ISSN: 0067-2904

Detection of FOXP-3 Expression in a Sample of Iraqi Cervical Cancer Patients Using Immunohistochemistry Technique

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Received: 20/11/2021 Accepted: 5/6/2022 Published: 30/12/2022

Abstract

This paper aims to find out if FOXP-3 was expressed in samples from Iraqi cervical cancer patients. Expression of FOXP-3 was detected in 55 cervical tissue samples by immunohistochemistry. Since thirty-five cases of aggressive cervical cancer were included, along with 20 normal samples used as controls. The nucleus and cytoplasm levels of FOXP-3 were counted, considering the ratio of positive cells and intensity. FOXP3 cytoplasmic staining was found in 27 out of 35 cases. Only 11 out of 35 samples displayed nuclear lymphocyte staining. Furthermore, four samples expressed this marker in both the nuclear and cytoplasm of the cervical cells. There is a highly significant difference in FOXP3 expression in the cytoplasm of malignant cells and lymphocytes compared to normal samples. Seven samples out of 11 cells correlated with lymph vascular invasion. These results show that tissue positive FOXP-3 possesses a possible diagnostic marker for Iraqi cervical cancer. FOXP3 is significantly expressed in cancer cells, and lymphocyte infiltrates [T-reg] compared to normal.

Keywords: FOXP-3 marker, cervical cancer, IHC, Nuclear expression, Lymphangiogenesis.

الكشف عن تعبير FOXP−3 في عينات من مرضى سرطان عنق الرحم العراقيين باستخدام تقنية الكيمياء النسيجية المناعية

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الخلاصة :

يهدف البحث الى معرفة ما إذا تم التعبير عن FOXP−3 في عينات من مرضيات سرطان عنق الرحم العراقيات. تم الكشف عن التعبير عن FOXP−3 في 55 عينة من أنسجة عنق الرحم بواسطة الكيمياء النسيجية المناعية. منذ اكتشاف 35 حالة إصابة بسرطان عنق الرحم الشديد، إلى جانب 20 عينة عادية تستخدم كعناصر سيطرة. تم حساب مستويات النواة والسيتوبلازم لـ FOXP−3 ، مع الأخذ في الاعتبار نسبة الخلايا الإيجابية والشدة. تم العثور على تصبيغ FOXP3 في 27 حالة من أصل 35 حالة. أظهرت 11

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عينة فقط من أصل 35 عينة تصبيغ الخلايا الليمفاوية النووية، كما كانت هناك 4 عينات عبرت عن هذه العلامة في كل من الخلايا النووية والسيتوبلازم لخلايا عنق الرحم. فرق كبير للغاية في تعبير FOXP3 في سيتوبلازم الخلايا الخبيثة والخلايا الليمفاوية مقارنة بالعينات العادية. سبع عينات من أصل 11 خلية ارتبطت بغزو الأوعية اللمفاوية. توفر هذه النتائج علامات على أن الأنسجة الإيجابية FOXP−3 تمتلك علامة تشخيصية محتملة لسرطان عنق الرحم العراقي. يتم التعبير عن FOXP3 بشكل كبير في الخلايا السرطانية والخلايا الليمفاوية التي تتسلل [T−reg] مقارنة بالمعدلات الطبيعية.

الكلمات المفتاحية: علامة FOXP−3 ، سرطان عنق الرحم ، IHC ، التعبير النووي ، Lymphangiogensis.

Introduction

Cervical cancer is one of the most frequent malignancies globally, and it is still the leading cause of mortality among women, especially in emerging nations[1]. The primary cause of cervical cancer death is cancer cells spread to other tissues and organs [2]. Many growth factors and receptors regulate cervical cancer invasion and metastasis [3]. Carcinogenic types of human papillomavirus (HPV) cause most cases of cervical carcinoma and are highly abundant in young women [4].

Certain cofactors have been included in the development of cervical cancer because of the high prevalence of HPV infection and the low incidence of cervical cancer among women worldwide [5]. These cofactors acteria such as *Chlamydia trachomatis* and *Neisseria gonorrhea*, fungi such as *Candida albicans*, parasites such as *Trichomonas vaginalis*, and viruses such as *herpes simplex* [3]-[5]. Sexual behavioral patterns, such as sexual intercourse at an early age, multiple sexual partners and low socioeconomic status, have also been described as important cofactors for HPV infection [6].

