RESEARCH ARTICLE

Tim-3 Up-regulation in Patients with Gastric Cancer and Peptic Ulcer Disease

Mahdieh Naghavi-Alhosseini[1,2], Mohsen Tehrani[1,2], Abolghasem Ajami[1,2], Alireza Rafiei[1,2], Tarang Taghvaei[3], Laleh Vahedi-Larijani[4], Hadi Hossein-Nataj[1], Hossein Asgarian-Omran[1,5]*

Abstract

Background: T-cell immunoglobulin and mucin domain protein-3 (Tim-3), an inhibitory immunoregulatory receptor, has been recently implicated in tumor biology and tumor-associated immune suppression. In the present study, expression of Tim-3 was evaluated in gastric cancer (GC) and peptic ulcer disease (PUD) at both mRNA and protein levels. **Methods:** A total of 133 gastric tissue biopsies, comprising 43 from GC cases, 48 from PUD and 42 from non-ulcer dyspepsia (NUD) serving as controls were collected. Additionally, non-neoplastic adjacent tissue biopsies were also obtained from 6 patients with GC. Infection with *Helicobacter pylori* was determined by the rapid urease test for all participants and H&E staining was conducted for GC and PUD patients. Tim-3 relative mRNA expression was determined by SYBR Green based Real-Time PCR using β-actin as a reference gene. Tim-3 protein expression was also studied by immunohistochemistry in 7 GC, 7 PUD and 10 NUD tissue samples. **Results:** Tim-3 was expressed at higher levels in GC (p=0.030) and PUD (p=0.022) cases compared to he NUD group. Among paired samples obtained from gastric cancer patients, tumor tissues showed elevated Tim-3 expression (p=0.019) in comparison with adjacent non-neoplastic biopsies. Tim-3 mRNA findings were supported by detection of more Tim-3 protein in cancerous (p=0.002) and ulcerative (p=0.01) tissues than in controls. Tim-3 was similarly expressed in *H. pylori* positive and negative cases.**Conclusion:** Higher Tim-3 expression in patients with gastric cancer and peptic ulcer implies that it might be involved in immune regulation and establishment of these gastrointestinal diseases. Targeted immunotherapy by blocking of inhibitory receptors like Tim-3 could be a promising approach for gastric cancer treatment.

Keywords: Gastric cancer- peptic ulcer disease- *Helicobacter pylori*- Tim-3

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Introduction

Peptic ulcer disease (PUD) and gastric cancer (GC) are common diseases worldwide (Everhart et al., 1998; Correa and Schneider, 2005). PUD is usually accompanied with a low health-related quality of life, while GC is the fourth most common cancer in the world and the second leading cause of cancer-related death (Portal-Celhay and Perez-Perez, 2006; Li et al., 2014b). A number of risk factors like genetics, chronic inflammation, infections, pernicious anemia, smoking and nutrition have been attributed to predisposition to GC and among them infection with *Helicobacter pylori* is mostly specified (Li et al., 2012; Karimi et al., 2014). Most individuals infected with *H. pylori* carry and spread the bacterium while they are asymptomatic, while others develop one of the two clinical outcomes, PUD and GC. The reasons for developing these two extreme outcomes have not been clearly understood (Amieva and El-Omar, 2008). *H. pylori* infection causes a chronic inflammation of the gastric mucosa, which gradually progresses through the premalignant changes to form a favorable microenvironment for tumor initiation and establishment (Moss and Blaser, 2005). Association of chronic gastric inflammation and dysregulation of the immune system components with progression to gastric malignancy has been well recognized and documented (Suarez et al., 2006).

