Clinicopathological Features' Correlation and Genes' Expression of *NEAT1, lincRNA-ROR* and *Oct4* in Iranian Patients with Gastric Cancer

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Abstract

Purpose

Long non-coding RNAs (LncRNAs) play a critical role in the initiation and development of Gastric Cancer(GC). The aim of this study was to consider the expression of *NEAT1*, *lincRNA-ROR* and *Oct4* and finally evaluate the correlation between their expression and clinical characteristics in Iranian patients with GC.

Methods

This cross-sectional study was performed on 41 gastric tumor tissue samples with matched normal adjacent tumor tissues. The RNA level of lncRNA *NEAT1* and *lincRNA ROR* and *Oct4* genes were assessed using the quantitative Real-time polymerase chain reaction. *B2M* was used as an internal control. Also, the relative expression of lncRNA *NEAT1* and *lincRNA ROR* compared *Oct4* in GC tissues was evaluated. The $2^{-\Delta\Delta Cq}$ method was used to determine the expression fold changes.

Results

A significant association was observed between the levels of *lincRNA ROR* and *Oct4* genes in gastric tumor tissues comparing normal adjacent tissues (Mean = 1.558, p= 0.014), (Mean = 3.337, p< 0.001) and (Mean= 4.385, p<0.001), respectively. In addition, clinicopathological data comparing with lncRNAs and *Oct4* mRNA expression levels in gastric cancer tissues showed no significant association. Here, we found that significant association between the levels of *lincRNA ROR* expression comparing *oct4* mRNA level in gastric cancer tissues (R=0.417; P=0.024).

Conclusion

Our results Showed that *lincRNA ROR* and *Oct4* have a significant association in gastric cancer. Also, our results indicate the first suggestion that *lincRNA ROR* expression correlated with *oct4* mRNA level in gastric cancer tissues.

Introduction

Gastric cancer (GC), a heterogeneous disease with the complicated mechanisms and geographical differences in the prevalence [1, 2], is the second common malignancy which is the most important leading cause of mortality among cancers worldwide [3]. In spite of reducing the trend of GC in most parts of the world, the impact of it on public health and burden of the disease is still remained [3, 4].

Helicobacter pylori (H. pylori) is a known environmental risk factors of GC that has various signaling pathways which reported to be contributed to the progress and development of GC. The combined effect of Helicobacter pylori infection and various lncRNAs on the increased risk of GC development has been studied [5, 6]. Several researches have looked into the relationship between lncRNAs and H. pylori pathogenicity in GC and have suggested that the correlational findings could be used for early intervention and treatment [7].

In order to better recognize the pathogenesis of GC and detect more actual biomarkers predicting the prognosis of patients, further investigations are required on all aspects of the disease. In recent years, it has been suggested that cancer stem cells (CSCs), known as tumor-initiating cells, play efficient role in tumorigenesis in a variety of human malignancies, including gastric cancer [8, 9]. According to a previous functional study, some genes with more attentions are involving in stem cell development such as octamer-binding transcription factor 4 (*Oct4*) as a major regulator in the progression of tumorigenesis and malignancy [10–14].

In embryonic stem cells, Oct4 has been identified as a genetic factor that regulates the transcription, modification of chromatin, regulation of long non-coding RNAs (lncRNAs) and microRNAs [15, 16]. *Oct4* is a key pluripotency programming agent which regulates the expression of lincRNA, the knockdown of *lncRNA ROR*, that inhibited the proliferation and invasion of gastric cancer stem cells [17, 18].

Previous research findings by Jen et al. suggested evidence of transcriptional regulator of *MALAT1* expression in lung cancer by the stemness transcription factor *OCT4*, that targeted enhancer regions of *MALAT1* and activated its expression, therefore leads to proliferation, migration and invasion of cancer cells at in-vitro experiments [17, 19]. Also, Jen et al. revealed lung cancer cells that have high expression of *NEAT1* and the *Oct4*-silenced cells re-formed with *NEAT1* promoted cell proliferation, migration and invasion [17]. However, our understanding about the role of *NEAT1* in the occurrence and development of GC are not fully clear. A number of studies have been found that knock-down of *MALAT1* could decreased cell, migration, proliferation and *MALAT1* downregulation indicate a reduce expression of genes such as *β-catenin*, *EMT*, *EZH2*, *Lin28* and *OCT4* [18].