Cervical cancer was the fourth most common cancer in women and the seventh most common cancer worldwide in 2012, with an estimated 528,000 new cases in developing countries, accounting for more than 12% of all female cancers. Eastern Africa [42.7], Melanesia [33.3], Southern [31.5], and Middle [30.6] Africa are high-risk locations with estimated ASRs of > 30 per 100,000. Australia/New Zealand [5.5%] and Western Asia [4.4%] have the lowest rates [7]. In Iraq, 10.74 million women between the ages of 15 and 64 are at risk of cervical cancer. According to current figures, 244 women are diagnosed with cervical cancer annually, with 159 dying from the disease. Cervical cancer is the 13th most prevalent cancer in Iraqi women and the 10th most common cancer among women aged 15 to 44. In 2016, the incidence rate was 1.08 percent, and the distribution and incidence rate [per 100,000 people] was 202 cases [1.41%][8].

Cervical cancer threatens 10.74 million Iraqi women aged 15 and up. Annually, 244 women are diagnosed with cervical cancer, with 159 dying from the disease, according to current statistics. Cervical cancer is the 13th most common disease among Iraqi women and the 10th most common cancer among women between the ages of 15 and 44. There is presently no information on the prevalence of HPV in Iraq's general population. Around 2.3 percent of women in Western Asia, including Iraq, have cervical HPV-16/18 infection at any given time, and HPVs 16 and 18 are responsible for 72.4 percent of invasive cervical malignancies [9]. Crude Cancer Distribution and Incidence Rate [Per100, 000 Population] by Site in Females, Iraq, 2016. There were 202 cases, with a percentage of 1.41 percent and an incidence rate of 1.08. [9]. FOXP also referred to as scurf, is a protein that plays a role in immune system responses [10]. One of the members of the FOX protein family, it works as a chief controller of the regulatory pathway in the function as well as in the development of regulatory T cells [11],[12],[13]. Alternation in the immune response down usually can be

caused by the regulatory T cells. In cancer, the immune system is prevented from attacking the cancer cells because of excess activity of regulatory T cells. In autoimmune diseases, autoimmune cells attack the body self-tissues because of the deficiency in the activity of regulatory T cells [14], [15]. FOX proteins are members of the forkhead/winged-helix family of transcriptional regulators, and they are considered to exert control through comparable DNA binding interactions during transcription. The *FOXP3* transcription factor in regulatory T-cell models is situated in promoters [gene promoters] included after stimulation of T-cell receptors, regulatory T-cells may decrease transcription of essential genes [16]. There are 11 coding exons in the human *FOXP3* genes. Exon-intron limits are comparable across the mouse and human genes ' coding areas. By evaluating the genomic sequence [particularly Xp11.23], the FOXP3 gene maps the X chromosomes p arm [10], [17, p. 3].

Methods

Patients and tissue samples

In this study, 55 samples were obtained from un-selected patients ages ranging [35-70] years from Baghdad Medical City, teaching laboratories/histopathology department divided into 35 blocks were cervical cancer, and 20 samples had no malignant lesions considered as control. Thirty-two samples were squamous cell carcinoma, and three blocks were adenocarcinoma. Al-Mustansiriyah University, College of Pharmacy approved the study.

Deparaffin and Immunohistochemistry staining of FOXP-3

Tissue sections [4 mm] that had been formalin-fixed and paraffin-embedded were de paraffinzed in xylene and rehydrated in absolute ethanol. Antigen retrieval was accomplished by boiling slides in a target retrieval solution/Dako [pH 9.0] for 10 minutes at 98°C in the microwave. My Bio Source /USA /Staining Detection Kit accordance with the manufacturer's instructions was utilized to detect the protein expression of FOXP-3. After that, the slides were rinsed twice with TBST buffer and then distilled water for 10 minutes. [0.1 percent Tween 20 Plus Tris-buffered saline]. After quenching the endogenous peroxidase-blocking agent in 0.3 percent H2O for 30 minutes, the slides were washed twice with TBST buffer for 5 minutes each time. After a 10-minute application of protein-blocking serum, tissue sections were incubated at 4°C for 6 hours in a humidified box with the primary antibody [1:200 dilution; My BioSource, USA] without washing. Then sections were treated with a secondary antibody [My Bio Source] for 30 min. Finally, all sections were visualized using chromogen DAB working solution followed by counterstained with Meyer’s hematoxylin and mount.

Scoring-Analysis

All stained slides were viewed under a light microscope at 10X, 40X, and 100X magnifications. Tang et al. [2017] reported that two pathologists independently rated the quantity and proportion of positive cells expressing FOXP-3 present within tumor-cell cytoplasm in 5 HPFs magnification, x40. Score 0 [no staining in tumor cells]; score 1+ [weak staining 10% of tumor cells]; score 2+ [moderate staining 10-50 percent of tumor cells]; and score 3+ [strong staining > 50% of tumor cells] were the four categories for protein expression of this marker. A score 1+/2+/3+ was considered a positive expression, but a score of 0 was a negative expression. In terms of the deep brown hue of this marker, the intensity was graded as negative staining, low, moderate, and high or strong staining [18]. In addition, according to Takenaka *et al.* [2013], the number of lymphocytes expressing *FOXP3* was performed manually in 10 high-power fields [HPFs; magnification, x40] [19].

