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Association of Nucleotide-binding Oligomerization Domain Receptors with Peptic Ulcer and Gastric Cancer

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ABSTRACT

Host innate immunity can affect the clinical outcomes of *Helicobacter pylori* infection, including gastritis, gastric ulcer, gastric adenocarcinoma, and MALT lymphoma. Nucleotide binding oligomerization domain (NOD)-1 and -2 are two molecules of innate immunity which are involved in the host defense against *H. pylori*. This study aimed to evaluate the effect of the expression level of NOD1 and NOD2 on the susceptibility to gastric cancer as well as peptic ulcer in individuals with *H. pylori* infection.

The gene expression levels of these molecules were compared in three groups of non-ulcer dyspepsia (NUD) as a control group (n=52); peptic ulcer disease (PUD), (n=53); and gastric cancer (GC), (n=39).

Relative expression levels of NOD1 in patients with GC were higher than those of NUD and PUD (p<0.001 and P<0.001, respectively). Similarly in case of NOD1, PUD group showed higher level of expression than NUD group (p<0.01). However, there was no significant difference between *H. pylori* -positive and -negative patients in NUD, PUD, or GC groups. Moreover, the expression levels of NOD2 showed no significant difference among NUD, PUD, or GC groups, while among *H. pylori*-positive patients, it was higher in GC group than NUD and PUD groups (p<0.05 and p<0.01, respectively). In addition, positive correlation coefficients were attained between NOD1 and NOD2 expressions in patients with NUD (R^2 Linear=0.349, p<0.001), PUD (R^2 Linear=0.695, p<0.001), and GC (R^2 Linear=0.385, p<0.001).

Collectively, the results suggest that the chronic activation of NOD1 and NOD2 receptors might play a role in the development of gastric cancer.

Keywords: Gastric cancer; NOD1 protein; NOD2 protein; Nucleotide-binding oligomerization domain; Peptic ulcer

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INTRODUCTION

Peptic ulcer disease (PUD) and gastric cancer (GC) are two major clinical problems,[1,2] PUD can lead to a low health-related quality of life and GC is the fourth most common cancer in the world and the second leading cause of cancer-related death.[1] *Helicobacter pylori (H. pylori*) infection is now identified as a major cause of both PUD and GC.[1,2] Although most *H. pylori*-infected individuals do not develop disease,[2-4] its chronic infection has been established to be related with several disease outcomes including gastritis, peptic ulcer, dysplasia, neoplasia, mucosa associated lymphoid tissue (MALT) lymphoma and invasive gastric adenocarcinoma.[5-7]

H. pylori antigens are recognized by the host receptors, so-called pattern recognition receptors (PRRs), which leads to the production of pro-inflammatory cytokines resulting in an inflammation.[6] Nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) are cytosolic pattern recognition receptors (PRRs)[8] which contain variable N-terminal domains (PYD, CARD and BIR).[9] NOD1 and NOD2 belong to NLR family and are composed of a central NOD domain, an N-terminal domain recruiting caspase (CARD) and a ligand-recognizing C-terminal domain called leucine rich repeat (LRR).[10] NOD1 detects muropeptide of gram-negative bacteria, while NOD2 recognizes bacterial components which are produced during synthesis or degradation of peptidoglycan.[11] Data from *in vivo* studies have shown that NOD1-impaired mice are more susceptible to infections caused by *H. pylori* strains with type IV secretion system.[12] Furthermore, one of the defense mechanisms of NOD2 is regulation of antimicrobial peptides against some microorganisms.[13] NOD molecules also play an important role in regulation of the chronic inflammatory conditions.[14] They recruit transcription factors, including nuclear factor (NF)-κB and STAT1 and, in consequence, induce tumor-related inflammation.[12]

Given the role of NODs in inflammation, we aimed to assess the NOD expression levels in various *H. pylori*-assocated pathologic conditions to determine the possible role of these molecules in the pathogenesis of these diseases.

