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Original Article

Transcriptional alteration of *NF-κB*-associated long noncoding RNAs in the stomach of *Helicobacter pylori*-infected and non-infected patients

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Abstract

Introduction: *Helicobacter pylori* could colonize the gastric mucosa and cause gastritis, ulcers and cancer. Numerous virulence factors have been identified in this bacterium that play important roles in promoting gastric disorders. Although the interaction of long noncoding RNAs (lncRNAs) with transcription, processing, and translation of genes associated with different diseases are described, their interaction with the inflammatory genes and *H. pylori* infection in the gastric tissue is not well known. This study compared changes in common NF-κB-regulatory lncRNAs in the gastric tissue of *H. pylori*-infected and non-infected patients with gastritis. Moreover, a link between the virulence entity of the strains and the transcriptional changes was analyzed.

Methodology: Two groups of infected and non-infected patients with chronic gastritis were included in the study. Genotyping of the *H. pylori* strains was done by PCR and relative changes in the expression of NF-κB and regulatory lncRNAs, lincRNA-p21, MALAT1, NKILA, were measured by relative quantitative real time-PCR.

Results: Transcriptional levels of MALAT1, lincRNA-p21, and NKILA genes decreased in the infected patients compared with the non-infected patients, which was significantly linked with increased NF-κB gene expression. Our results showed that a hypervirulent strain of *H. pylori* with oipA$^{\text{"on"}}$/HP-NAP^{+}/iceA1^{+}/iceA2^{+}/vacA s_1m_1/$s_1m_2^{+}$/cagA^{+} genotype can promote a higher level of NF-κB transcription in the inflamed tissue.

Conclusions: *H. pylori* infection could promote down-regulation of lincRNA-p21, MALAT1 and NKILA in the infected gastric tissue in correlation with NF-κB upregulation. More detailed studies are needed to show a link between the virulence genes and their impact on the regulation of lncRNAs in the stomach.

Key words: *Helicobacter pylori*; lncRNA; *NF-κB*; chronic gastritis.

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Introduction

Gastritis is inflammation of the gastric mucosa that could be chronic with infiltration of lymphocytes and/or plasma cells or severe with neutrophil infiltration. One of the known causes of gastritis is infection with *Helicobacter pylori* [1]. *H. pylori* is a human-specific bacterium that colonizes the stomach of more than half of the world's population [2]. During the infection course in the stomach, it could engage the immune system and induce chronic inflammation, which leads to chronic gastritis at its early stages. This infection can also lead to gastric atrophy, intestinal metaplasia, or gastric cancer in different patients, depending on the virulence entity and host conditions. Gastric cancer is the third leading cause of cancer-related death worldwide and *H. pylori* is considered as the main risk factor in its occurrence [3,4].

Different virulence factors, such as BabA, SabA, VacA, NAP, OipA, IceA and CagA are identified in *H. pylori* that play important roles in the stable establishment of the bacterium in the gastric mucosa and promotion of the histopathological changes in this tissue. VacA (vacuolating cytotoxin A) can initially inhibits T cell activation and IL-2 induction that will suppress local immunity against *H. pylori*; however, it also exerts a proinflammatory effect through induction

of NF-κB activity leading to chronic inflammation [5]. The role of CagA (Cytotoxic associated gene A) as an important virulence factor and known bacterial oncoprotein, has been extensively studied. This protein is involved in the induction of inflammation mainly through NF-κB activation and IL-8 production [6]. The presence of CagA is also associated with the development of gastrointestinal ulcers, severe gastritis, precancerous lesions and cancer [7,8]. IceA (Induced by contact with epithelium gene a) is constructed of two gene families of *iceA1* and *iceA2*. Studies have shown that infection with *iceA*-positive strains is associated with increased mucosal levels of IL-8 [9]. This factor can also be used as a marker for strains that provoke more severe inflammation and more severe wounds [10]. OipA (outer inflammatory protein) is an extracellular protein and in addition to acting as an adhesin protein, has been shown to be involved in the induction of IL-8, and increase in the bacterial colonization and neutrophil infiltration [9]. The functional status of *oipA* is regulated by a slipped-strand mispairing based on CT dinucleotide repeats that affect its reading frame, which causes the gene to be in the "On" (functional) or "Off" (non-functional) status [9]. HP-NAP (The neutrophil-activated protein of *H. pylori*) is a highly conserved protein in *H. pylori* that mediates the gastric tissue inflammation through its ability to stimulate of endothelial cells and production of oxygen radicals, proinflammatory cytokines and chemokines by the infiltrated neutrophils [11].