T-cell immunoglobulin and mucin domain-containing protein 3 (Tim-3) is known as a negative regulatory molecule which is mainly expressed on terminally differentiated Th1 cells, cytotoxic T-cells and innate immune cells. Tim-3 recognition by various ligands, like galectin-9, leads to a process of inhibitory and regulatory mechanisms in anti-tumor immunity and immune responses against chronic viral and bacterial infections (Freeman et al., 2010; Anderson, 2012; Gorman and Colgan, 2014). Different studies have demonstrated an obvious correlation between higher Tim-3 expression and

*[1]Department of Immunology, [2]Molecular and Cell Biology Research Center, [4]Department of Pathology, [5]Immunogenetic Research Center, School of Medicine, [3]Department of Gastroenterology, Gut and Liver Research Center, Mazandaran University of Medical Sciences, Sari, Iran. *For Correspondence: asgarianhossein@yahoo.com*

more extreme T-cells impairment and exhaustion (Jones et al., 2008). Tim-3 is highly expressed on tumor infiltrating lymphocytes and this is correlated with dysfunction in TNF-a, IL-2 and IFN-γ production (Sakuishi et al., 2010). Tim-3 expression has been evaluated in different solid and hematopoietic tumors such as lung (Zhuang et al., 2012), kidney (Yuan et al., 2014), melanoma (Wiener et al., 2007) and acute myeloblastic leukemia (Li et al., 2014a). Although the roles of Tim-3 in the context of its expression in tumor cells are largely unknown, there are some previous studies suggested that Tim-3 expression in tumor cells may be an independent prognostic factor and administration of anti-human Tim-3 antibodies is a promising approach for the improvement of cancer therapy. In prostate cancer, over-expression of Tim-3 has been proposed as a potential prognostic marker (Piao et al., 2013). Additionally, in vitro repressing of Tim-3 expression in a cervical cancer cell line using anti-sense strategy inhibited tumor cell migration and invasion (Cao et al., 2013). Although Tim-3 has comprehensively drawn researchers' attention for its negative regulatory role, such studies showed that this molecule could exert as a positive regulator on myeloid cells function (Kane, 2010). Enhancement of pro-inflammatory cytokines production and subsequent promotion of tissue inflammation was also reported by Tim-3 induction on DCs and macrophages (Anderson et al., 2007). Taken these considerations into accounts, it is now well accepted that inhibition of Tim-3 pathway by different methods can restore immune system capacity in both tumors and chronic microbial infections (Lee et al., 2010; Anderson, 2014).

Although the expression profile of Tim-3 has been evaluated in various malignancies, little is known about its expression pattern in GC patients. Given the role of suppression of the immune responses against *H. pylori* in the pathogenesis of both PUD and GC, in the current study, Tim-3 expression at mRNA and protein levels as well as its correlation with *H. pylori* infection was investigated in patients with gastric cancer and peptic ulcer disease.

Materials and Methods

Study Populations

Gastric biopsies were obtained from 43 patients with gastric cancer, 48 patients with peptic ulcer disease and 42 cases with non-ulcer dyspepsia (NUD) served as control group who underwent endoscopy for evaluation of their gastric problems at Imam Khomeini Hospital (Sari, Mazandaran, Iran) (Table 1). GC and PUD were diagnosed endoscopically and on the basis of morphologic and H&E staining. For 6 gastric cancer cases, non-neoplastic adjacent gastric tissue biopsies were also collected. Gastric tissue biopsies obtained from antrum and body of stomach in all three studied groups. None of the patients were received any chemotherapy treatments before sampling. The experimental procedure of the study was approved by Ethics committee of the Mazandaran University of Medical Sciences and written informed consents were obtained from all participants.

Determination of H. pylori infection

Rapid urease test was done for all samples when referred to endoscopy examination. In addition for gastric cancer and peptic ulcer patients, biopsy sections were stained for *H. pylori* detection and evaluated by a pathologist expert as well.

