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RESEARCH ARTICLE

Immunohistochemical expression of transformation growth factor (TGF-β1)in epithelial and stromal cells of Iraqi lung tumors .

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Manuscript Info *Abstract*

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Background: Lung cancer most common causes of cancer deaths in the United States and world. TGF-β Immunosuppressive cytokines ,induce regulatory properties in conventional CD4+ T cells , contribute to maintenance of lymphocyte homeostasis , and play a role in tumorgenesis**.** Sources of TGF-β1 in tumors vary and include the cancer cells themselves as well as various cells of the tumor stroma, with each source leading to context-dependent functional consequences. TGF- β has the power to act as a tumor suppressor (via its effects on proliferation, replication potential, and apoptosis) and as a tumor promoter (via its effects on migration, invasion, angiogenesis, and the immune system

Methods: TGF-β1 protein expression in lung tumor cells and stromal cells were studied by using immunohistochemical technique in 30 NSCLC Iraqi tissue samples and 22 benign tissue

Results: Significant diffrences (p= 0.0401,chi-3.877) of TGF-β1 expression was found 25/30(83.3%) in malignant samples compared to benign lesion cases 16/22(72.7%),while no differences have been found between the two group TGF-β1 expression in stromal cells. Frequency Score 3 and intensity significantly higher in benign tumor cells(63.64%,59.09%,) more than in malignant cells(53.33%, 53.3 3%).In stromal cells (score 3and intensity) , no differences have been found between malignant and benign lesion (43.33%versus 40.91%) .

Conclusions: Our data suggest a possible role of TGF-β1 in the pathogenesis of NSCLC and benign lesion .Increased expression of TGF- β1 may be associated with infiltration fibroblastic stroma which may together facilitate tumor development

INTRODUCTION

Lung cancer is the leading cause of cancer death, both in the United States and worldwide. It is estimated that lung cancer, constituted the most common causes of cancer deaths.(Youlden et al., 2008**) .** The majority of the cases now occur in the developing countries (55%). Lung cancer rates are particularly high in Central-Eastern and Southern Europe, Northern America and Eastern Asia.(Youlden et al., 2008& Ferlay et al.,2010). In Iraq,lung cancer consider the second cancer after breast with incidence rate about 4.96 /100,000 population, and first cancer causing deaths (13.82%)between Iraqi people(Iraqi Cancer Registry Team 2009)**.**

More recently, several investigators have studied an opposing aspect of the immune system, and the role of Foxp3 Treg (Woo et al.,2002 and Woo et al.,2001.). Karanikas et al.,(2008) provided unequivocal evidence that Foxp3 is expressed both at the transcript and protein level by tumor cells of various types (lung cancer, colon cancer, breast cancer, melanoma, erythroid leukemia, acute T-cell leukemia) ,expression correlated with the expression levels of IL-10 and TGFβ1.

TGF-β Immunosuppressive cytokines ,induce regulatory properties in conventional CD4+ T cells , contribute to maintenance of lymphocyte homeostasis , and play a role in tumorigenesis (Baratelli etal., 2010). TGF-β family includes three isoforms of TGF-β (TGF- β 1, TGF- β 2, and TGF-β 3). TGF- β 1 is the most abundant and universally expressed isoform; most studies have either examined or been performed with exogenous TGF- β 1. TGF- β is secreted into the extracellular matrix as a latent protein complex bound to a latency-associated protein and one of the isoforms of latent TGF- β binding protein(Lopez-Casillas et al.,1993) exert a wide spectrum of biological responses on a large variety cell types, e.g. regulation of cell growth, differentiation, matrix production, inflammation, host defense, and apoptosis (Letterio & Roberts ,1994; Wahl,1994).AlsoTGF-β plays a central role in the generation and function of $CD4^+$ $CD25^+$ Tregs (Chen etal.,2003)· .

Sources of TGFβ in tumors vary and include the cancer cells themselves as well as various cells of the tumor stroma, with each source leading to context-dependent functional consequences (Yang et al.,2008).