Chronic inflammation caused by *H. pylori* is associated with the silencing of tumor suppressor genes through epigenetic changes [12]. Among the mediating molecules produced in the inflammatory process are cytokines, chemokines, free radicals, prostaglandins, growth factors, and the metalloproteinase matrix. Genetic changes induced by these molecules, e.g., included DNA methylation and histone changes, are associated with many inflammatory and tumorigenic diseases. It seems that chronic infection with *H. pylori* could promote the epigenetic changes and deregulation of genes in the gastric tissue through the accumulation of proinflammatory cytokines and reactive species of oxygen and nitrogen; however, this interplay is not so clear yet [13,14]. Non-coding RNAs also play regulatory roles in cell homeostasis and differentiation. Understanding the interaction between *H. pylori* virulence factors and RNAs that are involved in the regulation of inflammatory pathways in the stomach could help us design new drugs against the disease. Apart from about 2% of the proteins encoding genes, most of the human genome is made up of non-encoded RNAs, which have been shown to play a very important and regulatory role in complex organisms. One of the important subfields of these transcripts is lncRNAs. These long non-coding RNAs are a group of RNA transcripts that are more than 200 nucleotides in length and generally cannot encode a specific protein. Like miRNAs, lncRNAs play a very important role in regulating the expression of target genes. This regulatory role can take the form of transcriptional changes, post-transcriptional processes, or epigenetic settings including miRNA binding, chromatin alterations, and gene interactions. Dysregulated lncRNA expression is associated with many diseases such colon, breast, lung, and stomach cancer, and acts as a tumor suppressor or oncogene in gastric cancer [15]. *NKILA*, *lincRNA-p21*, and *MALAT1* are among the lncRNA¬s that have a negative regulatory effect on NF-κB pathway. It was shown that NKILA could prevent the activation of NF-κB by inhibiting the phosphorylation of IκB in the NF-κB/IκB complex [16], while LincRNA-p21 regulates cellular responses to the p53 signaling pathway, which has anti-inflammatory properties. It can also interfere with the mRNA of the P65 molecule, a member of the NF-κB family, preventing its translation and thus preventing the activation of NF-κB [17]. MALAT1 regulates several important molecular signaling pathways, including NF-κB, which can lead to changes in important cellular processes, such as cell proliferation, cell death, cell cycle, migration, invasion, angiogenesis, and tumorigenesis. MALAT1 inhibits the activation of the NF-κB pathway through binding to p65/p50 subunits [18]. Although dysregulation of these lncRNAs is shown in some of human diseases, their roles in promotion of chronic gastritis and stomach cancer are still not well known. Further studies are needed to show the impact of *H. pylori* infection and its virulence entity on the expression of different LncRNAs in correlation with changes of inflammatory cytokines in the gastric tissue. In this study, transcriptional changes of *NKILA*, *lincRNA-p21* and *MALAT1*in response to *H. pylori* infection in the gastric tissue of the infected and non-infected patients with chronic gastritis, and the association of *H. pylori* virulence genotype in this interplay was investigated.

Methodology

Sampling

Gastric biopsy specimens were collected from the antrum of patients referred to the endoscopy unit of Firoozgar Hospital in Tehran between January and August 2019. One biopsy specimen from each patient

was transferred into a thioglycolate medium for the bacterial culture on a specific culture medium. The other samples from the same patients were provided for RNA extraction and transferred to the laboratory in RNAase free microtube in a nitrogen tank until storage in a -80 °C freezer.

Cultivation, identification and storage

To culture and isolate *H. pylori*, the samples were homogenized in thioglycolate culture medium and 100 µL of related suspension was inoculated to Brucella culture medium containing sheep blood (10%), antibiotics and antifungals. Brucella culture plates were placed in a jar at microaerophilic conditions and kept in the incubator at 37 °C for 3-5 days. Rapid urease, oxidase and catalase tests were done for the biochemical characterization of *H. pylori* isolates. All colonies grown in the subculture medium were transferred to the BHI broth storage medium containing glycerol and fetal calf serum at -80 ° C until DNA extraction provided that all biochemical tests were positive and the phenotypic identification and confirmation with polymerase chain reaction were done.

DNA extraction

DNA extraction of isolated bacteria was performed by alkaline lysis and boiling method according to its instructions as described by Saberi *et al.* [19]. In this method, grown colonies (10-20 mg) were poured into a microtubule containing sterile phosphate buffered saline (PBS) and stored at -20 °C. The samples were melted at room temperature, centrifuged at 6000 rpm for 5 minutes, then the pellets were mixed with 50-150 µL of NaOH (50 mM) and boiled for 20 minutes at 100 °C. The suspension was mixed with 20-50 µL of Tris-HCl (1 M, pH: 7.5) after a fast spin and centrifuged at 3000 rpm for 5 minutes. The supernatant containing pure DNA was transferred to a new tube. The concentration of extracted DNA samples was measured in OD_{260} nm (Nano Drop™ One Microvolume UV-Vis Spectrophotometers*)* and extracted DNA was stored at -20 °C until genotyping.

