



Evaluation of *NKILA* lncRNA and *NFKB* Genes Expression in an Iranian Gastric Cancer Patient Compared with Healthy Tissue and the Relationship between Clinicopathological Features

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Abstract

Background & Objective: Long non-coding RNAs play critical roles in the initiation and development of gastric cancer (GC). The aim of this study was to assess the expression of *NKILA* and *NFKB* genes and the relationship between their expressions with clinical characteristics in gastric cancer. Also, relative expression of lncRNAs *NKILA*, compared *NFKB* in GC tissues was evaluated.

Materials & Methods: This cross-sectional study was performed on 50 gastric formalin-fixed paraffin-embedded tumor tissue samples and 50 normal tissues. The RNA level of lncRNAs *NKILA* and *NFKB* genes was assessed using the quantitative real-time polymerase chain reaction. B2M was used as an internal control. The $2^{-\Delta\Delta Cq}$ method was used to determine the expression fold changes.

Results: A significant association was observed between the levels of *NKILA*, in gastric tumor tissues compared with normal tissues (Mean = 2.087, $p= 0.0484$). The relative expression of the *NFKB* gene revealed no statistically significant difference between the gastric tumor tissues compared with normal tissues ($P=0.3728$). In addition, clinicopathologic data compared with *NKILA* and *NFKB* mRNA expression levels in gastric cancer tissues showed no significant association. Here, we found no significant association between the level of *NKILA* expression compared with *NFKB* mRNA level in gastric cancer tissues ($R=0.03$; $P=0.2355$).

Conclusion: Our results showed that *NKILA* had a significant association with GC. Our findings also revealed that *NKILA* expression was not correlated with *NFKB* mRNA level in GC tissues.

Keywords: Gastric Cancer, *NKILA*, *NFKB*, Clinicopathological Feature, LncRNA

Introduction

Gastric cancer (GC) is the leading cause of cancer-associated death in men worldwide and has been reported as a common digestive system malignant tumor in the world (1). GC had a poor diagnosis, prognosis, and limited efficacy of therapies.

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Surgery is the main treatment that is mostly used for most patients in the advanced stage (2). However, even after surgical treatment, chemotherapy, radiotherapy, and molecular-targeted treatment, 5-year survival remains poor and most patients die from local recurrence or distant metastasis (3). Finding novel early detection methods of the tumor and identification of its specific molecular characteristics,



as well as discovering more knowledge about the pathogenesis of GC can help to manage public health and the treatment of GC.

Non-coding RNAs (ncRNAs) are commonly RNAs that do not encode a protein, but this does not mean that such RNAs do not have function nor contain information. Although most genetic information is commonly assumed to be transacted by proteins, recent evidence suggests that most mammalian genomes and other complex organisms are actually transcribed into ncRNAs (4). Indeed, the previous studies evaluating the lncRNA mechanisms showed that lncRNAs play important roles in regulating gene translation, transcription, post-transcription, and epigenetic modification (5-7). A number of studies have investigated the pathogenetic pathways of the Mammalian *NF-κB* family that have five various dimeric complexes, including RELA (also named p65), RELB, c-Rel, NF-κB1 p50, and NF-κB2 p52 (8, 9). According to a previous functional study, the expression of NFKB1 protein was upregulated but there are no significant changes between normal control and tumor tissues from mRNA expression, it is suggested that this discrepancy may be due to translational or post-translational regulation of the expression of NF-κB protein (10). *Helicobacter pylori* infection is well-established as one of the main risk factors for GC. Recent evidence indicates that *H. pylori* infection may be able to regulate NF-κB signaling (11). *NKILA* as a lncRNA interacts with the NF-κB signaling pathway to induce inflammation of cytokines such as TNF-α and IL-1 β in breast cancer cells (12). It has also been shown that *NKILA* plays an important role as a tumor suppressor in various human cancers by inhibiting the NF-κB signaling pathway (4). Previous studies have also reported that *NKILA* is negatively associated with metastasis and patient prognosis of breast cancer and Tongue squamous cell carcinoma (10, 12). Therefore, more research is needed to identify an NF-κB Interacting *NKILA* lncRNA at gastric cancer tissue. Here, the RNA level of *NF-κB* and *NKILA* in gastric tumor tissues will be investigated and

their association between *NKILA* expression and *NF-κB* and clinic-pathological features were evaluated.

