RESEARCH ARTICLE

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Clinical Significance of *TNFSF14*/LIGHT and *CD160* in Gastric Cancer and Peptic Ulcer Dyspepsia

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Abstract

Background: Previous studies have reported the role of the Herpes Virus Entry Mediator (HVEM) in various cancer including gastric cancer. However, the expression level and clinical significance of *CD160* and Tumor Necrosis Factor Ligand Superfamily Member 14 (*TNFSF14*) pathways in gastric cancer and gastric dyspepsia patients have remained unexplored. **Methods:** The study involved the collection of gastric tissue biopsies from 42 patients with non-ulcerative dyspepsia (NUD) as the control group, 43 gastric cancer (GC) patients, and 48 patients with peptic-ulcerative dyspepsia (PUD). All the patients were endoscopically examined at Imam Khomeini Hospital in Sari, Mazandaran, Iran. The expression levels of *TNFSF14* and *CD160* mRNA were assessed using quantitative real-time PCR (qPCR) with the SYBR Green method. Statistical analysis was performed to investigate the potential association between the clinical and experimental data. **Results:** Among the 133 gastric endoscopic biopsies examined, LIGHT exhibited a significant overexpression in GC patients (p-value < 0.01). Moreover, the expression of *TNFSF14* was higher in GC patients with stages I and II (p-value<0.05). Furthermore, GC patients with TNM stages III+IV were accompanied by high expression levels of LIGHT (p-value < 0.01) as well as *CD160* (p-value<0.05). The expression of *CD160* was also higher in younger adults with PUD (p-value<0.05). Whereas *TNFSF14* exhibited higher expression in older adults with GC (p-value<0.05). Furthermore, this research provided insights into the potential biological pathways and significant gene enrichment of *TNFSF14* and *CD160*, suggesting the potential role of *CD160* and *TNFSF14* in the regulation of immune system in GC and PUD. **Conclusion:** These findings suggest the possible role of LIGHT and *CD160* expression in gastric cancer patients in immune dysregulation toward gastric cancer. Targeted immunotherapy that harnessing co-stimulatory molecules like LIGHT and *CD160* could be a promising approach in the treatment of GC as well as potential GC tumor markers.

Keywords: Gastric Cancer- Dyspepsia- *CD160* antigen- Tumor Necrosis Factor Ligand Superfamily Member 14

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Introduction

Gastric cancer (GC) is one of the five most common cancers and the third leading cause of cancer-related deaths worldwide. GC and peptic ulcerative dyspepsia (PUD) are two common gastrointestinal diseases with high morbidity and mortality rates worldwide. On the other hand, PUD affects approximately 4% of the global population and is a major cause of hospitalization and healthcare costs [1-4]. The complex etiology and pathogenesis of GC and PUD involve a combination of genetic, environmental, and immunological factors [5]. Immune dysregulation is known to play a critical role in the development and progression of gastric cancer. The immune system interacts with cancer cells through various pathways, including immune checkpoints [6-8]. CD160 and TNFSF14 (also known as LIGHT) are two molecules in immune checkpoint pathways that may play a role in gastric cancer [9, 7, 10, 11]. *CD160* is a glycosyl phosphatidyl inositol (GPI)-anchored protein which is expressed in various immune cells, including natural killer (NK) cells, CD8+ T cells, and some subsets of CD4+ T cells. *CD160* is also involved in the regulation of the activation and function of NK cells and has been shown to

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promote their cytotoxicity against cancer cells [12-15]. On the other hand, *TNFSF14*/LIGHT is a member of the tumor necrosis factor (TNF) superfamily which is expressed in various immune cells, including T cells, B cells, and dendritic cells. *TNFSF14*/LIGHT is also involved in the proliferation and survival of cancer cells and the regulation of tumor-associated immune responses [16-18]. Studies have shown the involvement of *CD160* and *TNFSF14*/LIGHT in the development and progression of various types of cancer, including GC [19-23]. However, the precise mechanisms of the contribution of these molecules to gastric cancer have remained poorly understood.

This study evaluated the expression levels of *CD160* and *TNFSF14*/LIGHT genes in gastric cancer patients as well as the assessment of clinical histological characteristics. It was hypothesized that *CD160* and *TNFSF14*/LIGHT expression levels may serve as potential biological pathways in the progression and development of gastric cancer.

