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Immunohistochemistry Analysis for Interleukin-6 Expression from the Tumor Tissue

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Abstract: The cytokines including interleukins considered an important component of the body's immunity against inflammatory conditions and cancer diseases, and is an important factor in inducing the symptoms of wasting weight in cancer patients. Therefore, the knowledge and the study of these factors and their impact, production, particularly interleukin-6 gene expression and identification to recognize the presence and production of this factor in the tissues of patients through immunohistochemistry technique and gene expression. The study included 40 samples from different types of malignant tumor biopsy and (7) samples for benign tumor used as control. Immunohistochemistry (IHC) analysis revealed that there was an increase in the production of interleukin -6 which appeared as brown precipitate in the cytoplasm of cancer cell as positive staining , while, the benign tumor tissues (control) showed negative staining.

Keywords: IL-6, immunohistochemistry, tumor tissues

INTRODUCTION

Study for Wang *et al.* (2014) was determined the effect of overstating and administration alone or in combination on IL-6 levels in androgen –dependent prostate cancer LNCaP cells. While, the study of Carillo and Ippoliti (2006) were analyzed 168 prostatic carcinomas to define the role of IL-6, IL-10 and Hsp-90 in prostate cancer by progression the immunohistochemically expression for these proteins. For evaluate the predictive capacity of IL-6 in relation to clinical outcome Chen *et al.* (2013) were used clinical specimens from 85 patients with muscle-invasive and 50 patients with non-muscle invasive bladder cancer that selected for immunohistochemistry staining.

Chung *et al.* (2006) found the relationships between the positive expression of IL-6 and both clinical pathological factors and survival that evaluated at immunoreactivity of IL-6 in cancerous tissue that measured by immunohistochemically staining. Study of Dvorakova *et al.* (2006) confirmed expression of IL-6 in intestinal glandular epithelium in Barrett's esophagus tissue by the immunohistochemistry study.

Immunohistochemistry (IHC) is an important application of monoclonal and polyclonal antibodies to determine the tissue distribution of an antigen for health and disease, it is the utilization of monoclonal and polyclonal antibodies for the detection of specific antigens in tissue sections, specific tumor antigens are expressed up regulated in certain cancers, Therefore, immunohistochemistry widely used for diagnosis of cancers. Many of studies showed that IHC plays an important role in pathology and particularly in the subspecialties of oncologic pathology , neuropathology and hematopathology , The first IHC study was reported at 1941 but the principle of immunohistochemistry has existed since the 1930s(Ajura *et al.* ,2007). Kayamori *et al.* (2010) were examined the expression of IL-6 to revealed that was this expression not only in cancer cells but also in fibroblasts and osteoclasts at the tumor bone interface. While, Becker *et al.* (2005) were demonstrated that IL-6 can regulate the proliferation of intestinal epithelial cells (IEC) and inducing growth of dysplastic lesion in vivo as possible functional role of interleukin-6.

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were still covered with retrieval solution for 15 min on power (350 watts). Cool was done slowly at room temperature for at least 20 min.

C-Preparation of working solutions

Freshly prepared buffers was used in the preparation of all working solution, the materials used in this procedure were as the following:

- Blocking serum: This included 75 µl normal blocking serum stock with 5 ml PBS.
- Biotinylated secondary antibody: included 75 µl normal blocking serum stock with 5ml PBS and 25µl biotinylated secondary antibody stock.
- AB enzyme reagent: Which included 50µl reagent A (avidin) and 50µl reagent B (biotinylated HRP) and 2.5 ml PBS .mix and let stand for approximately 30 min.
- Peroxidase substrate: which included 1.6 ml distilled water and 5 drops 10x substrate buffer and 1 drop 50x DAB chromogen and 1 drop 50x peroxidase substrate.

Procedure

The procedure carried out at room temperature in humidified chamber as the following:

- Sections were incubated for 1 hour in 1.5% blocking serum in PBS (mixing bottle 1).
- Sections were incubated with primary antibody (1:50) for 4µl from primary antibody and 2ml from blocking serum at 4C overnight.
- Wash with three changes of PBS for 5 min each.
- Sections were incubated with biotinylated secondary antibody for 30 min.
- Wash with three changes of PBS for 5 min each.
- Sections were incubated with AB enzyme reagent for 30 min.
- Wash with three changes of PBS for 5 min each.
- Sections were incubated in 1-3 drops peroxidase substrate for 10 min.
- Wash with deionized water for 5 min.
- Sections were inserted in Mayer's hematoxylin stain for many seconds.
- The slides were rinsed gently in tap water for many seconds.
- The slides were rinsed gently with distilled water for 5 min.
- The sections were mounted using aqueous mounting medium such as paramount.
- The sections may be checked by light microscope.
- Scoring system for the different types of tumor tissue according to the Allred score, where measuring of proportion score (PI) and the intensity score (Is) for this tissues and then calculated the Allred score.