Statistics

Statistical significance was determined using the SAS [2012] tool, which was used to detect the effect of different components in research parameters. A P-value of 0.05 was considered significant, and a P-value of 0.01 was considered very significant when comparing

percentages using the Chi-square test.

Results

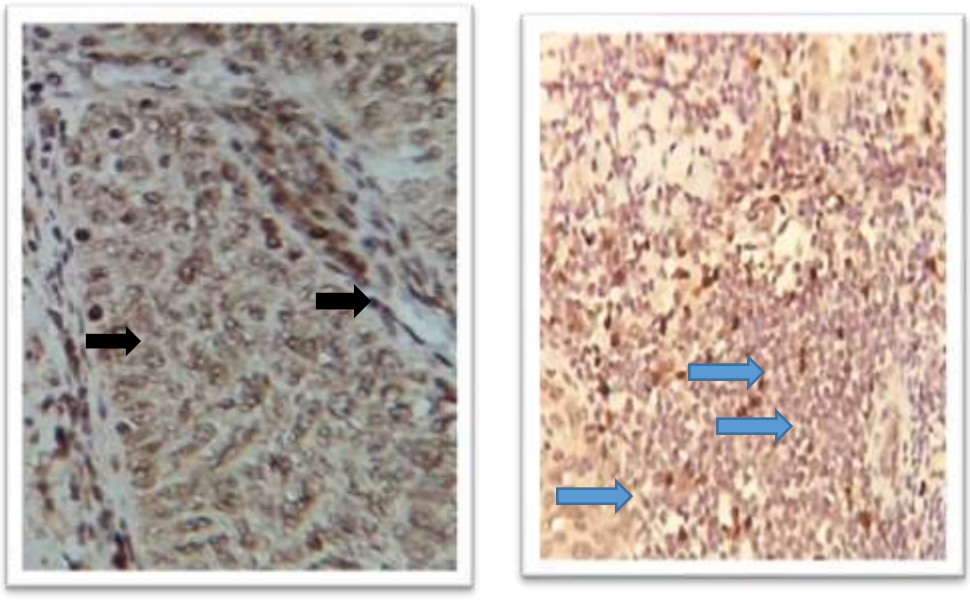
FOXP3 expression localization in cervical cancer cells, normal cervical cells and lymphocytes [T-reg] cells.

Table 1: Positive *FOXP3* antibody staining in Cytoplasm and Nuclear of cells and lymphocyte in cervical cancer and normal cells

Kinds of samples	Total number No.	Cancer cells-Cytoplasm No [%]	cells –Nuclear No [%]	Lymphocyte Nuclear No[%]
Malignant	35	27 [77.1]	4 [11.4]	11 [31.4]
Normal	20	0	9 [45]	3 [15]
Chi-Square [χ^2]		12.84 **	9.69 **	6.17 **
P-value		[0.0001]	[0.0001]	[0.0085]

