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Research Article

Immunohistochemical Expression of FOXP3+ CD4+ CD25+ Regulatory T Cells Suppress Anti-Tumor Immune Responses in Patients with Colorectal Cancer

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Abstract

Background/Aim: To evaluate the immunohistochemical expression of some regulatory T cells markers (FOXP3, CD4, and CD25) in colorectal carcinomas. **Materials and Methods:** This study was carried out on 50 patients with hisopathologically confirmed primary colorectal cancer. Two samples were collected from each patient: one sample from the tumor site and the other one from adjacent normally appearing colorectal tissues, as well as ten (20) colorectal tissues from control individuals with no cancer. From each block, 4 sections of 4 µm thickness were taken, 1 section was stained with hematoxylin and eosin (H and E) and the other 3 sections were stained immunohistochemical for FOXP3, CD4, and CD25. **Results:** FOXP3 expression was significantly increased in mass colorectal tissues in comparison to the marginal and control groups tissues. CD4 showed significantly higher expression in carcinoma than Marginal colorectal tissues and in the control group. Highly statistical significant differences were showed in study groups according to CD25 expression. Correlation between FOXP3, CD4, and CD25 expressions and clinicopathological factors was found between Stage of tumor FOXP3 and CD25 only. **Conclusion:** The distinct microenvironmental expression of FOXP3, CD4, and CD25 were in mass colorectal cancer in comparison to marginal CRC and apparently normal tissues. Reflect a gradient expression of markers in multiple T cell populations.

Keywords: CD4, CD25, FOXP3, colorectal cancer, Treg.

Introduction

Colorectal cancer is the third most common cause of cancer-related death in woman and the fourth leading cause of cancer mortality in males. Colorectal cancer scan be classified as inherited (due to genetic instability), inflammatory (due to presence of chronic inflammation of gastrointestinal tract, e.g., Crohn's disease) or sporadic, which accounts for more than 80% of all CRC (Vlado et al, 2013).

T cells in the gut lymphoid tissue are subject to substantial regulation to ensure that pathogenic microorganisms are eliminated while commensal bacteria are well tolerated. This regulation involves several T cell populations with inflammatory or regulatory functions and the balance of these can determine the outcome of an immune response to an infection (Littman & Rudensky, 2010).

Tumors arising in the gut may therefore be subject to different immune regulation than tumors in other sites since they have arisen in an immunomodulated environment. It is then likely that the gut-associated tumors, such as colorectal cancer, will have a unique immune microenvironment influencing tumor initiation and growth. There is considerable evidence that T cells are important in destroying tumors, and colorectal cancer patients with a high T cell infiltrate into the tumor are more likely to have a positive outcome (Galon *et al.*, 2006; Dahlin *et al.*, 2011).

Tumor-infiltrating lymphocytes (TILs) are considered to be the primary host immune response against solid

tumors. Early results have shown a correlation between survival and density of TILs in colorectal cancer (CRC) patients, particularly if the lymphocytes invade the glandular elements of the tumor (Pages *et al.*, 2005). Furthermore, there is accumulating evidence that the type of immune cells, rather than their sheer quantity, controls the efficiency of the host-versus-tumor immune response (Galon *et al.*, 2006).
T regulatory cells (Treg) function to restore immune homeostasis during chronic inflammation, and therefore it seems logical that Tregs would reduce the risk of inflammation- associated colon cancer by down-regulating inflammation (Nishikawa *et al.*, 2005; Erdman & Poutahidism, 2010).

On the other hand, that Tregs in cancer primarily suppress protective anticancer immune responses. This alteration of Tregs function, together with the tumor-helping chemokine milieu produced by the cancer tissue could support cancer progression (Nishikawa *et al.*, 2005; Erdman & Poutahidism, 2010). Tregs role in the carcinogenesis of inflammation associated-colon cancer and sporadic colorectal cancer is paradoxical due to conflicting results (Györgyi *et al.*, 2012). Sarah et al which observed CRC patients have an increased frequency of Treg and there is evidence that these Treg target anti-tumor immune responses (Sarah *et al.*, 2006).

