Original Article

Gene Expression Analysis of Two Epithelial-mesenchymal Transition-related Genes: Long Noncoding *RNA-ATB* and *SETD8* in Gastric Cancer Tissues

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Abstract

Background: Cancer is the second cause of death after cardiovascular diseases worldwide. Tumor metastasis is the main cause of death in patients with cancer; therefore, unraveling the molecular mechanisms involved in metastasis is critical. Epithelial-mesenchymal transition (EMT) is believed to promote tumor metastasis. Based on the critical roles of long noncoding *RNA-ATB* (*lncRNA-ATB*) and *SETD8* genes in cancer pathogenesis and EMT, in this study, we aimed to assess expression profile and clinicopathological relevance of these two genes in human gastric cancer. **Materials and Methods:** Quantitative real-time polymerase chain reaction was performed to assess these gene expressions in gastric cancer tissues and various cell lines. The associations between these gene expressions and clinicopathological characteristics were also analyzed. **Results:** Insignificant downregulation of *lncRNA-ATB* and significant upregulation of *SETD8* in cancerous versus noncancerous gastric tissues were observed. Among different examined cell lines, all displayed both genes expression. Except for a significant inverse correlation between the expression levels of *lncRNA-ATB* and depth of invasion (T) and a direct association between *SETD8* levels and advanced tumor grades, no significant association was found with other clinicopathological characteristics. **Conclusion:** *lncRNA-ATB* and *SETD8* genes may play a critical role in gastric cancer progression and may serve as potential diagnostic/prognostic biomarkers in cancer patients.

Keywords: *Gastric cancer, gene expression, long noncoding RNA-ATB, SETD8*

Introduction

Gastric cancer is the second most frequent cause of cancer mortality in the world.[1] It is the second and fourth most common cancer in Iranian men and women, respectively, and the first leading cause of cancer deaths in Iran.[2] Ordinary treatments have no effect on improving the survival for most Iranian patients with gastric cancer because it is being diagnosed at an advanced stages of disease.[3]

Epithelial-to-mesenchymal transition (EMT) is a process by which cell's features change from epithelial to mesenchymal, and it is known as a fundamental step for initiation of tumor cell metastasis. Various signaling pathways, transcriptional factors, and posttranscriptional regulatory networks such as long noncoding RNAs (lncRNAs) can motivate EMT process.[4]

lncRNAs are a heterogeneous group of ncRNAs, which are larger than 200 nucleotides, with no protein-coding duty.[3,5-7] These molecules are engaged in a broad range of biological and pathological processes such as cancer initiation, progression,[7] and metastasis.[5] Dysregulation of various lncRNAs in gastric cancer has been previously reported.[3,8,9]

A transforming growth factor-beta (TGF-β)-induced lncRNA, *lncRNA-ATB* (lncRNA activated by TGF-β), is a lncRNA with 2741 nucleotides in length which is expressed from ENSG00000249549 gene on 14 chromosome. *lncRNA-ATB* stimulates EMT through competitively binding to mir-200 family, thus insulating them away from their mRNA targets: *ZEB1* and *ZEB2*.[10,11] Dysregulation of *lncRNA-ATB* has been reported in several cancer types.[10-16] *SETD8* is a protein-coding gene encoding for an enzyme functioning as methyltransferase, in which its deregulation and role in cancer initiation, progression, and EMT has been also documented.[17,18]

Based on the important roles of *lncRNA-ATB* and *SETD8* genes in cancer pathogenesis, in the current study, we aimed

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to assess expression profile of these two genes in human gastric cancer. Furthermore, we explored the association between these two genes expression and clinicopathological parameters of human gastric cancer samples.