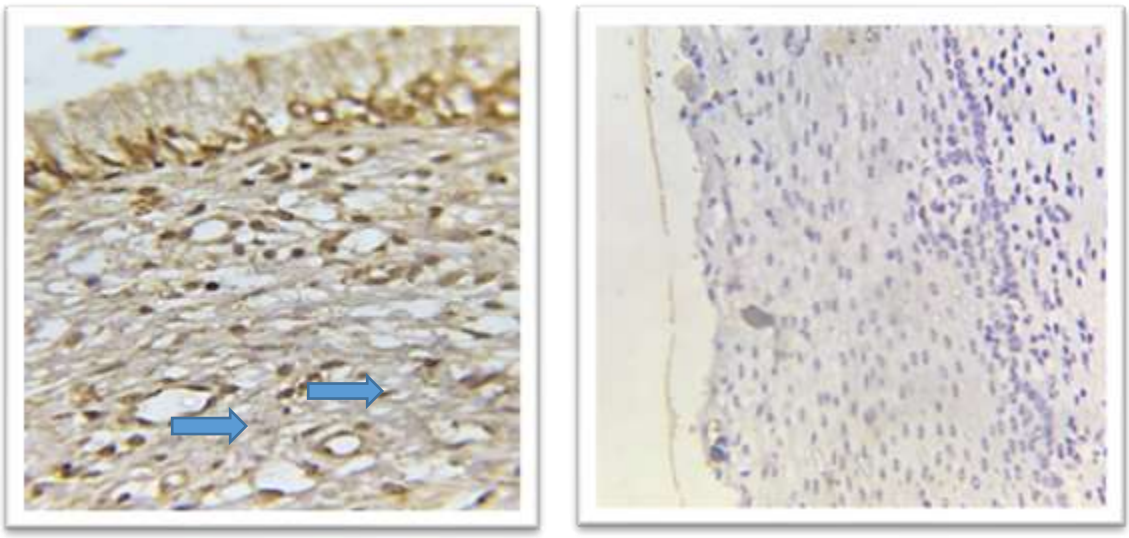
Regarding Table (1), Figures (1A, B and 2A, B), *FOXP3* cytoplasmic staining was seen in 27 cases of 35 [77.1 %] cervical cancer samples. Only 11 out of 35 samples [31.4 %] displayed nuclear lymphocyte staining; also, 4 [11.4%] samples expressed this marker in both nuclear and cytoplasm of the cervical cells. In addition, out of 20 normal cervical tissues, 9 [45%] samples presented nuclear staining in the luminal epithelium of cervical tissue compared to 4 [11.4%] of malignant cells with a P-value of 0.0001**. At the same time, there are 3 [15 %] cases with nuclear of lymphocyte and 8 [40 %] samples have no expression, as shown in Figure (1A, B). There is a highly significant difference in the *FOXP3* expression in the cytoplasm of malignant cells and lymphocytes compared to normal samples [0.0001**and 0.0085** respectively].

****HS: Highly significant at p < 0.01**



(A) **[B]**

Figure 1: A: Section of squamous cell carcinoma stained with anti *FOXP3* antibody showing cytoplasmic [➡] localization of anti-*FOXP3* in cancer cells [40X]. **B**: stained with anti *FOXP3* antibody showing nuclear [⟹] localization of anti-*FOXP3* in lymphocyte [T-reg] cells by using IHC staining technique [40X].



(A) **[B]**

Figure 2: A: section of Normal cells stained with anti *FOXP3* antibody showing nuclear localization of *FOXP3* in normal cells [40X]. **B**: stained with anti *FOXP3* antibody showing no expression localization of *FOXP3* in normal cells using IHC Technique [10X].

Association between *FOXP3* expression localization in cancer cells and T-reg infiltrate lymphocytes.

Table 2 showed 10 [37%] of the cases were positive *FOXP3* expression in nuclear of lymphocyte also positive in cytoplasm of cervical cancer cells, and this significant difference [P- value 0.0052 **] from one case [12.5%] which was negative cytoplasm expression.

Table 2: Interaction of IHC staining between *FOXP3* expression in Cytoplasm cancer cells and nuclear T-reg cells infiltrates cancer microenvironment.

FOXP3 expression localization	**Cancer cells - Cytoplasm**		**P-value**
	FOXP3 [+]	**FOXP3[-]**	
	Patient no. [%]	**Patient no. [%]**	
Total patients no.	27	8	
Lymphocyte -Nuclear			
FOXP3 +	10[37]	1[12.5]	0.0052 **
FOXP3 -	17[62.9]	7[75]	1.94 NS

Highly significant at p <0.01, significant at p < 0.05*, no significant at p >0.05**

FOXP3 expression in cervical cancer cells and normal cells sample according to the score.

Score 3 showed a significant difference in malignant samples 22.9 %, P-value 0.0037 compared to normal 0%. The results showed a high percentage of malignant samples that *FOXP3* expression in the cytoplasm in score 2 [34.3 %, $\chi 2$ 5.082, P-value 0.0261*] versus normal samples [20 %], but no significant difference was observed in malignant and normal samples [P-value 0.218] in score 1.

In normal samples,11 [55%] cases only among 20 cases of normal samples revealed no expression in the cytoplasm, as illustrated in Figures 3 and 4 A, B, and C.

