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A novel positive feedback loop of linc02042 and c-Myc mediated by YBX1 promotes tumorigenesis and metastasis in esophageal squamous cell carcinoma

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Abstract

Background: Long non-coding RNA (lncRNA) is a class of endogenous RNA with a length of more than 200 nucleotides, which is emerging as a pivotal player in cancer development and progression. However, the functional roles of many members in this class remain largely uncharacterized. In the present study, we explored the biological relevance of linc02042 in esophageal squamous cell carcinoma (ESCC).

Methods: qRT-PCR was used to detect the levels of linc02042 and c-Myc. Western blot was used to assess protein expression level. CCK-8 and Transwell assays were employed to test ESCC cell proliferation and invasion, respectively. The mice study including xenograft tumor and lung metastasis models was used to determine the role of linc02042 in vivo. RNA pull-down, ChIP and luciferase reporter assays were employed to test the relationship between linc02042, YBX1 and c-Myc.

Results: Linc02042 was found to be markedly upregulated in ESCC cell lines, tissues and plasma, and was closely correlated with malignant clinical features. Knockdown of linc02042 significantly inhibited ESCC cell viability and invasion in vitro as well as tumor growth and lung metastasis in vivo, whereas overexpression of linc02042 resulted in the opposite results. Mechanistically, linc02042 acted as a scaffold for YBX-1 binding to the 3'-UTR of c-Myc mRNA, leading to enhanced c-Myc mRNA stability, thereby facilitating ESCC growth and metastasis. Moreover, in turn, c-Myc was able to transcriptionally elevate linc02042 by directly binding to the E-box motif proximal to the transcription start site (TSS) of linc02042 promoter. Clinically, linc02042 was identified as an effective diagnostic and prognostic biomarker for ESCC patients, and its expression was strongly positively correlated with c-Myc expression in ESCC tissues.

Conclusion: Our data suggest that linc02042 plays an important tumor-promoting role in ESCC, which lays a foundation for considering it as a potential target for ESCC patients.

Keywords: Long non-coding RNA, ESCC, Biomarker, mRNA stability

Background

Esophageal cancer is a common aggressive malignancy with the seventh highest morbidity and mortality [1]. Every year, a large number of patients die from the disease worldwide, especially in China, with an average annual of about 150,000 deaths [2]. Esophageal squamous cell carcinoma (ESCC) is the major histological type of

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esophageal cancer, its 5-year survival rate is poor due to recurrence, metastasis or chemotherapy resistance [3]. Therefore, it is urgent to elucidate the pathogenesis of ESCC to provide new diagnostic and therapeutic targets for ESCC patients.

Long non-coding RNA (lncRNA) refers to a kind of endogenous RNA that is over 200 nucleotides in length and lacks protein coding potential [4]. lncRNA was initially considered as transcriptional noise, but subsequent studies have shown that many lncRNAs are only expressed in specific parts of the body in specific physiological states, or only in certain biological processes [5]. Knocking down of specific lncRNA could lead to phenotypic changes, thus proving that it has important biological functions. In fact, the current research on lncRNA covers almost all physiological and pathological processes, including the occurrence and development of cancer [6, 7].

It has been well documented that the mechanism of lncRNA functioning is mainly as a molecular sponge of microRNAs (miRNAs), in which lncRNA can directly bind to miRNAs and reduce the repressive effects of miRNAs on their different target genes [8]. However, in addition to this, a growing number of studies have shown that lncRNA can also directly bind to proteins to regulate gene expression, thus participating in the progression of cancer [9]. For instance, lncRNA GIAT4RA was shown to interact with LSH and block UCHL3-mediated deubiquitination of LSH, thereby inhibiting non-small cell lung cancer progression [10]. Zhang et al. reported that MEG3 directly bound to p-STAT3, resulting in p-STAT3 ubiquitination and degradation and inhibition of proliferation of cervical cancer cells [11]. However, this functional model of lncRNA/protein interaction in ESCC remains poorly understood.

A recent study performed genome-wide screening in ESCC and normal tissues and found that linc02042 was significantly upregulated in ESCC tissues [12], however, its molecular function and biological role are still not clear. Herein, we confirmed the overexpression of linc02042 in ESCC, and further elucidated its carcinogenic role as a protein binding partner.

Materials and methods

ESCC samples and cell lines

ESCC tissues and plasma in our study were all collected from Henan Provincial Chest Hospital, and patients who had received preoperative chemoradiotherapy were excluded. The detailed clinical data of patients are presented in Table 1. Routine follow-up was conducted every 3 months. All patients signed the informed consent. This study was approved by the Ethics Committee of Henan Provincial Chest Hospital. Five ESCC cell lines and one

Table 1 Correlation between linc02042 expression and clinicopathological features in ESCC patients (n = 98)

Parameters	All cases	linc02042 expression		p value
		Low (n = 49)	High (n = 49)	
Gender				
Male	58	28	30	0.681
Female	40	21	19	
Age (years)				
≤ 65	51	26	25	0.840
> 65	47	23	24	
Tumor size				
≤ 3	36	25	11	0.003
> 3	62	24	38	
Differentiation				
Well/moderate	41	26	15	0.024
Poor	57	23	34	
TNM stage				
I–II	58	35	23	0.014
III–IV	40	14	26	
Lymph node metastasis				
No	59	38	21	0.000
Yes	39	11	28	
Smoking				
No	37	20	17	0.532
Yes	61	29	32	
Drinking				
No	41	21	20	0.838
Yes	57	28	29	

normal esophageal squamous epithelium HET-1A cells were commercially obtained from Shanghai Institute of Biological Sciences, Chinese Academy of Sciences. They all cultured in DMEM medium supplemented with 10% fetal bovine serum in the incubator.