Materials & Methods

Patients

In this case-control study, 50 gastric formalin fixed paraffin embedded (FFPE) tumor tissue samples and 50 normal FFPE tissues were obtained from Aramesh Lab, Tehran, Iran. Histopathological tissue specimens were confirmed following evaluation by a pathologist. The participant had an Iranian ethnicity and detailed clinicopathological parameters provided the following data on each tumor's patients including age, sex, tumor grade, tumor stage, tumor size, *H.pylori* infection, were recorded. Non-Iranian patients and the ones undergoing chemotherapy or radiotherapy were excluded from the study. The tumor stage was determined using American Joint Committee on Cancer Staging Manual (7th edition) (13). This study was approved by the Ethics Committee of the Islamic Azad University of Tehran Medical Sciences - Faculty of Pharmacy and Pharmaceutical Sciences, Tehran, Iran (IR.IAU.PS.REC.1399.194).

Selection of Genes

Previously identified lncRNAs molecular epidemiologic studies were carefully reviewed. Based on certain criteria *lncRNANKILA* and its target *NF-κB* were selected based on previously demonstrated association with cancer.

RNA extraction and cDNA synthesis

Total RNA was extracted from the tumor samples of the patients using the RNeasy DSP FFPE RNA extraction kit (Qiagen Co, Germany). The RNA concentration was quantified by a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies) and its quality was determined by the A260/A280 and A260/A230 ratios. The concentrations of the samples were normalized and the 1 μ g of total RNAs were reverse-transcribed to cDNA using the RevertAid RT kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

Quantitative real-time PCR analysis

qPCR was performed using a PCR cycler (Rotor-Gene Q MDx; Qiagen GmbH). cDNA fragments were used as templates to amplify the *lncRNANKILA* and *NF- κ B* genes using SYBR® Premix Ex Taq™ (Takara Bio, Inc.), according to the manufacturer's protocol. The experimental protocol was performed as follows: i) Thermocycling conditions consisted of an initial activation step for 30 sec at 94°C, 35 cycles

at 94°C for 5 sec and 60°C for 35 sec; and ii) melting curve analysis. Primer sequences were designed for all the genes with GeneRunner Software and then the Primer-BLAST (NCBI) was used to check their specificity. The sequence of target primers was listed in Table 1. B2M gene is used as a normalizer endogenous gene. The 2- $\Delta\Delta$ Cq method was used to determine the expression fold changes (patient vs. normal)

Table 1. Primer sequences used for Real-time PCR

No	Gene Name		Sequence(5' to 3')	Primer size	GC%	$^{\circ}\text{Tm C}$
1	NKILA	Forward	TGATGATTCCAGCACAGACAG	21	53.62	58.02
		Revers	CACACACGAAGCCTCCTATG	20	55.00	58.35
2	NFkB	Forward	GTGGTGCCTCACTGCTAACT	20	55	59.96
		Revers	GGATGCACCCAGCTTCTGT	20	53.5	58.18
3	B2M	Forward	TGCTGTCTCCATGTTGATGTATTCT	25	40	56.98
		Revers	TCTCTGCTCCCCACCTCTAAGT	22	54	57.93

Statistical analysis

Statistical analysis was performed using SPSS 21 (IBM Corp., USA). Graphs were plotted using GraphPad Prism (v.5.04; GraphPad Software, Inc.), and the significance was determined using paired t-test in which $P < 0.05$ was considered as the significance level. The association between *lncRNA* and *NF- κ B* gene expression was assessed via the Spearman correlation test.

Results

General statistical information

In this study, 50 patients with the diagnosis

of GC were studied. 41 patients (82 %) were male, 10 patients (20 %) were smokers and the mean age of the patients was 60.18 ± 13.65 years. Alcohol habit was negative in all patients. Among the patients, 28 (56%) of the study population were positive infection by *H. pylori*. 50 healthy individuals with a mean age of 36.72 ± 14.86 years were studied. No treatment has been used for GC patients recruited in the current study. More details of demographic characteristics have been shown in Table 2.