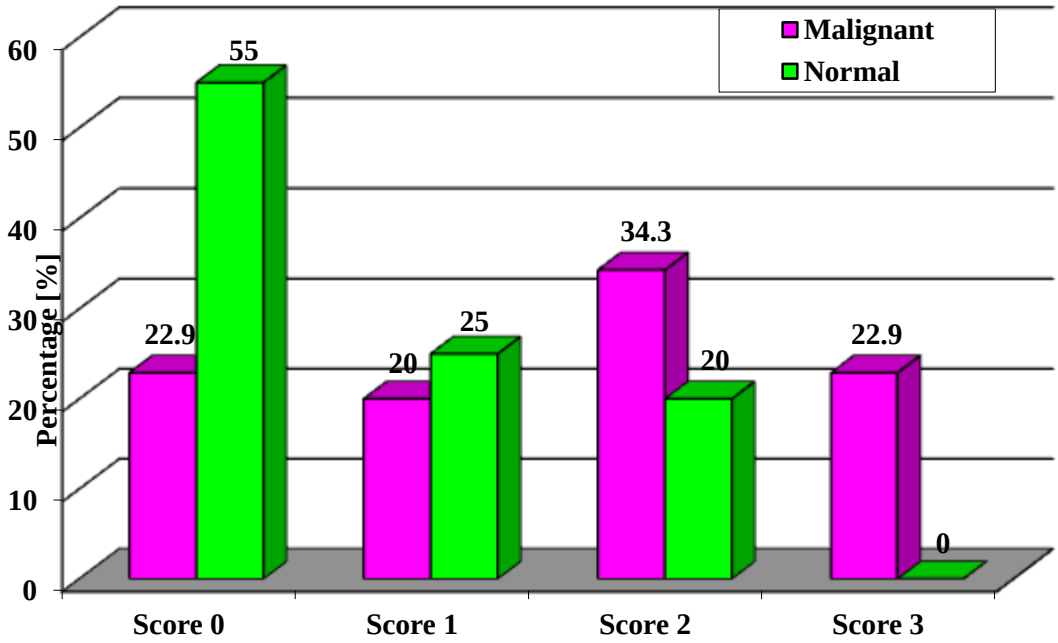
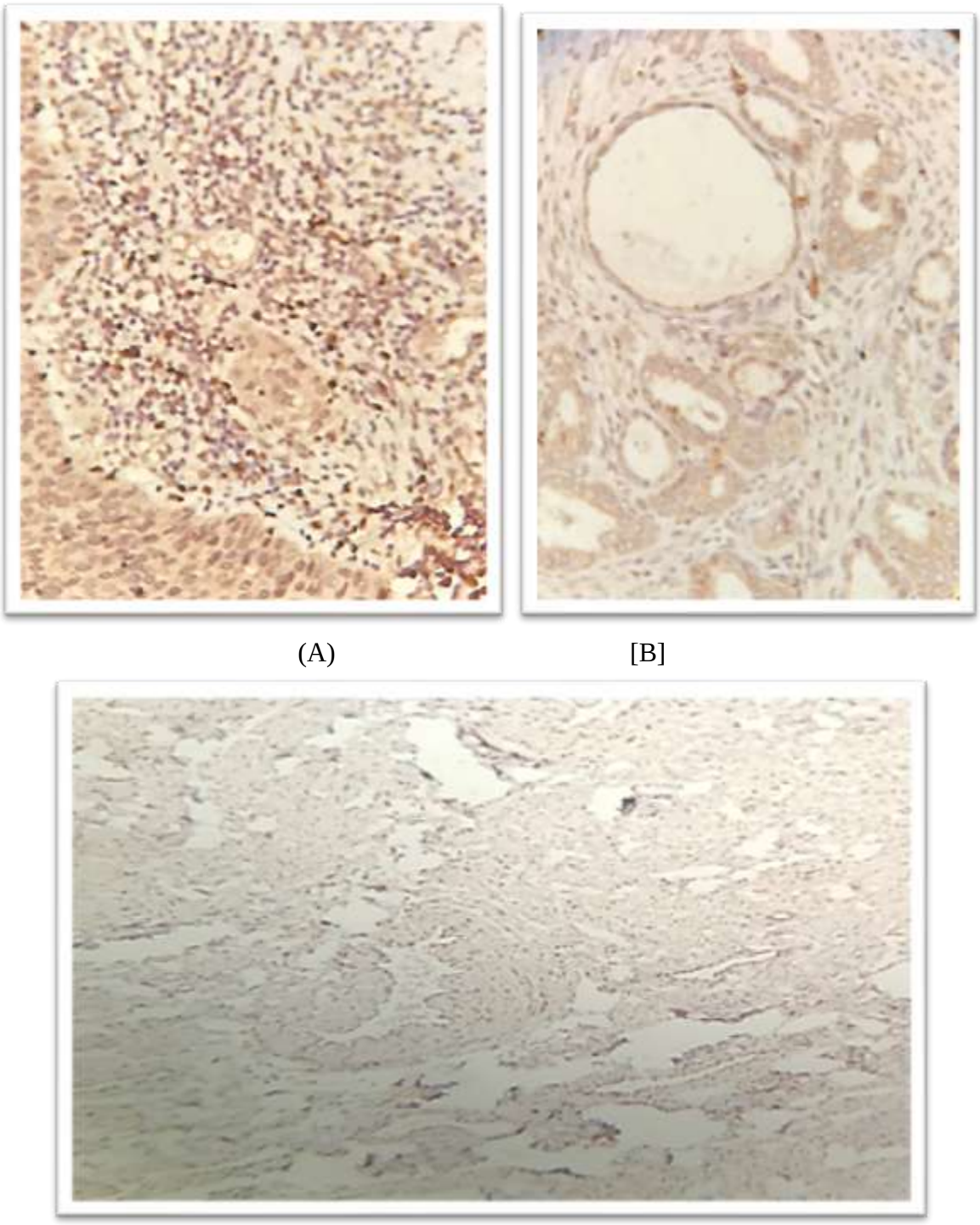