Results

Lonnorth *et al.* (1994) showed that IL-6 acted as a paracrine factors for stimulated by some other host derived factors because the tumor tissue produce highest concentrations of IL-6 in tumor cells as followed by inflammatory and endothelial cells that seem by the IHC staining. Paule *et al.* (2000) found no significant difference in the tumor size and grade between renal cell carcinomas with or without IL-6 expression, and demonstrated that IL-6 is expressed in 70% of primary tumors, however, a relatively large number of high grade tumors that is IL-6 expression by the IHC study.

Materials and Methods

The study included 40 samples obtained from different types of malignant tumor biopsy this include : Gastric (9) , Colon (8) as cachectic cancer patients and Lung (8), Thyroid (8) as non-cachectic cancer patients and (7) samples taken from benign tumor (as control) this include that is :Gastric (3) , Lung (1) , Colon (2) , Thyroid (1).

Immunohistochemistry Experiments (Cuello, 1993)

Immunohistochemistry for Interlukin-6

- Interlukin-6 immunohistochemistry kit was used from Santa Cruz biotechnology company (ABC staining system: Sc-2017) and IL-6 mouse monoclonal antibody (Sc-130326).

A- Removal of paraffin and rehydration

For IL-6 immunohistochemistry, 5 µm thick section were used from different types of biopsy, the paraffin were removed then the section dehydrated with 90%, 70% ,50% in ethanol alcohol at 5 min for each change.

Slides were placed in a 56-60 C oven for 60 min (Caution: oven temperature must not exceed 60 C). Then transferred to a xylene bath and two changes of xylene for 5 min each. The excess liquid was shook off and rehydrated slides in three changes of 99% absolute ethanol for 5 min each. The excess liquid was shook off then the slides placed in two changes of 95% ethanol for 5 min for each. The excess liquid was shook off and the slide placed s in 70% ethanol for 5 min. The excess liquid was shook off and the slides placed in distal water for 5 min.

B- Antigen Retrieval (unmasking of Antigen) Microwave retrieval:

The antigen retrieval solution was ready to use. The slides were washed with deionized distilled water and placed in a microwave-resistant plastic staining jar containing antigen retrieval solution. The microwave oven was operated on for 9 min on high power (750 watts) and made sure that the slides

The expression of IL-6 protein was localized in cytoplasm of the thyroid follicular tumor .its expression was detectable in (50%) and the Allred score was at (5.25) , the proportion score at (3.25) and the intensity score at (2.0) , this give an expression of intermediate staining Fig (1,2), while the benign tumor showed negative stain Fig(3).

As shown by the immunohistochemically investigation a brown color was precipitated in the cytoplasm which gave an indication of IL-6 positive stain.

1. Non-cachectic cancer patients
Thyroid tissue

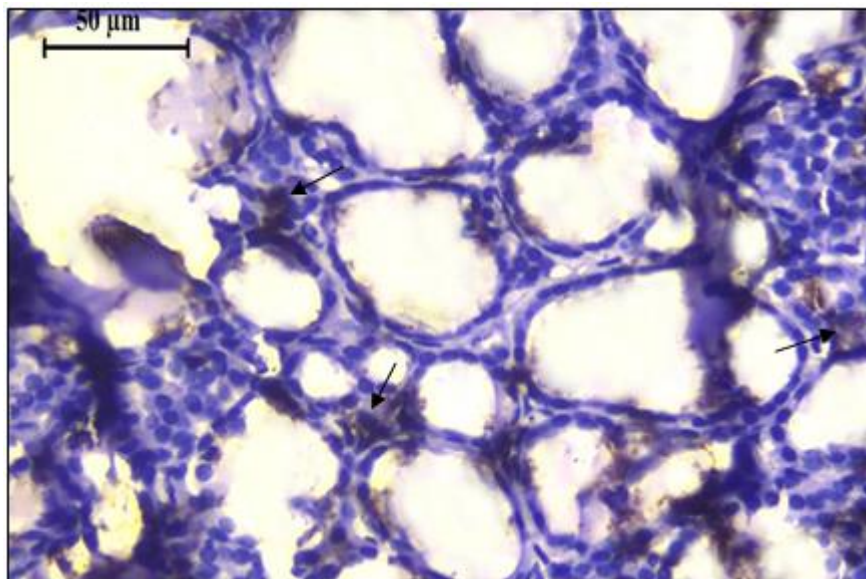


Figure (1): IL-6 immunohistochemistry for thyroid follicular tissue tumor, showing the positive staining for IL-6 expression in cytoplasm interfollicular cells (arrow).