**Table 2.** Demographic variables of the study population

Variable	Patients		Controls	
	Frequency	Percent	Frequency	Percent
Gender				
Male	41	82%	39	78%
Female	9	18%	11	22%
Smoking				
No	40	80%	38	76%
Yes	10	20%	12	24%
Stage				
I	9	18%		
II	2	4%		
III	21	42%		
IV	18	36		
Grade				
I	7	14%		
II	18	36%		
III	25	50%		
Tumor size				
>5	21	42%		
<5	29	58%		
H. pylori Infection				
positive	28	56%		
negative	22	44%		

Expression of lncRNANKILA and NF- κ B

In order to investigate the role of lncRNAs *NKILA* and *NF- κ B* in GC, the expression level of these genes was evaluated in GC tissues. The results showed that no significant difference

was observed in the expression level of *NF- κ B* compared to normal tissues (Mean = 1.102, $p=0.3728$). (Chart1A). The expression of *NKILA* was significantly down-regulated in GC tissues compared with normal tissues (Mean = 2.087, $p=0.0484$) (Chart1B).

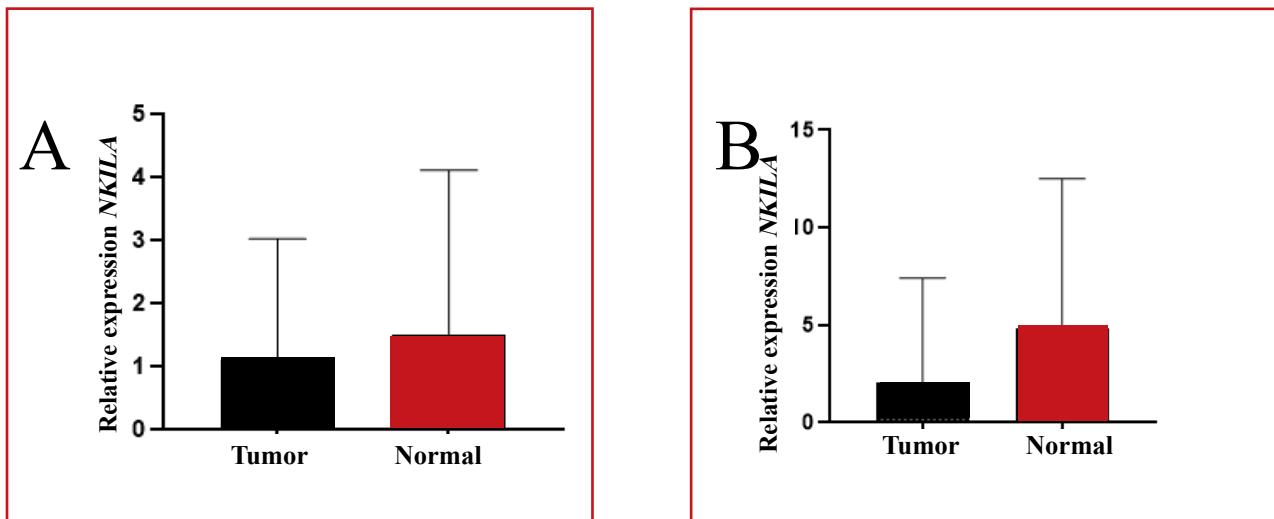


Chart 1. (A) comparison of the gene-relative expressions between the tumor tissue and the normal tissues. (A) *NF- κ B* expression, (B) *NKILA* expression. * $P<0.05$

Associations between the expression of lncRNANKILA and NF- κ B and clinical characteristics

The relative expression of *NF- κ B* demonstrated no statistically significant difference between grade I vs. II ($P=0.1187$), I vs. III ($p=0.0516$) and II vs. III ($p=0.8882$), respectively (Chart 2A). No significant associations were identified between the transcript level of *NF- κ B* and stage I&II vs III ($p=0.2419$), stage I&II vs IV ($p=0.1809$) and stage III vs IV ($p=0.8305$), respectively (Chart 2B). However, no significant associations were determined between the transcript level of *NF- κ B* and clinicopathological variable including size <5 &>5 cm groups and *H. pylori* infection positive & negative among GC patients ($p=0.5451$ and $p=0.6202$, respectively) (Chart2C-D).