Selection of primers for housekeeping and virulence factor genes

All the *H. pylori* isolates were confirmed using *glmM*-specific primers before genotyping experiments. The presence of virulence factors, including *oipA*, *HP-NAP*, *iceA1/A2*, *cagA* and *vacA s1m1/s1m2,* in the studied strains was performed using specific primers as shown in Table 1. PCR was performed in a reaction containing 12.5 µL of master mix 2X Ampliqon Company, Denmark, 0.5 µL of each primer, 0.5-1 µL of DNA and 10.5-11 µL of H_2O, in a total volume of 25 microliters. Amplification was done as follows: 1 cycle of initial denaturation at 94 °C for 5 minutes, 40 cycles of denaturation at 94 °C for 30 seconds, annealing at specified temperatures as was shown in Table 1 for 30

Table 1. The primer sequences of studied genes.

Gene	Primer Sequence	PCR Product Size (bp)	Annealing (°C)	Reference
glmM	F: GGATAAGCTTTTAGGGGTGTTAGGGG R: GCTTACTTTCTAACACTAACGCGC	249	61	20
HP-NAP	F: RTGCGATCGTGTTGTTTATG R: GATCGTCCGCATAAGTTAC	344	51	21
oipA	F: CAAGCGCTTAACAGATAGGC R: AAGGCGTTTTCTGCTGAAGC	428	55	22
iceA1	F: TATTTCTGGAACTTGCGCAACCTGAT R: GGCCTACAACCGCATGGATAT	719	59	23
iceA2	F: CGGCTGTAGGCACTAAAGCTA R: TCAATCCTATGTGAAACAATGATCGTT	662	57	23
VacA-s	F: CTGCTTGAATGCGCCAAAC R: ATGGAAATACAACAAACACAC	259-286	57	24
VacA-m	F: CAATCTGTCCAATCAAGCGAG R: GCGTCAAAATAATTCCAAGG	567-642	57	25
cagA	F: AATACACCAACGCCTCCAAG R: TTGTTGCCGCTTTTGCTCTC	397	55	26
NF-κB	F:TGCCAACAGATGGCCCATAC R: TGTTCTTTTCACTAGAGGCACCA	123	60	27
NKILA	F: AACCAAACCTACCCACAACG R: ACCACTAAGTCAATCCCAGGTG	108	60	28
MALAT1	F: AAGATGAGGGTGTTTACG R: AAGCCTTCTGCCTTAGTT	136	52	29
LincRNA-P21	F:GGGTGGCTCACTCTTCTGGC R: TGGCCTTGCCCGGGCTTGTC	80	64	17
Act-β	F: ATGTGGCCGAGGACTTTGATT R: AGTGGGGTGGCTTTTAGGATG	107	60	30

seconds, extension at 72 °C for 30 seconds, and 1 cycle of final extension at 72 °C for 10 minutes.

Sequencing of the oipA gene promoter region

To check whether *oipA* gene is at on or off status in its promoter region, PCR products of *oipA* gene were sent to Pishgam Company for sequencing by Sanger method. All the sequences were analyzed using CLC sequence viewer.

RNA extraction from biopsy specimens and cDNA synthesis

For RNA extraction, RNase-free consumables were used. RNA extraction of the gastric biopsy samples was done using TRIzol reagent (BlueZol, Iran) following the manufacturer's guidelines with some modifications. In summary, the cut tissue samples were mixed with 600 µL of solution BlueZol, Iran, and 150 µL of chloroform, and intense vortexing was performed for 15 seconds. The mixture was centrifuged at 12,000 rpm for 15 minutes at 4 °C. After transferring the colorless aqueous phase to another sterile tube, 400 µL of isopropranol was added and the mixture was frozen at -70 ° C for 30 minutes. The resulting RNA pallet was mixed with 1 mL of 80% ethanol and vortexed. After centrifugation for 5 minutes at 7500 rpm, each pellet was resuspended in 20-30 µL DEPC treated water, and stored at −70 °C after heat treatment in 60 °C for 5 minutes [31]. The extracted RNA concentration was measured by quantitative method (Nano Drop™ One Microvolume UV-Vis). DNase was used to remove any DNA contamination. All RNA extraction steps were performed to prevent possible contamination under the sterile hood and impregnated with DEPC solution. cDNA synthesis was done by cDNA synthesis kit according to the procedure provided by the company.