In normal cells, TGF-β, acting through its signaling pathway, stops the cell cycle at the G1 stage to stop proliferation, induce differentiation, or promote apoptosis. When a cell is transformed into a cancer cell, parts of the TGF-β signaling pathway are mutated, and TGF-β no longer controls the cell, These cancer cells will proliferate in addition the surrounding stromal cells (fibroblasts) also proliferate. Both cells increase their production of TGF-β. This TGF-β acts on the surrounding stromal cells, immune cells, endthelial and smooth-muscle cells. It causes immunosuppression and angiogenesis, which makes the cancer more invasive(Blobe et al.,2000). TGF- β has the power to act as a tumor suppressor (via its effects on proliferation, replication potential, and apoptosis) and as a tumor promoter (via its effects on migration, invasion, angiogenesis, and the immune system(Elliott&Blobe,2005

In this study malignant tumor and bengin lesion from lung were investigated to determine the protein expression of TGF-β1in tumors cell and stromal cells by using IHC.

Materials and Methods

Total of 30 paraffin blocks available for immunostaining from Iraqi lung cancer cases matched with 22 benign tumor cases were collected from Surgical Hospital& National Center for Early Detection of Cancer in Baghdad in Medical City/ Baghdad Hospital, diagnosis of these samples were confirmed by a pathologist in histopatholgy labs. in these two hospitals .Additional clinical and pathologic data were extracted from pathology reports of patients including age,gender, cancer type ,differentiation.

immunohistochemical procedure

Paraffin embedded sections of lung cancer & benign tumor were sectioned into 4 µm thicknesses using a microtome instrument. After deparaffinization in xylene ,hydration through a series of decreasing alcohol concentrations,the immune- histochemical procedure was performed target retrival solution (PH6) for 15min at 120ºcin microwave. immunoenzymatic staining were performed according to the manufacturer's instructions(abcam/canda). endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 10-15 minutes, tissues were washed in phosphate-buffered saline (PBS). Protein block was applied for5-10min, after washing with PBS, primary antibodies TGF-β1(abcam 66043)were diluted at 1:100 with Envision flex antibody diluents(Dako). 20 µl from diluent primary antibodyon tissue section then incubate the slides from 18- 24h.at 4ºc, washing with PBS. Two drops of Biolinyated goat anti human(abcam) from 20-30min were added followed by added two drops of streptavidin peroxidase also from 20-30min, Rinse the slides with BPS. 20ul from DAB chromogen(abcam) from5-10min was applied, washing with PBS. with hydrogen peroxide. The sections were then counter stained with hematoxylin,after amounted with aqueous mounting medium, the immunopositivity of the reaction was detected as brown staining observed by light microscopy.

Immunohistochemical Analysis

The number of TGF-β1 positive cells present in lung tumor sections were examined and counted manually in 10 high-power fields (HPFs; magnification, x400)for each sample by specialist histopathology. TGF-β1 expression tumor cells and stromal cells expression were scored as follow:0% negative stain, 1≤25%, 2 = 25–75% and 3 ≥

75%.The intensity of positive staining was grouped into low, moderate and strong according to deep of brown color of the marker(Gambichler et al., 2007and Elkak et al., 2003).

Statistical Analysis

The Statistical Analysis System- SAS (2010) was used to effect of different factors in study parameters. P value and Chi-square test were used to significant compare between percentage in this study.

TGF-β expression in NSCLC cancer and benign lung lesion:

Twenty five cases(83.3%)of lung cancer showed positive IHC expression of TGF-β in the cancer cells of lung cancer compared to 16(72.7%)cases of benign lesion.On the other hand ,21(70%)cases of lung cancer reveald positive stromal cells expression in the tumor cells compared to 15(68.2%)cases of benign tumor.Asignificant difference was noted between cancer cells expression in malignant and benign lesion $p<0.05$,while no significant difference in the stromal cells expression between malignant and benign lesions Figure(1).

TGF-β IHC percentage score and intensity in NSCLC cancer and benign lung lesion:

The highest score to be detected in lung tissue expression of TGF-β was score 3in 16 (53.33%)malignant cases and 14(63.64%) in benign lesion cases $p\leq0.05$. score 1,2 was high in malignant cells compared to benign $p\leq0.05$.The high intensity also predominated 13 cases(59.3%)of benign with a significant difference $p\leq0.05$,while Moderate intensity highly significant in malignant cases $p\leq0.01$ Table (1).The negative cases(score 0) in benign cases represented 27.27%(6 cases)and was significantly different from negative tumor cases. Stromal cell expression of TGF-β1 showed score 3 again to be the highest score in both malignant and benign lesions 13(43.3%) and 9(40.9%)with anon significant differences between them .High intensity on the other hand significantly dominated other intensity scores both in benign and malignant 10(45.45%) and 12(40%) respectively Table (2).