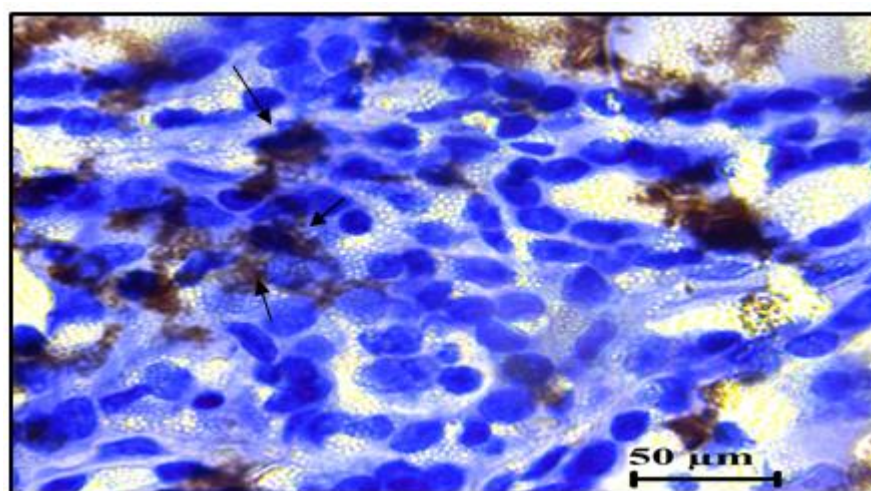


Figure (2): Thyroid gland section showing dense positive staining for IL-6 expression in interfollicular cells (arrow).

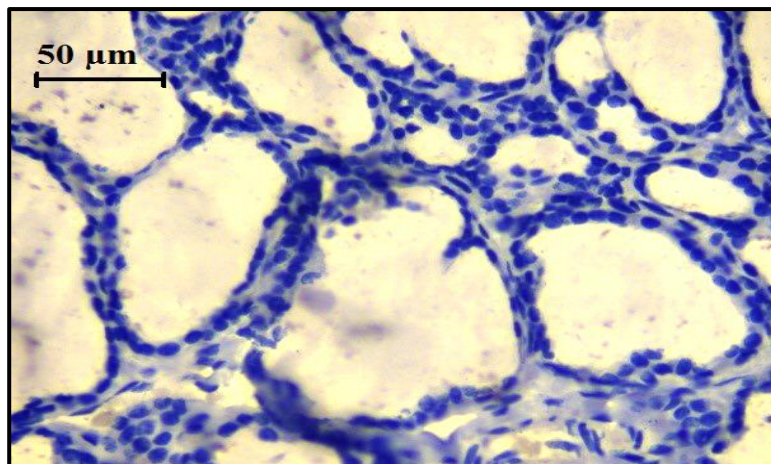


Figure (3): Thyroid gland section showing negative control staining for IL-6 expression.

at (3.00) , where the proportion score at (2.00) and the intensity score at (1.0) , Fig(4,5) , While , in figure (6) the instruction slide for immunohistochemistry for IL-6 positive expression in non-small lung tumor tissue .

Lung tissue

The expression of IL-6 protein was localized in cytoplasm of the lung tumor, the staining showed as brown precipitate in cytoplasm of cells that indicated a positive weakly staining. Its expression was detectable in (37.5%) and measuring the Allred score

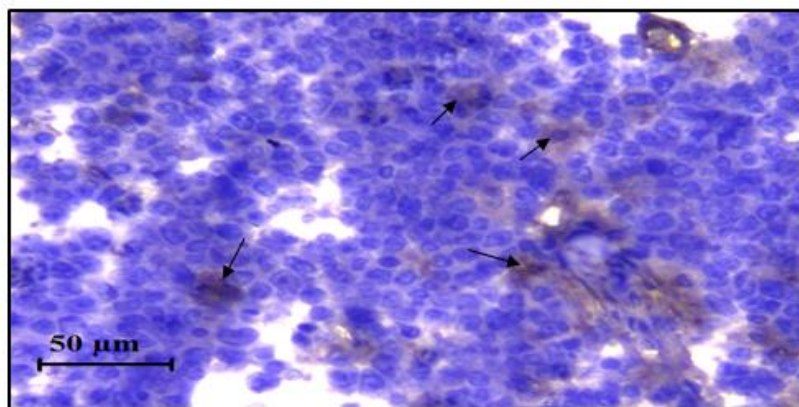


Figure (4): IL-6 immunohistochemistry for non-small lung tissue tumor non-cachexia cancer patients. Showing the positive staining for IL-6 expression in cytoplasm in adventitia connective tissue cells (arrow).