Primer efficiency and measurement of lncRNA transcription

Appropriate and specific primers targeting *NF-κB, MALAT1, lincRNA-p21, NKILA* and *ACTB* genes were selected using authentic articles and Gene Runner software. The absence of a dimer or loop, the existence of the appropriate melting temperature, and GC percentage of the primers were considered for their selection. The efficiency of primers was evaluated on diluted control synthesized cDNA samples using real-time PCR.

Relative quantitative real-time PCR

The expression level of *NF-κB, MALAT1, lincRNA-p21 and NKILA* genes in the *H. pylori*-infected compared with *H. pylori* non-infected patients with chronic gastritis was measured using SYBR green quantitative real-time PCR. *ACTB* gene was used as an endogenous gene as described before (28). The reaction mixture consisted of 0.5 µL of each primer, 12.5 µL RealQ plus Master Mix Green (Ampliqon, Denmark), 2 µL of cDNA, and distilled water up to the final volume of 25 µL. The thermal cycling conditions in Rotor Gene 6000 Corbett Sequence Detection System have comprised an initial denaturation step at 95 °C for 10 minutes, 40 cycles of denaturation at 95 °C for 30 seconds, annealing at 60 °C for 60 seconds, elongation at 72 °C for 60 seconds, and a final extension step at 72 °C for 3 minutes. All the tests were done in duplicate. To show the accuracy of the amplification for each gene, melting curve analysis and gel electrophoresis were done. Relative gene expressions for all samples were determined from the obtained Ct (Crossing Threshold) values and using the $2^{-\Delta\Delta ct}$ method ($2^{\wedge} - (\Delta ct \text{ of HP infected group} - \text{average } \Delta ct \text{ of HP non-infected group})$). Up- and down-regulation were defined based on RQ values ≥ 2 and ≤ 0.5, respectively.

Statistical analysis

Statistical analyses were performed using SPSS-26 software and GraphPad Prism software version 5. T-test and Mann-Whitney test were used to compare the expression relationship between the two groups. *p* value less than 0.05 was considered significant. Spearson coefficient was calculated and tested to analyze the relationship between participants' age and gene expression.

Table 2. Genotype diversity of *Helicobacter pylori* strains in patients with chronic gastritis.

Genotypes	Allelic variants	Number
A	$oipA^{+(on)}$/HP-NAP+/$iceA1^{+}$/$A2^{+}$/$cagA^{+}$/vacA s1m1/$s1m2^{+}$	3
B	$oipA^{+(on)}$/HP-NAP+/$iceA1^{-}$/$A2^{-}$/$cagA^{+}$/vacA s1m1/$s1m2^{+}$	2
C	$oipA^{+(on)}$/HP-NAP+/$iceA1^{+}$/$A2^{-}$/$cagA^{+}$/vacA s1m1/$s1m2^{+}$	5
D	$oipA^{+(off)}$/HP-NAP+/$iceA1^{+}$/$A2^{-}$/$cagA^{+}$/vacA s1m1/$s1m2^{-}$	2
E	$oipA^{+(off)}$/HP-NAP+/$iceA1^{+}$/$A2^{-}$/$cagA^{-}$/vacA s1m1/$s1m2^{-}$	2
F	$oipA^{+(off)}$/HP-NAP+/$iceA1^{+}$/$A2^{-}$/$cagA^{+}$/vacA s1m1/$s1m2^{+}$	1
G	$oipA^{+(off)}$/HP-NAP+/$iceA1^{+}$/$A2^{+}$/$cagA^{+}$/vacA s1m1/$s1m2^{-}$	1
Total		**16**

Results

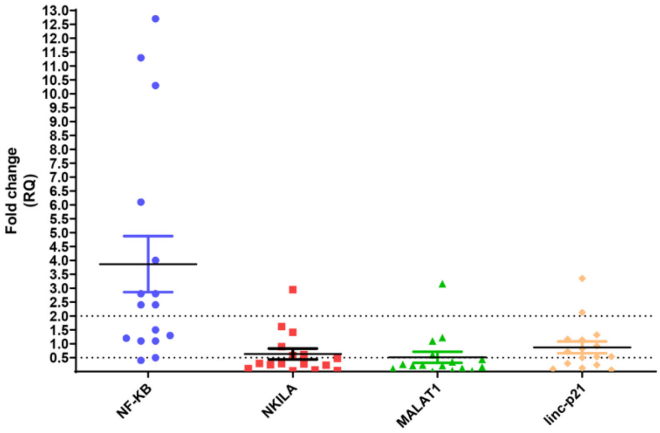
Demographic results

In this study, out of 168 patients who were subjected to endoscopy, patients were selected based on their histological findings and their history of infection with *H. pylori*. Accordingly, 32 patients with moderate chronic gastritis, including 16 patients infected (7 Males and 9 Females) and 16 non-infected patients (4 Males and 12 Females), were selected. The mean age of patients was 42.87 ± 10.77 in the infected group (HP-positive) and 47.93 ± 17.3 in the non-infected group (HP-negative).