Identification of the subcellular localization of linc02042

The subcellular localization of linc02042 was determined by Nuclear-Cytoplasmic isolation and fluorescence in situ hybridization (FISH) assays, which were respectively performed by using the Cytoplasmic & Nuclear RNA Purification (Norgen Biotek Corp, ON, CAN) and RiboTM Fluorescent In Situ Hybridization (RiboBio, Guangzhou, China) kits in accordance with the instructions from manufacturers.

Reverse transcription quantitative polymerase chain reaction (qRT-PCR)

Total RNA from ESCC tissues and cultured cells was extracted by Trizol reagent (Invitrogen, CA, USA) according to the standard protocol. Then, cDNA was synthesized using Superscript First-Strand Synthesis System

(Invitrogen), followed by PCR amplification and quantification using SYBR[®] Green qPCR SuperMix (Invitrogen) with specific primers. The expression level of genes relative to GAPDH were calculated by $2^{-\Delta\Delta C_t}$ method. The assay was repeated three times independently.

CCK-8 and Transwell assays

Cell viability was detected by CCK-8 assay using CCK-8 solution (Dojindo, Kumamoto, Japan) in accordance with the manufacturer's instruction. For cell invasion assay, the indicated cells were seeded onto 24-well culture plate mounted with Transwell chamber. After incubation for 2 days, the cells on the upper surface of the chamber were removed, and the cells on the lower surface were stained with crystal violet. The analysis was performed based on five random field under the microscope.

In vivo tumorigenicity and lung metastasis

The animal experiment was approved by the Committee on Animal Care of Henan Provincial Chest Hospital. For the xenograft tumor model, a total of 10 nude mice were randomly divided into two groups ($n=5$ per group), followed by subcutaneous injection of 1×10^7 linc02042-depleted or control KYSE30 cells into nude mice. Tumors were measured every week. In the fifth week, all mice were sacrificed and tumor tissues were collected and weighed. For the lung metastasis model, 1×10^6 linc02042-depleted or control KYSE30 cells were tail vein injected into nude mice ($n=5$ per group), and the lung metastatic nodules were counted 6 weeks after injection.

Western blot

Total protein from ESCC tissues and cultured cells was extracted by lysis buffer on the ice and separated on 10% SDS-PAGE gel. Then, the protein was transferred onto PVDF member and blocked by 5% non-fat milk powder for 30 min. The member was incubated with anti-c-Myc (#9402, CST, 1:1000 dilution) and anti-YBX1 (#9744, CST, 1:2000 dilution) primary antibodies at 4 °C overnight. The next day, the member was incubated with anti-rabbit IgG secondary antibody for 1 h at room temperature. Lastly, the member was exposed with ECL solution in the darkroom.

Luciferase reporter assay

The promoters of c-Myc and linc02042 were respectively cloned into pGL3-basic vector (Promega, WI, USA) and co-transfected with 5ng pRL-TK-Renilla into KYSE-30 and KYSE-150 cells using Lipofectamine 2000 (Invitrogen) as per manufacturer's protocol. After 48 h of transfection, the luciferase activity was detected by Dual-Luciferase Reporter Assay System (Promega) as per manufacturer's protocol.

RNA pull-down and RNA immunoprecipitation (RIP) assays

The linc02042 and anti-sense biotin-labeled probes were in vitro synthesized and labeled by using T7 High Yield RNA Synthesis Kit (Ambion, TX, USA) and RNA 3' End Biotinylation Kit (Thermo, Waltham, MA), respectively. After that, the probes were incubated with whole protein lysate extracted from KYSE-30 and KYSE-150 cells at 4 °C overnight. Subsequently, the protein-probe complex was incubated with BeaverBeads[™] Streptavidin magnetic beads (Beaver, Suzhou, China) for 2 h at room temperature. Then, the bead-probe-protein complex was washed six times and subjected for Western blot analysis. RIP assay was performed using Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, MA, USA) according to the manufacturer's instructions using anti-YBX1 (#9744, CST) antibody.

Chromatin immunoprecipitation (ChIP) assay

KYSE-30 and KYSE-150 cells were fixed with 1% formaldehyde for 10 min for the cross-link between protein and DNA, followed by quenching with 0.125 M glycine for 5 min. Then, DNA was sonicated into 200–1000 bp in length. The sample was incubated with anti-c-Myc (#9402, CST, 1:50) at 4 °C overnight, followed by incubation with ChIP-Grade Protein G Agarose Beads (#9007, CST) at 4 °C for 1 h, and the complex was washed and eluted by Elution buffer (1% SDS, 0.1 M NaHCO₃). Lastly, the enriched fragments were subjected for qPCR analysis.

