Expression of Th17/Treg related molecules in gastric cancer tissues

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Cite this article as: *Meng X, Zhu S, Dong Q, Zhang S, Ma J, Zhou C. Expression of Th17/Treg related molecules in gastric cancer tissues. Turk J Gastroenterol 2018; 29: 45-51.*

ABSTRACT

Background/Aims: *The function of regulatory T cells (Treg) and helper T cells 17 (Th17) related indexes, such as interleukin (IL)-6, IL-17, transforming growth factor (TGF)-β1, and forkhead box protein 3(FoxP3) in gastric adenocarcinoma tissues remains undefined. We investigated and analyzed the relevance of the proteins with the clinicopathological characteristics and the interactions among them in gastric cancer.*

Materials and Methods: *A total 68 gastric cancer patients and 40 healthy controls were enrolled. Immunohistochemistry (IHC) as well as quantitative real-time reverse transcription- polymerase chain reaction (RT-PCR) was used to determine the expression levels of IL-6, TGF-β1, IL-17, and FoxP3 in the prepared tissues. Statistical analysis included ANOVA and chi-square test.*

Results: *The expression levels of IL-6, IL-17, FoxP3, and TGF-β1 had significantly increased in cancer tissues compared to controls. Clinical staging of gastric cancer were correlated with the rise of IL-6, IL-17, FoxP3, and TGF-β1 levels expressed in cancer tissues. The expression level of TGF-β1 and IL-6 was positively related to that of IL-17 and FoxP3, similar to FoxP3 and IL-17 in gastric cancer tissues.*

Conclusion: *IL-6, TGF-β1, FoxP3, and IL-17 may promote the progression of gastric cancer individually or jointly and have complex interactions.*

Keywords: *Gastric cancer, interleukin-6, interleukin-17, transforming growth factor β1, regulatory T cells*

INTRODUCTION

Gastric cancer is among the fourth common malignancies with the second highest mortality rates caused by cancer in the world (1). One report has shown that the incidence and mortality of gastric cancer ranked second with 428,380 new cases and 339,308 cancer-related deaths in China (2). Chronic inflammation is one of the risk factors for gastric cancer particularly caused by *Helicobacter pylori* infection. The link between chronic inflammation and the risk of gastric cancer has become evident in recent years (3,4). However, the mechanisms remain unknown. Systemic and microenvironmental immunological change may be involved in this course.

Since their discovery, regulatory T cells (Treg) and helper T cells 17 (Th17) are defined as distinct subsets of CD4+ T cells. As major components of the adaptive immune system, Th17 as well as Treg are not only involved in the maintenance or inhibition of chronic inflammation, but also play important roles in various cancers, such as lung cancer, endometrial carcinoma, colorectal cancer, as well as gastric cancer (5-10). In our recent report, IL-17, mainly produced by Th17, may be the important promoting factor in the development and progress of gastric cancer and angiogenesis may be one of the underlying mechanisms (11).

Th17 and Treg may share some differentiating pathway from naïve CD4+ T cells. Transforming growth factor (TGF)-β1 is referred to be key factor in the generation of Th17 and Treg with or without IL-6. TGF- β1 alone promoted the generation of Treg through the induction of the transcription factor defined as forkhead box protein 3 (FoxP3), while CD4+ T cells will differentiate into Th17 by the combined action of IL-6 and TGF-β1 through orphan nuclear receptor (ROR) γt, signal transducer, and activator of transcription (STAT3) (12,13). Subsequently, the imbalance of Th17/Treg in gastric cancer has been focused on (10). However, the regulating mechanisms are still unknown.

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Received: **February 26, 2017** *Accepted:* **June 12, 2017**

DOI: **10.5152/tjg.2018.17114**

We aimed to investigate molecules that may play a role in the generation of Th17 and Treg or as their effectors in patients with gastric cancer. Our results try to shed light on the effect of these molecules in gastric cancer and the probable mechanisms.

