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Long Non-coding RNA *ZEB1-AS1* Promotes Tumorigenesis and Metastasis in Colorectal Cancer

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Abstract

Emerging evidence implicates that a large fraction of human genome was transcribed but the transcripts known as long non-coding RNA are not translated into proteins. They are contributing in different cellular processes, including cellular proliferation and apoptosis. LncRNAs were found to play critical roles in many diseases and act as key regulators in malignancies. In this study, we investigated the expression and clinical significance of *ZEB1-AS1* in colorectal cancer. In the present study, 64 samples including 32 samples of colorectal tumor and 32 matched tumor marginal samples were obtained from Iran National Tumor Bank (Tehran, Iran). RNA extraction was performed with Trizol reagent and cDNA was synthesized using MMULV reverse transcriptase enzyme. The expression of *ZEB1-AS1* in tumors and marginal tissues was determined by using real time PCR. We found that the expression level of *ZEB1-AS1* was much higher in tumor tissues compared to marginal samples. Moreover, in tumor group, *ZEB1-AS1* expression was significantly upregulated in high-grade tumors in comparison to low-grade ones. Additionally, we observed the expression of ZEB1-AS1 was much higher in lymph node metastatic tumors compared to non-lymph node metastatic ones. Our data showed that the *ZEB1-AS1* was dramatically overexpressed in colorectal cancer tissues. The results of the study revealed that the expression of *ZEB1-AS1* is correlated with tumor stage, lymph node metastasis and vascular invasion. Based on our findings, we suggested that *ZEB1-AS1* might be considered as a tumor marker with potential diagnostic, prognostic and therapeutic value for aggressive and metastatic colorectal cancers.

Keywords: *ZEB1-AS1*; Metastasis; Tumor marker; Colorectal cancer

Introduction

Colorectal cancer (CRC) is one of the most common cancers in the worldwide. It is the third most commonly diagnosed cancer in men in developed countries and the second most frequent cause of cancer related death in women (Brenner *et al.,* 2014; Ghafouri Sabzevari *et al.,* 2016). The prevalence of this cancer is very diverse in different geographical areas. More than 95% of colorectal cancer cases was observed in people aged 50 or older who commonly have other lifestyle-related conditions including type two diabetes mellitus and cardiovascular disease (Anderson *et al.,* 2015). Currently, the incidence of colorectal cancer in Asia is rising rapidly, each year, 1.9 million of people are diagnosed with this cancer, and more than half of them eventually die (Azeem *et al.,* 2015).

Long non-coding RNAs (lncRNAs> 200 nucleotides in length), are a new class of non-coding transcriptome in humans and do not have the protein-coding capacity (Nagano and Fraser, 2011). Recent studies have demonstrated that lncRNAs play an essential role in several important biological processes such as genomic imprinting, and cell proliferation (Fatica and Bozzoni, 2014; Gontan *et al.,* 2011; Liu *et al.,* 2017c). Therefore, dysregulation of lncRNAs can cause various human diseases, including cancer (Wapinski and Chang, 2011). The

aberrant expression of lncRNAs have been observed in various types of cancers such as prostate cancer, breast cancer, Cervical cancer hepatocellular carcinoma, melanoma, bladder cancer, gastric cancer and colorectal cancer (Deng *et al.,* 2017; Jannat Alipoor *et al.,* 2018a; Jannat Alipoor *et al.,* 2017b; Lemos *et al.,* 2016; Li *et al.,* 2017a; Liu *et al.,* 2017b; Rezanejad Bardaji *et al.,* 2018; Schmidt *et al.,* 2016; Wu *et al.,* 2017; Yang *et al.,* 2015). LncRNA ZEB1 antisense 1 (*ZEB1-AS1*) is a non-coding antisense transcript emanating from the promoters of *ZEB1* and it positively regulated the expression levels of *ZEB1* (Li *et al.,* 2016). Recent reports have been shown that *ZEB1-AS1* overexpressed and associated with poor prognosis in hepatocellular carcinoma, esophageal squamous cell carcinoma, glioma, and osteosarcoma (Li *et al.,* 2016; Liu and Lin 2016; Lv *et al.,* 2016; Wang *et al.,* 2015).

Based on the latest cancer registry statistics in Iran, colorectal cancer is the fifth most common cancer in males and the third most common cancer in females. The economic burden of colorectal cancer is substantial and is likely to increase over time in Iran owing to the current trend in colorectal cancer incidence. Diagnosis of the disease at a late stage is major reasons that promote mortality in colorectal cancer patients. Therefore, deciphering the molecular mechanisms underlying the initiation and progression of colorectal cancer, especially the genetic and epigenetic alterations, will lead to the identification of novel diagnostic biomarkers and the development of new therapeutic strategies. In current study, we investigate the potential expression of *ZEB1-AS1* in colorectal cancer.