Statistical analysis

All statistical results were analyzed by SPSS 22.0 software (IBM, NY, USA) and figures were generated by Graphpad Prism v5 software (Graphpad, CA, USA). Differences were determined by Student's t test for two groups and by one-way ANOVA for three or more groups. $p \leq 0.05$ was considered significant. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Results

Linc02042 expression is significantly upregulated in ESCC

We collected 98 pairs of ESCC and adjacent normal tissues to detect linc02042 expression. As shown in Fig. 1a, linc02042 was notably increased in ESCC tissues as compared to normal tissues. And this upregulation was also observed in five ESCC cell lines (Fig. 1b). We then evaluated the relationship between linc02042 expression and clinicopathological features of ESCC patients, the results showed that high linc02042 was positively correlated with malignant features, including larger tumor size, poor differentiation, advanced TNM stage and lymph node metastasis (Table 1). Further, we found that linc02042 was also overexpressed in ESCC plasma in

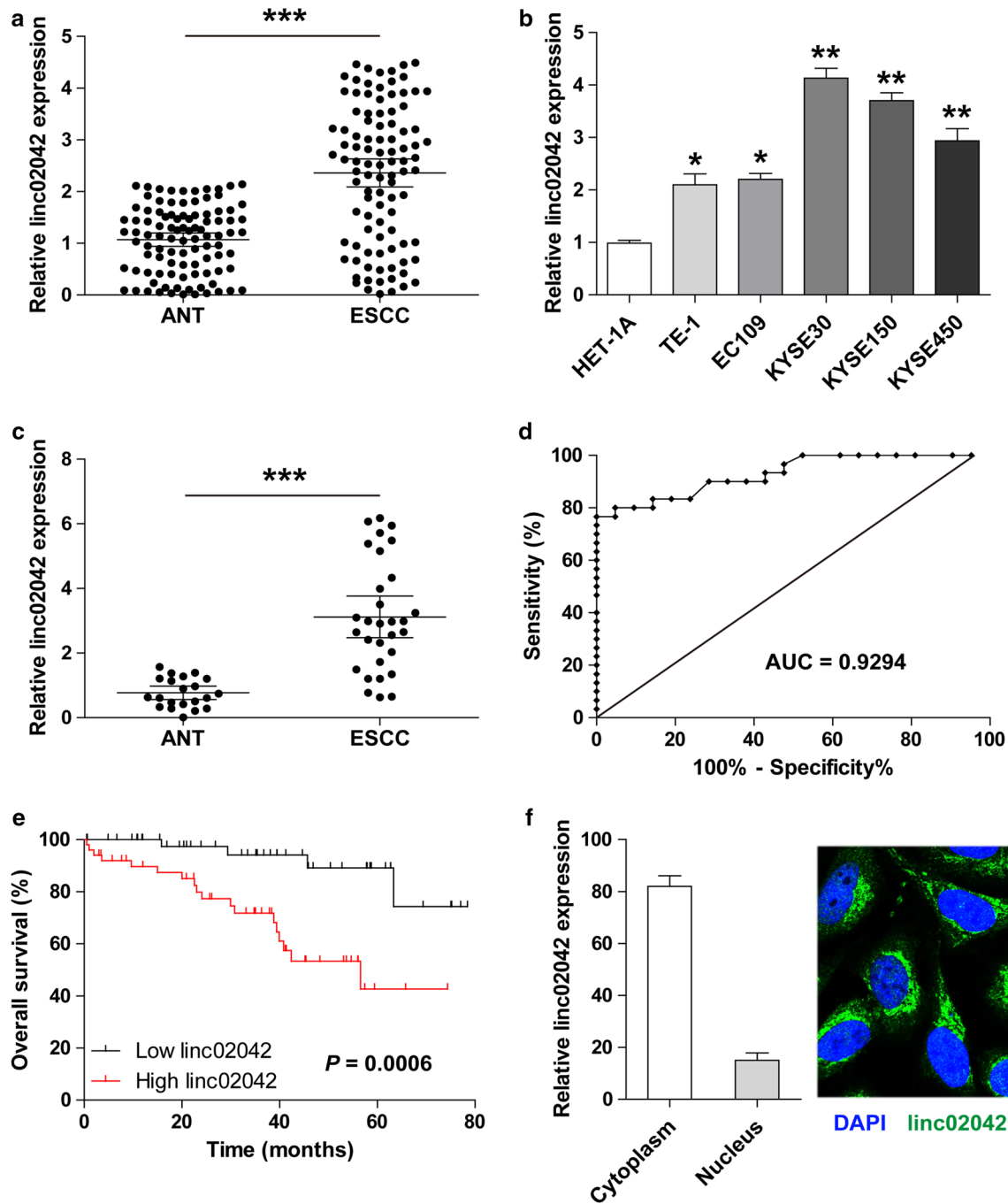


Fig. 1 Linc02042 is upregulated in ESCC. **a, b** qRT-PCR analysis of linc02042 expression in ESCC tissues (n = 98) and cell lines. **c** qRT-PCR analysis of linc02042 expression in plasma samples from ESCC patients and healthy controls (n = 30). **d** The ROC curve showing the diagnostic value of plasma linc02042 level. **e** The survival curve showing the prognostic value of linc02042 in ESCC. **f** The location of linc02042 detected by qRT-PCR analysis and FISH assay in HET-1A cells. **p* < 0.05, ***p* < 0.01, ****p* < 0.001

comparison to healthy control plasma (Fig. 1c), and the receiver operating characteristic (ROC) curve showed that the area under the curve (AUC) value was 0.9294 (95%CI 0.8638 to 0.9949) (Fig. 1d), implying that plasma