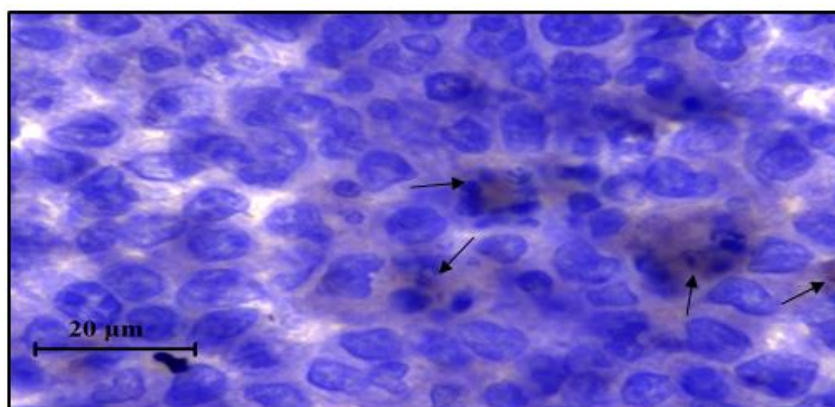


Figure (5): IL-6 immunohistochemistry for lung tissue tumor non-cachexia cancer patients. Showing the positive staining for IL-6 expression in cytoplasm in adventitia connective tissue cells (arrow).

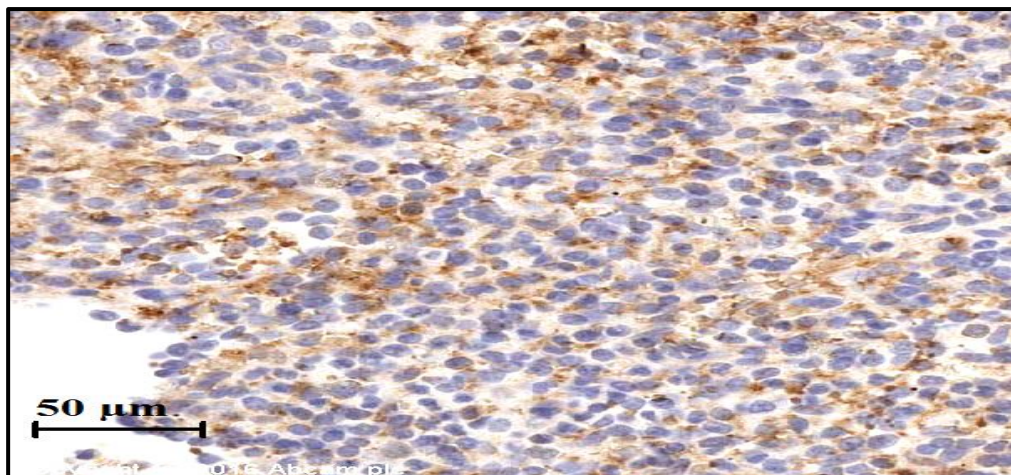


Figure (6): The instruction slide for immunohistochemistry for IL-6 positive expression in non-small lung tumor tissue (Santa Cruze company kit).

2. Cachectic cancer patients

Gastric tissue

The expression of IL-6 protein was localized in cytoplasm of the gastric (pyloric) tumor the brown precipitation found in the cytoplasm of surface epithelial cells, lamina propria cells, and in pyloric gland cells from mucosa layer, that indicated a positive staining .Its expression was detectable in (58.33%) and measuring the Allred score at (5.25), where the proportion score at (3.25) and the intensity score at (2.0) Fig (7, 8, 9). In contrast, the benign gastric tumor tissue showed negative stain Fig (10).

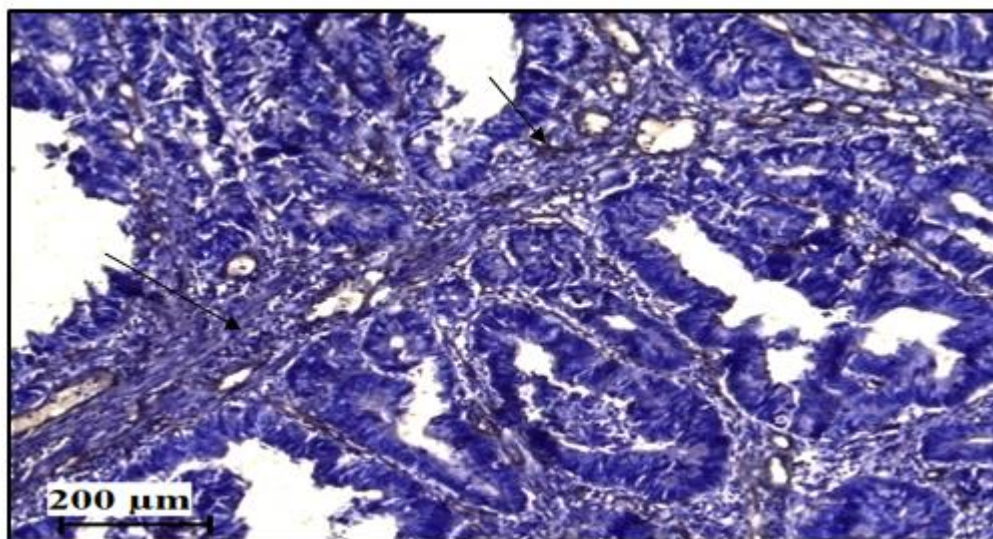


Figure (7): Section from malignant gastric tumor showing the positive staining for IL-6 expression in pyloric region (arrow).