MATERIALS AND METHODS

Patients

Patients with dyspepsia who underwent esophago-gastro-duodenoscopy at Imam Hospital or Tooba Outpatient Clinic (Mazandaran University of Medical Sciences, Sari, Iran) were enrolled in the study. All tissue samples were taken from the gastric antrum between January 2012 and December 2013. The study was approved by the Ethics Committee (No: 91229-251191) of Mazandaran University of Medical Sciences. Clinical history, demographic data, and written informed consent were taken from all study subjects. None of subjects had a history of chronic inflammatory or autoimmune disorders, received non-steroidal anti-inflammatory drugs (NSAIDs) during past two weeks, or had a history of *H. pylori* eradication therapy. None of patients with GC had surgery, radiotherapy, chemotherapy, or any other form of medical interventions before sample collection. Three tissue samples were taken from each patient during endoscopy. One of the tissue samples was applied for the rapid urease test and the second one was fixed and processed for routine histopathological examination. The third tissue sample from each patient was preserved in RNAse inactivating solution (RNAlater, Qiagen, Germany) for RNA extraction. Based on the endoscopic and histopathological assessments, tissues were divided into three groups of non-ulcer dyspepsia (NUD) as a control group (n=52); PUD, (n=53), and GC, (n=39). The histological grade of the gastric tumors was determined on the basis of differentiation.[15] The presence of *H. pylori* infection was determined by the rapid urease test and histopathological examination (including Giemsa staining). Patients were considered as *H. pylori*-positive if the results were positive by at least one method and *H. pylori*-negative if the results by both methods were negative. Patients in all three groups were then divided into two subgroups of *H. pylori*-positive and -negative. Table 1 shows the demographic data of patients as well as the histopathologic grading of gastric tumors.

RNA Isolation and cDNA Synthesis

Each tissue specimen was homogenized using mortar and pestle at room temperature. Total RNA was extracted from the dissected tissues using commercial

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Table 1. Demographic characteristics and gastric tumor grade of the individuals with *H. pylori* infection categorized in three groups; NUD, PUD and GC.

Variables		NUD (n=52)[a]	PUD (n=53)[b]	GC (n=39)[c]
Age (mean±SD)		47.1±14.6	54.4±18.5	71.4±10.6
Sex	**Male**	13(25.0%)	24(45.3%)	32(82.1%)
	Female	39(75.0%)	29(54.7%)	7(17.9%)
H. pylori	**Positive**	30(57.7%)	36(67.9%)	20(51.3%)
	Negative	19(38.8%)	16(30.2%)	17(43.6%)
	ND[d]	3(5.8%)	1(1.9%)	2(5.1%)
Gastric Tumor Grade	**G1**			3(7.7%)
	G2			11(28.2%)
	G3			20(51.3%)
	G4			0(0%)
	ND[d]			5(12.8%)

[a]NUD: Non-ulcer dyspepsia [b]PUD: Peptic ulcer disease [c]GC: Gastric cancer [d]ND: Not defined

RNA extraction kit (RNeasyMinikit, Qiagen, Germany), according to the manufacturer's instructions.

The quantity and quality of extracted RNAs were assessed using nanodrop spectrophotometer (Thermo Fisher Scientific Inc., USA) and agarose gel electrophoresis, respectively. RNA (1µg) was reverse-transcribed into complementary DNA (cDNA) using the RevertAid First-Strand cDNA Synthesis Kit (Fermentas, Germany) primed with random hexamer primer according to the manufacturer's protocol.

Quantitative Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR)

The sequences of NOD1, NOD2 and Hypoxanthine-guanine phospho-ribosyl-transferase (HGPRT), as a normalizer, were obtained from the GenBank database (Table 2). Primers for amplification of HGPRT as well as NOD1 and NOD2 were designed using the Beacon designer 7 software and synthesized by TIBmol Company (Germany) (Table 2).