Materials and Methods

Representative cancerous and noncancerous tissues

A total of 38 paired tissue specimens were tested for gene expression, of which 38 were cancerous and 38 were neighboring noncancerous samples of gastric from the same patients. In case of *lncRNA-ATB* gene, 37 paired samples were tested. Biological materials were provided by the Iran National Tumor Bank which is funded by the Cancer Institute of Tehran University, for Cancer Research.[19-21] All patients provided written informed permission to the Iran Tumoral Bank before the participation, and all experimental procedures were accepted by the Ethics Committee of Isfahan University of Medical Sciences. The clinical staging of the cancerous specimens was done based on the seventh edition of the American Joint Committee on Cancer (AJCC) staging manual for stomach.[22]

Cell lines and cell cultivation

The human liver hepatocellular carcinoma cell line (HepG2) was cultivated in RPMI-1640 (Gibco Life Technologies, Karlsruhe, Germany), supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 10 μg/ml streptomycin. The human embryonic kidney 293 cells, human pulmonary carcinoma cells (A542), human breast cancer cell (MCF7), and human umbilical vein endothelial cells (HUVECs) were cultivated in the high glucose DMEM (Gibco Life Technologies, Karlsruhe, Germany), enriched with 15% fetal calf serum, 100 U/ml penicillin, and 10 μg/ml streptomycin.

Total RNA isolation and cDNA synthesis

Total RNA was extracted from powdered gastric cancer tissues using TRIzol® Reagent (Invitrogen, Carlsbad, California, United States) as stated in manufacturer's protocol (Trizol Reagent. Catalog numbers: 15,596-026 and 15,596-018). One percent agarose gel electrophoresis was used to determine the integrity of the RNA. Nanodrop instrument (Nanolytik, Düsseldorf, Germany) was used for determining RNA concentration by measuring optical density at 260 nm. DNase treatment was performed using DNase set (Fermentas, Vilnius, Lithuania) for erasing genomic DNA. cDNA synthesis was performed using PrimeScriptTM RT reagent Kit (TaKaRa, Kusatsu, Shiga, Japan) as stated in manufacturer's protocol.

Quantitative real-time polymerase chain reaction and DNA sequencing

The expression level of *lncRNA-ATB* and *SETD8* genes was tested by quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) in comparison to β-Glucuronidase (*GUSB*) as a reference gene.[23] The primers for the target genes were designed with GeneRunner software, version 4.0, and tested by basic local alignment search tool (BLAST [http://blast.ncbi.nlm.nih.gov/Blast.cgi]) for unity attachment to genome. Primers were as follows for *lncRNA-ATB* forward: ACGTCTTGTGCTTGGATCCTA, for *lncRNA-ATB* reverse: CGCAAATACAGACAGGTCACC, with an amplicon size of 227 bp for *lncRNA-ATB,* primers for *SETD8* forward: CGAGCTGCAGTCTGAAGAAAG and for *SETD8* reverse: TTTCTTGGCGTCGGTGATC with an amplicon size of 184 bp for *SETD8*. Real-time PCR was done with RealQ Plus ×2 Master Mix, green (high ROX) (AMPLIQON, Odense M, Denmark) by an Applied Biosystems StepOnePlus™ instrument. The PCR amplification conditions included of first denaturation at 95°C for 10 min, 40 cycles of denaturation at 95°C for 15 s, annealing at 61°C for *lncRNA-ATB* and *SETD8* and at 60°C for *GUSB* genes for 60 s, then extension for 15 s at 72°C. Moreover, to be ensured that the actual genes of interest (*lncRNA-ATB* and *SETD8*) are getting specifically amplified, the PCR products of some samples were sequenced with an Applied Biosystems 3730XL sequencer (Macrogen, Seoul, South Korea).

Statistical gene expression analysis

The ΔC_t method (C_t of gene of interest-C_t of housekeeping gene) was used to quantify the relative levels of gene expression. All experiments were done at least two or three times and expressed as means ± standard error of mean. To determine normal distribution of samples, Kolmogorov–Smirnov test was performed. Student's *t*-test, analysis of variance, and Chi-square were done to examine statistical significance. SPSS software, version 16.0, was used for statistical analyses and $P < 0.05$ was considered statistically significant.