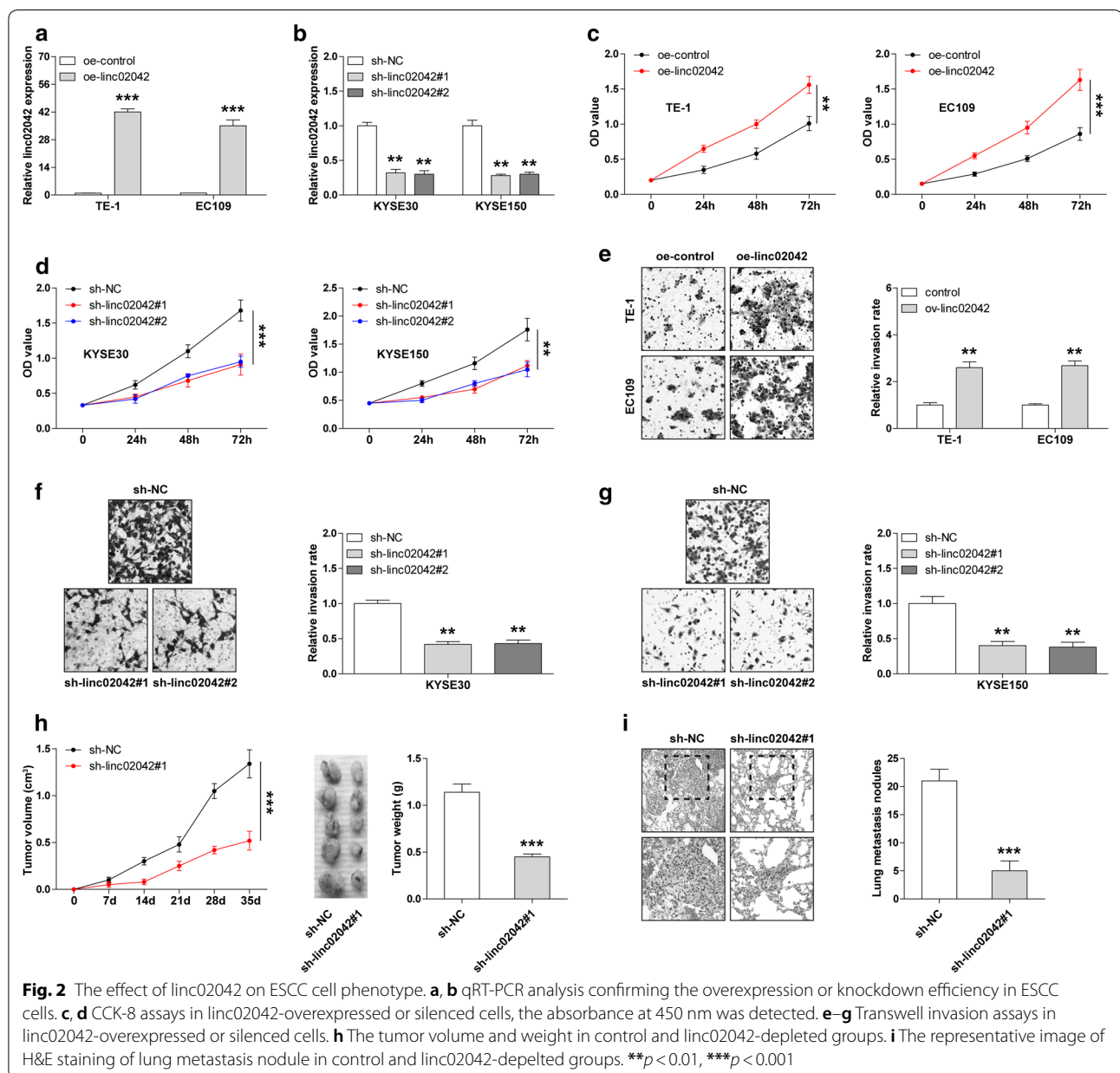
linc02042 has an excellent diagnostic efficacy for ESCC. Kaplan–Meier plotter displayed that patients with high linc02042 had shorter overall survival time than those with low linc02042 (Fig. 1e). Besides, we assessed the

subcellular localization of linc02042 by using qRT-PCR and FISH assays, and the results showed that linc02042 was predominantly located in the cytoplasm (Fig. 1f). These above data suggest that linc02042 is frequently overexpressed in ESCC and may play an essential role in the pathogenesis of ESCC.