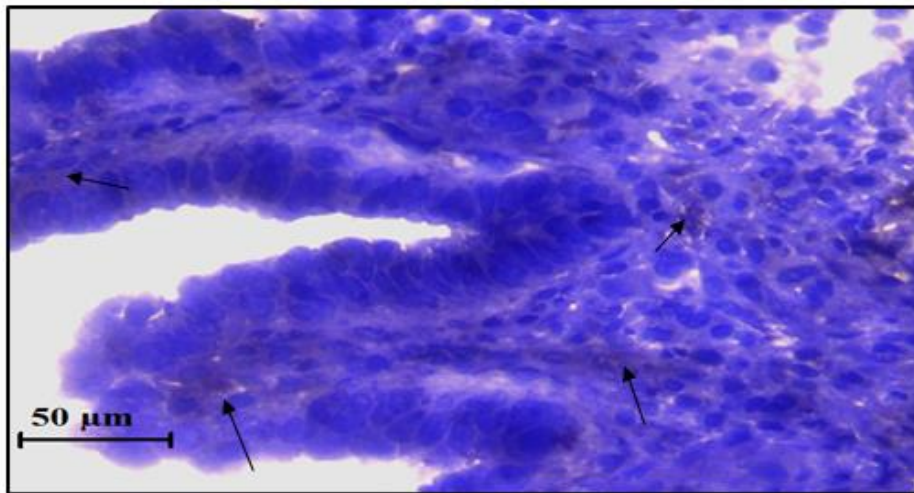


Figure (8): Another section from malignant gastric tumor taken in the lamina propria. Note the presence of positive stain of IL-6 (arrow).

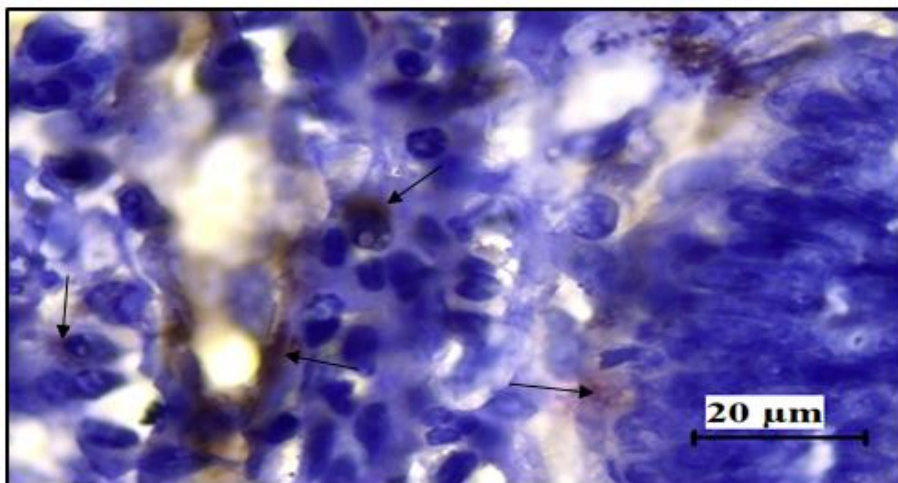


Figure (9): Section from malignant gastric tumor taken in gastric gland. Showing the positive staining for IL-6 expression in cytoplasm of pyloric gland cells (arrow).

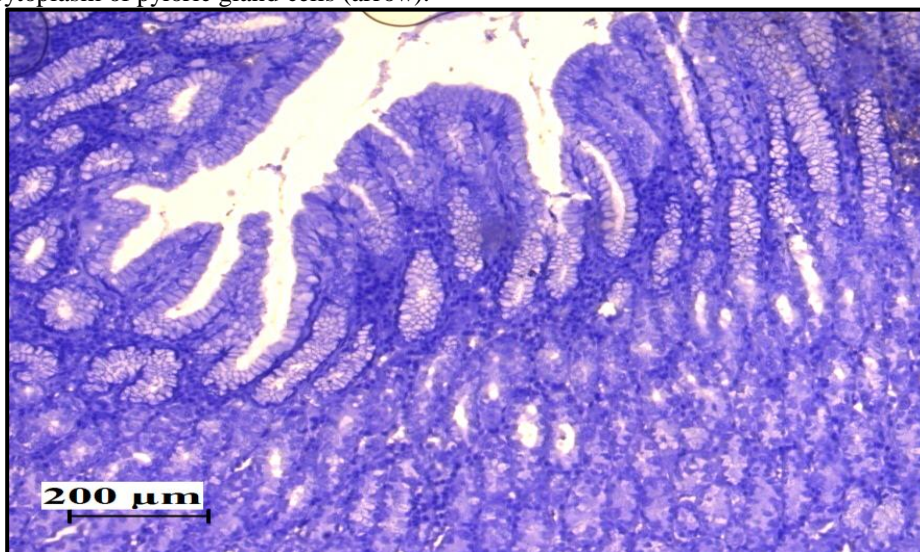


Figure (10): Section from malignant tumor taken from the gastric showing negative control staining for IL-6 expression.