Characteristics of H. pylori genotypes

Results of PCR using glmM primer confirmed the entity of all 16 isolates of the HP-positive samples (Table 2). Results of genotyping for the bacterial isolates based on *oipA*, *HP-NAP*, *iceA1/A2*, *cagA* and *vacA* s_1m_1/s_1m_2 showed the following genotypes: A: $oipA^{+(on)}/HP\text{-}NAP^{+}/iceA_1^{+}/A_2^{+}/cagA^{+}/vacA\ s_1m_1/s_1m_2^{+}$ (3 strains); B: $oipA^{+(on)}/HP\text{-}NAP^{+}/iceA_1^{-}/A_2^{-}/cagA^{+}/vacA\ s_1m_1/s_1m_2^{+}$ (2 strains); C: $oipA^{+(on)}/HP\text{-}NAP^{+}/iceA_1^{+}/A_2^{-}/cagA^{+}/vacA\ s_1m_1/s_1m_2^{+}$ (5 strains), D: $oipA^{+(off)}/HP\text{-}NAP^{+}/iceA_1^{+}/A_2^{-}/cagA^{+}/vacA\ s_1m_1/s_1m_2^{-}$ (2 strains); E: $oipA^{+(off)}/HP\text{-}NAP^{+}/iceA_1^{+}/A_2^{-}/cagA^{-}/vacA\ s_1m_1/s_1m_2^{-}$ (2 strains); F: $oipA^{+(off)}/HP\text{-}NAP^{+}/iceA_1^{+}/A_2^{-}/cagA^{+}/vacA\ s_1m_1/s_1m_2^{+}$ (1 strain) and G: $oipA^{+(off)}/HP\text{-}NAP^{+}/iceA_1^{+}/A_2^{+}/cagA^{+}/vacA\ s_1m_1/s_1m_2^{-}$ (1 strain). Among the virulence factors, *oipA*, *HP-NAP, iceA1, ice A2,* and *cagA* were detected in 62.5%, 100%, 62.5%, 25%, and 81.25 % of the strains, while $iceA_1^{+}/A_2^{+}$ and *iceA* negative strains were detected in 25% and 12.5% of them, respectively. Although *vacA* was detected in all of the isolates, diversity in its allelic variants was shown among the studied patients

Figure 1. Scatter plot of *NF-κB, MALAT1, lincRNA-p21* and *NKILA* expression in the gastric biopsy samples of *H. pylori*-infected patients with chronic gastritis.



	NF-κB Relative expression Mean ± SD	NKILA Relative expression Mean ± SD	MALAT1 Relative expression Mean ± SD	Linc-p21 Relative expression Mean ± SD
HP-positive patients	2.4 ± 4.03	0.39 ± 0.77	0.28 ± 0.79	0.62 ± 0.85

The transcriptional levels in the non-infected group were considered as reference. Decreased expression of lncRNAs was associated with increased expression level of NF-κB. Up- and down-regulation were defined based on RQ values ≥ 2 and ≤ 0.5, respectively (Dashed lines). *oipA*$^{+}$ and *oipA*$^{-}$ indicates *oipA* "on" and "off" status according to the sequencing data. *vacA* $s_1m_1/s_1m_2^{+}$ represent *H. pylori* strains with s_1m_1 or s_1m_2 alleles. *vacA* $s_1m_1/s_1m_2^{-}$ are related to *vacA* negative variants. Three distinct allelic forms of *iceA* (A_1^{+}; A_2^{+}, $A_1A_2^{+}$) are shown among the strains with different genotypic patterns. The genotypic pattern of *oipA on status* /$iceA_1^{+}$/$iceA_2^{+}$/$CagA^{+}$/*vacA* $s_1m_1/s_1m_2^{+}$ showed the highest *NF-κB* expression among other genotypes. Relative gene expressions for all samples were determined from the obtained Ct (Crossing Threshold) values and using the $2^{-\Delta\Delta ct}$ method ($2^{\wedge}$ - (Δct of HP infected group – average Δct of HP non-infected group)). Up- and down-regulation were defined based on RQ values ≥2 and ≤ 0.5, respectively (Dashed lines).

Table 3. Sequence of oipA gene promoter region in the studied samples.