It has been suggested that levels of *NEAT1* are involved in the regulation of cell differentiation, proliferation and invasion of gastric cancer [20]. However, the regulation of transcription of lncRNAs by *Oct4* in many tumorigenesis cases is still unknown. Up to recent years, many studies on lncRNAs have been focused on the underlying results and mechanisms of lncRNAs and their potential as prognostic and diagnostic markers [5, 21–26]. However, little is known about the transcriptional level and their association with other transcription factors such as Oct4 in gastric cancer tissues.

The aim of this study was to evaluate the expression of *NEAT1*, *lincRNA-ROR* and Oct4 and their relationship with clinical characteristics in Iranian patients with GC. in this regard, relative expression of lncRNA *NEAT1, lincRNA ROR* compared with *Oct4* in individual samples evaluated too.

Materials And Methods

Patients

In this designed case-control study, 41 tissue samples of GC with matched normal tissues adjacent to the tumor were prepared from Iranian patients who underwent surgical resection at Imam Reza Hospital, Tehran, Iran, between January 2016 and April 2018. The provided tissue samples transferred to the laboratory in liquid nitrogen immediately following removal through surgery and stored at 80˚C.

Histopathological diagnosis of tissue specimens were confirmed by a pathologist. Detailed clinicopathological parameters including age and sex of enrolled patients, tumor grade, stage and size, history of h pylori infection were recorded according to the unique questionnaire. The tumor stage was determined using American Joint Committee on Cancer Staging Manual (7th edition) [27].

genes' selection

The literature review of effective genes in the progress of patients with GC, resulted in the gene expression of lncRNA *NEAT1, lincRNA ROR and Oct4*.in this regards the mentioned genes selected for evaluating their correlation in Iranian GC population.

RNA extraction and cDNA synthesis

Total RNA was extracted from the tumor samples of the patients using the Total RNA extraction mini kit (Favorgen, Cat No. FABRK001, Iran). The RNA concentration was quantified by a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies) and its quality was Measured by the A260/A280 and A260/A230 ratio. The concentrations of the samples were normalized and the 1 µg of total RNAs were reverse transcribed to cDNA using the RevertAid RT kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The samples of cDNA stored at -70 ˚C for further evaluations.

Quantitative real-time PCR (qRT-PCR) analysis

qRT-PCR was performed using a PCR cycler (Rotor-Gene Q MDx; Qiagen GmbH). cDNA fragments were used as templates to amplify the *lncRNAs* and *Oct4* 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. Table 1 shows the primer sequences of considered genes. *B2M* gene 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

Primers name		Sequence (5'–>3')	GC%	Tm	Reference
LincRNA ROR	Forward	CCAGGACAATGAAACCAC	53.19	60	[48]
	Reverse	AGGAGCCCAAAGTAACAG	53.69		
NEAT1	Forward	ATCGGCAGGTTGGGACTTAG	55.00	60	Designed
	Reverse	TCCTCACACGTCCATCTCC	57.89		
Oct4	Forward	TATTCAGCCAAACGACCATCT	42.86	60	[49]
	Reverse	ACGAGGGTTTCTGCTTTGC	52.63		
β2M	Forward	TGCTGTCTCCATGTTTGATGTATCT	40	60	[48]
	Reverse	CTCTGCTCCCCACCTCTAAGT	57.1		

Statistical analysis

Statistical analysis was performed using spss software version 21 (IBM Corp., USA) and Data was plotted 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 significant. The association between *lncRNAs* and *Oct4* genes expression was assessed via spearman correlation test.

Ethical approving

This study was approved by the Ethics Committee of the Research Center for Cancer Screening and Epidemiology, AJA University of Medical Sciences, Tehran, Iran (IR.AJAUMS.REC.1398.128). All of the patients provided assigned inform consent prior the surgery about their desire of enrolling in this study.