Linc02042 promotes ESCC cell proliferation and invasion both in vitro and in vivo

To explore the biological function of linc02042, we generated the stable linc02042-overexpressed and -silenced ESCC cell lines by using pCDH-CMV-MCS-EF1-Puro

lentiviral vector (Fig. 2a, b). The CCK-8 results showed that overexpression of linc02042 markedly strengthened cell viability (Fig. 2c), while linc02042 knockdown dramatically weakened cell viability (Fig. 2d). Similarly, exogenous linc02042 expression enhanced cell invasiveness (Fig. 2e), whereas depletion of linc02042 resulted in an opposite effect (Fig. 2f, g). Further, we established in vivo tumorigenesis and metastasis models by subcutaneous and caudal vein injection of linc02042-depleted KYSE30 cells into nude mice, respectively. The results showed that the tumor volume/weight and lung metastasis nodules in sh-linc02042 group were less than those in control

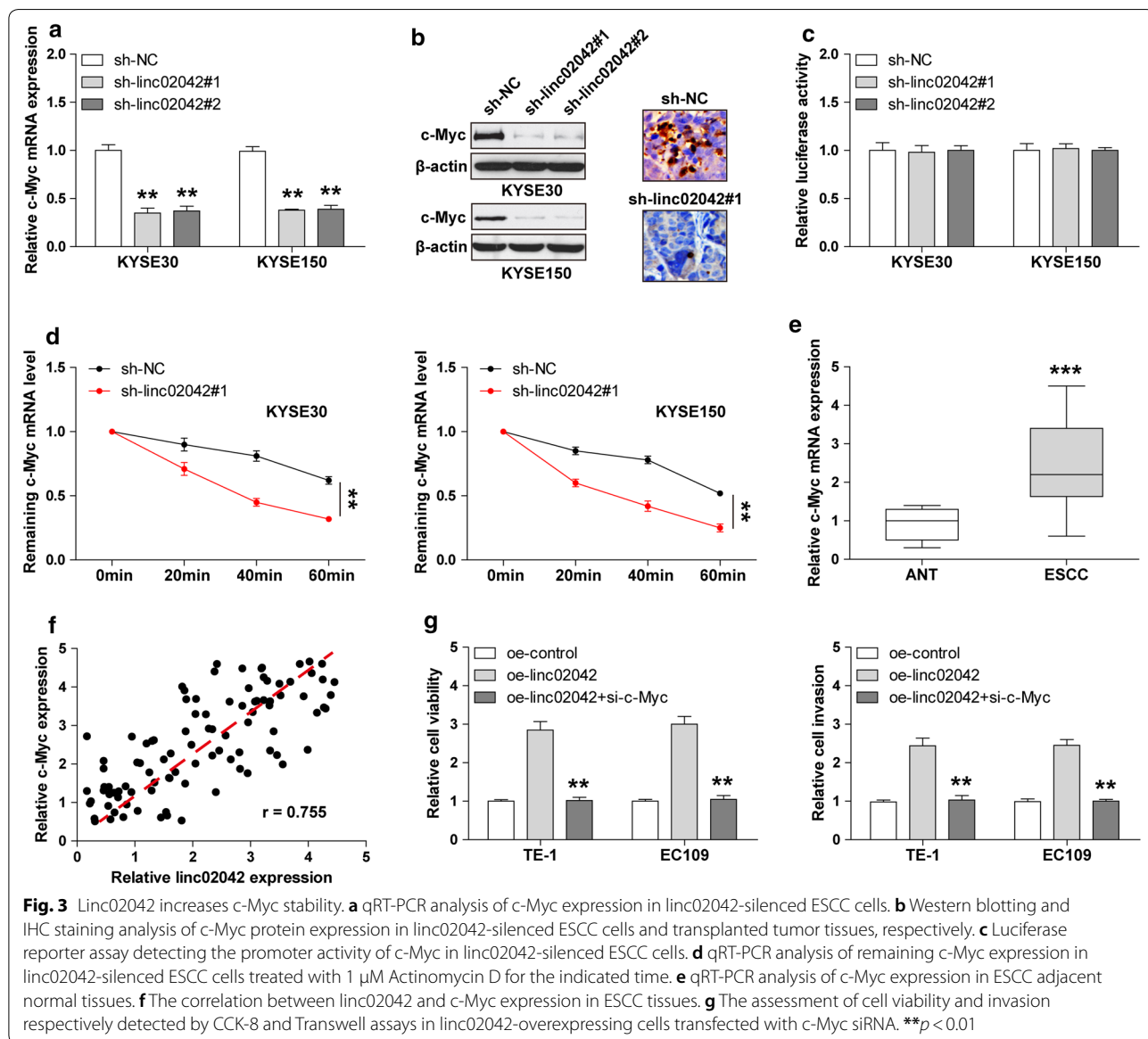


group (Fig. 2h, i). These findings indicate that linc02042 enhances ESCC cell aggressive phenotype.