Real-Time PCR for detection of Tim-3

Total RNA was extracted from all fresh gastric tissue biopsies using Qiagen RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's protocol. The quality of isolated RNA was checked by nano-spectrophotometer (WPA, England) and electrophoresis. Complementary DNA (cDNA) was reverse-transcribed from total RNA using the Thermo Scientific Revert Aid first strand cDNA synthesis kit (Thermo Scientific, USA). Real-Time PCR was performed using 2X Thermo Scientific Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific, USA) reagent in an iCycler iQ5 Real-Time PCR system (Bio RAD, USA) with the following primers: Tim-3, forward: GAC TTC ACT GCA GCC TTT CC, reverse: GAT CCC TGC TCC GAT GTA GA; β-actin, forward: CCT TCC TGG GCA TGG AGT CCT, reverse: TGG GTG CCA GGG CAG TGA T. The PCR reactions were amplified at 95°C for initial denaturation followed by 40 cycles at 94°C for 30 seconds, 61°C (Tim-3) and 57°C (β-actin) for 30 seconds, and 72°C for 30 seconds. The PCR amplicon sizes were 201 bp and 174 bp for Tim-3 and β-actin, respectively. Relative expression level of Tim-3 mRNA was determined with $2^{-\Delta Ct}$ value.

Analysis of Tim-3 protein by Immunohistochemistry (IHC)

Formalin-fixed and paraffin-embedded tissues of gastric biopsies were cut into 2-3 μm sections and mounted on to poly L-lysine coated slides. Specimens were deparaffinized, rehydrated and then heat-induced epitope retrieval was conducted by immersing slides in 10 mmol citrate buffer (pH 6.0) and boiling the buffer for 10 min in a pressure cooker. Endogenous peroxidase activity was quenched by 3% H2O2 for 30 minutes at room temperature. All slides were blocked with normal goat serum (DAKO, Denmark) for 15 minutes at room temperature in a humid chamber. Following blocking, all sections were subsequently incubated overnight at 4°C with anti-human Tim-3 polyclonal primary antibody (1:300 diluted, Antibodies-online, Atlanta, USA). After four times washing, the sections were incubated with biotinylated corresponding secondary antibody (Santacruz, USA) for 1h at RT. The Santacruz ABC staining system was used for the avidin-biotin complex method according to the manufacturer's instructions. For negative controls rabbit IgG was included in the immunostaining procedure. The sections were counterstained with hematoxylin, dehydrated through ethanol series, cleared in xylene and then mounted. All slides were analyzed by a pathologist and the semi-quantitative H-Score system analysis was used to assess staining intensity and percentage of the positive stained cells (Jiang et al., 2013) .

The H-Score was calculated by the following equation H-Score = $\sum$Pi(i) (i = 0,1,2,3, Pi =0~100%). i defines the intensity of staining designated as no staining = 0, weak staining = 1, moderate staining = 2 and strong staining = 3.

Pi represents the percentage of stained cells which varies from 0 to 100. Therefore, the H-Score ranges from 0 to 300, H-Score >0 is considered as positive staining and H-Score = 0 is considered as a complete negative staining.

Statistical analysis

All statistical analyses were performed using SPSS 21 for windows. One way ANOVA was used to compare the statistical difference between three groups and Mann Whitney U or Independent sample t tests were applied to compare differences between two groups. Furthermore, the Tim-3 expression difference between tumoral and corresponding non-neoplastic adjacent tissues was analyzed by Paired sample t test and Wilcoxon matched signed-rank test. The Spearman and Pearson correlation tests were appropriately applied to analyze the correlation of Tim-3 expression with clinical features of patients. P-values<0.05 were considered significant.

Results

Expression of Tim-3 mRNA in gastric tissues obtained from patients with gastric cancer, peptic ulcer and non-ulcer dyspepsia

Tim-3 mRNA expression in gastric tissues from three studied groups was evaluated using semi-quantitative Real-Time PCR assay. The housekeeping gene β-actin was also amplified in all samples and the mRNA expression results were represented as the ratio of Tim-3 to β-actin. To more validate and check the reproducibility of the data