Quantitative real-time PCR was performed using 96 well plates (Bio-Rad Laboratories Inc., USA) in a volume of 20 µl containing Maxima SYBR Green/ROX qPCR Master Mix (2X) (Thermo Scientific, USA), 10 pico mols of each of forward and reverse primers, and appropriate amounts of cDNA. The samples were denatured at 95°C for 10 minutes, and amplified using 40 cycles of 95°C for 30 seconds (s), 54°C for 30 s, and 72°C for 30 s using iQ5 real-time thermal cycler (Bio-Rad Laboratories Inc, USA). Cycle of threshold (Ct), a cycle of reactions in which amplification curve crosses the threshold and significant increase is observed in fluorescence, was measured. A mean Ct value for each duplicate measurement was calculated. Relative gene expression was then calculated using "ΔCt method and a reference gene" in the following manner for each sample: Ratio (reference/target) $=2^{Ct\ (reference) - Ct\ (target)}$.

Statistical Analysis

Statistical analysis was performed using the SPSS statistical package (SPSS, Chicago, IL, USA). The results were evaluated by independent-samples t-test, Mann-Whitney U test and Pearson and Spearman correlation tests where appropriate. Findings were considered significant when *p*-values were<0.05. The results presented in the text and tables represent geometric mean in case of $2^{\Delta Ct}$ and mean±standard deviation (SD) in case of other variables.

RESULTS

Fifty-two patients with NUD, 53 with PUD, and 39 with GC were enrolled in this study (Table 1).

NOD1 and NOD2 Relative Expressions

In order to measure the expression levels of NOD1 and NOD2, cDNA form each tissue sample was individually amplified using the primer pairs for NOD1 and NOD2 as well as for HGPRT as a normalizer. Relative expression levels of NOD1 and NOD2 were

then calculated by ΔCt method using a reference gene as mentioned above. As Figure 1 shows, relative expression levels of NOD1 were higher in GC patients than those of NUD and PUD groups (p<0.001 and p<0.001, respectively) (Figure 1 and Table 3). Moreover, PUD patients showed higher expression levels of NOD1 compared to NUD group (p<0.01) (Figure 1 and Table 3). However, there was no significant difference between *H. pylori*-positive and -negative patients in NUD, PUD, or GC groups (Table 4).

Moreover, the expression levels of NOD2 showed no significant difference among NUD, PUD, or GC groups, while among *H. pylori*-positive patients, it was higher in GC group than NUD and PUD groups (p<0.05 and p<0.01, respectively) (Tables 3 and 4).

Table 2. The sequence and product size of the primers used for amplification of NOD1, NOD2 and HGPRT genes in NUD, PUD and GC subjects.

Gene	GenBank Accession Number	Primers (5'-3')[a]	Product Size (bp)
NOD1	NM_006092.2	**For.** CAC TGT TCT CAG ACT CAG CGT A	194
		Rev. TGT TTT TTC CCA GTT TAA GAT G	
NOD2	NM_022162.1	**For.** GCA AGG CTC TGT ATT TGC G	194
		Rev. GTT ATT CCC CAG CCT CAA TG	
HGPRT	NM_000194.2	**For.** CTA ATT ATG GAC AGG ACT GAA CG	211
		Rev. TTG ACT GGT CAT TAC AAT AGC TC	

For, Forward primer; Rev., reverse primer.

Table 3. The relative mRNA expression levels of NOD1 and NOD2 molecules in NUD, PUD and GC groups are shown as ΔCT and $2^{\Delta CT}$.

	Group	Number of samples	Relative mRNA expression		*p*-value
			ΔCT (mean±SE)	$2^{\Delta CT}$ (Geometric mean)	
NOD1	NUD	50	-9.60±0.49	0.0012	0.001[a]
	PUD	52	-8.10±0.28	0.0035	<0.001[b]
	GC	38	-6.08±0.34	0.0148	<0.001[c]
NOD2	NUD	50	-9.54±0.57	0.0013	0.512[a]
	PUD	44	-9.35±0.33	0.0015	0.073[b]
	GC	32	-8.32±0.49	0.0031	0.084[c]

NUD: Non-ulcer dyspepsia PUD: Peptic ulcer disease GC: Gastric cancer

[a]Compared between NUD and PUD groups. [b] Compared between PUD and GC groups. [c] Compared between NUD and GC groups.

