

Linc-ROR and its spliced variants 2 and 4 are significantly up-regulated in esophageal squamous cell carcinoma

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ABSTRACT

Objective(s): Similar characteristics of molecular pathways between cellular reprogramming events and tumorigenesis have been accentuated in recent years. Reprogramming-related transcription factors, also known as Yamanaka factors (OCT4, SOX2, KLF4, and c-MYC), are also well-known oncogenes promoting cancer initiation, progression, and cellular transformation into cancer stem cells. Long non-coding RNAs (lncRNAs) are a major class of RNA molecules with emerging roles in stem cell pluripotency, cellular reprogramming, cellular transformation, and tumorigenesis. The long intergenic non-coding RNA ROR (lincRNA-ROR, linc-ROR) acts as a regulator of cellular reprogramming through sponging miR-145 that normally negatively regulates the expression of the stemness factors NANOG, OCT4, and SOX2.

Materials and Methods: Here, we employed a real-time PCR approach to determine the expression patterns of *linc-ROR* and its two novel spliced variants (variants 2 and 4) in esophageal squamous cell carcinoma (ESCC).

Results: The quantitative real-time RT-PCR results revealed a significant up-regulation of *linc-ROR* ($P=0.0098$) and its variants 2 ($P=0.0250$) and 4 ($P=0.0002$) in tumor samples of ESCC, compared to their matched non-tumor tissues obtained from the margin of same tumors. Our data also demonstrated a significant up-regulation of variant 4 in high-grade tumor samples, in comparison to the low-grade ones ($P=0.04$). Moreover, the ROC curve analysis demonstrated that the variant 4 of ROR has a potential to discriminate between tumor and non-tumor samples ($AUC=0.66$, $P<0.05$).

Conclusion: Our data suggest a significant up-regulation of *linc-ROR* and its variants 2 and 4 in ESCC tissue samples.

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Introduction

Esophageal cancer is the 6th most cause of cancer-related death, and also it is the 8th most common cancer in the world (1, 2). Two main subtypes of esophageal malignancies, esophageal squamous cell carcinoma (ESCC; a common form of the cancer with a high incidence in the East) and adenocarcinoma (ADC; a rare form of the cancer with a high incidence in the West) have a five-year survival rate of less than 10%, and both of them are highly lethal (3). Golestan province in Northeast of Iran is one of the highest risk areas of ESCC form in the world. In this area, ESCC subtype is accounting for ~90% of all types of esophageal cancers (4).

Forced ectopic expression of OCT4, SOX2, KLF4, and c-MYC (known as Yamanaka factors; OSKM) could reprogram somatic fully differentiated cells to

an embryonic-like or pluripotent state (5, 6). Recent studies indicated OSKM factors in cancer initiation and progression; supporting the hypothesis that cellular reprogramming events and tumorigenesis processes share similar regulatory mechanisms (7). In line with the hypothesis, recent evidences suggest that cancer could be considered as a reprogramming-like process (8).

Long non-coding RNAs (lncRNAs) are a new class of RNA molecules which comprises the most proportion of human transcriptome. LncRNAs have emerged as main players in regulating key cellular pathways promoting proliferation, stem cells self-renewal, and cellular reprogramming events (9-13). Altered expression signatures of a large number of lncRNAs have been reported in several human malignancies (14, 15). The exact molecular

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mechanisms of lincRNAs functions have not yet been fully elucidated. However, some of their well-known functions in promoting cancer progression and maintaining stem cell pluripotency carry out through association with chromatin remodeling complexes and transcription factors (9, 13, 16).

Long intergenic ncRNA ROR (lincRNA-ROR, linc-ROR) is a multi-exon lincRNA gene located in the genomic region of human 18q21.3. Linc-ROR was initially introduced by Loewer and colleagues as a regulator of reprogramming (ROR) in human induced pluripotent stem cells (iPSc) (17). OCT4, SOX2, and Nanog transcription factors directly bind to the promoter region of linc-RoR gene to induce its expression. Interestingly, linc-RoR shares miR-145-response elements with that of the aforementioned transcription factors; and hence, it refrains microRNA-mediated suppression of OCT4, SOX2, and NANOG transcripts to maintain self-renewal and pluripotency status of iPSc (18).

Here, we explored a potential expression alteration of linc-ROR and its newly identified splice variants (2 to 5) in ESCC tissue samples. Since variants 3 and 5 had no expression levels in ESCC samples, we continued the work with linc-ROR variants 2 and 4.