Results

General statistical information

In this study, 41 patients with the diagnosis of gastric cancer were enrolled. Out of them 34 patients (82.9%) were male (male to female ratio: 4.8), 7 (17.1%) were smokers, none of the patients were alcoholic drink and the mean age of the patients was 60.32 ± 14.185 years old.

Among the patients, 23 (56.1%) cases were positive for infection by *H. pylori*. Four (9.8%) patients diagnosed as positive Lymphatic Invasion of the cancer and 34 patients (82.9%) was negative.

The tumor grade of the 3, 16 and 22 patients, was determined as I, II and III respectively and also 4 (9.8%) of them were presented with tumors stage I, 22 (53.7%) with tumors stage III and 15 (36.6%) with stage IV. Out of considered patients, tumor

size of 16 (39.0%) cases was ≤ 5 cm and 20 (48.8%) patients had tumor size of >5 cm. Patients in the present study did not receive any preoperative treatment, and those who were undergoing chemotherapy or radiotherapy were eliminated. The more details of demographic characteristics are in Table 2.

Table 2
characteristics of enrolled patients

Characteristics		Sex			Mean age at DX (years)	Risk factors			Tumor size	
		M	F	M/F ratio		Smoking	h. pylori infection	Lymphatic invasion	≤ 5 cm	>5 cm
Total patients		34 (82.9%)	7(17.1%)	4.8	60.32 ± 14.185	7 (17.1%)	23 (60.5%)	4 (10.5%)	16 (44.4%)	20 (55.6%)
Tumor stage	I	2 (50%)	2 (50%)	1	53.25 ±18.78	-	-	-	-	1 (100%)
	III	18 (81.8%)	4 (18.2%)	4.5	59.77±14.86	2 (9.1%)	15 (68.2%)	2 (9.1%)	12 (54.5%)	10 (45.5%)
	IV	14 (93.3%)	1 (6.7%)	14	63±12.06	5 (33.3%)	8 (53.3%)	2 (13.3%)	4 (30.8%)	9 (69.2%)
Tumor grade	I	1 (33.3%)	2 (66.7%)	0.5	44.333±7.23	-	-	-	-	-
	II	14 (87.5%)	2 (12.5%)	7	60.12±14.62	3 (18.8%)	8 (50%)	-	10 (62.5%)	6 (37.5%)
	III	19 (86.4%)	3 (13.6%)	6.3	62.63±13.54	4 (18.2%)	15 (68.2%)	4 (18.2%)	6 (30%)	14 (70%)

M, male; F, Female; M/F, Male to Female

Expression of lncRNA NEAT1, lincRNA ROR and Oct4 **in the tissue samples**

To explore the role of lncRNA *NEAT1, lincRNA ROR and Oct4* in GC, expression levels quantified in GC tissues. The differences in the expression levels of *NEAT1* in the tumor sample between adjacent normal tissues were not statistically significant (p= 0.117) (Fig. 1A). However, gene expression analysis showed significant increased difference between *lincRNA ROR* samples compared to adjacent normal tissues (Mean = 3.337, p< 0.001) (Figure 1B). Also, there was significant upregulation difference of Oct4 level between the samples of GC patients compared with adjacent normal tissues observed (Mean= 4.385, p<0.001) (Fig. 1C).

Lack of associations between the expression of lncRNAs **and clinical characteristics**

In order to further evaluate the role of lncRNA *NEAT1, lincRNA ROR and Oct4* in gastric cancer, the associations between the RNA levels of the gene and several clinicopathological features including tumor stage, grad, size and H. pylori infection, were also investigated.

there were no significant association between the transcript level of *NEAT1* and tumor stages I&III and IV, tumor grades I&II and III, H. pylori infection and tumor size ≤5 & >5 cm (P= 0.911, p= 0.303, p=0.626 and p= 0.076, respectively) (Fig. 2A-D). No significant associations also were determined between the transcript level of *lincRNA ROR* and clinicopathological variable including stages I&III and IV, grades I&II and III, H. pylori infection and tumor size ≤5 & >5 cm (P= 0.756, p= 0.971, p=0.344 and p= 0.715, respectively) (Fig. 2E-H). In addition, the statistical analysis between Oct4 mRNA expression and clinicopathological features groups revealed no significant association (P= 0.363, p= 0.253, p=0.219 and p= 0.198, respectively) (Fig. 2I-L).