Figure 3: percentage of positive cancer cells *FOXP3* expression in cervical cancer and normal samples according to the score. Score 0**:** No stained cells. Score 1: Weak staining, < 10% cervical cancer cells stained, score 2: Moderate staining, 10–50% cervical cancer cells stained, score 3: Strong staining, > 50% cervical cancer cells stained.

According to the score, nuclear anti-FOXP3 staining in lymphocyte [T-reg] of cervical cancer and normal tissues

This new data showed a high percentage in nuclear FOXP3 expression in score 1[22.9 %] of malignant samples more than normal samples 3[15%], but without significant difference [$\chi 2$ 2.09, P-value 0.088]. The expression of FOXP3 in T-reg cells was high in score 2 [8.6%] against normal samples with significant difference [$\boldsymbol{\chi 2}$**=** 4.38, P-value 0.0461*]. No samples showed expression in scores of 0 and 3, neither malignant nor normal samples. 17[85%] samples among 20 normal samples revealed no FOXP3 expression in nuclear T-reg cells, as summarized in Figures 4 A, B, C and 5.



(A) [B]

[C]

Figure 4: A: Section of squamous cell carcinoma stained with anti FOXP3 antibody showing cytoplasmic localization of anti-FOXP3 high expression in cancer cells with lymph vessel using IHC [40X]. **B:** Section of squamous cell carcinoma stained with anti FOXP3 antibody showing cytoplasmic localization of anti-FOXP3 low expression in cancer cells with lymph vessel using IHC [40X]. **C:** Section of squamous cell carcinoma stained with anti FOXP3 antibody showing no expression in cancer cells using IHC [10X]

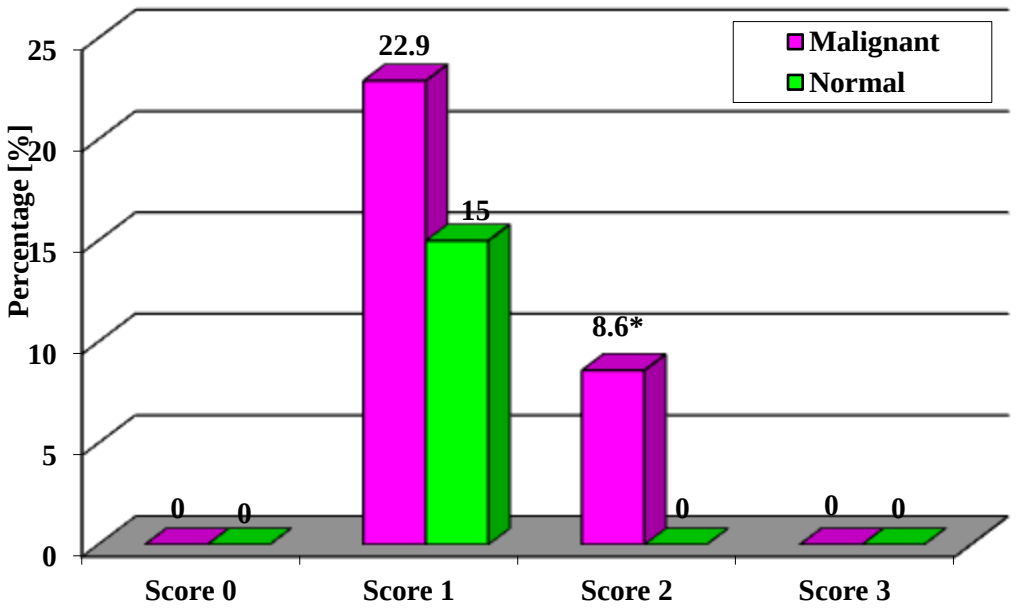


Figure 5: Percentage of positive lymphocyte *FOXP3* expression in cervical cancer and normal samples according to the score. Score 0: no stained cells, score 1: 1-25% stained cells, score 2: 26-50% stained cells, score 3: 51_100 % stained cells.