Materials and Methods

Clinical ESCC samples

Fresh tumor and non-tumor tissue biopsies were obtained from the biobanks of Gastroenterology and Hepatology Research Center (Golestan University of Medical Sciences) and Buali (Avicenna) Research Institute (Mashhad University of Medical Sciences). The clinical samples were classified in two groups: 30 tumor biopsy samples obtained from patients with ESCC and 30 apparently normal tissue biopsies were taken from the margin of same tumor biopsies. Histopathological characteristic were evaluated according to the American Joint Committee on Cancer criteria and TNMG procedure for stage and grade classification (Table 1). The whole experimental procedure was approved by the Ethics Committee of Tarbiat Modares University and Golestan University of Medical Sciences. Written consents had been also obtained from all patients prior to surgical removal of their tumors.

RNA extraction and cDNA synthesis

Total RNA was extracted from both frozen tumor and non-tumor clinical samples, using Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA) and according to the manufacturer's instructions. To remove any potential contamination with DNA molecules, the extracted RNA was treated with a DNase enzyme (Takara, Japan) in an RNase-free condition. We then synthesized the first strand of complementary DNA (cDNA) by using the

Hyperscript RT reagent Kit (GeneAll, South Korea) and random hexamer primers (Takara, Japan), as described by the manufacturers.

Quantitative real-time PCR

Bioinformatics analysis was used to design specific PCR primers (Figure 1A, Table 2) to amplify the main and the variants 2 and 4 of linc-ROR (with the GenBank accession numbers of NR_048536.1, AB844430.1, and AB844432.1, respectively), using Gene Runner (version 3.05), PerlPrimer (version 1.1.21), and Oligo (version 7.56) softwares. Real Q plus 2x master mix Green (Ampliqon, Denmark) supplemented with ROX dye was used for all quantitative real-time PCR reactions. As an endogenous control, the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH, NM_002046.4) transcript was quantified, and the expression of linc-ROR and its variants were normalized to its expression level. All real-time PCR reactions were carried out by the ABI STEP ONE real-time PCR systems (Applied Biosystems, Foster City, CA) using the following cycling conditions: initiation at 95 °C for 15 min, amplification for 40 cycles with denaturation at 95 °C for 15 sec, annealing and extending at 63 °C for 55 sec. Moreover, we confirmed the authenticity of the PCR products by melt curve analysis and direct sequencing.

Statistical analysis

All real-time PCR experiments were carried out in duplicates. The LinReg PCR software (version 11) was used to calculate the exact PCR efficiency, and the real-time PCR data was adjusted based on these efficiencies. In order to calculate the expression fold change of the interest genes, the expression level of them in each clinical sample was normalized to that of GAPDH gene, as an internal control. We then normalized the expression of candidate genes in tumor samples to the matched non-tumor ones ($2^{-\Delta\Delta CT}$ method). To discriminate between tumor and

Table 1. The clinico-pathological characteristics of the patients with esophageal squamous cell carcinoma

Case number	Clinic-pathological Characteristics
65/26	Age (median)
16/14	Sex (male/female)
1/12/2/2/9/4	Stage (I/IIA/IIB/ IIA-III/III/unknown)
5/17/5/3	Grade (I/II/III/unknown)
16/10/4	Location (middle/lower/unknown)
1/1/3/1/20/1/3	Tumor invasion (T1/T1a/T2/T2a/T3/T4/unknown)

13/11/3/3 Regional lymph node (N0/N1/Nx/unknown)

Table 2. The list of primers used to specifically amplify the main and variants 2 and 4 of linc-ROR as well as the internal control, GAPDH

Transcript	Primer	sequence	PCR product (bp)
Linc-ROR	F	ACAAGGAGGAAAGGGCTGAC	124
	R	TTCTGGAAGCTAAGTGCATG	
AB844430 variant 2	F	AAGCAGCTGTGACCTGGC	179
	R	TCTGGCACCTAGCATAGCAC	
AB844432 variant 4	F	CAGCCAAAAATATCGTCAGAGT	265
	R	TCTTACTTAGCGACAATGCCATC	
GAPDH	F	ATGGGGAAGGTGAAGGTCC	108
	R	GGGGTCATTGATGGCAACAATA	