Relative expression of lncRNA NEAT1, lincRNA ROR **and** Oct4 **in individual samples.**

In order to determine association between the expression of the lncRNA *NEAT1*, *lincRNA ROR* and the *Oct4* gene, the relative expression of these genes was compared in each set of the samples. No significant association was observed between the levels of *NEAT1*comparing *occt4* in gastric cancer tissues (R=0.244; P=0.185). In addition, we observed significant association between *LincRNA ROR* and *Oct4* (R=0.417; P=0.024) (Fig. 3).

Discussion

With the advances in medicine and life science, still GC remains a worldwide public health concern [28]. So, it is essential to exploring novel effective molecular mechanisms of GC progression for tumorigenesis prevention or improvement survival rate. Accumulating evidence demonstrates that aberrantly expressed lncRNAs are implicated in GC tumorigenesis, progression and these lncRNAs involved in numerous cell signal pathways and act as either tumor suppressors or oncogenes [24–26].

The current study found that *lincRNA ROR* and *Oct4* mRNA level in GC tissues comparing normal adjacent tissues showed significant association, but *NEAT1* level in GC tissues comparing normal adjacent tissues showed no significant association. Also, clinicopathological data comparing with lncRNAs and *Oct4* expression levels in GC tissues showed no significant association. Another important finding was that significant association between the levels of *lincRNA ROR* expression comparing *oct4* mRNA level in gastric cancer tissues.

Oct4 (*POU5F1*) is an important stem cell transcription factor in the maintenance of self-renewal and are essential in embryogenesis and pluripotency [29]. Increasing evidence over the past decades indicated that the Oct4 is also overexpressed in various tumors stem cells and suggested that Oct4-positive tumor cells have correlation with clinical prognosis, chemo resistance and lymph node metastasis [30]. In addition, post-transcriptional alteration of *Oct4* disturbs its activity and further study needs to determine the role of *Oct4* in gastric cancer and its clinical relevance, as well as finding correlation with lncRNAs in patients tumor tissue have remained controversial [31]. Additionally, Helicobacter pylori infection, one of the important causes of gastric cancer, has been shown to increase the mRNA level of *Oct4* through Wnt/β-catenin signaling pathway in human gastric tumor cells [32].

Shuai Wang (2016) and et al revealed that *lincRNA-ROR* caused upregulation of Oct4 stemness transcriptional factor. Their data confirmed that *lincRNA-ROR* was related with core stemness transcriptional factors and the pluripotent state of Gastric CSCs [18]. However, few researches have been conducted the clinical significance and biological mechanisms of *lincRNA-ROR* in gastric cancer. Previously, it has been found that *lincRNA-ROR* RNA level was significantly associated with tumor depth, tumor size, TNM stage, lymph node metastasis and gastric cancer patients' overall survival [33]. Another important recently finding shows that *lincRNA-ROR* expression levels are positively related with increased multidrug resistance and high level of *lincRNA-ROR* is a poor prognostic factor for patients with gastric cancer. knockdown of *lincRNA-ROR* reduced multidrug resistance-associated protein 1 (MRP1) mRNA level and increased apoptosis of drug-resistant gastric tumor cells in response to adriamycin (ADR) and vincristine (VCR) treatment [34].

Previous research by Jayu Jen et al, has indicated that *Oct4* interacted by the promoter or enhancer regions of various lncRNAs, Jayu Jen and colleagues confirmed that *Oct4* enhancer activities of *MALAT1* and potentiated promoter activity of *NEAT1* and they suggested that upregulation of Oct4-mediated *NEAT1* may play critical roles in embryonic or tumor stemness maintenance in lung cancer cells [17]. In HepG2 cells, the relationship between *MALAT1* and *Oct4* has been showed that *MALAT1* suppression significantly decreased the expression levels of transcription factors Oct4, which these outcomes showed that *MALAT1* could promote the stem-like properties of liver cancer cells [35].