Materials and Methods

Study Population

Patients underwent endoscopy to evaluate their gastric discomfort at Imam Hospital or Tooba Outpatient Clinic during 2017-2019. Gastric tissue biopsies were sampled from the antrum and body of the stomach of all patients. According to histopathological examinations, patients were divided into three groups: non-ulcer dyspepsia (NUD, n=42) with normal histology which served as the control group, peptic-ulcerative dyspepsia patients (PUD n=48,) and gastric cancer (GC, n=43) patients. The study was approved by Ethics Committee of Mazandaran University of Medical Science (Sari, Iran). All participants signed informed consent. Biopsy samples of patients with gastric cancer, non-ulcer dyspepsia, and peptic ulcer disease were stained for Helicobacter pylori detection followed by pathologist evaluation. Patients with autoimmune and immunodeficiency diseases, as well as those receiving chemotherapy, chemo-radiotherapy, or immunotherapy, were excluded from the study. The staging system of gastric cancer was determined according to the UICC/AJCC manual by a pathologist.

Quantification of CD160 and LIGHT using Real-Time PCR

Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. The concentration and optical density of extracted samples were determined by a Nanodrop spectrophotometer (WPA, England), while their integrity was assessed by visualization on a 1% agarose gel. The RNA samples were stored at -70 °C for further experiments. The RNA samples were reverse transcribed and cDNA was synthesized using 2.5 µg of total RNA, random primers, and a cDNA Synthesis Kit (Yekta Tajhiz, Teh, IR) following the manufacturer's protocols. The integrity of each cDNA preparation was assessed by the qPCR assay of the HPRT gene expression as an internal control gene with appropriate primers/probes and subsequent visualization on the agarose gel. The relative expression levels of LIGHT (CD258) and *CD160* mRNA were evaluated by quantitative real-time PCR (qPCR) using the SYBR Green. Real-time PCR was carried out using the 2X Thermo Scientific Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific, USA) reagent in a T100 Thermal Cycler Real-Time PCR system (Bio-Rad, USA) with the following primers: LIGHT, forward: CTGATACAAGAGCGAAGGT, reverse: GCTGAGTCTCCCATAACA; *CD160*, forward: TCCATTCTATTCACAGAGACAG, reverse: ACTGAGAGTGCCTTCATTATG; β-actin, forward: CCTTCC TGGGCATGGAGTCCT, reverse: TGGGTGCCAGGGCAGTGAT. The PCR reactions were amplified at 95°C for initial denaturation followed by 40 cycles at 94°C for 30 seconds, 58°C (LIGHT), 60°C (*CD160*), and 57°C (β-actin) for 30 seconds, and 72°C for 30 seconds. The PCR amplicon sizes were 106 bp, 93 bp, and 174 bp for LIGHT, *CD160*, and β-actin, respectively. The relative expression levels of other mRNAs were determined by the 2-ΔCt method [24]. The β-actin house-keeping gene was amplified in all studied samples to ensure the reliability and consistency of the Real-Time PCR data. The mRNA quantification results were then reported as the ratio of target genes to β-actin, which is a robust and valid approach to validate the reproducibility of the Real-Time PCR data.

Statistical analysis

Depending on the data distribution and the type of variables, both parametric and non-parametric statistical tests were utilized to analyze the data. Specifically, one-way ANOVA and the Kruskal-Wallis tests (followed by Dunn's test) were used to compare differences among the study groups. The t-tests (or Mann-Whitney tests, as appropriate) were also employed to evaluate differences between the two groups. The significance level was set at $P < 0.05$. The data were presented as means ± standard error of the mean (SEM).

Results

Clinical and Para clinical Parameters of the study population

The study population included 42 patients with non-ulcer dyspepsia (NUD) as the control group, 48 patients with peptic-ulcerative dyspepsia (PUD), and 43 patients with gastric cancer (GC). The mean age was significantly higher in the GC group (71.23 ± 10.74 years) compared to the NUD (47.52 ± 15.91 years) and PUD (56.21 ± 15.00 years) groups. The gender distribution varied across the groups, with the GC group having more male participants (47 males) compared to the NUD (11 males) and PUD (21 males) groups.

Helicobacter pylori infection status was available for 115 out of the 133 total participants. Positivity rates were 57.5% (24 out of 42) in the NUD group, 75% (36 out of 48) in the PUD group, and 48.8% (21 out of 43) in the GC group. Regarding tumor grade in the GC group, 4 patients had grade I, 16 had grade II, and 30 had grade III tumors. The mean tumor size in the GC group was 5.5 ± 2.79 cm, with a range of 2-13cm (Table1).