In addition, the statistical analysis between *NKILA* RNA expression and clinicopathological features of groups revealed no significant association between the grad I vs. II ($P=0.7835$), I vs. III ($p=0.8777$) and II vs. III ($p=0.5319$), respectively (Chart2E). The results showed that no significant associations were identified between the transcription level of *NKILA* and stage I&II vs III ($p=0.4814$), stage I&II vs IV ($p=0.2889$) and stage III vs IV ($p=0.5898$), respectively (Chart2F). However, no significant associations were determined between the transcript level of *NKILA* and clinicopathological variable including size <5 &>5 cm groups and *H. pylori* infection positive & negative among GC patients ($p=0.1356$ and $p=0.1578$, respectively) (Chart 2G-H).

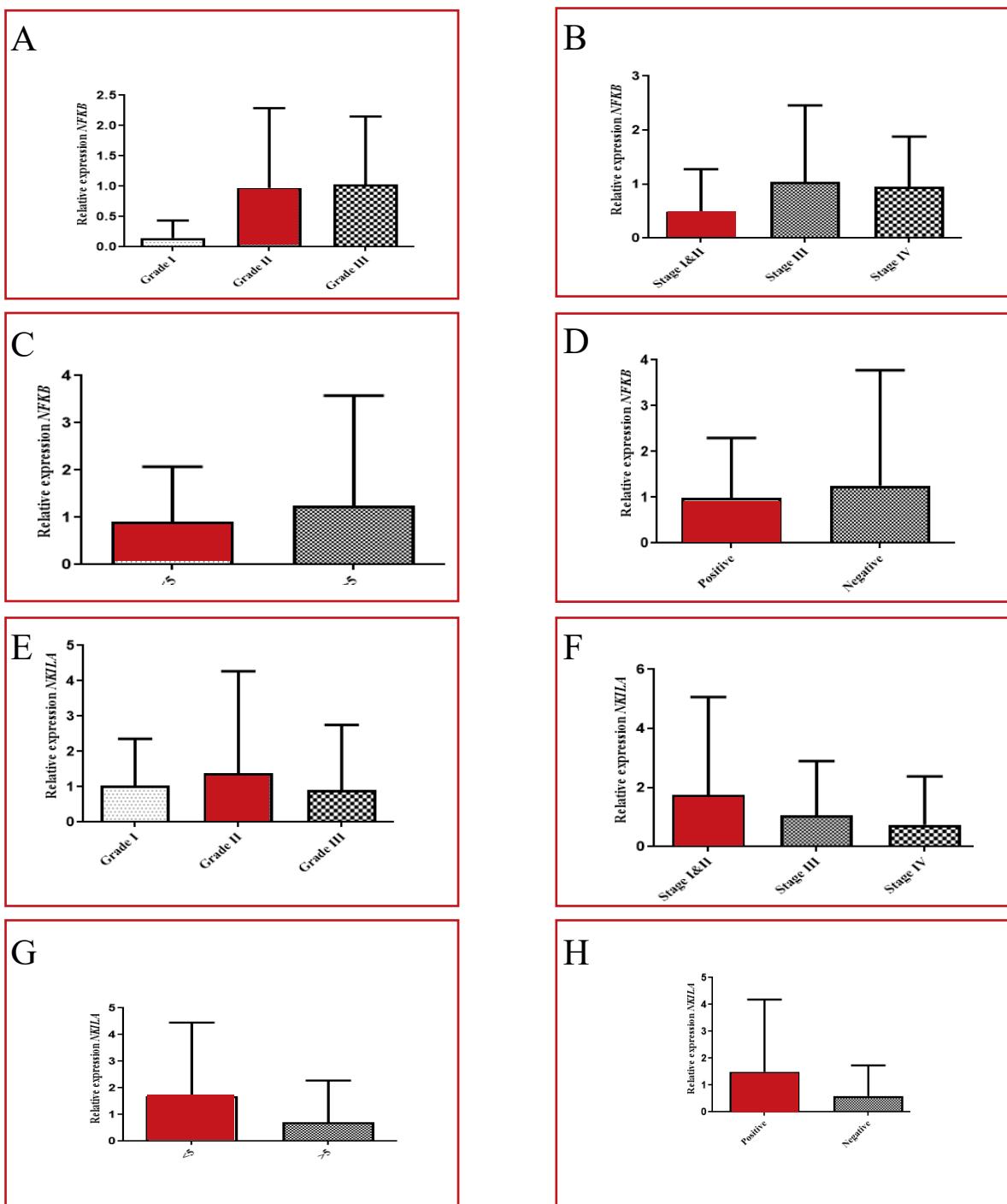


Chart 2. Relative RNA expression between the *NKILA* and *NF- κ B* genes with clinicopathological feature. (A) Relative expression of *NKILA* between the different grades of gastric cancer tissues, (B) Relative expression of *NKILA* between the stage of the tumor tissue, (C) Relative expression of *NKILA* between the two-tumor size group ≤ 5 , > 5 cm, (D) Relative expression of *NKILA* between the negative and *H. pylori*-positive