Linc02042 increases the stability of c-Myc mRNA

Through analyzing the TCGA ESCC database using cBioPortal software, we found that linc02042 was positively linked with c-Myc, a well-known proto-oncogene. Consistently, c-Myc mRNA as well as protein levels were significantly decreased in linc02042 knockdown ESCC cells in comparison with control cells (Fig. 3a, b). Moreover, the IHC staining results showed that less c-Myc positive cells were observed in linc02042-depleted transplanted tissues compared to control tissues (Fig. 3b). To test whether linc02042 affected c-Myc

expression at the transcriptional level, we performed the luciferase reporter assay by inserting the promoter of c-Myc into pGL3-basic vector. As shown in Fig. 3c, the promoter activity of c-Myc was not affected after depletion of linc02042, implying that c-Myc was controlled by linc02042 at the post-transcriptional level. We then tested the mRNA stability via treating cells with Actinomycin D, a transcription inhibitor. Surprisingly, knockdown of linc02042 shortened the half-life of c-Myc mRNA from more than 60 min to about 35 min (Fig. 3d). Besides, we found that c-Myc expression was substantially overexpressed in ESCC tissues (Fig. 3e) and strongly positively correlated with linc02042 expression ($r=0.755$) (Fig. 3f). Importantly, overexpression of linc02042 could not



enhance cell viability and invasiveness in the absence of c-Myc (Fig. 3g). These data suggest that c-Myc is indispensable for the tumor-promoting function of linc02042 in ESCC.

YBX1 mediates the regulation of linc02042 on c-Myc

Given that YBX1 acts as an important regulator of c-Myc mRNA stability via binding to its 3'-UTR [13] and that numerous lncRNAs have been found to be bound by YBX1 [14], we thus inferred that linc02042 might increase c-Myc mRNA stability via YBX1. To confirm this inference, we first test the possibility of binding between linc02042 and YBX1 by using RPISeq database, the results showed that linc02042 has a strong possibility of direct interaction with YBX1 (RF classifier = 0.88, SVM classifier = 0.9) (Fig. 4a). Next, we performed the RNA pull-down assay, as shown in Fig. 4b, endogenous

YBX1 was abundantly enriched by linc02042 probe. And RIP assay coupled qRT-PCR analysis further confirmed this interaction, in which more linc02042 was immunoprecipitated by YBX1 antibody as compared with IgG antibody (Fig. 4c). However, linc02042 knockdown did not affect YBX1 protein expression (Fig. 4d). Importantly, we found that YBX1 was abundantly enriched on the 3'-UTR of c-Myc mRNA, but this phenomenon was eliminated in the absence of linc02042 (Fig. 4e), suggesting that linc02042 is required for the binding of YBX1 to c-Myc mRNA. Further, overexpression of linc02042 could not increase c-Myc expression after depletion of YBX1 (Fig. 4f), and the enhanced aggressive phenotype caused by linc02042 overexpression was substantially blocked by YBX1 knockdown (Fig. 4g). These results indicate that linc02042 and YBX1 form a dimer complex that regulates c-Myc expression.