Colon tissue

In sections obtained from malignant colon tumor showed high degree of positive staining in the mucosa. Its expression was detectable in (70.75%) and measuring the Allred score at (5.66) , where the proportion score at (2.66) and the intensity score at (3.0) ,Figures(11,12,13,14).

While in patients with benign tumor colon tissues showed no stain precipitation, Figure (15).

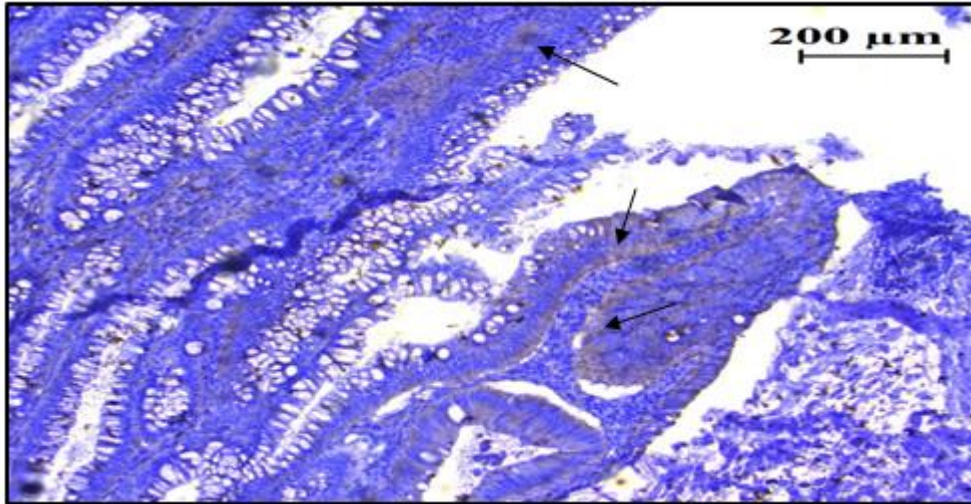


Figure (11): Section from malignant colon tumor showing the positive staining for IL-6 expression in cytoplasm of lamina propria(arrow).

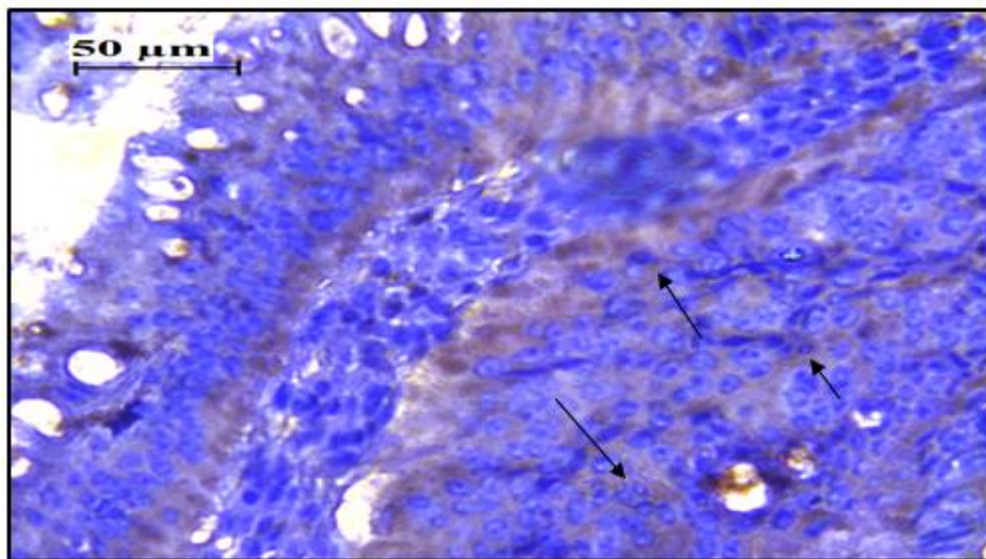


Figure (12): Another section taken from malignant colon tumor showing the positive staining for IL-6 expression in cytoplasm of lamina propria(arrow).