patients, (E) Relative expression of *NF- κ B* between the different grade of gastric cancer tissues, (F) Relative expression of *NF- κ B* between the stage of the tumor tissue, (G) Relative expression of *NF- κ B* between the two-tumor size group ≤ 5 , > 5 cm, (H) Relative expression of *NF- κ B* between the negative and *H. pylori*-positive patients.

Relative expression of lncRNAs *NKILA* and *NF- κ B* in individual samples

In order to determine whether there is an association between the expression of the lncRNAs *NKILA* and

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NF- κ B genes, the relative expression of these genes was compared in each set of samples. No significant association was observed between the levels of *NKILA* and comparing *NF- κ B* in GC tissues ($R=0.03$; $P=0.2355$) (Chart 3).

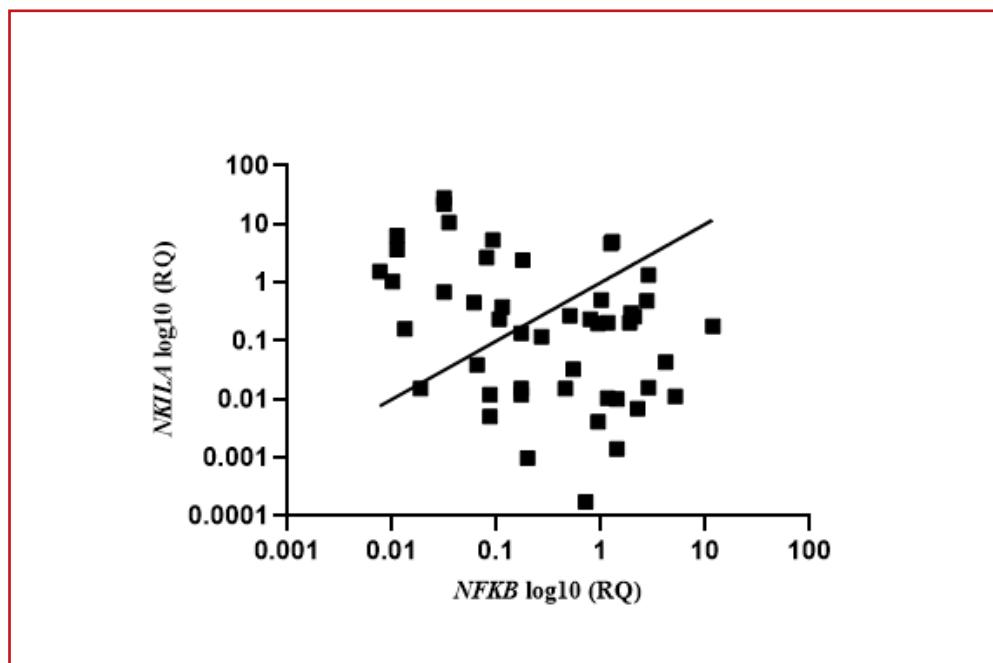


Chart 3. Linear regression analysis was performed to evaluate the correlation of expression levels between the lncRNA *NKILA* and *NF- κ B* genes in tumor tissues. The extent of RNA expression is presented as log₁₀ transformed values. The R- and P-values are presented for each analysis. *NKILA*, NF-KappaB Interacting LncRNA; *NF- κ B*, nuclear factor kappa B; RQ, relative quantification.