Table 1. The Table Presents the Major Clinical and Para Clinical Findings of the Study Populations. Note: *, Out of 133 participants, information on H. pylori infection was available for 115 individuals; **, Tumor Grade and Tumor size are specific to gastric cancer patients.

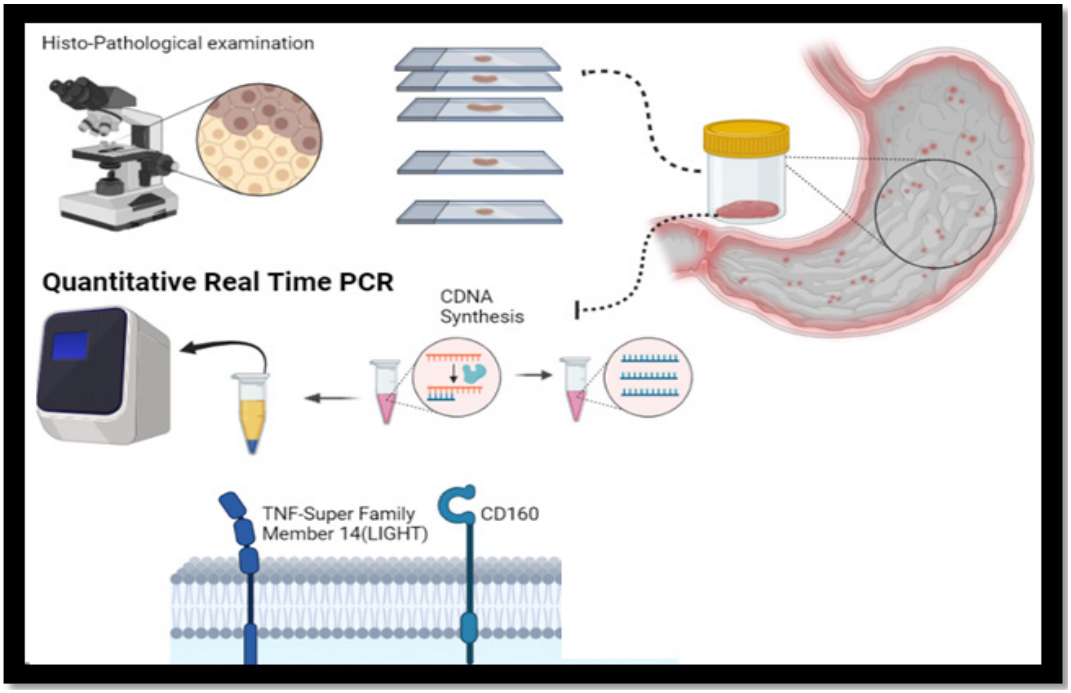
Variables	Scale	Non-Ulcer Dyspepsia	Peptic Ulcer Disease	Gastric Cancer
Study samples (N)	Male	11	21	47
	Female	31	27	25
Age (year)	Mean ± SD	47.52 ±15.91	56.21 ± 15	71.23 ± 10.74
	Range	19-77	27-87	50-90
H. pylori infection*	Positive	24	36	21
	Negative	13	11	10
Tumor Grade**	I	_	_	4
	II	_	_	16
	III	_	_	23
Tumor size, cm**	Mean ± SD	_	_	5.5 ± 2.79
	Range	_	_	2-13
	Samples (N)	_	_	24

TNFSF14 is significantly overexpressed in gastric cancer patients

The expression levels of target mRNAs, including *CD160* and LIGHT were quantified in biopsy-derived gastric tissues of patients diagnosed with gastric cancer, peptic ulcer dyspepsia (PUD), and non-ulcer dyspepsia (NUD) using the quantitative Real-Time PCR assay. Analysis of 133 gastric specimens revealed significantly higher expression levels of LIGHT in the GC group compared to the controls (P-value <0.01). No significant difference was observed in the expression of *CD160* relative to the control group in the study groups (Figure 1). Results also indicated no significant difference in the expression of *CD160* and LIGHT in terms of sex, H. pylori infection, and the presence of blood vessel invasion, lymphatic invasion, or perineural invasion.