A recent study has revealed that CD4+CD25+ Foxp3+IL-17+ T cells in CRC tissue express TGF-β and IL-10, the functional molecules of Tregs. This subset of T cells suppresses anti-tumoral peripheral CD8+ T cells in a tumor antigen-specific manner, therefore contributing to cancer development (Ma & Dong, 2011).

The aim of the present study is to evaluate the immunohistochemical expression of FOXP3, CD4, and CD25 in mass and marginal colorectal cancer tissues.

Materials and Methods

The study was designed as a retrospective one. Colorectal tissues were obtained from fifty patients with colorectal cancer as paired tissue specimens by dissection of tumor and tumor-adjacent apparently normal tissues. Specimens belong to the period from June 2010 until November 2013. From each patient two blocks was taken formalin fixed, paraffin embedded colorectal adenocarcinoma (colectomy specimens) and twenty cases from individual colon tissue (proved by colonoscopic and histopathological examination) were proved to be free from any significant pathological changes were considered as a negative control groups for this study. Mass, margin, and colonoscopy blocks were collected from the archives of histopathology laboratories of Teaching Laboratories of the Medical City/Baghdad and Teaching Al-Hussein hospital/ Kerbala Holy.

The diagnosis of these tissue blocks were based on the obtained pathological records of these cases from hospital files as well as histopathological laboratories records. A confirmatory histopathological re-examination of each obtained tissue blocks was done.
From each block, 4 sections of four µm thick sections were made and sticked on positively charged slides were taken. One section was routinely deparaffinized with standard xylene and hydrated through graded ethanol in water, stained with HE, and covered with a coverslip. And the other 3 sections were stained immunohistochemically using three steps—indirect streptavidin method for Monoclonal Mouse Anti-Human CD4 (Cat. Number: AM421, BioGenex, Netherlands), Monoclonal Mouse Anti-Human CD25 (Cat. Number: AM453, BioGenex, Netherlands), and Monoclonal Mouse Anti- Human FOXP3 (Cat. Number: ab22510, abcam, England). BioGenex Ab ready to used (CD4 and CD25) have been optimally diluted for used with theses reagents and were not required further dilution, while optimal dilution of FOXP3 was 4:50 from concentrated primary antibody. We used the tonsil tissue as a positive control of FOXP3, CD4, and CD25. Technical negative control for three markers was obtained by omission of primary antibody. Brown nuclear staining of FOXP3 is considered as a positive reaction. Brown cytoplasmic staining of endothelial cells of CD4 and CD25 is considered as a positive reaction.

Scoring of immunohistochemical staining

The immunohistochemical forkhead box protein 3 (FOXP3) staining in colorectal cancer was evaluated under light microscopy at X100, X400, and X1000. The counting of positive cells was performed at X1000. The extent of FOXP3+ protein was scored as follows: No positive cells, **(0),** 1-25% positive cells, **(1+),** 26-50% positive cells **(2+),** 51-100% positive cells **(3+)**,(Takenaka et al, 2013).

Quantification of CD4, and CD25 proteins expression were evaluated under light microscopy at X100, X400, and X1000. The counting of positive cells was performed at X1000. The following score was adopted according to a laboratory protocol of king's College

Hospital (London):- **Score 0 (Negative):** No stained cells. **Score 1 (+):** The positive cells (stained) represented 10% total cells. **Score 2 (++):** The positive cells (stained) represented more than 10% to 30% of total cells. **Score 3 (+++):** The positive cells (stained) represented more than 30% to 50% of total cells. **Score 4 (++++):** The positive cells (stained) represented more than 50% of total cells.

Statistical analysis

Data were analyzed using Statistical Package for Social Sciences (SPSS program) version 18 and Microsoft Office Excel 2007. Chi-square test was used to detect the significances between variables of our study. P-value was considered significant when < 0.05.