Discussion

One of the most frequent malignancies, gastric cancer (GC) has the highest death rate in the world. Non-coding RNAs, such as miRNAs and lncRNAs, have been shown to play important roles in GC carcinogenesis, although the mechanism is yet unclear. Numerous studies have suggested the role of lncRNAs in tumorigenesis, metastasis, invasion and angiogenesis of cancer (14-16). In addition, some lncRNAs such as *MALAT1* and *LINC00668* have been identified as diagnostic biomarkers for GC (17). One of these lncRNAs is *NKILA*, which plays a functional role in various types of cancers such as breast cancer (12), lung cancer(18), esophageal squamous-cell carcinoma (19) and colorectal cancer (4). In some research it was reported that *NF- κ B* interacting lncRNA (*NKILA*) was remarkably reduced in many breast cancers, which is associated with cancer metastasis, invasion and poor patient prognosis (20).

The results showed a significant decrease in the expression of *NKILA* in GC patients comparing with the control group. In addition, the expression level of *NKILA* and *NF- κ B* genes had no significant association with grades, stages, tumor size and *H.pylori* infection. Therefore, it can be suggested that GC is the result of interaction with environmental factors and aberrant expression of several genes (21). Afrough et al. (21) revealed that there was no significant association in the expression level of *NKILA* gene between GC and non-cancerous tissues, however, the expression level of *NKILA* gene was higher in patients with a positive family history of cancer and was lower in *H. pylori*-infected tissues.

The expression levels of different genes have been shown to upregulate following the silencing of *NKILA* gene expression and enhance with increasing *NKILA* expression, such as inhibition



of *TWIST1* expression in tongue squamous cell carcinoma by *NKILA* (10). In another report, Ke et al. (22) indicated that both transcription and protein levels of *NF-κB* target genes such as *CCND1*, *TWIST1*, *MMP9* and *XIAP* were altered by *NKILA* gene silencing. Huang et al. (23) demonstrated that *NKILA*, *NF-κB*-interacting lncRNA, increases tumor immune evasion by sensitizing T cells to activation-induced cell death. A previous functional study has shown that *NKILA* can inhibit metastasis by blocking IKK phosphorylation sites and thus repressing *NF-κB* (10). *NKILA* has also been reported to act as an inhibitor of *NF-κB* signaling by inhibiting the phosphorylation of IκB and p65 (24). Jiang et al. (7) showed that *NKILA* downregulated in CRC cell lines and tissues, which is associated with clinical progression of CR. However, the mechanism of action of *NKILA* in GC is still unknown. *NF-κB* has been identified as a key factor in cancer and the regulation of a wide range of biological, immune, and inflammatory responses (25). *NF-κB* is target transcription factors that regulate the expression of genes involved in many processes that play a key role in the development and progression of cancer, such as proliferation, migration, and apoptosis (9). In recent years, many studies have been conducted on the mechanisms of *NF-κB* signaling and a tight connection between cancer development and *NF-κB* activity in tumor cells. The results of previous studies indicate that *NF-κB* can be an interesting therapeutic target for cancer treatment (7, 26). In colorectal cancer, *NF-κB* activation leads to invasion and metastasis of cancer cells (24, 27). Thus, lncRNAs can interact directly with the functional domains of signaling proteins, and act as a class of *NF-κB* modulators to suppress cancer metastasis. Therefore, we examined *NKILA* lncRNA association with *NF-κB* mRNA in GC tissue, however, no significant association was found between them in GC tissues, which may be due in part to limitations in the number of cases studied. Further large-scale studies still need to be done.

In conclusion, our findings show that there were no significant interactions between lncRNA *NKILA* and the *NF-κB* pathway in regulation of GC. *NKILA* may be a potential diagnostic biomarker in GC. In addition, *NKILA* may serve as a novel prognostic marker and therapeutic target in GC. However, the detailed mechanisms of *NKILA*-induced suppression of GC progression were not investigated in the present study and further confirmation of the current results requires more evidence from prospective multi-center studies.

Statement of Ethics

The patients were of Iranian descent and provided written informed consent for the present study prior to the sampling procedure. The procedure for this study was approved by the Ethics Committee of the Islamic Azad University of Tehran Medical Sciences - Faculty of Pharmacy and Pharmaceutical Sciences, Tehran, Iran (IR.IAU.PS.REC.1399.194), and followed the ethical guidelines of The Declaration of Helsinki (1975).

Acknowledgment

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Conflict of Interest

The authors declare no conflict of interest.

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