Discussion

TGF-β1 is highly expressed in the lungs of some individuals at risk for lung cancer(Takizawa et al.,2001) Moreover, abnormally high levels of TGF-β1 and loss of negative regulatory signaling in response to TGF-β have been described in lung cancer and were associated with enhanced tumorgenicity and reduced survival (Barthelemy-Brichant et al.,2002; Anumanthan et al., 2005).

In both cancer cell and stromal cell was observed higher positive TGF-β1 expression in cancer more than bengin Figure (1), Few study carried on TGF-β1 expression in cancercells area and stromal area. Richardsen et al., (2012) who agreement with present findings ,they found that the expression of TGF- β1 was highest in the tumor cell areas of both primary tumors and metastases (both $p < 0.001$) more than stromal area .In spite of that high stromal expression of TGF- β1 in primary tumors was associated with increased mortality (HR 5.2, 95% CI 1.1-24.0, $P = 0.035$). This may indicate that stromal expression of TGF- β1 is plays an important role in the early stage of tumor progression.also TGF-β1-positive cells are predominantly observed in the tumor itself (Imai et al.,2013)

TGF-β1 signalling initiates with binding and activating specific dual cell-surface receptors (type I and II) that have intrinsic serine/ threonine kinase activity(Letterio and Roberts,1998). Reduced TGFβRII expression in human NSCLC is associated with more aggressive tumor behavior and inflammation that is, at least partially, mediated by increased TGFβ1 expression (Malkoski et al., 2012).

The findings of the present study were showed that high percentage score(3) and intensity expression of TGF-β1predomenant in tumor cells and stromal cells of both malignant and benign Table (2 and 3).

Gambichler et al.,(2007),they reported that high mean percentage TGF-β1 IHC staining in Basal cell carcinoma (BCC) versus (2.1)in non lesional skin. However, staining for TGF–β1, Smad4, and Smad7 did not show substantial differences between BCC and non-lesional skin. percentage of CD4+, CD25+, Foxp3+,IL-10+, and TGF-β+ expression was observed in limited colorectal tumors (I/II) as compared to advanced tumors (III/IV) and normal tissue (Kim et al.,2013). Allred score for TGF-β1 expression was significantly higher ($P = 0.0017$) in the invasive adenocarcinoma (MIA) with fibrous stromal invasion (Imai et al.,2013).

In mouse model, TGFbRII deletion increased the number of benign and malignant lung lesions per tumor section, also increases total TGF-β1and proliferation and local inflammation(Malkoski et al.,2012). Whene cancer

cells will proliferate in addition the surrounding stromal cells also proliferate. Both cells increase their production of TGF-β. This TGF-β effect on the surrounding stromal cells, immune cells, endthelial and smooth-muscle cells. It causes immunosuppression and angiogenesis, which makes the cancer more invasive(Blobe et al.,2000)..

In human lung increased TGF-β ligand produced by TGFbRII-negative tumors may recruit other inflammatory cells, for example,CD3-positive T lymphocytes, to the local environment where these cells could promote lung tumor progression(Bierie, Moses,2010). The inflammation was likely a result of secreted proinflammatory cytokines TGF-βwhich induce development of regulatory T cells and T-helper (TH)17 lymphocytes leading to facilitate tumor Growth(Forrester et al.,2005; Ruffell et al.,2010). These findings may explaination of the high TGF-β percentage score(3)and intensity expression of cancer cell and stromal cells in both malignant cases and benign lesion cases Table(2)and (3).Also tumor microenvironment including variable cells (fibroblast,immune cells) and stroma may induce transition the epithelial cell from premalignant to malignant.

Conclusions:

Our study consider the first study in Iraq to investigation of the TGF-β1 expression by using IHC in both tumor cells and tumor stromal cells in NCSLC tissue and benign lesion. Our data suggest a possible role of TGF- β1 in the pathogenesis of NSCLC and benign lesion .Increased expression of TGF- β1 may be associated with infiltration fibroblastic stroma which may together facilitate tumor development.High percentage score(3) and intensity in tumor cells and stroma cells related with lung tumor progression. Further study will need to investigate the TGF-β expression related with stage of tumor compared to normal tissue.