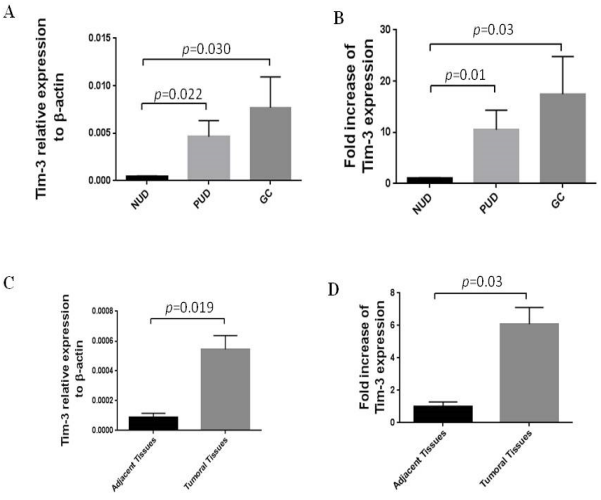

Figure 1. Expression Profile of Tim-3 mRNA in Gastric Biopsies. A. Relative expression of Tim-3 mRNA in gastric biopsies obtained from cases with gastric cancer (GC), peptic ulcer disease (PUD) and non-ulcer dyspepsia (NUD). B. Fold increase of Tim-3 expression in gastric cancer and peptic ulcer patients compared to non-ulcer dyspepsia group. C. Relative expression of Tim-3 mRNA in gastric cancer patients compared to their non-neoplastic adjacent tissues. D. Fold increase of Tim-3 expression in gastric cancer patients compared to their non-neoplastic adjacent tissues. Relative expression of Tim-3 is represented as $2^{-\Delta Ct}$ value after normalization with β-actin as an internal control. The Y axis shows the Mean+SEM value. P-values less than 0.05 were considered significant.

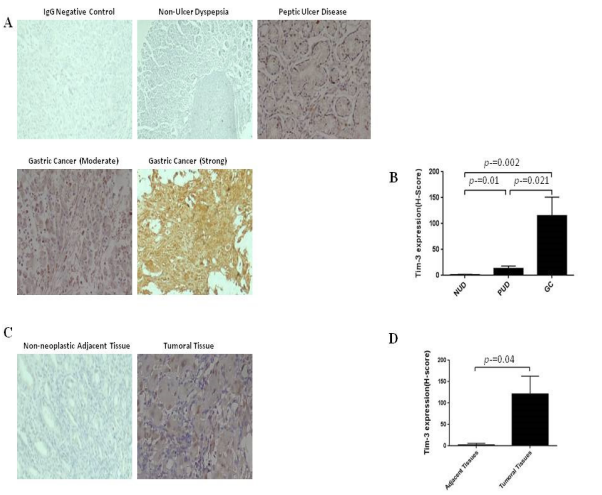

Figure 2. Immunohistochemical Findings of Tim-3 Protein Expression in Gastric Biopsies. A. Immunohistochemistry staining shows strong nuclear and cytoplasmic Tim-3 expression in gastric tumoral biopsies (GC), weak or moderate staining in peptic ulcer disease (PUD) and no staining in non-ulcer dyspepsia (NUD) control group. B. Quantitative H-Score system of immunohistochemistry results indicate significant higher Tim-3 expression in gastric cancer tissues compared to peptic ulcer and non-ulcer dyspepsia biopsies. Patients with peptic ulcer disease showed more Tim-3 expression than non-ulcer dyspepsia group. C. Immunohistochemistry staining of Tim-3 in tumoral and non-neoplastic adjacent biopsies obtained from a patient with gastric cancer which shows higher Tim-3 expression in tumoral biopsies. D. Quantitative H-Score system of immunohistochemistry results confirm more Tim-3 expression in tumoral tissues compared to their non-neoplastic adjacent biopsies. The Y axis shows the Mean+SEM value. P-values less than 0.05 were considered significant.