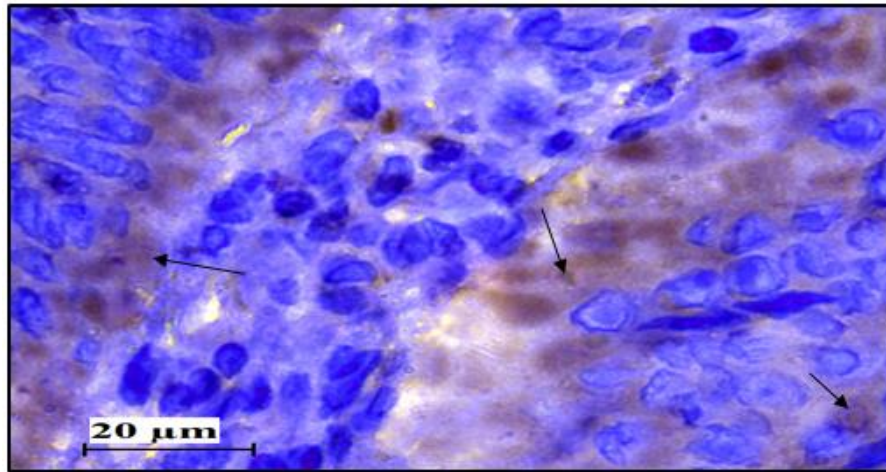


Figure (13): Section from malignant colon tumor showing the positive staining for IL-6 expression in cytoplasm of lamina propria (arrow).

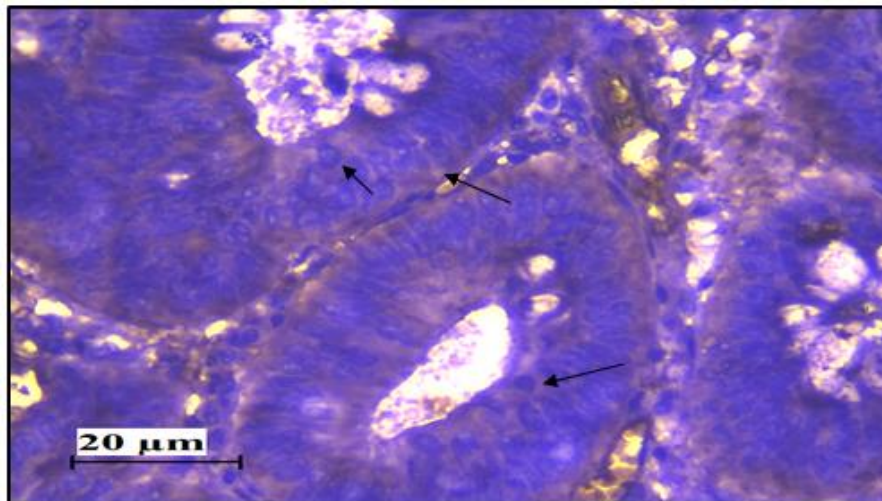


Figure (14): Section from malignant colon tumor, showing the positive staining for IL-6 expression in cytoplasm colon gland cells (arrow).

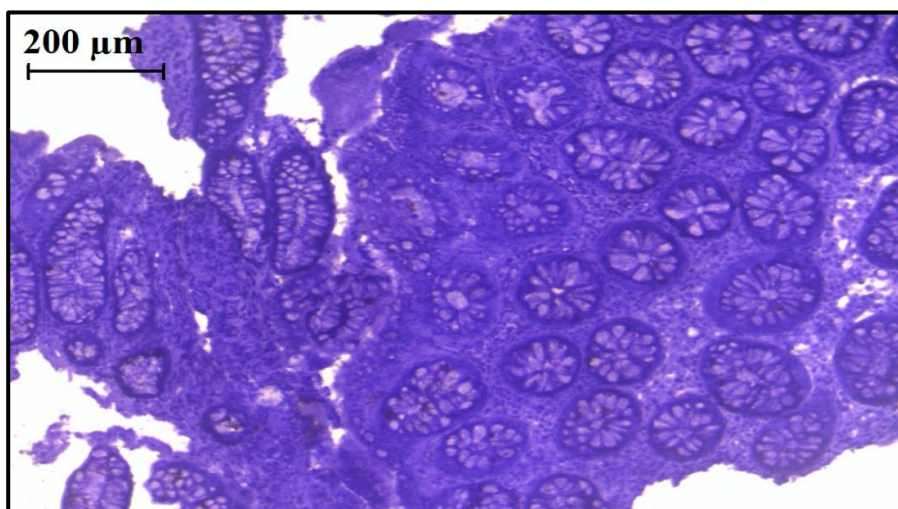


Figure (15): Colon tissue tumor showing the negative control staining for IL-6 expression.