Some previous studies have shown the important clinical outcome of *NEAT1* in gastric cancer [20, 36–46]. In contrast to earlier findings, no evidence of significant *NEAT1* overexpression in our study detected. Jing-wei Fu et al found that overexpressed levels of *NEAT1* in gastric cancer tissues and cell lines significantly increased and associated with clinical stage, lymph node metastasis, distant metastasis and histological type [36]. Farbod Esfandi et al, explore associations of *NEAT1* in gastric cancer samples compared with adjacent noncancerous tissues, patients' clinicopathological data and their potential as diagnostic biomarkers. The results of Farbod Esfandi and colleagues study show that significant associations between site of primary

tumor and relative expression of *NEAT1* in cancer samples compared with adjacent noncancerous tissues [42]. meta-analysis by Jian Fang et al, have suggested that High *NEAT1* expression is facilitates tumorigenesis of various human cancers and can be used as poor prognosis biomarker in cancer patients [47]. However, most of the studies were assessed by Jian Fang et al, meta-analysis conducted in China; hence, differences may happen between ethnic groups [47].

In conclusion, our data offer the first suggestion that *lincRNA ROR* expression correlated with *oct4* mRNA level in gastric cancer tissues. However, a further study with more focus on *lincRNA ROR* molecular mechanisms in association with *oct4* mRNA level in gastric cancer tissues and cell lines is therefore suggested.

Declarations

Acknowledgement

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Conflict of Interest

The authors declare no conflict of interest.

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Figures

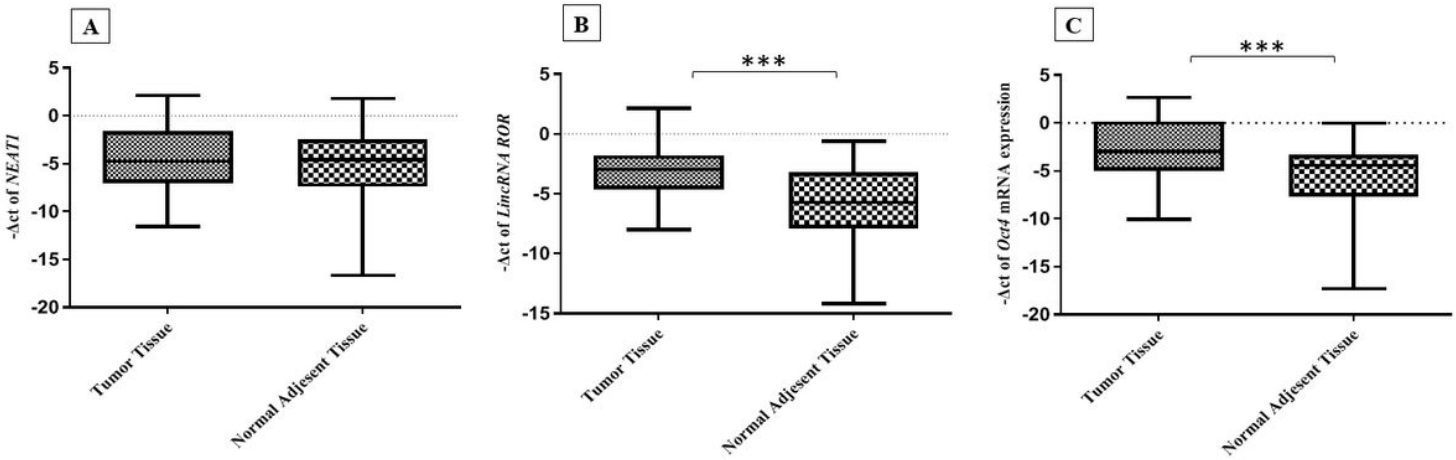

Figure 1

Real-time quantitative PCR analysis of NEAT1, lincRNA ROR and occt4 expression in gastric tissue (A) Relative mRNA expression of NEAT1 in GC comparing adjacent normal groups (B) Relative mRNA expression of lincRNA ROR in GC comparing adjacent normal groups (C) Relative mRNA expression of Oct4 in GC comparing adjacent normal groups. GC, Gastric cancer; (*P < 0.05) (**P < 0.01) (***P < 0.001).