Differential expression of CD160 and TNFSF14 in advanced stages of gastric cancer

To investigate the expression profile of *CD160* and *TNFSF14* across different stages of gastric cancer, the expression levels of these genes were compared in patients with TNM stages I+II (early-stage) and III+IV (advanced-stage) and the non-ulcer dyspepsia (NUD), and the control group. The results showed significantly higher levels of *CD160* and *TNFSF14* gene expression in the GC patients at advanced stages (TNM stages III+IV) compared to the NUD group. In contrast, patients with early-stage gastric cancer (TNM stages I+II) exhibited higher levels of *TNFSF14* expression, while their *CD160* expression was similar to that of the control group. Moreover, no statistically significant differences in gene expression levels between stages I & II and stages III & IV, for both *TNFSF14* and *CD160* (Figure 2).



Graphical Abstract

Table 2. Highly Correlated Genes Co-Express With TNSF14 Identified with ARCHS4 RNA-seq Gene-Gene Co-Expression Matrix Table displaying the names, p-values, and q-values, and overlap genes of significant terms in the Ontology (GO Biological Process 2021) library. The q-value is an adjusted p-value calculated using the Benjamini-Hochberg method for correction of multiple hypotheses testing. Only the top 10 significant relevant biological processes and their associated genes are displayed in this table. (the full table CSV file containing all significant results is provided in supplementary)

Index	Name	P.value	Adjusted p.value	Odds ratio	Overlap genes
1	negative Regulation of T cell mediated cytotoxicity (GO:0001915)	0.000006669	0.0001848	123.06	*PCPRC*
2	T-helper cell lineage commitment (GO:0002295)	0.000006669	0.0001848	123.06	*SLAMF6*
3	Regulation of lymphocyte activation (GO:0051249)	8.15E-09	5.75E-07	104.68	*CD84*
4	Regulation of T-helper 17 cell lineage commitment (GO:2000328)	0.0003664	0.00482	101.51	*CD84*
5	Positive regulation of natural killer cell mediated cytotoxicity directed against tumor cell target (GO:0002860)	0.0003664	0.00482	101.51	*LILRA2*
6	Positive regulation of natural killer cell mediated immune response to tumor cell (GO:0002857)	0.0003664	0.00482	101.51	*LILRB1*
7	T-helper 17 cell lineage commitment (GO:0072540)	0.0003664	0.00482	101.51	*CCL5, CCL4*
8	Positive regulation of natural killer cell mediated cytotoxicity (GO:0045954)	4.92E-10	7.63E-08	90.67	*ITGAX*
9	Negative regulation of NLRP3 inflammasome complex assembly (GO:1900226)	0.0005113	0.006105	81.2	*CD84*
10	Regulation of natural killer cell chemotaxis (GO:2000501)	0.0005113	0.006105	81.2	*CD84*

Differential expression of CD160 and TNFSF14 genes in relation to age and tumor size

The expression levels of *CD160* and *TNFSF14* genes were compared in patients with non-ulcerative dyspepsia (NUD), peptic ulcerative dyspepsia (PUD), and gastric cancer (GC) across different age groups. A significant difference was found in *CD160* gene expression of PUD patients over and under or equal to the median age of