Results

Fifty colorectal cancer specimens from patients were evaluated. Thirty one of 50 patients was male (62%) and nineteen (32%) were female. Median age at diagnosed stage was 53.5 were ranged from twenty six years to eighty three years. All patients had stages A, B and C according to Duke stage. Table (1).

Table 1: Characteristic features of patients.

Characteristic	N	%
Number of patients	**50**	**100**
Sex		
Male	31	62
Female	19	32
Age (years)		
<50	24	48
>50	26	52
TNM		
Duke A	6	12
Duke B	13	26
Duke C	29	58
Duke D	2	4
Tumor location		
Right colon	17	34
Left colon	23	46
Rectum	10	20
Degree of differentiation		
Well	16	32
Moderate	31	62
Poorly	3	6

FOXP3 was detected in 33/50 (66%) of mass colorectal tissues, 23/50 (64%) of margin colorectal tissues, and in 8/20 (40) of control group. No significant difference (P>0.05) was found among study groups. A high percentage (36.4%) was found in score II of mass group and score III (47.9%) of margin group. Table (2).

Table (2): Frequency distribution of immunohistochemistry results of FOXP3 protein according to the signal scoring.

Foxp3 signal scoring		Colorectal Mass Tissues (n=50)		Colorectal Marginal Tissues (n=50)		Apparently Healthy control tissues (n=20)		P-value1
		No.	%	No.	%	No.	%	
Negative		17/50	34	27/50	54	12/20	60	0.1
Positive		33/50	66	23/50	46	8/20	40	
Scoring	**I**	10/33	30.3	7/23	30.4	2/8	25	**P-value 2**
	II	12/33	36.4	5/23	21.7	2/8	25	0.1
	III	11/33	33.3	11/23	47.9	4/8	50	

The score II of immunohistochemical staining of the tumors was higher with anti-CD4 than score I and III in mass and margin colorectal tissues were 44.8% and 36.8 % respectively. Whilst in normal control group only two cases were found with score I positive for anti-CD4. Statistical significant difference (P<0.05) was found among study groups according to the scoring signal. Table (3). The highest score to be detected in colorectal cancer expression of CD25 was score II in 10/27 (37%) mass tissues and 8/14(57.2%) in margin group, and 2/2 (100%) in apparently healthy control group. Statistical significant difference (P<0.05) was found among study groups according to the scoring signal. Table (4).

Table (3): Frequency distribution of immunohistochemistry results of Anti-CD4 marker according to the signal scoring.

CD4 signal scoring		Colorectal Mass Tissues (n=50)		Colorectal Marginal Tissues (n=50)		Apparently Healthy control tissues (n=20)		P-value1
		No.	%	No.	%	No.	%	
Negative		21/50	42	31/50	62	18/20	90	0.02*
Positive		29/50	58	19/50	38	2/20	10	
Scoring	I	5/29	17.3	6/19	31.6	2/2	100	P-value2
	II	13/29	44.8	7/19	36.8	0/2	0.0	0.001**
	III	11/29	37.9	6/19	31.6	0/2	0.0	
	IV	0/29	0.0	0/16	0.0	0/2	0.0	

Table (4): Frequency distribution of immunohistochemistry results of Anti-CD25 marker according to the signal scoring.

CD25 signal scoring		Colorectal Mass tissues (n=50)		Colorectal Marginal tissues (n=50)		Apparently Healthy control tissues (n=20)		P-value1
		No.	%	No.	%	No.	%	
Negative		23/50	46	36/50	72	18/20	90	0.001*
Positive		27/50	54	14/50	28	2/20	10	
Scoring	I	8/27	29.7	5/14	35.7	2/2	100	P-value2
	II	10/27	37.0	8/14	57.2	0/2	0.0	0.001*
	III	9/27	33.3	1/14	7.1	0/2	0.0	
	IV	0/27	0.0	0/14	0.0	0/2	0.0	

There was no significant correlation between histopathologic factors of colorectal cancer with CD4 expression. There was a significant positive correlation between stage of tumor in colorectal carcinoma with FOXP3 and CD25 expressions. Table (5) .