Sample	Sequence of the signal peptide coding region of the *oipA* gene	Status
46	ATGAAAAAAGCTCTCTTACTCTCTCTCTCTCTCTCGTTCTGGCTCCACGCTGAA…	On
67	ATGAAAAAAGCTCTCTTACTCTCTCTCTCTCTCTCGTTCTGGCTCCACGCTGAA	On
69	ATGAAAAAAGCTCTCTTACTAACTCTCTCTCTCTCGTTCTGGCTCCACGCTGAA	On
75	ATGAAAAAAGCCCTCTTACTAACTCTCTCTCTCTCGTTCTGGCTCCACGCTGAA	On
86	ATGAAAAAAGCTCTCTTACTAACTCTCTCTCTCTCGTTTTGGCTCCACGCTGAA	On
88	ATGAAAAAAGCCCTCTTACTCTCTCTCTCTCTCGTTTTGGCTCCA…TGA*[a]	Off
90	ATGAAAAAAGCTCTCTTACTAACTCTCTCTCTCTCTCGTTGTGGC…TAG*	Off
125	ATGAAAAAAGCTCTCTTACTCTCTCTCTCTCTCTCTCGTTCTGGG…TAG*	Off
129	ATGAAAAAAGCTCTCTTACTAACTCTCTCTCTCTCGTTCTGGCTCCACGCTGAA	On
132	ATGAAAAAAGCCCTCTTACTAACTCTCTCTCTCTCTCTCTCTCTCGTT…TGA*	Off
133	ATGAAAAAAGCTCTCTTACTCTCTCTCTCTCTCTCTCGTTCTGGC…TGA*	Off
145	ATGAAAAAAGCTCTCTTACTAACTCTCTCTCTCGTTCTGGCTCCACGCTGA*	Off
146	ATGAAAAAAGCTCTCTTACTAACTCTCTCTCTCTCGTTCTGGCTCCACGCTGAA	On
152	ATGAAAAAAGCTCTCTTACTAACTCTCTCTCTCTCGTTCTGGCTCCACGCTGAA	On
155	ATGAAAAAAGCTCTCTTACTAACTCTCTCTCTCTCGCTTTGGCTCCATGCTGAA	On
160	ATGAAAACCTTGATAAATCTTCTCTTACTAACTCTCTCTCTCTCGCTCTGGATCCAC	On

[a.] (∗) indicates stop codon in *oipA* nucleotide sequence, representing the "Off" functional status.

(s_1/m_1: 25%; s_1/m_2: 50%; s_2/m_2: 25%; s_2/m_1: 0%). In this study, strains with *vacA* s_1m_1 or *vacA* s_1m_2 genotype were defined as a general *vacA*s_1m_1/s_1m_2 genotype.

Sequencing of the oipA gene promoter region

Analysis of the number of repeats and their effects on the reading pattern of *oipA*, showed 10 strains at "on "status and 6 strains at "off" functional status. Details about the nucleotide sequences of the *H. pylori* strains are shown in Table 3.

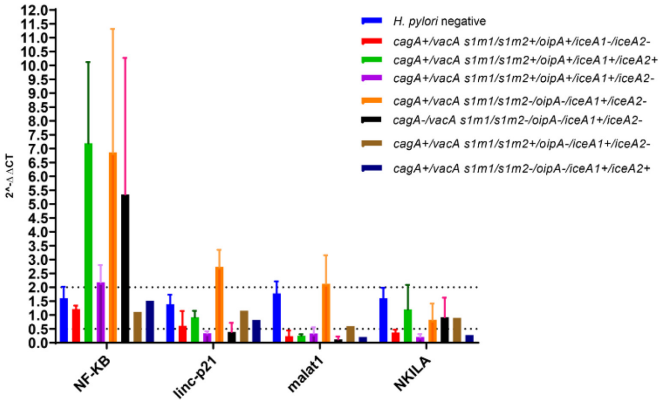
Expression and correlation of NF-κB, linc-p21, MALAT1 and NKILA genes in two groups of patients

The relative expression of *NF-κB, NKILA, LincRNA-p21* and *MALAT1* was evaluated using real-time-PCR technique. Alteration in the expression levels of these genes is shown in Figure 1. Accordingly, while the mean relative expression of *NF-κB* showed a significant increase in the infected group, a decrease in the mean relative expression of *MALAT1*, *NKILA,* and *Linc-p21* was shown in these patients. Our results showed a correlation between transcriptions of the studied genes, where a significant relationship between the expression of *Linc-p21* and *MALAT1* genes and between the *NF-κB* and *NKILA* was measured (*p* values of 0.005 and 0.045, respectively).