Results

Optimization of real time-polymerase chain reaction

Prior to the quantitative analysis, optimization procedures were done to achieve a specific amplicon for *lncRNA-ATB* and *SETD8* genes in both conventional and real-time RT-PCR reactions on cancerous gastric tissue specimens. As it was demonstrated in previous studies that both *lncRNA-ATB* and *SETD8* are expressed in MCF-7 breast cancer cell line,[11,18] we applied the cDNA of MCF-7 cell line for optimization procedures. Agarose gel electrophoresis of the amplified product of *lncRNA-ATB* and *SETD8* demonstrated a specific band with the expected sizes for both genes. In addition, in real time process, unique melting curves without primer dimers for each of the examined genes confirmed the specificity of amplicons (data not shown). BLAST of the results of PCR product sequencing against human transcripts showed 100% identity with corresponding transcripts.

Expression of long noncoding *RNA-ATB* and *SETD8* genes in various cultivated cells, human cancerous, and noncancerous gastric specimens

According to RT-PCR results, five of five examined cell lines displayed *lncRNA-ATB* and *SETD8* expressions [Figure 1]. To determine the expression levels of *lncRNA-ATB* and *SETD8* genes in human gastric tissues, cDNAs were synthesized for all specimens and real-time PCR was performed using specific primers for *lncRNA-ATB, SETD8*, and *GUSB* genes. Relative expression of target genes was detected by subtracting its C_t value to that of the *GUSB* gene. The results of real-time qRT-PCR experiments demonstrated that while the relative expression of *lncRNA-ATB* gene was high in some examined tumor tissues, its expression was low in some others. Figure 2 shows the relative mean expression of *lncRNA-ATB* gene in cancerous and noncancerous gastric tissue samples. As expected from the results, statistical analyses failed to show any significant difference between the *lncRNA-ATB* gene expression of cancerous specimens and that of the noncancerous ones. Relative quantitation of the expression levels of *SETD8* in gastric adenocarcinomas showed that the relative levels of its transcripts were significantly increased (P = 0.018) in cancerous tissues compared with adjacent noncancerous tissues: 0.88 ± 0.66 versus 2.78 ± 0.59, respectively, as shown in Figure 3.

Association between long noncoding *RNA-ATB* and *SETD8* expression and clinicopathological characters in gastric cancer

To examine the clinical consequences of the *lncRNA-ATB* and *SETD8* expressions, the correlation between clinicopathological status of gastric cancerous specimens and levels of *lncRNA-ATB* and *SETD8* expression was calculated. Except for a significant inverse correlation between the expression levels of *lncRNA-ATB* and depth of invasion (T) (P = 0.0002), no significant association was found with other clinicopathological characteristics for both examined genes (data not shown).

As in other previous studies in which lncRNA expression in cancerous tissues was classified as high or low according to the median value of lncRNA expression, gastric cancer patients were further divided into two groups based on the median value of relative gene expression in cancer tissues. The correlations between the *lncRNA-ATB* and *SETD8* expression levels and the clinicopathological characters of gastric cancer patients are summarized in Tables 1 and 2. The results suggested a relationship between high *SETD8* level and advanced tumor grades (P = 0.01). Overall, no significant association was observed between *lncRNA-ATB* and *SETD8* expression level and any other clinicopathological factors.

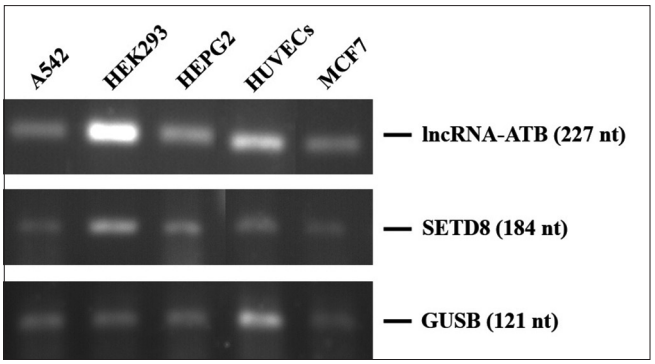

Figure 1: Expression of long noncoding *RNA-ATB*, *SETD8*, and *β-Glucuronidase* transcripts in various cell lines. Electrophoresis of long noncoding *RNA-ATB*, *SETD8*, and *β-Glucuronidase* polymerase chain reaction products on the agarose gel showed a unique band with expected sizes for each gene for all cell lines

