 n(%) p=0.07		IL-10 n(%) p=0.2	
Histological Classification	Positive n=7	Negative n=23	Positive n=8	Negative n=22	Positive n=5	Negative n=25
Diffuse n=18	4 (57.1)	14 (60.9)	7 (87.5)	11(50.0)	4 (80.0)	14 (56.0)
Intestinal n=7	3 (42.9)	4 (17.4)	0 (0.0)	7 (31.8)	0 (0.0)	7 (28.0)
Mixed n=5	0 (0.0)	5 (21.7)	1 (12.5)	4 (18.2)	1 (20.0)	4 (16.0)

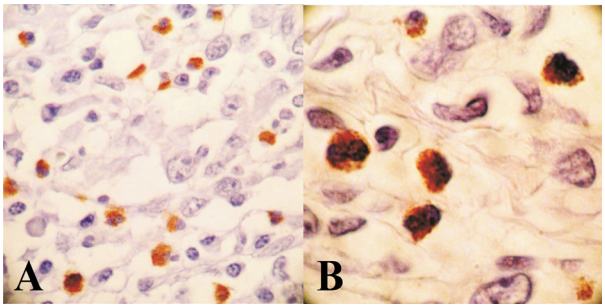

Figure 3. Immunodetection of the Expression of IL-10 Cytokine in Section Paraffin of Biopsies of Patients with Diffuse Gastric Adenocarcinoma. is Observed High Positivity in the Cytoplasm of a Large Number of Tumor Cells by Immune Peroxidase Test Using Anti-IL-10 Moab and Signals IL-10 are Reveled in Brown Precipitate. A) 40X, B) 100X

with an age range of 14-80 years were analyzed, with an average of 58.34 and a standard deviation of 14.7 (Kolmogorov-Smirnov 0.83, p = 0.49). 23 patients (76.7%) underwent total gastrectomy and only 7 patients (23.3%) subtotal gastrectomy. The type of intervention was in relation to the location of the tumor, total gastrectomy (GT) was performed for proximal gastric lesions including resection of the spleen and distal subtotal gastrectomy (distal GST) for lesions of the antrum. Based on the Lauren classification 18 cases (60%) of patients with diffuse adenocarcinoma, 7 cases (20%) of intestinal and 5 cases (17%) of mixed (Table 1) were studied. The inflammatory reaction was evaluated according to Sidney classification, that consist of four levels based on the number of macrophages and lymphocytes field with findings of no assessable inflammatory reaction in 2 cases, mild in 14 cases, moderate in 13 cases and severe in 1 case (Table 2). The clinical stage was assessed with the TNM classification, including 5 biopsies (16.6%) were stage I, 8 cases (26.6%) for stage II, 9 cases (30%) for stage III and 8 cases for stage IV (26.6%) patients (Table

Table 3. This Chart Shows the Inflammatory Infiltrate, Present in the Study of the Biopsies of Patients with Gastric Cancer, According to Sidney Classification

Cytokines	IL-2 n(%) p=0.3		IL-4 n(%) p=0.5		IL-10 n(%) p=0.7	
Inflammatory Infiltrate	Positive n=7	Negative n=23	Positive n=8	Negative n=22	Positive n=5	Negative n=25
Light n=14	5 (71.4)	9 (39.1)	4 (50.0)	10 (45.5)	3 (60.0)	11 (44.0)
Moderate n=13	2 (28.6)	11 (47.8)	4 (50.0)	9 (40.9)	2 (40.0)	11 (44.0)
Severe n=1	0 (0.0)	1 (4.3)	0 (0.0)	1 (4.6)	0 (0.0)	1 (4.0)
No evaluation n=2	0 (0.0)	2 (8.7)	0 (0.0)	2 (9.0)	0 (0.0)	2 (8.0)

3). Th2 cytokines (IL-4, IL-10) positive were in 87.5% and 80% of cases of diffuse gastric cancer (Table 1), with slight inflammatory infiltrate and moderate (Table 2) the highest percentage was detected in female patients. The IL-2 the highest percentage of positivity was in diffuse gastric cancer (Table 1), stage I-II (Table 3) in males with slight inflammatory infiltrate. The IL-2 was detected in the cytoplasm of tumor cells in diffuse and intestinal gastric cancer (Fig. 1, brown precipitate) and IL-4 and IL-10 only in the cytoplasm of tumor cells of diffuse gastric cancer (Fig. 2, 3 brown precipitate). TGFβ was negative, at all stages and types of gastric cancer not shown. Statistically significant differences in stages I-II, for 100% of the IL-2 positive ($p = 0.009$) were found 87.5% of IL-4 positive ($p = 0.005$) and 100.0% of the IL-10 positive ($p = 0.009$).