MATERIALS AND METHODS

Patients

Sixty-eight gastric cancer patients from January 2012 to December 2013 at our hospital were enrolled. They were aged between 28 to 82 years with the mean±standard deviation (SD) age of (60.9±11.5) years. Clinicopathological features of these patients are summarized in Table 1. None of the patients received radiotherapy, chemotherapy, or other medical interventions in this study. Forty control participants underwent gastroscopy for health examination including 22 males and 18 females, with the average age±SD of 55.4±6.2 years. No statistical differences in sex and age were noted between patients and controls. This study was authorized by our hospital's medical ethics committee. Informed consents were signed by all subjects in this study.

Table 1. Clinicopathological features of patients with gastric cancer

	Number of patients	%
Total number of patients	68	
Age		
Mean (range)	60.9 (28~82)	
Sex		
Male	47	68%
Female	21	32%
Location		
Cardiac	11	16%
Non-cardiac	57	84%
Differentiation		
Well-moderate	20	29%
Poor	48	71%
TNM stage		
I	11	16%
II	21	30%
III	24	35%
IV	12	19%

TNM: tumor, node

Tissue samples

Tissue samples of the stomach from the patients were collected during surgery, and those from controls were obtained by endoscopic biopsy from the gastric antrum. Samples were fixed in 4% paraformaldehyde and embedded in paraffin for histopathological analysis according to our previous literature (11).

Immunohistochemistry

The sections of samples were stained using techniques described previously (11). Primary antibodies were used as follows: polyclonal rabbit anti-human IL-6 and polyclonal rabbit anti-human FoxP3 (1:100), polyclonal rabbit anti-human IL-17 (1:150), polyclonal rabbit anti-human TGF-β1 (1:200; Biosynthesis Biotech, Beijing, China). Phosphate-buffered saline (PBS) was used to replace primary antibody as negative control.

Scoring system for immunohistochemistry

Light-microscopic analysis was performed by counting positively stained cells in 5 separate regions under 400× high-power magnifications. The total score was calculated as the sum of two parts: (1) percentage of immunopositive cells (0=no positive cells, 1, <25%; 2, 25%~50%; 3, 51%~75%; 4, >75%) and (2) staining intensity (0, negative; 1, weak; 2, moderate; 3, high). Scores of 0 was regarded as negative (-), 2~3 as weak (+), 4~5 as moderate (++), and 6~7 as strongly positive (+++).

Quantitative real-time reverse transcriptase-polymerase chain reaction

Total RNA was extracted and cDNA was prepared as described previously (14). Overall, 1μL cDNA was amplified using polymerase chain reaction (PCR) in a 20 μL reaction mixture containing Ssofast EvaGreen Supermix (BIO-RAD, Hercules, CA, USA). Forward and reverse primers used are provided in Table 2. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an endogenous reference gene for relative quantification. Data were analyzed using the comparative ΔCt method with normalization to GAPDH mRNA in each sample.

Statistical analysis

The statistical Package for Social Sciences version 17.0 software (SPSS Inc.; Chicago, IL, USA) was used for anal-

Table 2. Real-time PCR primers of investigated genes

Gene	Primer sequence
IL-6	forward: 5′- GAT GAG TAC AAA AGT CCT GAT CCA - 3′;
	reverse: 5′- CTG CAG CCA CTG GTT CTG T - 3′
TGF-β1	forward: 5′-GTC CAC AGC CAT TGA CCT TT-3′
	reverse: 5′-ACC GCT CAC TTC CAG AGA GA-3′
IL-17	forward: 5′ -CGA TCC ACC TCA CCT TGG A-3′
	reverse: 5′-TCC CAG ATC ACA GAG GGA TAT CTC TC-3′
FoxP3	forward: 5′ -CAG CAC ATT CCC AGA GTT CCTC-3′
	reverse: 5′ -GCG TGT GAA CCA GTG GTA GATC- 3′
GAPDH	forward: 5′-ATT CCA CCC ATG GCA AAT TC-3′
	reverse, 5′-GCA TCG CCC CAC TTG ATT-3′

IL-6: interleukin-6; TGF-β1: transforming growth factor beta 1; IL-17: interleukin-17; FoxP3: forkhead box protein 3; GAPDH: glyceraldehyde phosphate dehydrogenase; PCR: polymerase chain reaction