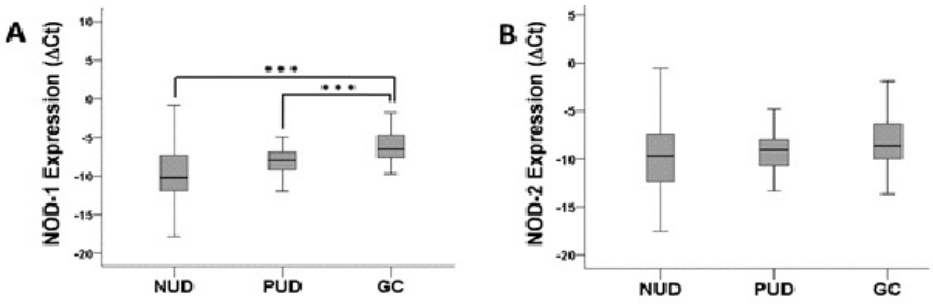

Figure 1. Comparison of mRNA expression levels of NOD1 (A) and NOD2 (B) in patients with NUD, PUD, and GC
To clarify the role of NOD1 and NOD2 in pathophysiology of dyspeptic disorders, gene expression levels of these molecules in dyspeptic patients were compared. Relative expression levels of NOD1 and NOD2 were calculated by "ΔCt method using a reference gene (HGPRT)" relative expression levels of (A) NOD1 were higher in GC patients than those of NUD and PUD groups. (B) The expression levels of NOD2 showed no significant difference among NUD, PUD, or GC groups. NUD (Non ulcer dyspepsia); PUD (Peptic ulcer dyspepsia); GC (Gastric cancer); NOD (Nucleotide-binding oligomerization domain).

Table 4. The relative mRNA expression levels of NOD1 and NOD2 in NUD, PUD and GC groups in both *H. pylori positive* and *H. pylori* negative categories.

	Group	*H. pylori*	Number of samples	Relative mRNA expression		*p* value
				ΔCT (Mean±SE)	$2^{\Delta CT}$ (Geometric mean)	
NOD1	NUD	Positive	32	-9.85±0.62	0.0011	0.001[a]
		Negative	17	-9.58±0.73	0.0013	
	PUD	Positive	34	-7.99±0.30	0.0038	0.003[b]
		Negative	16	-8.56±0.62	0.0027	
	GC	Positive	20	-6.35±0.41	0.0122	<0.001[c]
		Negative	16	-5.87±0.63	0.0171	
NOD2	NUD	Positive	32	-9.56±0.72	0.0013	0.688[a]
		Negative	16	-10.19±0.90	0.0008	
	PUD	Positive	30	-9.31±0.40	0.0016	0.009[b]
		Negative	13	-9.55±0.64	0.0013	
	GC	Positive	18	-7.44±0.57	0.0057	0.029[c]
		Negative	13	-9.55±0.81	0.0013	

NUD: Non-ulcer dyspepsia PUD: Peptic ulcer disease GC: Gastric cancer

[a]Comparison between *H. pylori*-positive NUD and PUD groups.

[b]Comparison between *H. pylori*-positive PUD and GC groups.

[c] Comparison between *H. pylori*-positive NUD and GC groups.