According to the Allred score, the present study showed the highest levels of staining were in the Colon malignant tumor, since the percentage of the positive staining was 70.75%, followed by Gastric malignant tumor which reached 58.33%. Which the lowest score was in lung malignant tumor which was 37.5% (Table-1).

The result showed that cancer cells from cachectic patient tissues gave strong signal for IL-6 antibody in comparison with tissue sections of non-cachectic patient tissues.

Table (1): The scoring of different types of tumor tissue to the IL-6 had shown according for the Allred score formula.

Type of cancer	Number	Main of Proportion Score	Main of Intensity Score	Allred Score (0-8)	Positive staining 100%
Gastric	9	3.25	2.0	5.25	(58.33%)
Thyroid	8	2.25	2.0	4.25	(53.1%)
Lung	8	2.0	1.0	3.0	(37.5%)
Colon	8	2.66	3.00	5.66	(70.75%)

Discussion

The human interleukin-6 is a glycoprotein contain of 184 amino acids that depending on the degree of glycosylation that is a molecular weight between 21-28 KDa(Burger, 2013). The interleukin -6 involved in the differentiation and regulation of immune response and regulation of various cellular functions this include proliferation, apoptosis and angiogenesis (Culig *et al.*, 2005).

Furthermore, this protein is synthesized by different cell types such as B and T cells, monocytes, macrophages, fibroblasts, endothelial and mesothelial cells, keratinocytes, mast cells, stromal cells, and in some nerve cells and certain tumor cells (Kishimoto *et al.*, 1995). Another main source of IL-6 is adipose tissue as to a pleiotropic cytokine (Fain *et al.*, 2004).

The interleukin-6 as known to be a cytokine with many multiple functions to regulation of hematopoiesis, induction of acute phase reactions and inflammation as well as bone, cartilage , and lipid metabolism , it is not involved in B cells only but also in T cells immune responses (Mihara *et al.* ,2012; Kishimoto *et al.*, 1995). The IL-6 gene expression under normal physiological conditions, that mostly induced by stimulation cause an inflammatory response for the TNF- α and β , IL-1, bacterial endotoxin and lipopolysaccharide, virus infection, and interferons (Kishimoto *et al.* ,1995).

The immunohistochemically analysis of the present study gave evidence that IL-6 expression was highest

in cachexia patients with digestive malignant tumor (e.g. Colon and Gastric).

This study was in agreement with the study of Tan *et al.* (2013) that showed interleukin-6 expression in lung cancer tumorigenesis. This study was in agreement with Martignoni *et al.* (2005) they show the strong present for IL-6 immunoreactivity in the cytoplasm of pancreatic cancer cells for the cachectic cancer patients compare with the same type of tumor for the non-cachectic cancer patients.

Kishimoto (2010) reported that the increase of IL-6 production contributed to the pathogenesis of many chronic inflammatory and autoimmune diseases and the IL-6 import factor in variety of human diseases states including cardiovascular diseases, sepsis, fever, cachexia, insulin resistance, osteoporosis, and neuro disorders.

The oncogene – associated inflammatory can be leads to production of inflammatory cytokines such as interleukin-6 (IL-6) (Bayliss *et al.*, 2011; Ancrile *et al.*, 2007). However, there is association of the IL-6 with increased risk of lung cancer (Chen *et al.*, 2013; Bai *et al.*, 2013). Many of studies have that correlated high circulating IL-6 levels with poor survival of lung cancer patients (Songur *et al.*, 2004; Wojcik *et al.*, 2010).

Chen *et al.* (2013) studied the correlated state of IL-6 expression with higher clinical stage; the higher recurrence was blocked in the study of bladder cancer tissue which showed over expressed for IL-6 in this

tissue compared with non-malignant bladder tissue. Moreover, IL-6 expression was significantly increased in advanced stage as compared to early stage of gastric cancer (Wang *et al.*, 2013). And colorectal carcinoma (Chang *et al.*, 2006). Also IL-6 expression has been observed to be higher in the primary tumor tissues than the adjacent normal tissues in prostate cancer (Engelhardt *et al.*, 2014). Breast cancer (Labovsky *et al.*, 2015). And esophageal squamous cell carcinomas (Chen *et al.*, 2013a; Chen *et al.*, 2013b).

Several studies correlated high circulating IL-6 levels with poor survival of lung cancer patients (Songur *et al.*, 2004; Wojcik *et al.*, 2010). Because the production of inflammatory cytokines such as IL-6 that correlated with oncogene-associated inflammation (Bayliss *et al.*, 2011; Ancrile *et al.*, 2007).

While Kobawala *et al.* (2015) considered the overexpression of IL-6 in papillary thyroid cancer patients was significantly and linearly correlated with large tumor size, presence of capsular invasion and extra-thyroidal extension of tumors, this current observed significant higher in cytoplasmic expression of IL-6 protein in the primary tumors of PTC patients than in the patients with benign thyroid disease patients.

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