Table (5): Correlation between FOXP3, CD4 and CD25 expression and histopathological characteristics in colorectal cancer patients.

Histopathological Characteristics		FOXP3		CD4		CD25	
		+	-	+	-	+	-
Cancer Grading	Well differentiated	9	6	9	6	9	6
	Moderately differentiated	22	9	18	13	16	15
	Poorly differentiated	2	1	2	1	2	1
P –value		0.6		0.9		0.8	
Site of tumor	Right side	11	6	11	6	10	7
	Left side	13	10	11	12	13	10
	Rectum	9	1	7	3	4	6
P –value		0.1		0.3		0.6	
Stage of tumor	A	3	2	4	1	1	4
	B	8	4	7	5	11	1
	C	22	9	16	15	14	17
	D	0	2	2	0	1	1
	P –value	0.04*		0.3		0.01*	

Discussion

FOXP3 have an important regulatory role in development, phenotype, and functional maintenance of Treg cells (Marzano et al, 2009). Indeed, over expression of FOXP3 in Treg cells could impede immune surveillance for tumor and inhibit an effective immune response to autologous tumor cell antigens, thereby promoting tumor growth and invasion (Marzano et al, 2009; Ye et al, 2013).

Various solid tumors studies are proved a high density of tumor infiltrating Foxp3+ Treg in the tumor microenvironment, including ovarian (Sato et al, 2005), pancreatic (Hiraoka et al, 2006), and hepatocellular carcinoma (Kobayashi et al, 2007).

However, several researches demonstrated conflicting results (Carreras et al, 2006; Salama et al, 2009) and it should be noted that not all FOXP3+ TILs are Tregs, since T-cell receptor (TCR) activation of conventional T cells may induce the transient expression of FOXP3 without suppressive properties (Martin et al, 2010).

Recently, many clinical data from lung (Tao et al, 2012), breast (Ladoire et al, 2011; Miki et al, 2013), pancreatic (Hinz et al, 2007), hepatocellular (Wang et al, 2010), and urinary bladder cancer (Winerdal et al, 2011) as well as melanoma (Ebert et al, 2008) provided first evidence for a Foxp3 expression in tumor cells.

Karanikas et al, 2008, who that provided in their study evidence that cancer cells of various types of tumor cell lines (lung cancer, colon cancer, breast cancer, melanoma, erythroid leukemia, acute T-cell leukemia) express Foxp3 mRNA as well as Foxp3 protein was detected in all tumor cell lines, albeit in variable levels, not related to the tissue of origin.

Expression of Foxp3 by cancer cells would enable them to downregulate effector T cell responses directed against the tumor. This would give clinical evidence for an effective mechanism of a direct tumor-derived evasion from immunological destruction in CRC (Kim et al, 2013).

In this study, immunohistochemistry was used to detect FOXP3 expression in colorectal tissues. Present study concordance with newly study that which found the expression of FOXP3 was more common in colorectal cancer tissues than in normal colorectal tissues by using IHC technique, likely indicating the increased likelihood of Treg cell localization within tumor tissue (Liu et al, 2014).

Our findings are consistent with previous study which found that FoxP3_ TILs were significantly enriched in primary colon cancers compared with autologous normal colonic mucosa (Frank et al, 2009).

At the same technique used in the present study, there is a study has shown an increase in the Foxp3+ expressing were found in 61 out of 65 tumors of the patients (n= 61/65, 93.8%) (Kim et al, 2013).

Similar to our results the marker of regulatory T cells (Foxp3) expression was significantly higher in colon tumors than in autologous normal colon tissues (Sabine et al, 2008).

In newly research to study the demethylated status of the Treg-specific demethylated region (TSDR) of the FOXP3 gene was reported to be a potential biomarker for the identification of nTregs. Which found significantly, demethylation rate of the TSDR (DMR-TSDR) in colon tumor tissues was higher than DMR adjacent normal tissues (Zhuo et al, 2009).