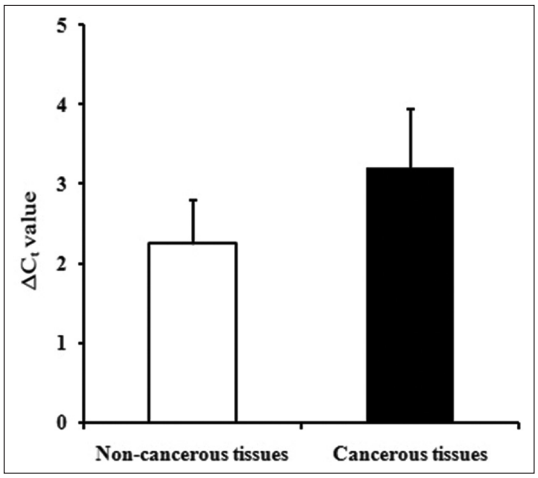

Figure 2: Expression level of long noncoding *RNA-ATB* in gastric cancerous and adjacent noncancerous specimens (n = 37). A smaller ΔC_t value demonstrates higher expression. Data are expressed as means ± standard error of mean. Error bars represent standard error of mean

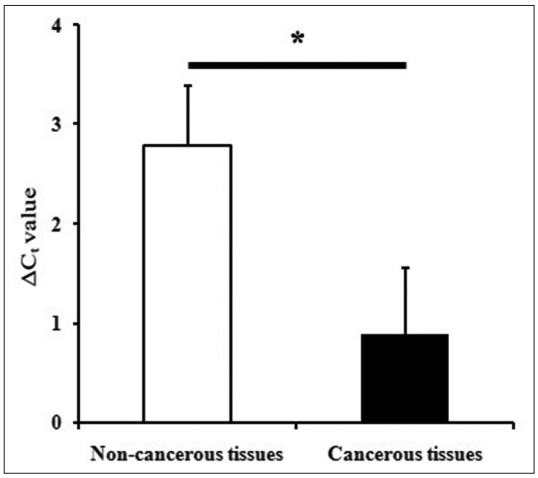

Figure 3: Expression level of *SETD8* in gastric cancerous and adjacent noncancerous specimens (n = 38). A smaller ΔC_t value demonstrates higher expression. Data are expressed as means ± standard error of mean. *Value that is statistically significant. Error bars represent standard error of mean

Discussion

In the current study, we examined the *lncRNA-ATB* gene expression profile in various cultivated cells and a series of cancerous and noncancerous gastric tissue specimens. Real-time qRT-PCR showed that *lncRNA-ATB* was expressed

Table 1: Relationship between long noncoding *RNA-ATB* expression levels (as divided into two groups based on the median of ΔC_t) and clinicopathological characters of cancerous gastric samples

Characteristics	Numbers (#37)	*lncRNA-ATB* expression		*P*
		Low (higher ΔC_t than median) (#18)	High (smaller ΔC_t than median) (#19)	
Sex				
Male	22	10	12	0.37
Female	15	8	7	
Age (years)				
≥70	17	10	7	0.16
<70	20	8	12	
Depth of invasion (T)				
T2	2	0	2	0.24
T3-T4	35	18	17	
N classification				
NX-N0	7	2	5	0.06
N1	19	8	11	
N2-N3	11	8	3	
M classification				
MX	7	2	5	0.20
M0	22	11	11	
M1	8	5	3	
TNM stage				
I-II	18	8	10	0.33
III	11	5	6	
IV	8	5	3	
Perineural invasion				
Negative	8	6	2	0.06
Positive	29	12	17	
Lymphatic invasion				
Negative	13	5	8	0.24
Positive	24	13	11	
Tumor size (cm)				
≥5	28	15	13	0.22
<5	9	3	6	
Tumor grades				
I	9	5	4	0.35
II	9	5	4	
III	19	8	11	
Tumor types				
Diffuse	18	8	10	0.37
Intestinal	19	10	9	