Association between *FOXP3* Immune Marker Expression and Clinicopathologic Parameters of cervical cancer.

The number and percentage of positive tumor cells with cytoplasm staining were cited in a study of IHC data of FOXP3 related to some clinic pathologic parameters and categorized to Negative and positive expression according to the [18], which considered score 0,1 as negative FOXP3 expression while scoring 2,3 as positive. The *FOXP3* positive expression was 20 [57.1%] while FOXP3 negative was 15 [42.85%] 35 malignant samples were found among the total number of samples. The data summarized in Table 3 showed demographic criteria association with FOXP3 expression patterns. There was no significant difference in positive and negative FOXP3 expression in the cytoplasm between the age and differentiation groups [P=0.1451 and 0.652], respectively, but the Squamous cell carcinoma [85%] showed a significant difference [P-value = 0.0273*] versus Adenocarcinoma [15%] in positive group. Positive expression of FOXP3 marker association with lymph vascular invasion was lower [55%] than in negative group [60%] while positive lymph node metastases samples appeared highly associated with positive FOXP3 expression group [40%] with significant difference χ^2 8.57, p-value= 0.0035 * versus negative FOXP3 expression group [13.3%]. There are 7 samples out of 11 [63.63 %] T-reg cells in positive groups correlate with lymph vascular invasion.

Table 3: Clinic pathological parameters associated with positive cancer cells FOXP3 expression in cervical cancer cells samples.

Clinical parameters NO. / Variable		Negative expression =15 No [%]	Positive expression =20 No [%]	Chi-Square [χ^2]	P-value
Age [year]					
> 50	10	4[26.6]	6[30.4]	2.07 NS	0.1451
≤ 50	25	11[73.3]	14[70]		
Differentiation					
Well + moderately	31	13[86.6]	18[90]	1.39 NS	0.652
Poorly	4	2[13.3]	2[10]		
Histological type					
Squamous carcinoma	32	15[100]	17 [85]	5.382 *	0.0273
Adenocarcinoma	3	0	3[15]	S	
Lymph vascular invasion					
Yes	20	9[60]	11[55]	1.237	0.0966
No	15	6[40]	9[45]	NS	
Lymph node metastases					
Yes	10	2[13.3]	8[40]	8.57 **	0.0035
No	25	13[86.6]	12[60]	HS	

**** HS: Highly significant at p <0.01, *S: significant at p < 0.05, NS: no significant at p >0.05**

Discussion

FOXP3 was considered a particular T-reg cell immune marker and plays a significant role in the growth and function of Treg cells [20]. *FOXP3* expression has been found recently in cancer patients peripheral blood, lymph nodes, and tumor microenvironment, not only in T cells. The *FOXP3* positive T-reg cells in liver cancer are abundant, and *FOXP3* has been discovered to promote cancer metastasis and be linked with bad cancer prognosis [21]. In ovarian cancer patients local lymph nodes, *FOXP3* was found over-expressed in T-reg cells and strongly linked to bad prognosis [22]. However, there is little information available in the world about this marker concerning its expression in cervical cancer in Iraq.

In the current investigation, cytoplasmic expression of FOXP3 was the most common pattern in cervical cancer cell samples. While the predominant pattern of *FOXP3* expression in normal cases was nuclear, this marker was expressed in both cytoplasm and nuclear cases. According to the expression level in the cytoplasm of cancer cells and lymphocytes. In malignant vs normal situations, there is a highly substantial difference. Figures [1 A, B], [2 A, B], and [1 A, B].

When comparing cervical cancer cells to normal cells, Huang et al. [2016] found a lot of FOXP3 protein in the cytoplasm but very little in the nucleus [23]. Foxp3 expression was identified in 66 percent [33/50] of cervical cancer tissue, with cytoplasm being the most common [18]. In addition, other studies go in line with recent data in breast cancer that cytoplasmic expression was also predominant [24]. In lung cancer, a significant association

between nuclear and cytoplasmic *FOXP3* expression in both cases of lung cancer and benign lesions was observed using IHC [25].

This indicates that FOXP3 protein produced in the cytoplasm is inactive unless expressed in the final functional site, the nucleus. Um, *et al*. [2011] and Feng *et al*. [2012] had both confirmed the expression of *FOXP3*in the nuclei and cytoplasm of lung cancer [26], [27]. Generally, the association between cancer cell expression localization nuclear or cytoplasm and lymphocytic [T-reg] expression suggests a different function of *FOXP3* expression in both types of cells. Increasing the frequency of FOXP3 lymphocytes in the tumor microenvironment may enhance invasion and metastasis by suppressing the immune response to tumor cells [26].