Relationship between NF-κB expression and H. pylori virulence genotypes

To determine a possible relationship between transcriptional levels of *NF-κB* and the regulatory genes with *H. pylori* genotypes, statistical analysis using the Mann-Whitney method was done. As it was shown in Table 4, strains that carried all the virulence factors (*oipA-"on" status /iceA*$_1$*/A*$_2^+$*/HP-NAP*$^+$*/cagA*$^+$*/vacAs*$_1$*m*$_1$*/s*$_1$*m*$_2^+$) induced higher levels of *NF-κB* expression in comparison to the strains with defective genotypes (*p* value: 0.05). There was no significant relationship between genotypes and lncRNA expression levels. As was shown in Figure 2, the genotypic pattern of *oipA "on"*

Figure 2. Diversity in the transcription of NF-κB associated lncRNAs in the gastric biopsies of H. pylori-infected patients and their link with virulence genotypes.



status/*iceA*$_1$+/*A*$_2^+$/*cagA*$^+$/*vacA* s_1m_1/s_1m_2+ showed the highest *NF-κB* expression among other genotypes. Moreover, genotype *oipA "on"* status/*iceA*$_1^+$/*A2*$^-$ */cagA*$^+$/*vacA* $s_1m_1/s_1m_2^+$ presented the lowest expression of regulatory lncRNAs among other genotypes.

Discussion

In this study, we compared differences in the expression of lncRNAs, *NKILA, lincRNA-p21*, and *MALAT1*, in two groups of *H. pylori*-infected and non-infected patients with chronic gastritis. According to our results, the relative decrease in the expression levels of *MALAT1, lincRNA-p21,* and *NKILA*, as negative regulators in the NF-κB inflammatory pathway, was detected in the infected patients, while a significant increase in NF-κB transcription was measured. This reduction was statistically significant for *MALAT1* and *NKILA*.

Few studies have been performed on the expression of lncRNAs in the gastric tissue in association to *H. pylori* infection. Esfandi *et al.* examined the expression of several lncRNAs including MALAT1 in gastric tumor tissues compared to adjacent normal tissues and did not report a significant relationship in terms of

Table 4. Relationship between virulence factors and expression of studied genes.

Genotype	NF-κB, *p* value	Linc-p21, *p* value	MALAT1, *p* value	NKILA, *p* value
***oipA*$^{(On)}$** (N[a.] = 10) ***oipA*$^{(Off)}$** (N = 6)	0.8	0.1	0.2	0.3
***iceA1*+** (N = 14) ***iceA1*-** (N = 2)	0.3	0.6	0.8	1
***iceA2*+** (N = 4) ***iceA2*-** (N = 12)	0.1	0.3	0.9	0.6
***oipA*$^{(on)}$/*iceA1*$^+$/*iceA2*$^+$/*cagA*$^+$/*vacA* s_1m_1/m_2^+** (N = 3) **Other genotypes** (N = 13)	**0.05**	0.3	0.8	0.5

[a.] N, number of the *H. pylori* isolates with defined allelic variants for each virulence factor.

MALAT1 expression between the two groups [32]. Another study was conducted by Chaleshi *et al.* on 41 gastric tumor tissues in comparison with adjacent normal tissue and compared the expression of *MALAT1* in these tissues. The findings of this study showed an increase in *MALAT1* expression in gastric tumor tissue compared with the normal tissue. Moreover, they showed that the expression of this gene was decreased in patients infected with *H. pylori* compared to non-infected patients, which was not statistically significant [33]. Afrough *et al.* examined the expression of several LncRNAs associated with the NF-κB pathway, including *NKILA* in gastric cancer tissues compared to their adjacent normal tissues. They did not find a significant relationship in *NKILA* expression between these groups, but a significant decrease was shown in the expression of *NKILA* in the gastric tissue of *H. pylori-infected* patients compared with non-infected patients (*p value*: 0.01) [34]. According to our knowledge, no study has been performed on the expression of *LincRNA-p21* in gastric tissue. However, results of a study on epithelial cells of intestinal tumor compared with the non-cancerous ones showed a significant decrease in lincRNA-p21 expression in the tumor cells [35].

Gastric epithelial cells can respond to *H. pylori* infection through several pathways. One of the most important and well-known pathways is NF-κB activation, which can result in the production of large amounts of IL-8 from the gastric epithelial cells. Downstream target genes of the NF-κB pathway are involved in many physiological and pathological processes. Studies show that this inflammatory pathway can be activated by CagA, VacA, lipopolysaccharides, peptidoglycans, ureases, and OMPs. The results of our study showed that the expression of NF-κB gene in *H. pylori-infected* patients was nearly 2.4 times higher than the non-infected patients, which could indicate the role of *H. pylori* in activating the NF-κB inflammatory pathway. Other studies show activation of the NF-κB inflammatory pathway as a result of increased inflammation and invasion and infiltration of leukocytes into the infected area in the infected patients with *H. pylori*. The virulence entity of the strain infecting the gastric tissue could determine the fate of this interplay. *H. pylori* strains with *oipA*$^{(on)}$/*iceA1*$^{+}$/*iceA2*$^{+}$/*cagA*$^{+}$/*vacA* s1m1/m2^{+} genotype showed significantly higher levels of *NF-κB* expression compared with other genotypes in our study. In support of our finding, the study by Ying *et al.* showed that incubation of the MKN-45 cell line with *cagA*+ *H. pylori* strains could significantly increase the rate of invasion. This effect was significantly reduced by an NF-κB inhibitor called NS-398 or PDTC[36]. In another study by Shibata *et al.*, alteration in the gene expression profile of infected AGS cell line with *cag*PAI+ *H. pylori* and its association with two important *cag*PAI-activated signaling pathways, NF-κB and ERK, were examined. The results showed increased levels of expression in more than 80% of the studied genes (566 of 641 genes), including *IL-8*, *RelA* and *Rac1*, which was significantly higher compared with the *cagE* mutant strain. *NF-κB* specific inhibitors (APDC) could decrease the expression of 367 genes, including *IL-8*, significantly [37].