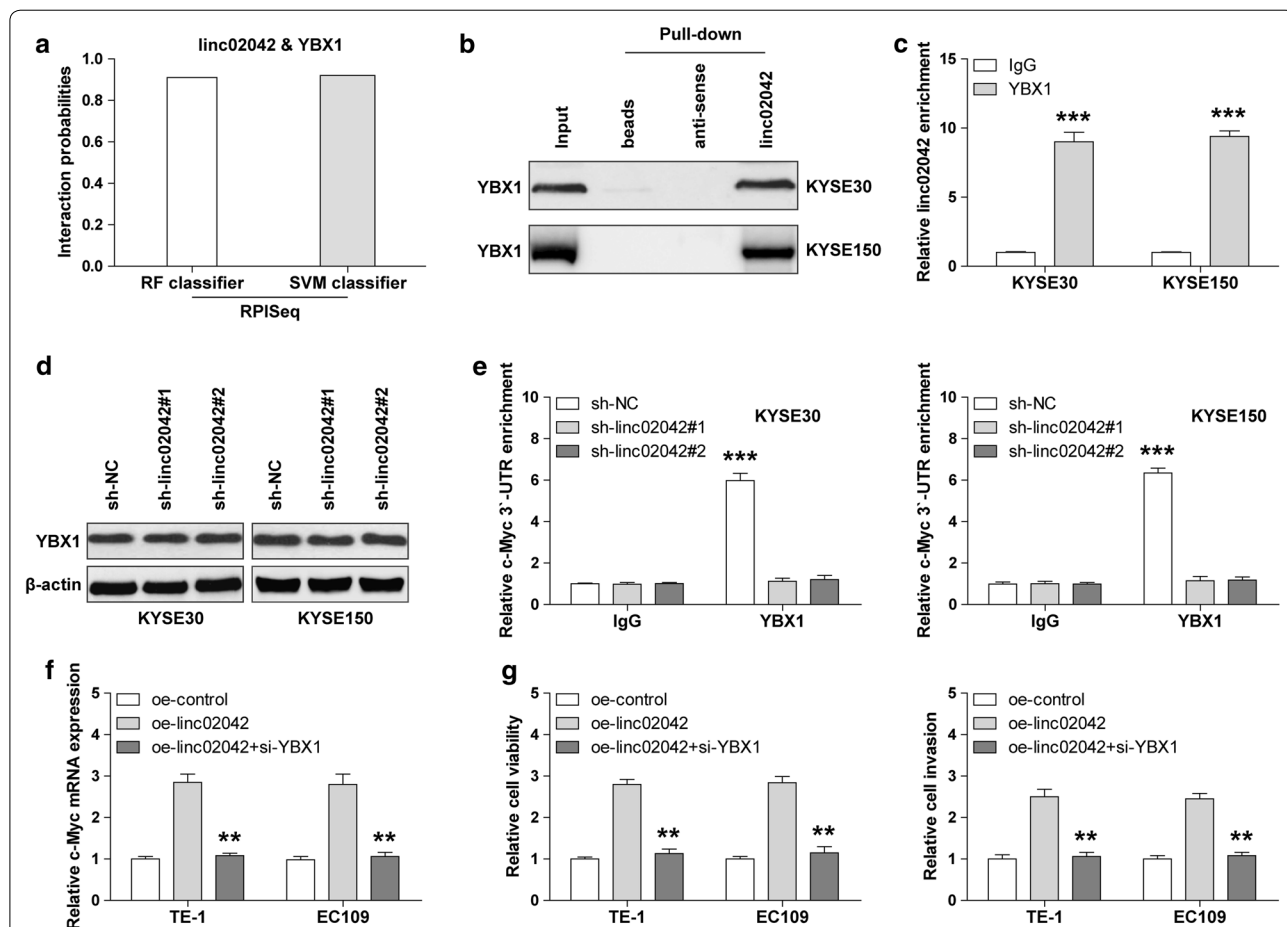
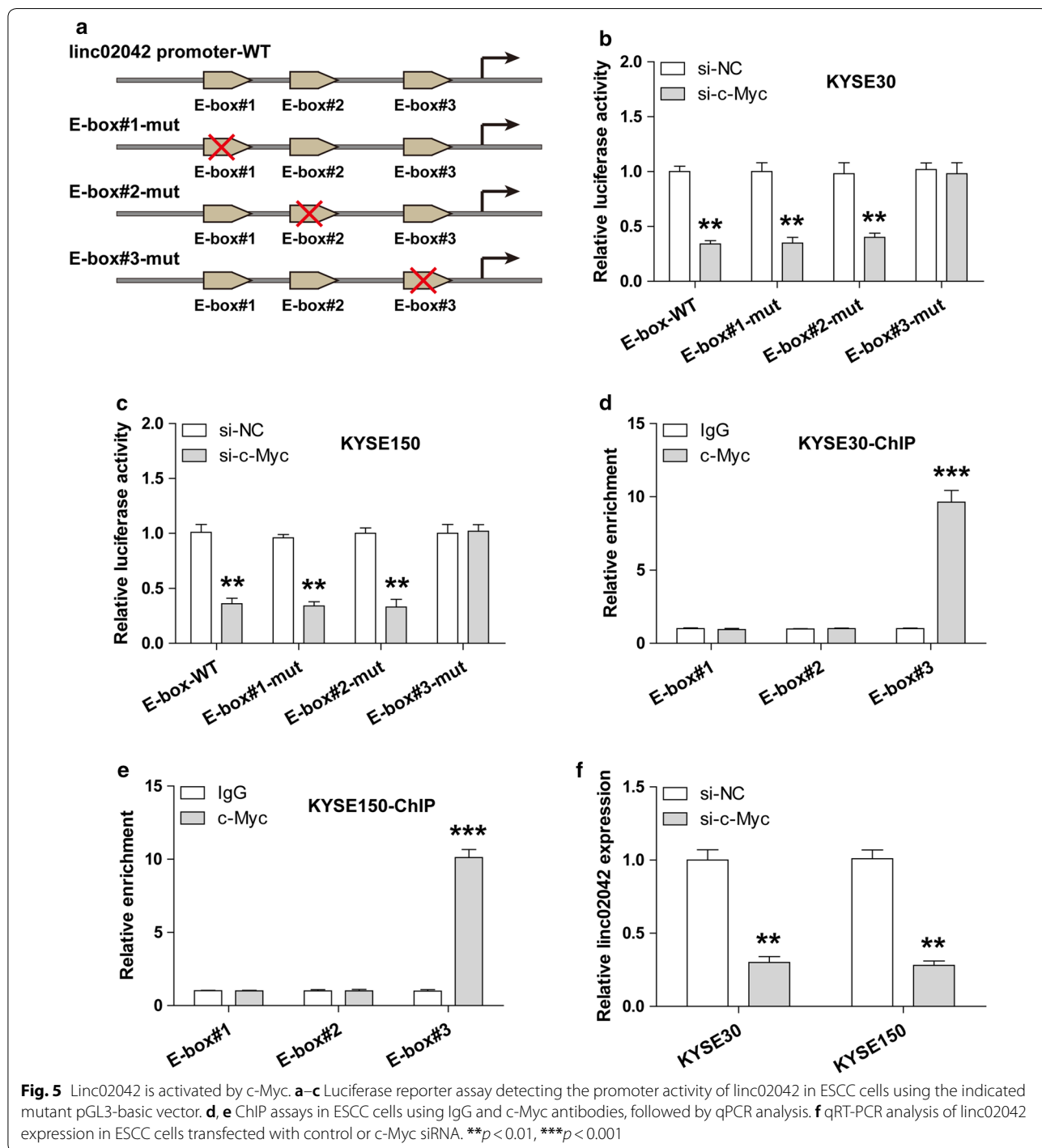