lncRNA-ATB: Long noncoding RNA-ATB, TNM: Tumor node metastasis

in all examined cell lines. In gastric tissue samples, cancerous tissues had relatively lower *lncRNA-ATB* expression levels than noncancerous ones, although the difference was not statistically significant. We also revealed that *lncRNA-ATB* levels were inversely associated with depth of invasion (T). A previous report by Saito *et al*.,[14] in patients with gastric cancer, showed that high level of *lncRNA-ATB* expression was associated with vascular invasion, lower survival rates, and poorer prognosis. They also reported *lncRNA-ATB* expression levels in all eight examined GC lines. A critical difference between the Saito's study and our own is that they only collected cancerous tissue samples, and not any noncancerous gastric tissues. Yuan *et al*.[11] discovered the *lncRNA-ATB* gene and measured its expression level in a set of hepatocellular carcinoma (HCC) tissues. Their result showed that *lncRNA-ATB* transcript level is higher in the HCC tissues compared to the paired adjacent noncancerous hepatic tissues. There was also a direct correlation between *lncRNA-ATB* levels and the metastasis-related clinicopathological characteristics of HCC samples. They also assessed the gene expression in SMMC-7721 hepatoma cells, the normal liver cell line QSG-7701, breast cancer cell line MCF7, and colorectal cancer cell line SW480. All cells had basic levels of *lncRNA-ATB* expression before treatment with TGF-β and showed a significant increase in its levels after TGF-β treatments, further confirming that *lncRNA-ATB*

Table 2: Relationship between *SETD8* expression levels (as divided into two groups based on the median of ΔC_t) and clinicopathological characters of cancerous gastric samples

Characteristics	Numbers (#38)	*SETD8* expression		*P*
		Low (higher ΔC_t than median) (#19)	High (smaller ΔC_t than median) (#19)	
Sex				
Male	23	10	13	0.25
Female	15	9	6	
Age (years)				
≥70	16	9	7	0.37
<70	22	10	12	
Depth of invasion (T)				
T2	2	0	2	0.24
T3-T4	36	19	17	
N classification				
NX-N0	5	2	3	0.39
N1	18	10	8	
N2-N3	15	7	8	
M classification				
MX	8	2	6	0.08
M0	23	12	11	
M1	7	5	2	
TNM stage				
I-II	20	10	10	0.17
III	11	4	7	
IV	7	5	2	
Perineural invasion				
Negative	8	4	4	0.50
Positive	30	15	15	
Lymphatic invasion				
Negative	13	7	6	0.50
Positive	25	12	13	
Tumor size (cm)				
≥5	29	15	14	0.50
<5	9	4	5	
Tumor grades				
I	11	9	2	0.01*
II	10	4	6	
III	17	6	11	
Tumor types				
Diffuse	17	6	11	0.09
Intestinal	21	13	8	