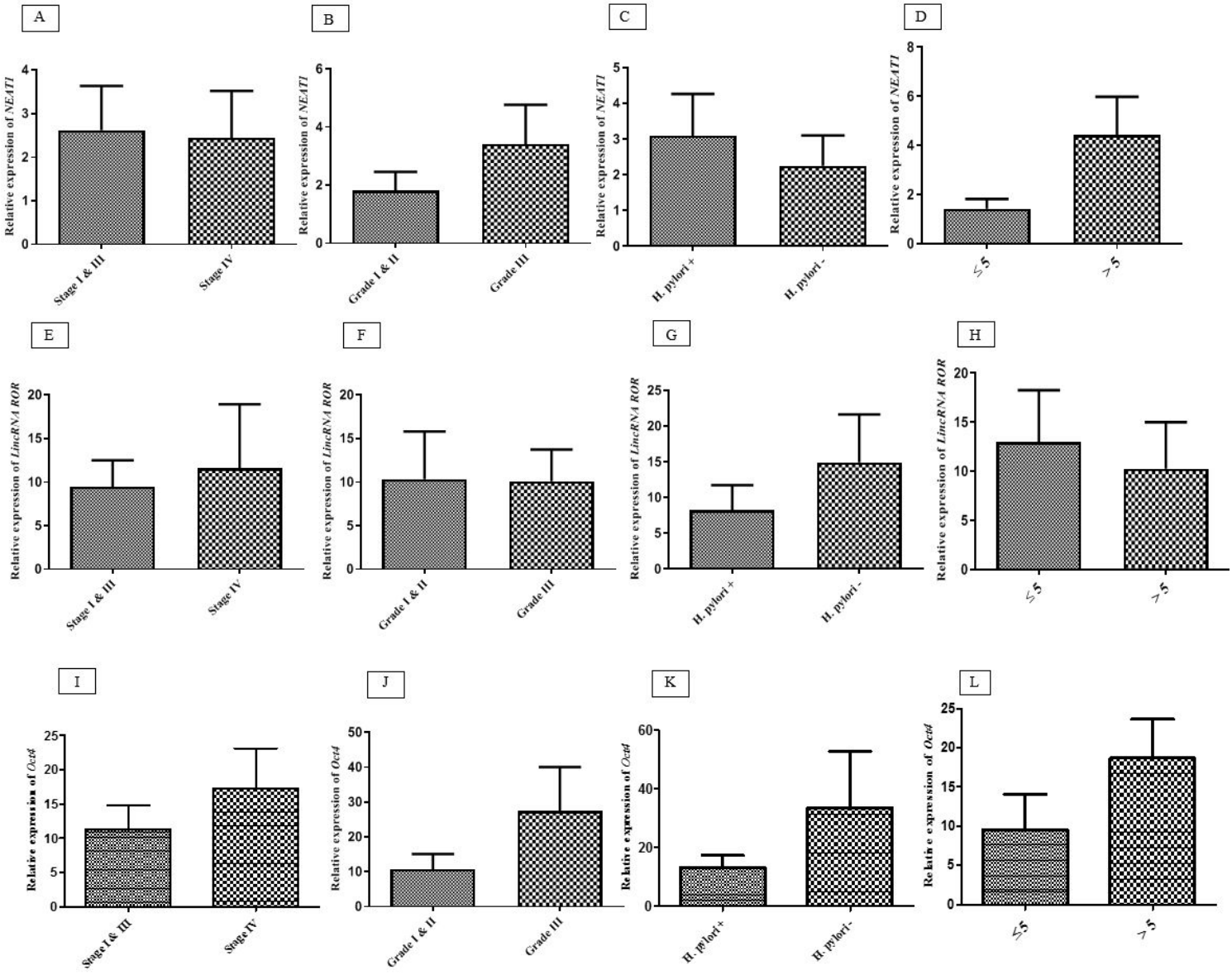

Figure 2

Relative RNA expression between the MALAT1, NEAT1, lincRNA ROR and occt4 genes with clinicopathological features. (A-D) Relative expression of NEAT1 between the different clinicopathological variable including I&III and IV, grad I&II and III, H. pylori infection positive & negative and tumor size <5 & >5 cm (P > 0.05). (E-H) Relative expression of LincRNA ROR between the different clinicopathological variable including I&III and IV, grad I&II and III, H. pylori infection positive & negative and tumor size <5 & >5 cm (P > 0.05). (I-L) Relative expression of Oct4 