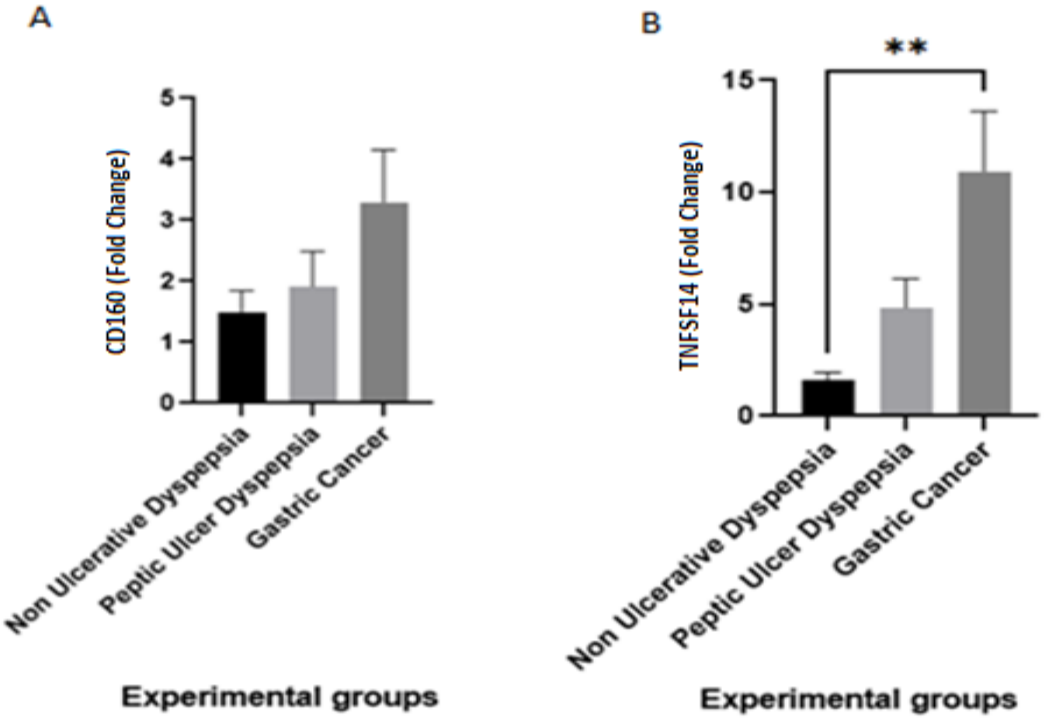


Figure 1. Expression profiles of CD160 and TNFSF14 (LIGHT). Quantitative PCR was used to measure the relative expression of CD160 in gastric cancer (GC), peptic ulcer dyspepsia (PUD), and non-ulcer dyspepsia (NUD) groups. Figure 1A shows the relative expression of CD160 in the experimental groups compared to the NUD group as a control. B. The relative expression of TNFSF14 (LIGHT) is presented among the three study groups, illustrated TNFSF14 (LIGHT) overexpression in tissue samples from GC patients compared to NUD. The graphs show the data with Mean ± SEM values, and statistical significance was considered at P. values <0.05.\

Table 3. Genes co-expressed with CD160 Identified with ARCHS4 RNA-seq Gene-Gene Co-Expression Matrix Table displaying the names, p-values, and q-values, and overlap genes of significant terms in the Ontology (GO Biological Process 2021) library. The q-value is an adjusted p-value calculated using the Benjamini-Hochberg method for correction of multiple hypotheses testing. Only the top 10 significant relevant biological processes and their associated are displayed in this table. (the full table CSV file containing all significant results is provided in supplementary)

Index	Name	P.value	Adjusted p.value	Odds ratio	Overlap genes
1	Regulation of natural killer cell mediated cytotoxicity (GO:0042269)	$1.57E^{-12}$	3.04E-10	78.57	*NCR1 NCR3 KLRK1 SLAMF6 CRTAM CD226 KLRD1 KLRC1*
2	Regulation of lymphocyte activation (GO:0051249	$8.15E^{-09}$	5.75E-07	104.68	*FCRL3 CCL5 IK2F3 LAT CRTAM*
3	Positive regulation of natural killer cell mediated cytotoxicity (GO:0045954)	$4.92E^{-10}$	7.63E-08	90.67	*NCR3 KLRK1 CRTAM CD226 KLRD1 SLAMF6*
4	Positive regulation of natural killer cell mediated immunity (GO:0002717)	$1.27E^{-09}$	1.64E-07	74.65	*NCR3 KLRK1, CRTAM KLRD1, CD226 SLAMF6*
5	Negative regulation of T cell mediated cytotoxicity (GO:0001915)	0.000006669	0.0001848	123.06	*PTPRC KLRD1 KLRC1*
6	T-helper cell lineage commitment (GO:0002295)	0.000006669	0.0001848	123.06	*SPN LY9 SLAMF6*
7	Regulation of immune response (GO:0050776)	$3.24E^{-20}$	2.52E-17	28.94	*CD96, KLRB1 SH201A, CRTAM CD3G, SLA2 PTPN22, SPN NCR1, NCR3 KLRK1, CASP8 CD40LG, KLRF1 CD226, SLAMF6 KLRD1, KLRC1 CLEC20*
8	T-cell activation (GO:0042110)	$3.23E^{-14}$	1.25E-11	33.78	*ITK, CASP8 PTPRC, TNFSF14 CD28, CRTAM RHOH, CD28 CD3G, PTPN22 LAT, NLRC3 SLA2*
9	Immune response-activating cell surface receptor signaling pathway (GO:0002429)	$1.11E^{-07}$	0.0000054	55.07	*NCR3 CSAR2 LILRA2 SLA2 LAX1*
10	Positive regulation of leukocyte mediated cytotoxicity (GO:0001912)	$1.76E^{-09}$	1.95E-07	41.53	*NCR3 KLRK1 SLAMF6 CD226 KLRD1 IL12RB1*