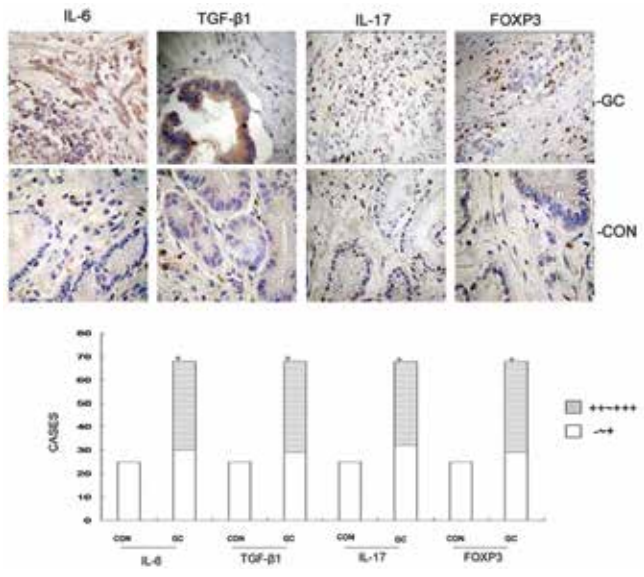

Figure 1. Increased expression of IL-6, TGF- β1, FoxP3, and IL-17 in GC tissues. Representative IHC staining of IL-6, TGF- β1, FoxP3, and IL-17 in cancer tissues and controls (magnification 200×) are shown in the upper part. Overexpression of IL-6, TGF- β1, FoxP3, and IL-17 was seen in more patients compared to controls (*, P<0.01).
GC: gastric cancer; CON: controls; IL-6: interleukin-6; IL-17: interleukin-17; TGF-β1: transforming growth factor beta 1; FoxP3: forkhead box protein 3; IHC: immunohistochemistry

ysis. Descriptive data were recorded as mean±SD. The mean between different groups was compared using analysis of covariance (ANOVA). The relation between investigated indexes and tumor staging was assessed using the chi-square test. Spearman correlation coefficient and linear regression coefficient were calculated to evaluate the interaction among the expressions of IL-6, IL-17, FoxP3, and TGF-β1. A P value <0.05 was identified as statistically significant.

RESULTS

Increased expression level of IL-6, TGF-β1, FoxP3, and IL-17 in gastric cancer tissues

The expression of IL-6 and TGF-β1 was localized in the cytoplasm of mononuclear cells and tumor cells, IL-17 in the cytoplasm of mononuclear cells, and FoxP3 in the nuclei of mononuclear cells. In the control tissues, only few infiltrated mononuclear cells had a low degree of positive staining, and the normal gastric epithelia were negative to staining (Figure 1). Protein and mRNA expression levels of IL-17, IL-6, FoxP3, and TGF-β1 were higher in cancer tissues compared to controls (Figure 1,2).

Relationship expression of IL-6, TGF-β1, FoxP3, and IL-17 in gastric cancer tissues and the tumor, node, and metastasis stage

The expression levels of IL-6, TGF-β1, FoxP3, and IL-17 were associated with the cancer tumor, node, and metastasis (TNM) stage. Higher expression levels of the above indexes were observed in the advanced stage compared to the early stage and showed an increasing trend with the TNM stage (Figure 3,4).

Interactions among the expression level of IL-6, TGF-β1, FoxP3, and IL-17 in gastric cancer tissues

The expression level of IL-6 and TGF- β1 was positively related with that of FoxP3 and IL-17 in cancer tissues. Correlation between the expression of IL-17 and FoxP3 also exhibited statistical significance (Table 3, Figure 5)

DISCUSSION

Th17 cells, distinguished based on IL-17-producing ability, have been shown to contribute to the induction and propagation of inflammation including autoimmune tissue injury. Much of the pro-inflammatory functions of Th17 cells have been attributed to the secretion of IL-17 (15,16). Tregs, as an immunosuppressive subset of T lymphocytes, were found to express the signature FoxP3 transcription factor, which is critical for their development and regulatory functions (17). Treg-mediated immune suppression and tolerance to self-components