between the different clinicopathological variable including I&III and IV, grad I&II and III, H. pylori infection positive & negative and tumor size <5 & >5 cm (P > 0.05). GC, Gastric cancer; H. pylori, Helicobacter pylori

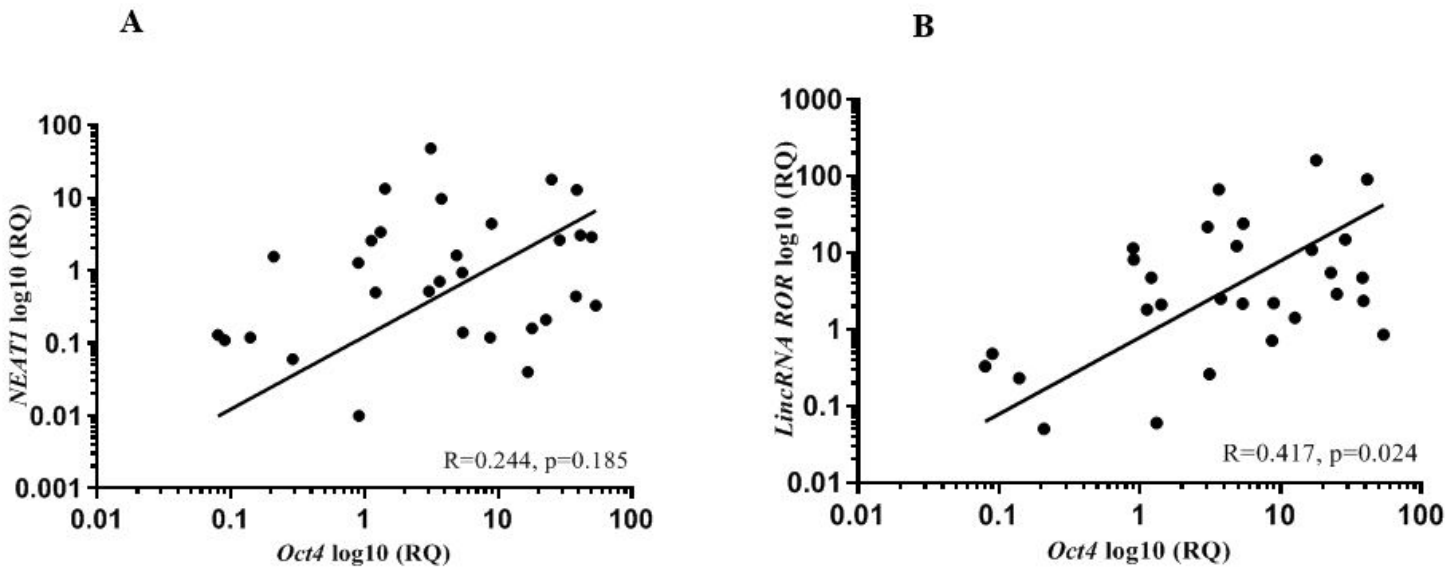

Figure 3

Association analyses using a linear regression between NEAT1, lincRNA ROR and occt4 expression in gastric tumor tissues compared with healthy adjacent tissues. (A) Association analyses using a linear regression between NEAT1 and Oct4. (B) Association analyses using a linear regression between LincRNA ROR and Oct4. RQ, relative quantification