Materials and methods

Tissues samples

A total of 32 colorectal tumor tissues and their paired adjacent non-tumor tissues were obtained from Iran National Tumor Bank which was founded by Cancer Institute of Tehran University of Medical Sciences (Tehran, Iran). The samples had been immediately snap-frozen in liquid nitrogen and stored at -185 ºC until being used for RNA extraction. The project was approved by the Ethics Committee of Kerman Graduate University of Technology. Prior to participation, the patients' written informed consents were obtained by the Iran National Tumor Bank. The clinicopathological parameters of colorectal patients are shown in table 1.

Table 1. The association of *ZEB1-AS1* expression with clinicopathological factors of colorectal cancer patients

Clinicopathological parameters	Number of cases	*ZEB1-AS1* expression		P-value
		Low	High	
Age (years)				0.177
≤65	15	5	10	
>65	17	5	12	
Gender				0.456
Male	16	3	13	
Female	16	7	9	
Tumor size (cm)				0.765
≤5	13	2	11	
>5	19	8	11	
Histologic grade				0.046
I, II	9	3	6	
III, IV	23	7	16	
TNM stage				0.009
I, II	9	6	3	
III, IV	23	4	19	
Depth of tumor				0.123
T1,T2	15	3	12	
T3,T4	17	7	10	
Lymphatic invasion				0.008
Negative	9	5	4	
Positive	23	5	18	
Vascular invasion				0.045
Negative	11	4	7	
Positive	21	6	15	
Perineural invasion				0.43
Negative	20	8	12	
Positive	12	2	10	

*$p < 0.05$

RNA extraction

Total RNA was extracted by Trizol solution (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The quantity and quality of the extracted RNA was measured by UV spectrophotometry (Cary 60, Australia) at 260 nm and visual observation of samples loaded on 2% agarose gel electrophoresis respectively.

cDNA synthesis and quantitative real-time PCR

RNase-free DNase (Fermentas, Lithuania) treatment of isolated RNA was done for eliminating co-purified genomic DNA according to the manufacturer's instructions. The first strand of cDNA was synthesized in the presence of 1 µg RNA, 200 U/µL MMLV reverse transcriptase (Fermentas, Lithuania), 20U RNase inhibitor, dNTP mix (final concentration of 1 mM) with random hexamer priming in a 20 µL reaction volume. Quantitative PCR was performed using SYBR Green qPCR MasterMix 2X (Yekta Tajhiz, Iran) on Rotor-Gene 3000 instrument (Corbett Life Science, Valencia, CA, USA). The amplification profile was denatured at 95˚C for 2 minutes, followed by 40 cycles of denaturation at 95 ˚C for 30 seconds, annealing and extension at 60 ˚C for 30 seconds. The specific primers of ZEB1-AS1 (135 bp) and β-actin (120 bp) were as follow respectively:
5'-CTATCGGAGTTGGAAAGGGAC -3'
5'-ATCTACTAAGGAGGCTGCTG-3'
5'-ACCACCTTCAACTCCATCATG -3'
5'- CTCCTTCTGCATCCTGTCG -3'
The specific primers were designed by Gene Runner software and we used the BLAST software to ensure that primers are specific. β-actin was used as an internal control gene and ΔCT values were normalized to β-actin levels. All procedures were repeated for three times.

Statistical analysis

The differences in gene expression between the two groups were evaluated using t-test, which was performed by SPSS ver. 22.0 software and REST program. A *p*-value less than 0.05 was considered statistically significant.

Results

LncRNA *ZEB1-AS1* expression in CRC tissues

In first phase of current study, we investigated the potential expression of *ZEB1-AS1* RNA in 32 CRC tissues and paired marginal samples using RT-qPCR. The expression of β-actin was determined as an internal control for standardizing of potential sampling errors. The melting curve was obtained as a single peak, which indicates that there is only one PCR product (Fig. 1B). To confirm the identity of the PCR product, it was loaded on an agarose gel and we observed only one specific band which was proved the specificity of PCR product. (Fig. 1A). The results showed that *ZEB1-AS1* expression was significantly higher in CRC tissues in comparison to normal marginal samples (Fig. 2A, $P < 0.01$).