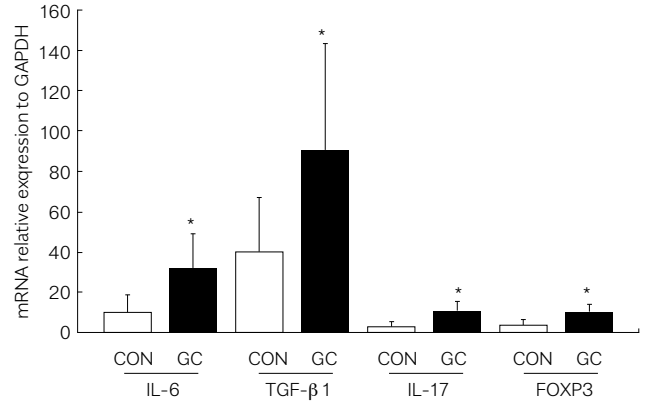

Figure 2. mRNA expression of IL-6, TGF- β1, FoxP3, and IL-17 in GC tissues were higher compared to controls (*, P<0.01).
GC: gastric cancer; CON: controls; IL-6: interleukin-6; IL-17: interleukin-17; TGF-β1: transforming growth factor beta 1; FoxP3: forkhead box protein 3

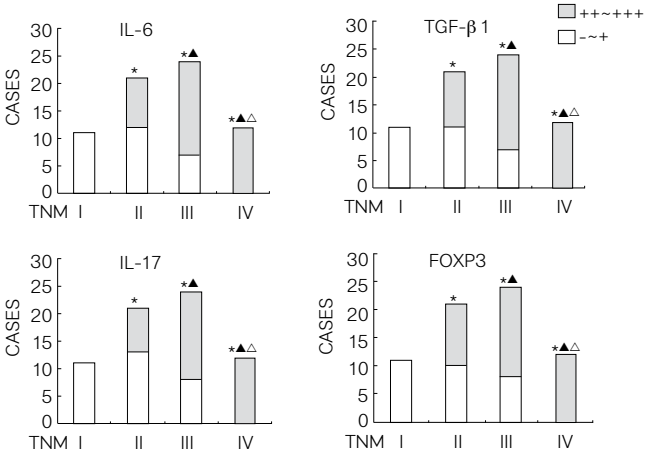

Figure 3. Expression of IL-6, TGF- β1, FoxP3, and IL-17 was significantly higher in the advanced stage than in the early stage, as calculated by IHC.*, compared with TNM I, P<0.01; ▲, compared with TNM II, P<0.01; Δ, compared with TNM III, P<0.01.
IL-6: interleukin-6; IL-17: interleukin-17; TGF-β1: transforming growth factor beta 1; FoxP3: forkhead box protein 3; IHC: immunohistochemistry; TNM: tumor, node, and metastasis

Table 3. Spearman rank correlation between the investigated indexes by IHC

Indexes	Correlation coefficient (r_s)	p
IL-6 vs IL-17	0.872	<0.001
TGF-β1 vs IL-17	0.717	<0.001
IL-6 vs FoxP3	0.824	<0.001
TGF-β1 vs FoxP3	0.864	<0.001
IL-17 vs FoxP3	0.751	<0.001

IL-6: interleukin-6; TGF-β1: transforming growth factor beta 1; IL-17: interleukin-17; FoxP3: forkhead box protein 3; IHC: immunohistochemistry