*Statistically significant. TNM: Tumor node metastasis

is regulated by TGF-β. *lncRNA-ATB* expression has also been evaluated in patients with colorectal cancer. Its high expression levels was associated with greater tumor size, depth of tumor invasion (T), lymphatic invasion, vascular invasion, lymph node metastasis, hematogenous metastasis, and poorer outcomes.[12] In a recent report, high *lncRNA-ATB* expression level was documented in trastuzumab-resistant SKBR-3 cells and breast cancer tissues. Trastuzumab resistance and invasion-metastasis cascade in breast cancer is mediated by sponging role of *lncRNA-ATB* for miR-200c and thus increasing ZEB1 and ZNF-217 and then inducing EMT.[15] The first study which reported downregulation of *lncRNA-ATB* in tumoral tissues was published on 2015.[13] A set of pancreatic cancerous and adjacent noncancerous pancreatic tissues were included in the study and the results showed that *lncRNA-ATB* transcript level was significantly lower in pancreatic cancerous tissues compared with the paired adjacent noncancerous tissues. Their results also showed that *lncRNA-ATB* expression levels were significantly and inversely correlated with lymphatic metastasis, neural invasion, and clinical stage. However, *lncRNA-ATB* expression did not correlate with other clinicopathological characteristics. *lncRNA-ATB* expression in colon cancer tissues was reported to be significantly higher than matched adjacent mucosa. There was also a direct association between levels of *lncRNA-ATB* and pN and AJCC stages. Overall survival and disease-free survival in cases

with both high *lncRNA-ATB* expression and low *E-cadherin* expression were also significantly lower than patients with low *lncRNA-ATB* levels. Evaluation of expression of *lncRNA-ATB* in three high-invasive colon cancer cells and three low-invasive colon cancer cells showed higher expression levels in metastatic cell lines.[16] Taken together, our result on gastric cancer is more consistent with the previously published report by Qu *et al*.[13] who reported a remarkably downregulated levels of *lncRNA-ATB* in pancreatic tissues. As Qu *et al*. commented, these findings revealed that *lncRNA-ATB* might act as an oncogene or cancer suppressor in a tissue-dependent manner.[13]

The histone methyltransferase (HMTs) family of proteins consists of enzymes that methylate lysine or arginine residues on histone tails as well as other proteins. The role of many HMTs in tumorigenesis and progression of human cancers have been documented.[24] As a member of HMTs, *SETD8* has a well-defined role in the TP53 pathway, thus regulating cell proliferation and genome stability.[25] SETD8 monomethylates protein P53 at lysine 382 and represses its proapoptotic and cell cycle arrest functions.[25] The role of *SETD8* in promoting EMT and metastasis has been well documented.[18] Expression analysis of *SETD8* in a set of breast tissues revealed a positive correlation between higher *SETD8* levels and metastatic capacity of breast cancer tissues.[18] In a study by Takawa *et al*.,[17] overexpression of *SETD8* in numerous types of cancers including bladder cancer, non-small cell lung carcinoma, small cell lung carcinoma, chronic myelogenous leukemia, HCC, and pancreatic cancer has been reported. In the current study, we observed a significantly higher expression of *SETD8* in gastric cancer tissues compared to noncancerous ones. Our data are in agreement with the proliferation-promoting role of *SETD8* in other cancer types.[17,18] An association between *SETD8* expression levels and higher tumor grades was observed in our study. In the same vein, *SETD8* expression level has been correlated with the grade of malignancy in bladder cancer.[17] Several studies have assessed the cancer-predisposing functions of a microRNA binding site within 3'UTR of *SETD8* (rs16917496).[26-30] It was shown in HCC and non-small cell lung cancer that the *SETD8* CC genotype is correlated with reduced SET8 protein levels and longer survival.[29,31]

Conclusion

We investigated the clinicopathological relevance of two EMT-related genes: *lncRNA-ATB* and *SETD8* in a series of gastric cancer tissues. Our study demonstrated that *lncRNA-ATB* expression was partly decreased in gastric cancer and inversely correlated with the depth of invasion (T). We further showed that *SETD8* level was augmented in gastric cancerous tissues and this elevation was associated with higher tumor grades. These data showed that *lncRNA-ATB* and *SETD8* genes may play a critical role in gastric cancer progression and may serve as potential diagnostic/prognostic biomarkers in cancer patients. However, further studies are needed to reveal the molecular mechanisms of *lncRNA-ATB* and *SETD8* in gastric cancer pathogenesis.

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Conflicts of interest

There are no conflicts of interest.

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