obtained from Real-Time PCR, intra-assay and inter-assay quality control experiments were performed for both Tim-3 and β-actin. Coefficient of variation (CV) indices of intra-assay and inter-assay analysis were 0.01 and 0.01 for Tim-3 and 0.03 and 0.02 for β-actin, respectively. As shown in figures 1A and 1B, Tim-3 was highly expressed in gastric tissues obtained from patients affected with GC (p=0.030) and PUD (p=0.022) compared to NUD tissues. There was no significant difference for Tim-3 expression between patients with GC and PUD. To explore the Tim-3 profile in tumor microenvironment, six tumoral tissues and their corresponding non-neoplastic adjacent biopsies were analyzed for Tim-3 expression. Tim-3 mRNA was significantly more expressed in tumoral tissues compared to their corresponding non-neoplastic adjacent tissues (p=0.019, Figures 1C and 1D). No significant correlations were observed between Tim-3 expression and different clinicopathological features represented in Table 1.

Tim-3 protein expression in gastric tissues obtained from patients with gastric cancer, peptic ulcer and non-ulcer dyspepsia

Immunohistochemistry assay was applied to evaluate Tim-3 protein expression and confirm the mRNA results obtained from Real-Time PCR. Protein expression

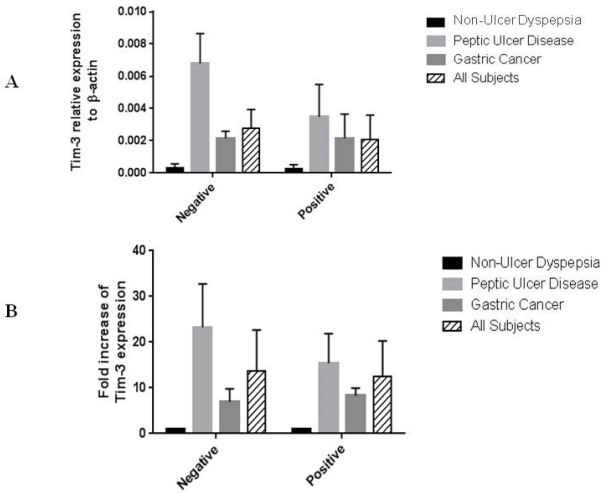

Figure 3: Correlation of Tim-3 mRNA expression with *H. pylori* infection. A. Similar Tim-3 mRNA expression in *H. pylori* positive and negative cases affected with gastric cancer, peptic ulcer or non-ulcer dyspepsia. All enrolled samples were also divided in two groups based on the *H. pylori* infection. No significant differences of Tim-3 expression were found between patients with or without *H. pylori* infection. B. Fold increase of Tim-3 expression in *H. pylori* positive and negative cases. Relative expression of Tim-3 is represented as $2^{-\Delta Ct}$ value after normalization with β-actin as an internal control. The Y axis shows the Mean+SEM value.

analysis was performed for 7 GC tissues, 7 PUD samples and 10 NUD gastric tissues. For six GC patients, the paired non-neoplastic adjacent tissues were also available for IHC staining. Tim-3 was clearly up-regulated in gastric tissues obtained from patients with GC and PUD compared to NUD stomach tissues (Figures 2A). When Tim-3 protein expression was further evaluated by semi-quantitative H-Score system, GC tissues displayed higher Tim-3 expression compared to control samples (p=0.002) and PUD specimens (p=0.021) (Figure 2B). Tim-3 protein was also more expressed in patients with PUD in comparison with control group (p=0.01). In agreement with the mRNA results, Tim-3 protein was also more detected in tumoral tissues compared to their corresponding non-neoplastic adjacent biopsies (p=0.04, Figures 2C and 2D). Tim-3 protein expression was found to be significantly correlated with mRNA expression results (r=0.535, p=0.006) which demonstrates Tim-3 protein expression was statistically higher in gastric biopsies with higher mRNA expression.

Tim-3 is similarly expressed in H. pylori positive and negative samples

Infection with *H. pylori* was confirmed in 67.7%, 76.5% and 63.8% of GC, PUD and NUD groups, respectively. No significant correlation was found between *H. pylori* infection and Tim-3 expression in all three studied groups (Figure 3).