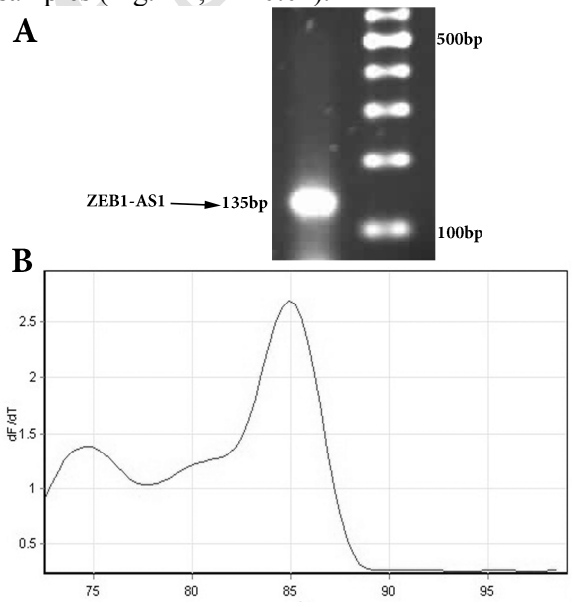

Fig. 1. The specific primers used for the proliferation fragment of *ZEB1-AS1*: (A) single-band of PCR product on 2% agarose gel which was stained with ethidium bromide; (B) Melting curve analysis of ZEB1-AS1 in RT-qPCR reaction.

Correlation of lncRNA ZEB1-AS1 expression with clinicopathological features of CRC tissues

In next phase of our study, we analyzed the association of *ZEB1-AS1* expression with

clinicopathological characteristics of CRC patients. Our results revealed that the expression of *ZEB1-AS1* was significantly upregulated in advanced tumor stage CRC tissues (P < 0.01, Fig. 2B). Furthermore, our data showed that the expression of *ZEB1-AS1* transcript was higher in high-grade CRC tissues in comparison to low-grade ones (*P*<0.05, Fig. 2C). Moreover, the expression of *ZEB1-AS1* was much higher in CRC tissues with lymphatic metastasis compared with CRC tissues with negative lymphatic status (*P* = 0.008, Fig. 3A). Additionally, our finding revealed that *ZEB1-AS1* expression markedly upregulated in vascular invasion CRC tissues (*P*= 0.045, Fig. 3B). Finally, gene expression analysis exhibited that the expression of *ZEB1-AS1* transcript in CRC tissues had no significant association with other parameters, such as age, gender, tumor size and perineural invasion (Table 1).

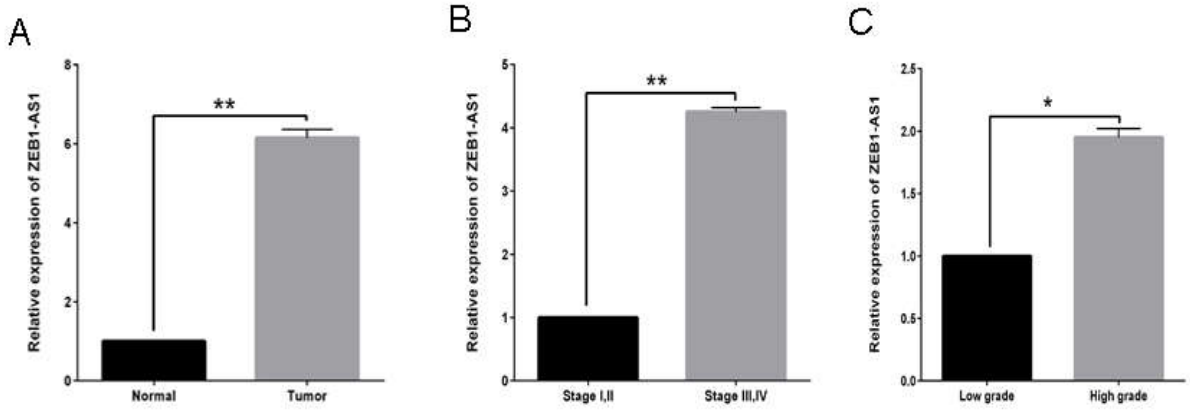

Fig. 2. The relative expression of *ZEB1-AS1* in CRC tissues: (A) *ZEB1-AS1* expression in tumor and adjacent normal tissues; (B) *ZEB1-AS1* expression in low grade (I, II) and high grade (IIII, IV) tumor tissues; (C) Relative expression of *ZEB1-AS1* in different tumor stages. Data are shown as mean±SD (*p < 0.05; **p < 0.01).