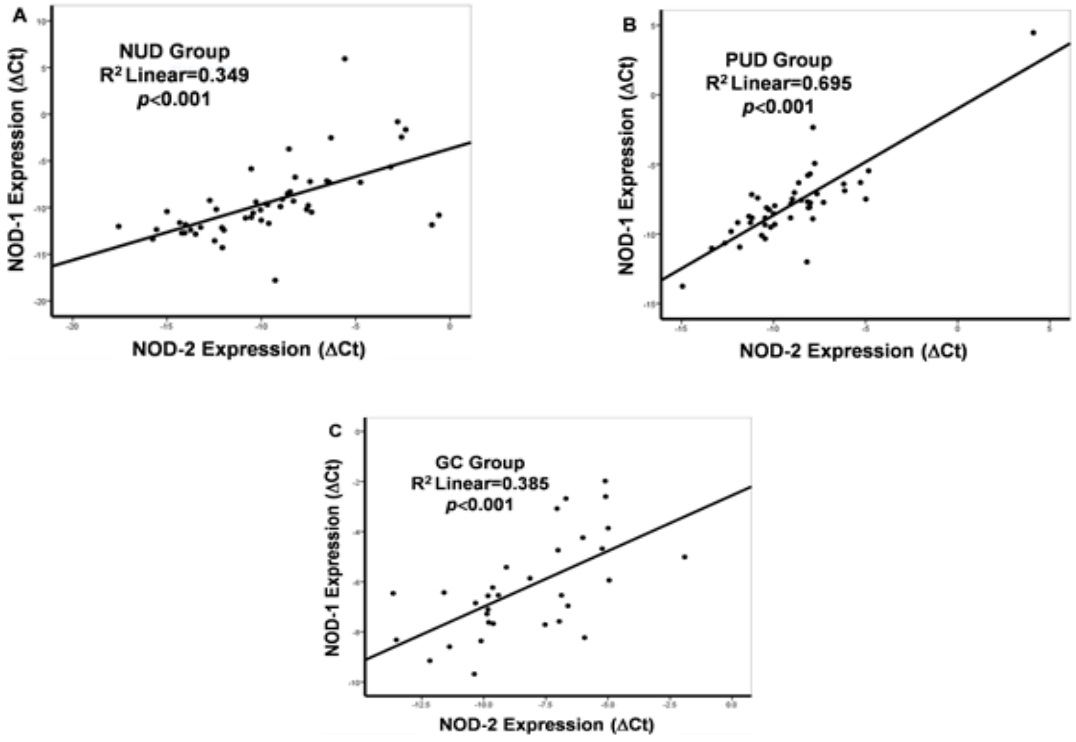

Figure 2. Linear correlations between the expression levels (ΔCT) of NOD1 and NOD2 in patients with (A) NUD, (B) PUD, and (C) GC.

A: The strength of association between the expression levels of NOD1 and NOD2 in NUD patients was analyzed using the Spearman rank order correlation test. Significant correlation coefficients were attained.

B: The strength of association between the expression levels of NOD1 and NOD2 in PUD patients was analyzed using the Spearman rank order correlation test. Significant correlation coefficients were attained.

C: The strength of association between the expression levels of NOD1 and NOD2 in GC patients was analyzed using the Spearman rank order correlation test. Significant correlation coefficients were attained.

Positive Correlations between NOD1 and NOD2 Expression

The strength of association between the expression levels of NOD1 and NOD2 was further analyzed using the Spearman rank order correlation test, and the correlation coefficient was calculated. Significant correlation coefficients were attained between NOD1 and NOD2 expression in patients with NUD (R^2 Linear=0.349, p<0.001), PUD (R^2 Linear=0.695, p<0.001), and GC (R^2 Linear=0.385, p<0.001) (Figure 2).