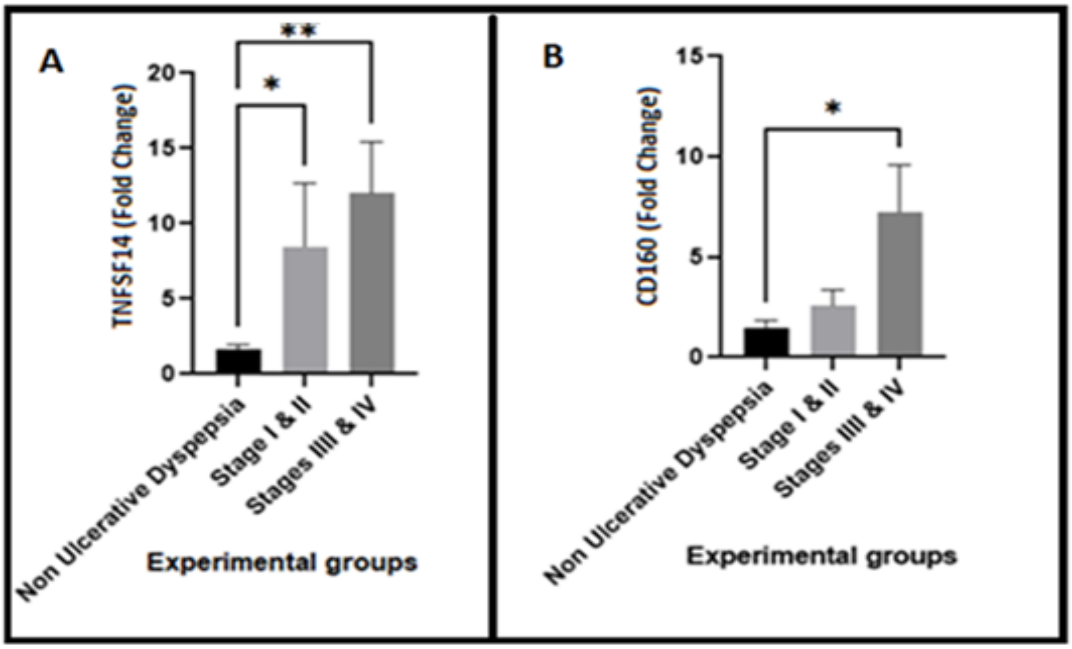


Figure 2. Expression Levels of TNFSF14/LIGHT and CD160 in Gastric Cancer Patients with Different TNM Stages Compared to the NUD Control Group. (A) Expression levels of TNFSF14/LIGHT in gastric cancer patients with TNM stages I+II (early-stage), TNM stages III+IV (advanced-stage), and NUD control group. The Kruskal-Wallis test showed a significant difference in TNFSF14/LIGHT expression levels among the three groups ($p < 0.01$). Pairwise comparisons revealed significantly higher expression of TNFSF14/LIGHT in gastric cancer patients with TNM stages I+II compared to the NUD control group ($p < 0.05$) and in gastric cancer patients with TNM stages III+IV compared to the NUD control group ($p < 0.01$). (B) Expression levels of CD160 in gastric cancer patients with TNM stages III+IV (advanced-stage) and NUD control group. The Kruskal-Wallis test showed a significant difference in CD160 expression levels between the two groups ($p < 0.05$). Data are presented as mean ± standard deviation. Pairwise comparisons were performed using Dunn's test. **p<0.01, *p<0.05. NUD, non-ulcer dyspepsia.