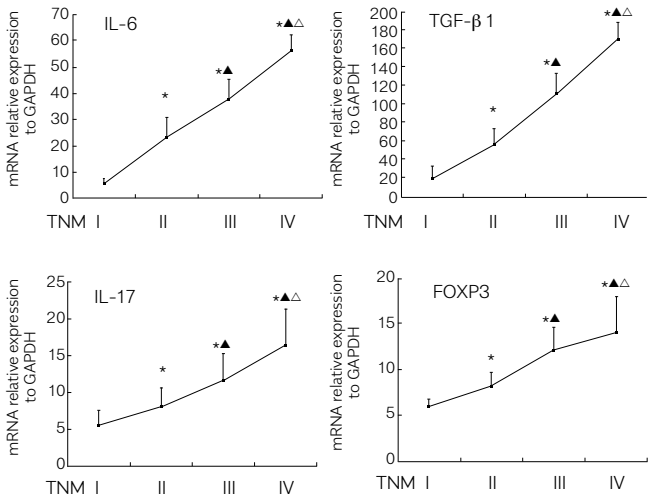

Figure 4. Expression of IL-6, TGF- β1, FoxP3, and IL-17 was significantly higher in advanced stage than that in early stage, as calculated by qRT-PCR.*, compared with TNM I, P<0.01; ▲, compared with TNM II, P<0.01; Δ, compared with TNM III, P<0.01.
IL-6: interleukin-6; IL-17: interleukin-17; TGF-β1: transforming growth factor beta 1; FoxP3: forkhead box protein 3; TNM: tumor, node, and metastasis; qRT-PCR: quantitative reversed transcriptase-polymerase chain reaction

have been identified at least by contact-dependent suppression or by the secretion of anti-inflammatory cytokines, such as IL-10 and TGF-β (18,19). Since the occurrence of tumor is closely related to chronic inflammation, different regulation patterns of Th17 and Treg may influence the inflammatory background in tumor microenvironment. Thus, Th17 and Treg may act on the formation, growth, and expansion of tumors.

Th17 and Treg have been studied in many malignancies including gastric cancer. The expression of Th17 and Treg in the diseases is related to the factors that influence their differentiation (20,21). Moreover, the function of Th17 and Treg in the diseases depends on the effectors that are synthesized and secreted by them (10, 11). The correlation between the factors that induce their differentiation and their effect factors in the disease has not been reported systematically.

In this study, we found that the expression levels of IL-6, TGF-β1, FoxP3, and IL-17 in gastric cancer were higher compared to the controls. There were positive correlations between these factors and the clinical stage of gastric cancer. In tumor tissues, there were similar interactions among the expression of IL-6, TGF-β1, FoxP3, and IL-17.

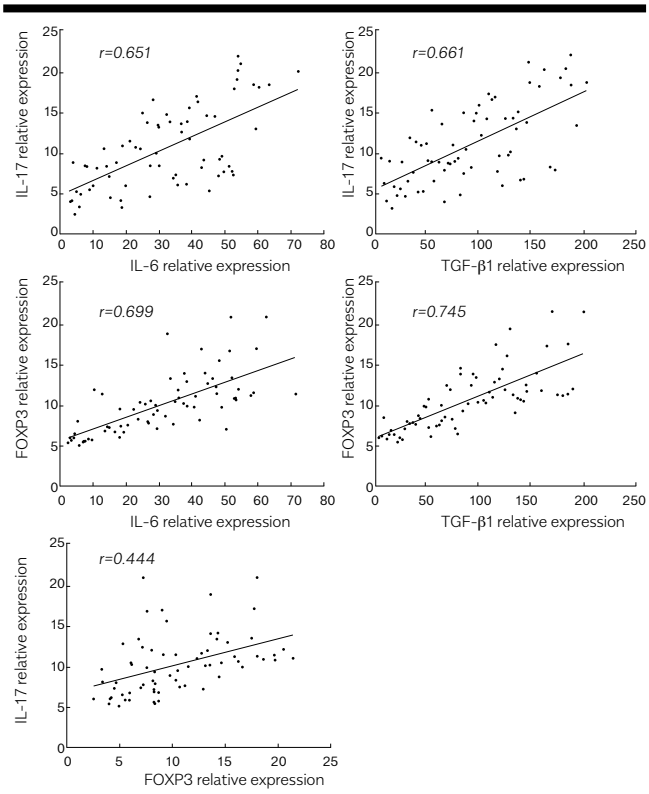

Figure 5. Correlation analysis of mRNA expression of IL-6, TGF- β1, FoxP3, and IL-17 in GC tissues. Both axes represent the relative expression level of investigated genes.
GC: gastric cancer; IL-6: interleukin-6; IL-17: interleukin-17; TGF-β1: transforming growth factor beta 1; FoxP3: forkhead box protein 3

IL-17 along with IL-6 is a pro-inflammatory cytokine and has been detected in various cancers including gastric cancer. Several researches have reported that overexpression of IL-17 and IL-6 was not only observed in gastric cancer compared to controls, but also positively related with the staging of gastric cancer, consistent with the results of this study (11,22,23). These results suggest that IL-17 and IL-6 may be the motivating factors in tumor progression. The pathogenic role of IL-17 and IL-6 in malignancies may be mediated by stimulating the inflammatory cells, such as neutrophils, macrophages, fibroblasts, and endothelial cells, inducing angiogenesis and activating the small signaling G proteins -Rho protein (11,19,24-26).