Discussion

In the context of chronic infections and cancer which accompanied with persistent antigen exposure and inflammation, T cells may remarkably alter to an immunological state termed "exhaustion" characterized by several features, such as loss of effector functions, expression of multiple inhibitory receptors and decreased proliferation (Wherry and Kurachi, 2015). Tim-3 is expressed on the cell surface of various immune cells and tumoral tissues and has been indicated to interact with soluble or cell associated ligands (Yeung et al., 2011). It is generally believed that Tim-3 is one of the main exhaustion markers of T cells that functions as a suppressive receptor in antitumor or antiviral responses. Studies demonstrated that Tim-3 marks the most suppressed or dysfunctional population of CD8+ T cells in both solid and hematologic malignancies (Wherry, 2011).

We observed a significant over-expression of Tim-3 mRNA in GC and PUD patients. In addition, Tim-3 protein expression was evaluated by immunohistochemistry in 7 GC tissues, 7 PUD samples and 10 NUD gastric tissues. Similar to mRNA, Tim-3 protein was markedly over-expressed in patients with GC and PUD compared to controls. Our findings were in consistent with previous studies regarding Tim-3 expression in different cancers (Wiener et al., 2007; Zhuang et al., 2012; Li et al., 2014a; Yuan et al., 2014; Zhu et al., 2016). Immunohistochemical detection of Tim-3 showed a clear positivity of protein expression in melanoma cells and melanoma surrounding mast cells (Wiener et al., 2007). Moreover, the presence of Tim-3 was also confirmed by Real-Time PCR and flow cytometry in two WM35 and HT16B-M1melanoma cell

Table 1. Major Clinical and Paraclinical Findings of Study Populations

Variables		Non-Ulcer Dyspepsia	Peptic Ulcer Disease	Gastric Cancer
Study samples (N)	Male	11	21	36
	Female	31	27	7
Age (year)	Mean±SD	47.52±15.91	56.21±15	71.23±10.74
	Range	19-77	27-87	50-90
H. pylori infection*	Positive	24	36	21
	Negative	13	11	10
Tumor Grade**	I	-	-	2
	II	-	-	8
	III	-	-	15

H. pylori* infection information was available for 115 from 133 patients;Tumor grade was not available for some patients with gastric cancer

lines (Wiener et al., 2007). In a study by Yang Cao et al, the analysis of cervical tumor tissue sections revealed higher Tim-3 expression in cervical intraepithelial neoplasia and cervical cancer in comparison to chronic cervicitis (Cao et al., 2013). At the current time, there are limited and controversial reports regarding the importance of Tim-3 molecule in gastric cancer. While a recent study have demonstrated higher Tim-3 expression on tumor infiltrated T-CD4+ and T-CD8+ of patient with GC (Cheng et al., 2015), the other one declared lower expression of Tim-3 protein in GC tissues compared to normal paired mucosa (Jiang et al., 2013). Despite many published data to understand the involvement of Tim-3 in tumor immunity, the roles of Tim-3 expression in tumor cells have not yet been well defined. Huang et al. showed that Tim-3 expression on endothelial cells facilitates the onset, growth, and dissemination of lymphoma by suppressing the activation of CD4+ T cells and Th1 polarization (Huang et al., 2010). We can hypothesize that Tim-3 over-expression in gastric tissues works in a similar way and may introduce one of the various mechanisms used by the tumor cells for immune evasion. Future studies on interaction of Tim-3 on tumor cells with its ligands such as galectin-9 and HMGB1 could provide more understandings about the role of Tim-3 pathway on deviation of anti-tumor immune responses in malignancies. Wiener et al., (2007) have reported a mechanism involved in the regulation of Tim-3 expression in tumor cells through TGF-β1 which TGF-β1 cause Tim-3 up-regulation and local immunosuppression. As TGF-β plays an important role in invasion and metastasis of gastric cancer (Fu et al., 2009; Achyut and Yang, 2011), thus alteration of Tim-3 expression profile in gastric cancer cells may originate from the secretion of TGF-β from infiltrated lymphocytes or gastric tumor cells during the process of gastric cancer progression.