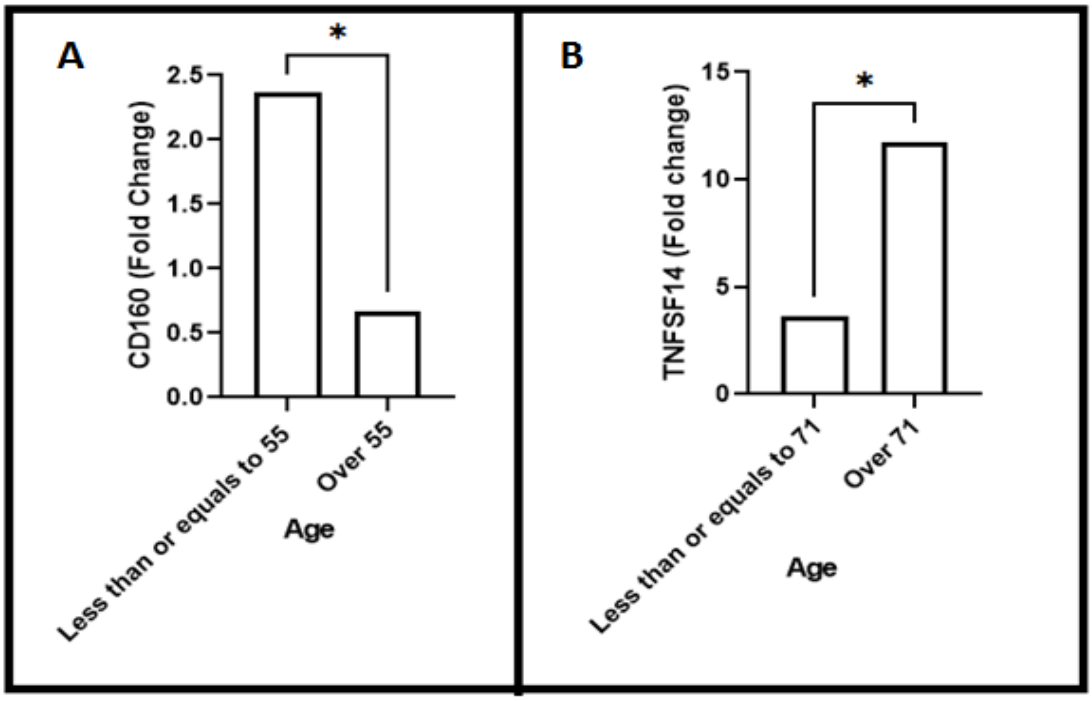


Figure 3. Comparison of CD160 and TNFSF14 Differential Gene Expression Levels Across Age among Patients with Peptic Ulcerative Dyspepsia (PUD), and Gastric Cancer (GC). A. Box plots show the median gene expression levels with significant differences with a p-value < 0.05*. Significant differences in CD160 gene expression were found between PUD patients over and under or equal to the median age of 55 years (p-value < 0.05), with higher expression in the younger PUD group. B. Significant differences in TNFSF14 gene expression were found between GC patients over and under or equal to the median age of 71 years (p-value < 0.05*), with higher expression in the older GC group. No significant differences were found in other comparisons (data not shown).

55 years (p-value < 0.05), with higher expression in the younger adults. A significant difference was also observed in *TNFSF14* gene expression of GC patients over and under or equal to the median age of 71 years (p-value < 0.05), with higher expression in the older GC subjects (Figure 3). No significant difference was detected in the expression of *TNFSF14* and *CD160* genes in GC patients with tumor size over and under or equal to the median of 5.5 cm (data are not presented).

*Enrichment Analysis Reveals Potential Biological Processes Associated with TNFSF14 and CD160 Expressio*n

Enrichment analysis was performed using Enrichr® (https://maayanlab.cloud/Enrichr/) with the GO Biological Process 2021 ontology to gain insights into the potential biological pathways associated with *TNFSF14* and *CD160*. Concerning *TNFSF14*, a significant enrichment of genes was found to be involved in various biological processes, including negative regulation of T cell-mediated cytotoxicity (GO:0001915), T-helper cell lineage commitment (GO:0002295), negative regulation of leukocyte degranulation (GO:0043301), and positive regulation of gamma-delta T cell activation (GO:0046645). Furthermore, positive regulation of natural killer cell chemotaxis (GO:2000503) and positive regulation of myelination (GO:0031643) were significantly enriched (Table 2).

In the case of *CD160*, a significant enrichment of genes was involved in various biological processes, including regulation of natural killer cell-mediated cytotoxicity (GO:0042269), regulation of lymphocyte activation (GO:0051249), positive regulation of natural killer cell-mediated cytotoxicity (GO:0045954), and positive regulation of natural killer cell-mediated immunity (GO:0002717). Additionally, negative regulation of T cell-mediated cytotoxicity (GO:0001915), T-helper cell lineage commitment (GO:0002295), and T cell activation (GO:0042110) were significantly enriched (Table 3).

These findings suggest that both *TNFSF14* and *CD160* may be involved in the regulation of immune responses, particularly those mediated by T cells, natural killer cells, and myeloid cells.