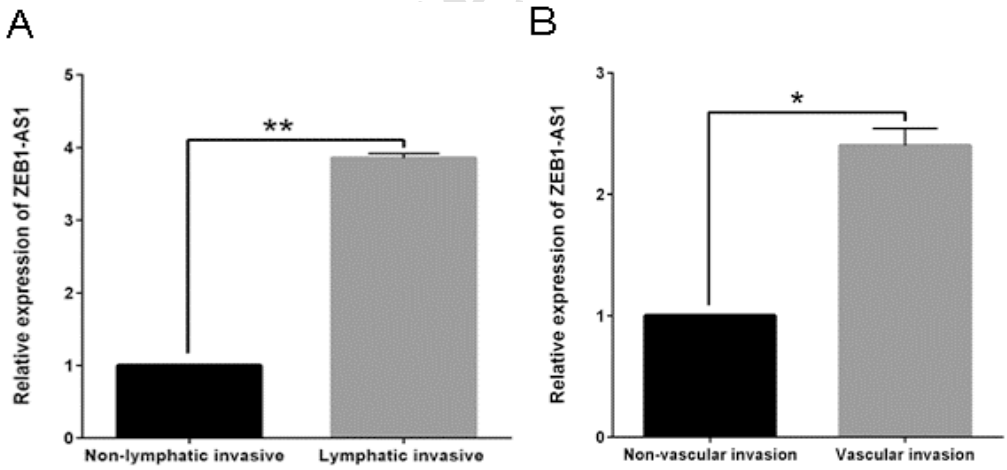

Fig. 3. Relative *ZEB1-AS1* expression with metastatic lymph nodes tumors: (A) The expression of *ZEB1-AS1* was significantly higher in metastatic lymph nodes tumors rather than non-lymph node metastases tumor tissues; (B) *ZEB1-AS1* expression was upregulated in vascular metastatic tumor tissues in comparison to non-vascular invasive tumor specimens. Data are shown as mean±SD (*p < 0.05; **p < 0.01).

Discussion

Colorectal cancer was one of the most commonly diagnosed malignancies. Identification of molecular markers, epigenetic changes, and new pharmacological properties improve the diagnosis and treatment status of CRC patients. The molecular mechanisms involved in the progression of CRC remain mostly unclear. Accumulating evidence showed

that lncRNAs played key roles in the regulation of multiple biological processes such as cell proliferation and apoptosis (Liu *et al.,* 2017c). Because of the crucial role of lncRNAs in various cellular activities, a large volume of studies has been devoted to clarify the role of these molecules in various cancers (Fatima *et al.,* 2015). One of these lncRNAs is the *ZEB1-AS1*, which appears to be a major factor in the progression and metastasis of different cancer types. Previous studies have shown that ZEB1-AS1 is expressed in some cancers and is probably involved in the tumorigenic process. ZEB1-AS1 expression has been reported in several cancer types including hepatocellular, osteosarcoma, lung, glioma and prostate cancers (Li *et al*., 2016; Liu and Lin, 2016; Lv *et al*., 2016; Wang *et al*., 2015). In this study, 32 patients with colorectal cancer have been studied for expression of this lncRNA in tumor and paired normal adjacent tissues.

The results of this study showed that *ZEB1-AS1* is upregulated in CRC tissues in comparison with adjacent normal tissues. These finding is consistent with previous reports, and therefore *ZEB1-AS1* can be considered as a potential biomarker for colorectal cancer. Recent report showed that *ZEB1-AS1* promoted lung cancer progression through up regulating Cyclin D1 and c-myc via activating Wnt/β-catenin pathway (Li *et al.,* 2017b). Additionally, *ZEB1-AS1* was found to act as oncogene in osteosarcoma through binding to p300 in the *ZEB1* promoter region which leads to induce an open chromatin structure, and activate the transcription of *ZEB1*. Moreover, *ZEB1-AS1* was determined to function as a molecular sponge for miR-200s and consequently inhibit ZEB1 translation (Liu and Lin, 2016; Liu *et al.,* 2017a). In consistent with previous studies, our findings showed that the expression of *ZEB1-AS1* was upregulated in advanced tumor stage, high-grade, lymph node metastasis, and vascular invasion CRC tissues. Therefore, we suggested *ZEB1-AS1* transcript might be contributed in the tumorigenic and metastatic processes of colorectal cancer.

In conclusion, our findings showed that *ZEB1-AS1* expression is increased in colorectal cancer and its expression is associated to pathological parameters of CRC patients such as tumor stage, grade, and metastasis and invasion status of CRC tissues. Altogether, our data suggest that ZEB1-AS1 might be contributed in colorectal cancer progression and could be considered as a novel tumor marker for diagnosis and treatment of colorectal cancer.

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