To more explore Tim-3 expression profile in tumor microenvironment, non-neoplastic adjacent gastric biopsies were also checked for Tim-3 expression in parallel to their tumoral tissues. Higher Tim-3 expression in tumoral biopsies showed more immunosuppressive milieu in tumor microenvironment due to the presence and pressure of cancerous cells which control the signature and response of the surrounding immune cells. It is important to mention that since in this study the expression of Tim-3 was investigated in crude tumor cell extracts and tissue biopsies, complementary experiments such as double staining with Tim-3 and immune cells markers (CD3, CD4) are needed to demonstrate the expression pattern of Tim-3 in tumor microenvironments. Combinatorial targeting of Tim-3 and PD-1 with blocking antibodies dramatically delayed tumor growth in mice bearing CT26 and the function of tumor infiltrated lymphocytes was restored when treated with anti-Tim-3 and anti-PD-1 antibodies (Sakuishi et al., 2010). Regarding the more expression of Tim-3 in gastric cancer, targeted immunotherapy by blocking of Tim-3 could be a useful promising approach for gastric cancer treatment.

Since both GC and PUD have common etiologic causes, the expression of Tim-3 was also evaluated in PUD patients to show whether Tim-3 expression may modulate the local immune responses in PUD development. Our results clearly showed that Tim-3 expression was significantly higher in patients with PUD in both mRNA and protein levels compared to controls. To our knowledge, this is the first study which has investigated Tim-3 expression in patients with PUD. As protein expression analysis showed more Tim-3 expression in GC patients compared to PUD, it may be due to the advanced gastric pathological problems in GC. These findings was also declared by a previous study demonstrating higher Tim-3 protein expression in GC in comparison to gastritis (Cheng et al., 2015).

Helicobacter pylori is defined as a major risk factor for development of peptic ulcer and gastric cancer. However, we did not find any significant correlation between Tim-3 expression and *H. pylori* infection even when analysis was performed in all three studied groups or each group separately. Our finding is in concordant with recent study showing that Tim-3 expression had no correlation with presence of *H. pylori* infection (Jiang et al., 2013). A possible explanation is that all *H. pylori* variants do not prime the same immune responses as highlighted in previous reports (Portal-Celhay and Perez-Perez, 2006). The presence or absence of the cag-PAI gene, a gene responsible for production of the most important *H. pylori* virulence factor, is the major disease-related genetic difference in *H. pylori* isolates that its significant association with severe gastritis, atrophic gastritis, peptic ulcer disease and distal GC were observed and reported in previous studies (Lochhead and El-Omar, 2007; da Costa et al., 2015). Therefore, the genetic differences in *H. pylori* strains may affect the recruitment of the various immune cells as well as the expression of the immune regulatory molecules like Tim-3. Interestingly, the prevalence of *H. Pylori* infection was similar between three studied groups in our study. To explain this similarity, it is important to mention that there is a high prevalence of *H. pylori* infection in some regions such as north of Iran. Additionally, based on the previous studies although *H. Pylori* is one of the most important risk factors for PUD and GC development, approximately 1-3% and 10% of the infected individuals develop gastric adenocarcinoma and peptic ulcer disease, respectively (Saccà et al., 2014).

Taken together, Tim-3 up-regulation in the gastric mucosa of GC and PUD patients suggest that chronic inflammation and immunoregulatory mechanisms in the gastric mucosa could be the initial steps of gastric cancer or peptic ulcer development. Targeting immune checkpoint inhibitory receptors to restore the potential anti-tumor activities of tumor infiltrated immune cells could be helpful in the immunotherapy approaches of gastric cancer.

Statement conflict of Interest

The authors state no conflict of interest.

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