RESEARCH ARTICLE

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

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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 the 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...
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-α, IL-2 and IFN-γ production (Sakushi 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 \textit{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 \textit{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 \textit{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 \textit{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 RNasy 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 $2^{-\Delta\Delta Ct}$ 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 $\text{H-Score} = \sum Pi(i) \times (i=0,1,2,3, Pi=0\text{~to}100\%). i$ defines the intensity of staining designated as no staining = 0, weak staining = 1, moderate staining = 2 and strong staining = 3.
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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 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.

**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 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 1. Expression Profile of Tim-3 mRNA in Gastric Biopsies.](image1)

![Figure 2. Immunohistochemical Findings of Tim-3 Protein Expression in Gastric Biopsies.](image2)
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-M1 melanoma cell

| 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
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Tim-3 expression in gastric cancer is one of the most important risk factors for PUD and GC development. However, 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 (Sacca 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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