Discussion

The present study aimed to investigate the expression profile of *CD160* and *TNFSF14* genes in the tumor microenvironment of gastric cancer patients. The results showed the significant overexpression of *TNFSF14* in both advanced and early stages of gastric cancer, suggesting the possible involvement of *TNFSF14* in the development and progression of gastric cancer which is consistent with previous studies reporting *TNFSF14* implication in various cancer types [7, 25, 13]. Although the origin of these changes was not explored in the current study, other researchers have suggested the possible involvement of *TNFSF14* in cancer through multiple mechanisms such as affecting tumor-infiltrating T lymphocytes, activating the non-canonical NF-kB pathway, vascular normalization, and generation of tertiary lymphoid structure [26-29].

Regarding *CD160*, the study found no significant difference in its expression levels between control and gastric cancer groups, indicating that *CD160* may not be a major contributor to the pathogenesis of gastric cancer. Advanced-stage gastric cancer patients, however, exhibited significantly higher levels of both *CD160* and *TNFSF14* compared to the control group. This finding is consistent with the results of previous studies that found elevated mRNA levels of multiple immune checkpoints in CRC tumor tissues, suggesting immune evasion as a potential mechanism of tumor growth and progression in advanced gastric cancer [30].

Additionally, this research did not uncover any noteworthy correlation between the expression of *CD160* or *TNFSF14* and various clinical and para-clinical parameters, except for a significant connection in *TNFSF14* expression levels in gastric cancer patients across different age ranges, as well as in *CD160* expression levels in peptic-ulcerative dyspepsia patients across different age ranges.

The results of this research are in line with previous human and animal studies reporting age-related alterations in the expression of *TNFSF14* across various tissues [13, 31, 32]. Likewise, other researchers represented chronic inflammation related to aging (inflammaging) as a key player in several molecular and cellular aspects involved in gastric cancer as well as other age-related diseases [33-36]. Moreover, *TNFSF14* was significantly associated with various biological processes using Enrichr® [37, 38, 4], including negative regulation of T cell-mediated cytotoxicity, T-helper cell lineage commitment, negative regulation of leukocyte degranulation, positive regulation of gamma-delta T cell activation, and positive regulation of natural killer cell chemotaxis. These findings suggest that *TNFSF14* may be involved in modulating the immune response in gastric cancer.

On the other hand, *CD160* was significantly associated with regulation of natural killer cell-mediated cytotoxicity, regulation of lymphocyte activation, positive regulation of natural killer cell-mediated cytotoxicity, and positive regulation of natural killer cell-mediated immunity. Additionally, negative regulation of T cell-mediated cytotoxicity, T-helper cell lineage commitment, and T cell activation were significantly enriched. These findings indicate the probable role of *CD160* in regulating the function of natural killer cells and T cells in the context of gastric cancer. Overall, our gene enrichment analysis provides insights into the potential biological pathways associated with *TNFSF14* and *CD160* in gastric cancer, which should be further explored in future studies.

The observations of this research are consistent with prior investigations which introduced the HVEM pathway and its corresponding ligands as potent mediators in various cancer types [39-42]. It is essential to emphasize that in addition to the valuable insights provided by this study, further investigations are required to comprehensively elucidate the role of *CD160* and *TNFSF14* in the advancement of gastric cancer. Subsequent research can delve into exploring the mechanisms of action of these genes in gastric cancer and assessing their potential as therapeutic targets.

In conclusion, this study offers novel insights into the expression profile of *CD160* and *TNFSF14* in the tumor microenvironment of gastric tissues and suggests that *TNFSF14* could be as a GC tumor marker and potentially consider for diagnostic or therapeutic purpose. However, further studies are needed to confirm our findings and elucidate the underlying mechanisms.

Author Contribution Statement

Abolghasem Ajami, Hossein Asgarian-Omran, Reza valadan, Saeed Taghiloo and Mohsen Tehrani designed the study, Mohsen Keykhosravi, Ahmad Najafi and Seyed Mohammad Javadzadeh collected the data, and conducted the experiments. Mohsen Keykhosravi, Islam Majd and Qasem Fatehi analyzed the data and wrote the manuscript.

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Data Availability

The data generated and analyzed during the current study are available from the corresponding author upon request.

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Ethical Approval and Considerations

This research was approved by the Ethics Committee of Mazandaran University of Medical Sciences, Sari, Iran. All participants provided written informed consent prior to enrollment.

Conflict of Interest

The authors declare that they have no conflicts of interest.

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