DISCUSSION

This study reveals that gene expression of NOD1 in GC group was significantly higher than those in PUD and NUD groups. Similarly, PUD samples expressed higher levels of NOD1 than NUD ones. These findings highlight the role of NOD1 in inflammation and cancer. In this regard, Kang et al. investigated the role of NODs in innate immune responses in a prostate epithelial cell line and showed that NOD1 had a distinctly high expression level in all tested cells. Moreover, their study proposed NOD1 expression as a part of the innate immune response by prostate epithelial cells which plays an important role in the development and progression of prostate cancer.[16] A possible mechanism for this is that NOD1 forms a signalosome complex through its CARD domain and employs RIP2 kinase, and thus increases the expression of inflammatory genes via activation of NF-κB.[11] If the inflammation becomes chronic, it might lead to malignancy.[19] On the other hand, Chen et al. showed that defects in NOD1 could increase apoptosis which can finally lead to the development of intestinal inflammation and colon cancer. Hence, they suggested a protective role for NOD1 in the pathogenesis of colon cancer.[17] In another study, Corriea et al. reported that presence of NOD1 in MCF-7 cells, a human breast adenocarcinoma cell line, induces apoptotic pathways which inhibit estrogen-dependent cell proliferation.[18] Moreover, NOD1 results in a moderate increase in caspase-9 through recognition of damaged cells; thereby participates in induction of cell death and control of cell growth.[18] Therefore, NOD1 signaling pathway serves as a protector in tumor incidence. The results of our study demonstrated the role of this molecule in gastric mucosal inflammation and cancer. In fact, it can be inclined that the activity of this molecule can lead to cancer if the tissue homeostasis is disrupted.[19] Taken together, it seems that NOD1 might play both inhibitory and progressive roles in cancers and that the expression and function of NOD1 might be different in various tissues and conditions. We further compared the expression levels of NOD1 in *H. pylori*-positive and -negative samples in each group of NUD, PUD, and GC; however, the results showed no significant difference. In this regard, one study assessed the expression levels of NOD1 protein in patients infected with *H. pylori* and it was observed that the level of NOD1 increased in the gastric mucosa of these patients.[20]

Another study evaluated the role of NOD1 in the killing of *H. pylori*. They showed the effective presence of this molecule in *H. pylori* infection.[21] In our study, we evaluated the gene expression levels of NOD1 and NOD2 in *H. pylori*-positive and -negative patients with NUD, PUD, and GC. Although NOD1 expression was higher in patients with GC than in those with NUD and PUD, it was not significantly different between *H. pylori*-positive and -negative GC patients. Thus, we can suggest that the tumor microenvironment in GC samples causes an over-expression of NOD1 regardless of *H. pylori* infection.

In the present study, the expression levels of NOD2 were not statistically significant among the three groups of NUD, PUD, and GC. Moreover, no significant difference in NOD2 gene expression was observed between *H. pylori*-positive and -negative patients. However, *H. pylori*-positive samples in GC group showed higher expression levels of NOD2 than those in NUD and PUD groups. This suggests a role for NOD2 in *H. pylori*-induced inflammation and gastric cancer, which is in agreement with the results of the other experiment conducted by Kent et al.[19]

We further analyzed the correlation between the expression levels of NOD1 and NOD2. The results showed significant correlations between NOD1 and NOD2 expression in patients with NUD, PUD, or GC. In conclusion, this study showed an over-expression of NOD1 in patients with GC compared to those with NUD or PUD regardless of *H. pylori* infection. Regarding NOD2 expression, *H. pylori*-positive samples in GC group showed higher expression levels of NOD2 than those in NUD and PUD groups.

The present study had several limitations: firstly, we were unable to determine the stage of the gastric tumor, since the study necessitated obtaining biopsies prior to surgery and on the other hand we had hardly

access to patients for the follow-up after the surgery. Secondly, the mean age of the normal group was lower than those of gastric cancer group. The reason was that in normal group we needed to examine those whose gastric tissue was normal and devoid of any signs of inflammation; however, the majority of older individuals with gastrointestinal symptom, at least, manifested the inflammation in their endoscopic presentation and did not meet the inclusion criteria. Hence, we had to include in normal group, younger individuals referring to the center, whose biopsies showed normal endoscopic manifestation.

In conclusion the results of the study suggest that the repeated activation of NOD1 and NOD2 receptors can lead to the development of GC. Investigating the functions of NOD1 and NOD2 in chronic inflammation of gastric epithelial cells would be helpful to reveal the exact role of these receptors in gastric mucosal inflammation and cancer.

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