In our study, the decrease that was observed in the transcription of regulatory lncRNAs was correlated with the increase in NF-κB transcription in the infected patients. Although the negative regulatory effect of lncRNAs on NF-κB expression was shown in previous studies [16,18,38,39], data about their link with infections is scant. Balloy *et al.* compared the expression profiles of lncRNAs in *Pseudomonas aeruginosa*-infected epithelial cells in patients with cystic fibrosis and the infected cells in non-cystic fibrosis patients. They showed 108 lncRNA specific for cystic fibrosis in response to *Pseudomonas aeruginosa* that could play a potential role in incompatibility and immune response in cystic fibrosis patients [40]. The results of the study by Yang *et al.* also showed different expression of lncRNAs stimulated by *Escherichia coli* infection causing meningitis in hBMEC cells infected with this bacterium [41]. Yi *et al.* studied different expression of lncRNAs in CD4^{+} T cells in latent and active tuberculosis infection and showed that this lncRNA is linked to the host immune response [42]. The expression of some lncRNAs in patients with *H. pylori* infection has also been studied. In a study by Polakovicova *et al.*, lncRNA XLOC004562, XLOC005912 and XLOC-000620 had the highest level of increase and lncRNAXLOC-004122 and XLOC-014388 had the highest level of decrease in the expression in infected GES-1 cell line with *H. pylori* after 24 hours. The reduction of two later lncRNAs was also confirmed in *H. pylori-positive* biopsy specimens [43].

Although there are studies that examined possible relationship between lncRNAs and *H. pylori* infection, the difference in the virulence entity of *H. pylori* strains and their impact on the expression of lncRNAs was not studied so far. Results of our study showed that the expression of lncRNAs regulating the NF-κB pathway in patients infected with oipA^{+} "on" status was reduced compared to patients infected with oipA^{+} "off" status;

however, this reduction was not statistically significant. This interplay could be demonstrated by the production of knockout strains in the absence and presence of other virulence factors. The presence of *iceA1/A2* showed no significant change in the expression of the lncRNA genes. Consistent with this finding, the study by Maeda *et al.* in Japan did not show a significant relationship between NF-κB activation and different *iceA1/A2* alleles, while the presence of *cagE* gene significantly increased NF-κB activation [44]. Doing studies on a higher number of samples is needed for any further conclusion on this subject. Our results showed a higher level of NF-κB expression in the strains with *oipA*$^{\text{"on"}}$/*HP-NAP*$^{+}$/*iceA1*$^{+}$/*iceA2*$^{+}$/*vacA* $s_1m_1/s_1m_2^{+}$/*cagA*$^{+}$ genotype compared to other genotypes. This result indicated the importance of virulence genotype in the activation of the NF-KB signaling pathway. *H. pylori* infection is one of the most common causes of gastrointestinal diseases. According to the findings in this study, *H. pylori* infection could promote downregulation of *lincRNA-p21, MALAT1,* and *NKILA* in the infected gastric tissue which is correlated with the upregulation of NF-κB. Although more detailed studies are needed to show link between virulence genes and the studied lncRNAs, our results showed that hypervirulent strains with *oipA*$^{\text{"on"}}$/*HP-NAP*$^{+}$/*iceA1*$^{+}$/*iceA2*$^{+}$/*vacA* $s_1m_1/s_1m_2^{+}$/*cagA*$^{+}$ genotype can promote a higher level of NF-κB transcription in the inflamed tissue. Studies in a larger population are needed to demonstrate this link, which could be mediated by their interaction with the regulating lncRNAs in the NF-κB pathway.

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Ethical approval

This study was approved by the ethical committee of the Research Center at Tehran University of Medical Science (accepted Number, IR.TUMS.SPH.REC.98-3-99-46160) and an informed consent form was obtained from all the patients.

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