FoxP3 is considered the key transcription factor of Treg, and TGF- β1 is one of the main effectors of Tregs (17). The expression of FoxP3 and TGF- β1 in gastric cancer tissues reflected the distribution and function of Treg in tumor microenvironment. Treg may suppress cytotoxic T- cell responses in the tumor microenvironment, thereby hindering the host anti-tumor immunity (27). We found that FoxP3 and TGF- β1 are abundantly expressed in gastric cancer than in normal tissues, particularly in the advanced stage. These results indicate that the progression of gastric cancer may be the outcome of immune escape partially caused by Treg accumulated at the lesion region.

Recent studies have elucidated the reciprocal developmental pathways for Treg and Th17 (15,28). IL-6 and TGF- β1 are both involved in the differentiation of Treg and Th17 from naïve CD4+ T cells. Gnerlich et al. (29) found that IL-6 might skew the balance toward Th17 cells in the tumor microenvironment in pancreatic cancer. Chen et al. (30) reported that Th17 and Treg were positively correlated with TGF-β and IL-6 in the peripheral blood of cervical cancer patients. Therefore, the distribution of Th17 and Treg may be influenced by the overexpression of TGF-β1 and IL-6 in cancer. Researches on the distribution of Th17/Treg in gastric cancer have increased in recent years, but the results are not consistent. Th17 and Treg may both exhibit ascending trend with TNM stage, or Treg rather than Th17 increases in advanced gastric cancer (10,31). There are also differences between the distribution of Th17 and Treg in peripheral blood and cancer tissues. Yamada et al. (32) reported that Treg predominated in peripheral blood mononuclear cells (PBMC), whereas Th17 was most abundant in cancer tissue. Our exploratory study revealed that the expression of TGF-β1 and IL-6 increased together with the expression of FoxP3 and IL-17 as tumor up to later stage, similar to the relevance between the expression of FoxP3 and IL-17. These results may be interpreted as more differentiation of Th17 and Treg in cancer tissues promoted by TGF-β1 and IL-6 separately or in combination. In addition, the increased expression of IL-17 and FoxP3 might have a synergistic effect on tumor progression. The mechanism of TGF-β1 and IL-6 affecting the imbalance of Th17 and Treg in gastric cancer needs to be determined through further investigation.

This study focused on the correlation between Th17 and Treg cell-related factors and gastric cancer. The direct effects of these factors on proliferation and apoptosis of gastric cancer cells were not investigated. Cytological

tests in vitro in the future may compensate for these limitations.

In conclusion, IL-6, TGF- β1, FoxP3, and IL-17 may promote the progression of gastric cancer. IL-17 and FoxP3 might have a synergistic effect on the development of gastric cancer. It is important to understand the mechanisms of immunoregulation in gastric cancer to develop novel treatment strategies or to improve the efficacy of standard therapy.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethic committee of Qingdao Municipal Hospital (Decision Date: 08.03.2011; Decision Number: 2011-15).

Informed Consent: Written informed consent was obtained from the patient who participated in this study.

Peer-review: Externally peer-reviewed.

Author contributions: Concept - X.Y.M., S.T.Z.; Design - S.T.Z., Q.J.D.; Supervision - C.H.Z., Q.J.D.; Funding - S.T.Z.; Materials – J.M.; Data Collection and/or Processing - X.Y.M., J.M.; Analysis and/or Interpretation - X.Y.M., C.H.Z.; Literature Review - Q.J.D., S.T.Z.; Writer - X.Y.M., S.T.Z.; Critical Review - S.T.Z., C.H.Z.

Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: This study was supported by grant from Beijing Key Laboratory for Precancerous Lesion of Digestive Diseases (Project No: 2012XHAB02).

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