Present finding were in agreement with previous study which used Tissue microarrays and immunohistochemistry to assess the densities of FOXP3_lymphocytes in tumor tissue and normal colonic mucosa and which revealed that the FOXP3_ Treg density was higher in tumor tissue compared with normal colonic mucosa (Salama et al, 2009).
However, our results, uncovering Foxp3 expression as a generalized feature of tumor cells, indicated that the determination of its functional consequences requires further elucidation to distinguish between FOXP3+ Tregs and FOXP3+ TILs.

Human natural regulatory CD4+ T cells comprise 5–10% of peripheral CD4+T cells. They constitutively express the IL-2Rα chain (CD25) and the nuclear transcription Foxp3. These cells are heterogeneous and contain discrete subsets with distinct phenotypes and functions (Camisaschi et al, 2010).

The identification of CD4+CD25+ T regulatory cells has been shown to play a crucial role in maintaining immunologic tolerance (Fontenot et al, 2005).

Marc et al, 2011, who proved in their study CD25 is not solely expressed on Treg cells but also on activated conventional CD4+ T cells, by using flow cytometry in Different Cancer Subtypes.

Recent data indicate that, at tumor sites, immune suppression is also due to the action of a subset of CD4+CD25+ Foxp3+ T cells that release IL-10 and TGF-β1 (Strauss et al, 2007; Ahmadzadeh et al, 2008).

A large body of data now associates increased frequencies of CD4+Foxp3+ T cells (Tregs) with a range of tumors (Betts et al, 2006). Previously, one study revealed that antitumor CD4+ T cell responses were reduced in patients with CRC before resection of the tumor; this phenomenon was related to Treg frequencies in peripheral blood, defined initially by high levels of CD25 expression on CD4+ T cells (Clarke et al, 2006), and more recently by expression of the transcription factor Foxp3 (Betts et al, 2012).

Recent research by using flow cytometry revealed that the relative proportion of CD4+ T cells that expressed Foxp3, conventionally classified as Tregs, was significantly higher within tumors compared with healthy colon (Scurr et al, 2014).

Holm et al, 2006, in their data indicated that although CD4+CD25+ T cells expressing Foxp3 are present within both lymphoid organs and the colon, subsets of IL-10-producing CD4+CD25+ T cells are present mainly within the intestinal lamina propria suggesting compartmentalization of the regulatory T cell response at effector sites.
A previous study by using flow cytometric analysis of fresh tumor from CRC patients showed that a significantly higher percentage of lymphocytes expressing CD25 tumour infiltrating lymphocytes than controls group (Maxwell-Armstrong et al, 1999).

One research carried out by Clarke et al, 2006, to compare the frequency of carefully defined CD4+ CD25+ FOXP3+ regulatory T cells by Flow cytometric analysis in CRC patients with healthy age-matched controls and IBD patients. They found in their study the CRC patients have an increased frequency of Treg and there is evidence that these Treg target anti-tumor immune responses.

Recent research to study the accumulation of CCR4 CTLA-4 FOXP3CD25 regulatory T cells in colon adenocarcinomas correlate to reduced activation of conventional T cells. They showed a correlation between increasing frequencies of regulatory T cells and decreasing frequencies of effector T cells (Svensson et al, 2012). According to Girardin et al, 2013, who observed in their study both inflammatory T cells (IL-171) and regulatory CD4 T cell populations (CD25 or CD25 FoxP3) were increased in frequency in tumor tissue compared with healthy bowel tissue.

In our study, we used a single IHC detection kits for detection of CD4, CD25 and FOXP3 expression, therefore we cannot say for sure that what we have found is actually Treg. For this reason, we recommend further studies using double IHC detection kits it is in order to give more accurate results and to enhance the results of the current study and discrimination between T-reg and T-conv cells infiltrating in microenvironment of colorectal cancer.

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