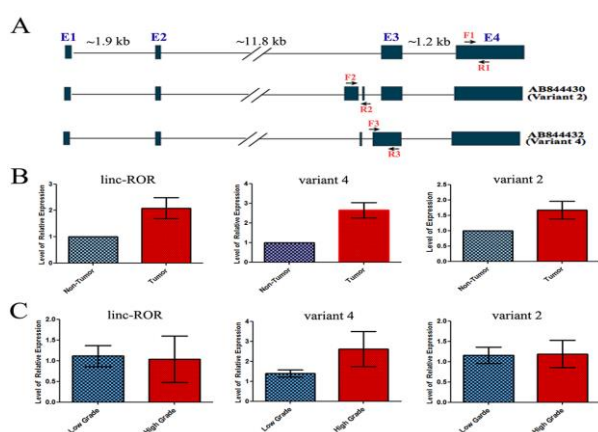


Figure 1. A) A representative schematic view of the genomic organization of linc-ROR and its spliced variants 2 and 4. The location of the forward and reverse primers used to amplify specifically each transcript is indicated with different set of arrows. B) Expression alteration of linc-ROR, and its splice variants 2 and 4 in ESCC tumor samples. The relative expression levels of linc-ROR and its splice variants 2 and 4 are represented in ESCC tumors vs. their matched non-tumor samples obtained from the same patients. An up-regulation of linc-ROR, and its splice variants in ESCC tumor vs non-tumor samples is evident. C) Relative gene expressions in high and low grades esophageal tumors. The red and blue boxes represent high grade (GIII) and low grade (GI and GII) tumor samples, respectively. Note that there is a significant up-regulation just for the linc-ROR variant 4 in high-grade tumors of ESCC

ESCC: esophageal squamous cell carcinoma

non-tumor clinical samples, as well as between different grades of tumor samples, receiver operating characteristic (ROC) curve analysis was plotted by Graphpad Prism (version 5.0.0.288).

Results

Up-regulation of linc-ROR in ESCC tissue samples

To quantify linc-ROR variants transcripts in ESCC samples, we designed specific primers on regions that were unique for each RNA transcript (Figure 1A). Our quantitative real-time RT-PCR data demonstrated a significant up-regulation of linc-ROR in tumor samples of ESCC, compared to the marginal non-tumor tissues obtained from the same patients (fold change 1/61, $P=0.0098$, Figure 1B). Next, we

investigated a potential association between linc-ROR expression and the grade of malignancy of ESCC

tumors. The data analysis, however, failed to show any association between the expression level of linc-ROR with the grade of malignancy of samples (Figure 2C). Moreover, there was no statistically significant difference of linc-ROR expression in tumor samples between different genders (data not shown).

Up-regulation of linc-ROR variants 2 and 4 in ESCC tissue samples

We next quantified the expression patterns of two novel variants of linc-ROR, variants 2 and 4, in ESCC tissue samples. Specific primers were designed in regions that were unique for each transcript (Figure 1A). The quantitative real-time PCR data revealed a significant up-regulation of variant 2 (fold change 1/67, $P=0.025$) and variant 4 (fold change 2/65, $P=0.0002$) in tumor specimens, in comparison to their paired non-tumor samples (Figure 1B). The data was unable to find any association between the expression levels of variant 2 with the grade of malignancy of the tumors (Figure 2B). In contrast, variant 4 had a significantly higher expression level in high grade (GIII) tumors, compared to the low grade (GI and GII) ones ($P=0.04$, Figure 1B). Moreover, there was no statistically significant difference between expression alteration of the linc-ROR variants 2 and 4 and the gender of patients (data not shown).

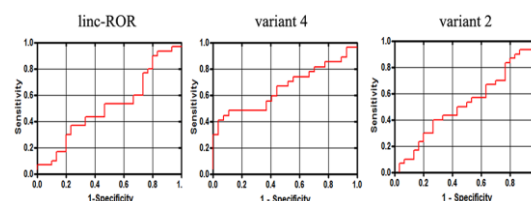


Figure 2. Analyzing the sensitivity and specificity of linc-ROR variants, as potential tumor markers of ESCC. ROC curve analysis demonstrated only a moderate sensitivity and specificity of linc-ROR spliced variant 4's expression level to discriminate between tumor and non-tumor states of ESCC tissue samples. Similarly, Area Under the Curve (AUC) was statistically significant only for variant 4 ($AUC=0.65$, $P=0.049$). An $AUC>0.70$ is considered as a good index for a biomarker with a high ability to correctly classify

tumor and non-tumor groups of samples. On the other hand, the ROC curve analysis failed to discriminate between high and low grade ESCC tumor samples