Discussion

This study describes the expression of cytokines IL-2, IL-4, 1L-10, TGFβ in biopsies of Mexican patients with gastric cancer in different stages of malignancy; we detected the expression of cytokines in patients female with gastric cancer stages I-II. We found that there were IL-2, IL-4, IL-10 significantly more positive expression in male and female patients with stage I and II with light and moderate inflammatory infiltrate with diffuse gastric cancer than those with gastric cancer in stage III and IV (Tables 2 and 3). It was also found that IL-4, IL-10 expression in patients with undifferentiated cancer than patients with moderate or high-level differentiation. These results suggest that positive detection of IL-4 and IL-10 expression may be used as a molecular marker for distinguishing the stage I and II of gastric cancer, as well as distinguishing undifferentiated and moderate cancer differentiation. As the IL10 represents an immunoregulatory cytokine with immunosuppressive anti-inflammatory activity and it can stimulate the cell NK (Moore et al., 2001; Mege et al., 2006), for this reason, we think, there may be active NK cells in stages I and II, that can detect and destroy tumor cells to induce them in a direct cytotoxicity offering the patient with gastric cancer higher survival rates. We have observed in mice implanted with gastric tumor cells a NK cells increased and decreased size of the tumor mass (manuscript in redaction). Jingting et al In preclinical studies in a mouse model of tumor growth in vitro with added NK cells and IL-2 cytokine, they have demonstrated a significant antitumor effect against a number of hematopoietic and solid tumors (Jingting et al 2013). However, contradictory results have been found in gastric carcinoma cells that can release IL-10, which has a suppressive activity on NK cells that could reduce secondarily the cytotoxicity of NK cells and increase tumor mass. (Szkaradkiewicz et al., 2010). Xiong-Fei in his paper said that since IL-10 can both reduce and enhance anti-cancer properties, it may be significant to explore the role of IL10 polymorphisms in the development of Gastric Cancer in different clinical stages, or Gastric Cancer of different subsites (Pan et al., 2013). Tanaka et al., (2008) demonstrated that the intraperitoneal with IL-10 treatment was able suppressed peritoneal dissemination of gastric cancer cells and reduce peritoneal metastasis and increase survival rate, in the inoculated mice. We found expression of IL-10 and IL-4 in patients with gastric cancer stages I-II in which, gastric cancer can be controlled with a light to moderate inflammatory infiltrate in Mexican population (Table 1 and 2). Different authors mention, that in cancer the presence of macrophages in the inflammatory infiltrate is closely related to progression and metastasis. During the startup of cancer the macrophages create an inflammatory environment that is mutagenic and induces progressively the tumor to malignancy. In the biopsies of Mexicans patients studied in stages I and II, we detected a high population of macrophages and tumor cells producing IL-4 IL-10 which in some cases, may be related to tumor development and can diminish the beneficial effects of therapy. We have detected active CD68 + macrophages in mice implanted with gastric tumor cells and production of IL4 at 15 days post implantation of tumor cells (manuscript in redaction). With an in vivo assay for invasive tumor cells in PyMT mouse model, in breast cancer cell xenografts, macrophages have shown to be required for tumor cell migration and invasion (Condeelis et al., 2006; Williams et al., 2016; Qian et al., 2010). Macrophage polarization to the invasion-promoting phenotype is in turn regulated by IL-4 synthesized by CD4+ T cells or tumor cells. In the absence of IL-4, macrophages are unable to promote invasion and migration of tumor cells, and metastasis is dramatically reduced in the PyMT model (DeNardo et al., 2009; Gocheva et al., 2006). Relative to IL-10, Jing Liang, found increased expression of IL 10 in patients with stages III and IV with low level differentiations possessed significantly higher positive detection ratios than patients with moderate or high-level differentiation in the Chinese population (Liang et al., 2011). Another cytokine that mediates suppression of immune response as

well as strong inhibition of epithelial-cell growth, Involves the transforming growth factor-β (TGF-β), (Letterio et al., 1998) which was not detected in any Mexican patient with stages I to IV, though, it has been demonstrated the presence of IL10 and TGFβ in culture supernatants of gastric tumor cells. Increased serum levels of IL-10 and TGF-β1 were described in advanced gastric cancer (De Vita et al., 1999; Nakumara et al., 1998; Na et al, 2010). These suggest that TGFβ produced by gastric cancer cells changes the morphology of mesothelial cells and may be closely associated with peritoneal dissemination of cancer cells (Na et al., 2010). Alterations in the receptor TGFβ have been associated in different types of cancer, such as colorectal cancers, gastric, breast, small cell lung carcinoma and endometrial carcinoma who have progressed to an invasive state. (Myeroff et al., 1995; Akiyama et al., 1997; Fynan et al., 1993; Sun et al., 1994; Peralta-Zaragoza et al., 2001). It is very important to identify the different cytokines th1, th2, th3, expressed or produced by tumor cells; to detect the immunosuppression degree induced in the patient by these tumors and could be neutralized using monoclonal antibodies, to have an effective immune response in the patient. We found something very important that is the predominance of diffuse gastric cancer (60%) and intestinal gastric cancer (23%) in the Mexican patients studied, which differs from what reported; by other studies (50% diffuse cancer and 50% intestinal cancer (Lauren. 1965). Whence is necessary and very important, to study a larger population of patients with cancer, to understand the role of the IL-4 and IL-10 cytokines in the immune response suppression induced by tumor cell in gastric cancer, to be used as molecular markers to distinguish stages I and II in which the cancer can be controlled, offering the patient a better quality of life and a longer survival rate.

Recommendations

It is necessary to introduce screening programs in our country, for early detection of gastric cancer surgical treatment and thus can provide better disease-free time and survival.

A clearer understanding of this immunoendocrinas network interactions could profoundly promote the treatment of this disease.

In addition, it is also necessary immunogenetic studies to detect antigens of histocompatibility; protective or flattering because each population is different and in the literature most of the studies that has been conducted is on the United States of America or European population.

Conflicts of interest

The authors declare no potential conflicts of interest.

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