Baratelli et al. [2010], suggested that the distinct expression of *FOXP3* in tumour cells and T-reg lymphocyte infiltration is influenced by factors present in the tumour microenvironment such as PGE2, COX-2 and TGF-β, (Table 2), [28]. In the current study, the score 2 of positive *FOXP3* immunostaining expression in cytoplasm localization in malignant cells was considerably higher in malignant samples compared to normal samples. In the lymphocytes infiltrate, scores 1and 2 were the high expression in malignant samples compared to normal, Figure [3 and 5]. In a breast cancer study, *FOXP3* expression was associated with elevated Ki-67 in breast cancer cells, meaning that *FOXP3* expression could encourage cancer cell proliferation [24]. In reality, FOXP3 was found in 93.2 percent [n=55] of lymphocytic infiltrates, potentially confirming the rationale that T-reg may consist of an escape mechanism for an immune reaction from cervical neoplasms. However, neither tumour cell specimens nor lymphocytic infiltrates affected survival associated with *FOXP3* staining [29].

New research on cervical cancer found that the expression of *FOXP3* was found in the cytoplasm and nucleus of malignant cervical cancer cells and the nucleus of T cells in a proportion of 91.67% [99/108]. Both markers were favorably associated with *FOXP3* expression in cervical cancer cells. On the one hand, these indicators can facilitate tumor immune escape by increasing the number of T-reg cells, hence promoting tumor development. High levels of both lead to the regulation of FOXP3 expression in cervical cancer cells, which influences cancer cell proliferation and apoptosis and affects the genesis and progression of tumors. High FOXP3 expression is linked to a better prognosis in cervical cancer patients, including an increase in T-reg cell counts and a higher rate of cancer cell expression [23]. Finally, Zuo et al. [2007] attributed genetic abnormality to reduced *FOXP3* expression in tumour cells, including deletions in exons 3 and 4 or alternative splicing [30].

Dimitrakopoulos *et al*., 2011 demonstrated that cytoplasmic expression of *FOXP3* reflected the ability of an epithelial cell, either benign or malignant to synthesize this protein. Since marker expression in the cytoplasm of any cell does not reflect positively on that cell to it [31]. By this, it is regarded as a non-specific reaction off course unless the protein [marker] has a functional cytoplasmic localization [19]. On the other hand, Karanikas *et al*., and 2008 cited that synthesize of the protein in any particular cell indicates of gene expression in that cell [32].

A new study looked into the relationship between cytoplasm FOXP3 expression and cervical cancer clinic pathological parameters. Scores 2 and 3 indicated positive expression (20/57.1%) in tissue samples, whereas scores 0 and 1 indicated negative expression (15/42.8%), as shown in Table 3. Positive expression is no significantly linked with age and

grade. In comparison, it was linked considerably with histological squamous carcinoma type. Lymph vascular invasion in the positive *FOXP3* group was slightly low, whereas lymph node metastases were significantly high in the positive group, especially that T-reg infiltrates are high in positive lymph vessel samples [7/11] that mean related to this marker with lymphangiogenesis in cervical cancer.

These findings support Tang et al. [2017], who found immunological reactivity of FOXP3 in cervical cancer in 87.5 percent of samples and clinical pathologic criteria based on positive and negative FOXP3 expression. Age, grade and tumor size were not correlated with *FOXP3* expression, while this marker was associated with phase and lymph node metastasis with $P<0.05$ [18]. In other studies, *FOXP3* expressions were also correlated with phase, lymph node metastasis, tumor size and HPV infection in patients with cervical cancer, they were not related to the degree of differentiation, pathology and age of patients with cervical cancer [33]. *FOXP3* was not associated with age, histology, differentiation and metastasis of the lymph nodes [23].

In lung cancer, *FOXP3* expression was significantly associated with age group > 61 both when this expression was studied in infiltrating lymphocytes or the tumor cells themselves, $p<0.05$. There was no significant difference according to gender, tumor types regarding the expression of *FOXP3* in the tumor cells or in infiltrating lymphocytes as well, $p>0.05$, while Moderately differentiated tumors showed a significant association with *FOXP3* expression. Kim *et al*. [2013] found that *FOXP3* expression mediated by cancer cells rather than by T-reg cells contributes to disease progression [34].

Although FOXP3 expression was high in cervical cancer cells and metastatic lymph nodes, more research is needed to determine how FOXP3 affects cancer cell activity. FOXP3 is involved in the development of cervical cancer and its spread.

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