ESCC: esophageal squamous cell carcinoma

The expression level of *linc-ROR* spliced variant 4 could discriminate between tumor and non-tumor states of ESCC samples

We performed ROC curve analysis to evaluate the suitability of *linc-ROR* and its variants (2 and 4) expression levels to discriminate between tumor and non-tumor states of ESCC clinical samples. The data represent AUC=0.512 ($P>0.05$), AUC=0.522 ($P>0.05$), and AUC=0.656 ($P=0.049$) for *linc-ROR*, variant 2, and variant 4, respectively (Figure 2). In contrast to *linc-ROR* and variant 2, variant 4 showed a better suitability to classify tumor and non-tumor samples of esophagus. However, its AUC score, slightly fell below the score needed for a good biomarker (AUC>70). Moreover, in evaluation of different grades of malignancy and expression of the *linc-ROR* and its variants, ROC curve analysis failed to discriminate high and low grades tumor samples (data not shown).

Discussion

Up-regulation of several lncRNAs including *HOTAIR*, *MALAT1*, *PCAT-1*, *UCA1*, and *SOX2OT* have been reported in ESCC (19-24). Based on numerous similarities between molecular pathways of tumorigenesis and reprogramming events and the fact that *linc-ROR* promotes cellular reprogramming, we hypothesized that this non-coding RNA could have a part in cancer initiation and/or progression processes. For this reason, we aimed to investigate its expression pattern in ESCC clinical specimens. Furthermore, we examined a potential correlation between its expression pattern and histopathological characteristics of the ESCC tumor samples.

Our data revealed that *linc-ROR* and its spliced variants 2 and 4 were up-regulated in ESCC tumors samples, compared to their corresponding non-tumors ones obtained from the margin of same tumors. Moreover, our data demonstrated a significant up-regulation of variant 4 (but neither *linc-ROR* nor variant 2) in high-grade ESCC tumors, suggesting a potential role for variant 4 in ESCC tumor progression. Despite a statistically significant up-regulation of the main and spliced variants of *linc-ROR* in ESCC tissue samples, the data obtained from ROC curve analysis demonstrated that only variant 4 has a moderate sensitivity and specificity in discriminating between tumor and non-tumor ESCC samples.

Our data on elevated levels of *linc-ROR* transcripts in ESCC samples, is in agreement with recent studies reporting an up-regulation of *linc-ROR* in several other somatic cancers including breast cancer and hepatocellular carcinoma (25, 26). Overexpression of *linc-ROR* caused an epithelial to mesenchymal transition (EMT) process in immortalized human

mammary epithelial cells, probably through preventing the degradation of miR-205 target genes, such as the inducer of EMT known as ZEB2 (25). *linc-ROR* is also implicated in breast cancer cells metastasis, and the induction and maintenance of stemness properties to the pluripotent and cancer stem cells (25). *linc-ROR* was also highly up-regulated in hepatocellular cancer cells. The extracellular *linc-ROR* derived from hepatocellular tumor cells could modulate the chemosensitivity of the tumor cells (26). Moreover, human *linc-ROR* is a strong negative regulator of P53 transcription factor, in response to DNA damage. In contrast to MDM2 that causes P53 degradation, *linc-ROR* suppresses P53 translation, and p53-mediated cell cycle arrest and apoptosis (27).

In contrast to the aforementioned studies claiming an up-regulation of *linc-ROR* in some cancers, Feng et al. reported a significant down-regulation of *linc-ROR* expression in glioma tumors. Accordingly, ectopic expression of *linc-ROR* diminished cell proliferation, inhibited *KLF4* expression, and decreased the capacity of tumorosphere formation in U87 glioma cell line. The latter findings suggested that *linc-ROR* may act as a tumor suppressor gene in glioma (28).

Conclusion

Our data highlighted the up-regulation of *linc-ROR* and its splice variants 2 and 4 in ESCC. Our data also propose the existence of more similarities in molecular pathways common between tumorigenesis and reprogramming events. Altogether, our data suggest that *linc-ROR* transcripts might have a potential role in tumor initiation and/or progression of esophagus cancer. However, this conclusion needs further functional analysis for experimental validation.

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