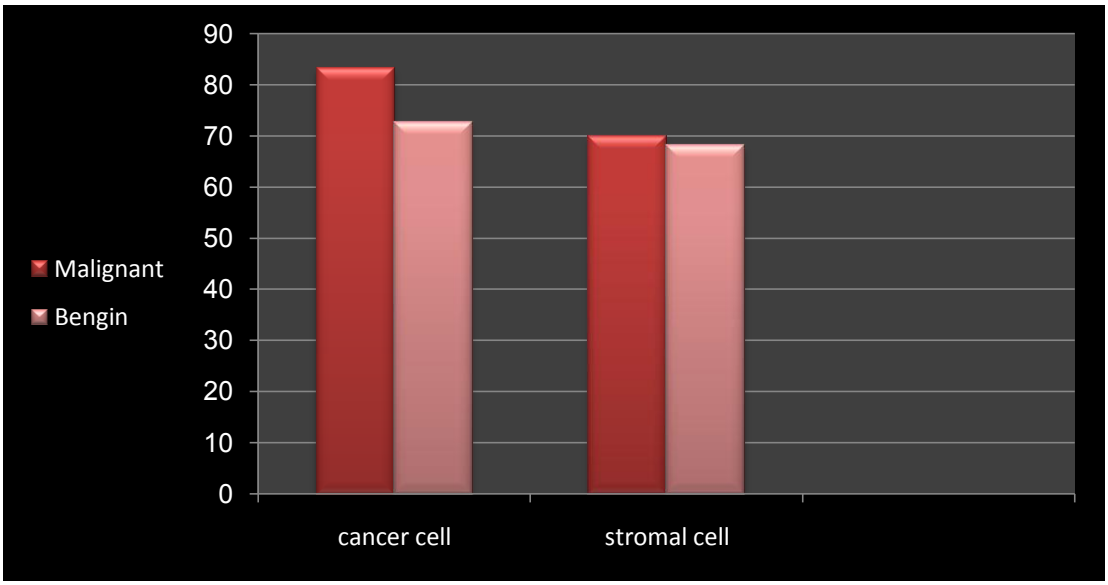

Figure 1: TGF-β1 positive expression localization in NSCLC and benign tissue samples

Table 2: Frequency of TGF-β1 expression (score and intensity)of tumor cells in malignant & benign tissue

tumor cells	Score								Chi-square value
	Ve		1		2		3		
	No.	%	No.	%	No.	%	No.	%	
Malignant	5	16.67	3	10.00	6	20.00	16	53.33	10.402**
Bengin	6	27.27	0	0.00	2	9.09	14	63.64	11.84 **
Chi-square value	--	4.89 *	--	4.22 *	--	4.19 *	--	4.24 *	---

* (P≤0.05), ** (P≤0.01).									
Tumor cells	Intensity								Chi-square value
	Ve		L		M		H		
	No.	%	No.	%	No.	%	No.	%	
Malignant	5	16.67	2	6.67	7	23.33	16	53.3 3	10.902**
Bengin	7	31.82	2	9.09	0	0.00	13	59.09	11.548**
Chi-square value	--	5.473 *	--	0.633 NS	--	7.25 **	--	1.025 *	---
* (P≤0.05), ** (P≤0.01).									

Ve-:negative,L:Low,M:Moderate and H:high

Table(3):Frequency of TGF-β1 expression (score and intensity)of stromal cells in malignant & bengin tissue

Stromal cells	Score								Chi-square value
	Ve-		1		2		3		
	No.	%	No.	%	No.	%	No.	%	
Malignant	9	30.00	5	16.67	3	10.00	13	43.33	9.603 **
Bengin	7	31.82	0	0.00	6	27.27	9	40.91	10.568 **
Chi-square value	--	0.244 NS	--	5.622 *	--	6.29 **	--	0.317 NS	---
* (P≤0.05), ** (P≤0.01).									
Stromal cells	Intensity								Chi-square value
	Ve-		L		M		H		
	No.	%	No.	%	No.	%	No.	%	
Malignant	9	30.00	5	16.67	4	13.33	12	40.00	10.69 **
Bengin	7	31.82	1	4.55	4	18.18	10	45.45	10.42 **
Chi-square value	--	0.244 NS	--	4.83 *	--	1.367 NS	--	1.255 NS	---
* (P≤0.05), ** (P≤0.01).									

Ve-:negative,L:Low,M: Moderate and H:high

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