Fig. 4 Linc02042 interacts with YBX1. **a** RPISeq database predicting the interaction probability between linc02042 and YBX1. Both RF and SVM classifier greater than 0.5 mean positive. **b** RNA pull-down coupled Western blotting analysis in ESCC cells. **c** RIP assays in ESCC cells with IgG and YBX1 antibodies, followed by qRT-PCR analysis of linc02042 expression. **d** Western blotting analysis of YBX1 protein expression in linc02042-depleted ESCC cells. **e** RIP assays in ESCC cells with IgG and YBX1 antibodies in linc02042-depleted ESCC cells. **f** qRT-PCR analysis of c-Myc expression in linc02042-overexpressed cells treated with YBX1 siRNA. **g** The assessment of cell viability and invasion respectively detected by CCK-8 and Transwell assays in linc02042-overexpressing cells transfected with YBX1 siRNA. ***p* < 0.01, ****p* < 0.001

c-Myc trans-activates linc02042 expression

Intriguingly, three E-box motifs (c-Myc binding site) were found on the linc02042 promoter, we thus assumed that linc02042 might be also regulated by c-Myc. To test this hypothesis, we constructed a series of pGL3-basic luciferase vector (Fig. 5a) and performed the luciferase

reporter assay. The results showed that silencing of c-Myc significantly reduced the promoter activity of wild-type vector in both KYSE30 and KYSE150 cells, whereas this phenomenon was eliminated after mutation of E-box#3 rather than E-box#1 or E-box#2 (Fig. 5b, c). Further, the CHIP assay results revealed that c-Myc directly bound to



E-box#3, but not E-box#1 or E-box#2 (Fig. 5d, e). Consistently, linc02042 expression was evidently decreased in c-Myc-silenced KYSE30 and KYSE150 cells in comparison to control cells (Fig. 5f). These data suggest that linc02042 is transcriptionally controlled by c-Myc.

Discussion

ESCC is a common malignant tumor with high heterogeneity, which makes its pathogenesis and progression extremely complicated. In the current study, we found a novel ESCC-related lncRNA, linc02042, which was significantly increased in ESCC tissues, cells and plasma. Gain and loss functional assays showed that linc02042 promoted ESCC cell proliferation and invasion both in vitro and in vivo. Stepwise mechanism studies revealed that linc02042 directly interacted with YBX1 and increased the binding of YBX1 to c-Myc 3'-UTR, resulting in potentiating c-Myc mRNA stability, thereby facilitating ESCC malignant progression. Therefore, our data highlight the importance of lncRNA in ESCC and also advance the understanding of the regulatory mechanism of proto-oncogene c-Myc.

A large number of studies have reported that non-coding RNA is an effective cancer biomarker, especially lncRNA with length over 200 nt. For example, lncRNA MALAT1 [15], PIK3CD-AS1 [16], linc-ZNF469-3 [17] and LINC01133 [18] were respectively identified as diagnostic or prognostic biomarkers of colorectal cancer, hepatocellular carcinoma, triple negative breast cancer and gastric cancer. Herein, ESCC patients with high linc02042 expression displayed shorter survival time than those with low linc02042 expression, implicating the prognostic value of linc02042 in ESCC. In addition, we also found that plasma linc02042 was notably over-expressed in ESCC patients in comparison with healthy controls, and the AUC value is 0.9294, indicating that plasma linc02042 level is an excellent non-invasive diagnostic biomarker of patients with ESCC. Further investigation in large-scale samples is needed to confirm the clinical implication of linc02042.

Increasing evidence suggests that lncRNA participates in cancer occurrence development by acting as a protein-binding partner [19]. Herein, we found that linc02042 could directly bind to YBX1 by performing RNA pull-down and RIP assays. YBX1 is a multifunctional oncoprotein involved in cancer cell growth, metastasis and chemotherapy resistance that can modulate mRNA stability by bind to AU-rich elements (ARE) on mRNA 3'-UTR [20]. To date, numerous lncRNAs have been identified to interact with YBX1, such as HOXC-AS3 [21] and LINC00312 [22], and YBX1 was significantly upregulated in human cancers [23]. However, in this study, the interaction between linc02042 and YBX1 did not increase

YBX1 protein level, but rather recruited YBX1 to the 3'-UTR of c-Myc mRNA to stabilize c-Myc. This notion was confirmed by the RIP assay in which linc02042 knockdown substantially decreased the binding of YBX1 to c-Myc 3'-UTR. c-Myc is a well-known oncogene that activates a variety of oncogenic signaling pathways [24]. c-Myc is shown to be frequently overexpressed in multiple cancers, and its expression is tightly controlled by different factors at the transcription or post-transcription level [25]. Up to now, only a few studies focused the regulatory role of lncRNA on c-Myc mRNA stability. Here, we found that linc02042 increased c-Myc mRNA stability, and the pro-tumor effect of linc02042 was practically disappeared in the absence of c-Myc, suggesting that c-Myc is required for the function of linc02042 in ESCC. Besides, silencing of YBX1 evidently blocked the increased c-Myc level caused by linc02042 overexpression, indicating that linc02042 regulates c-Myc mRNA stability in an YBX1-dependent mechanism.

Of note, c-Myc is also a key transcription factor that regulates gene expression via directly binding to the E-box motif on the promoter [26]. Many genes have been identified as the downstream targets of c-Myc, including lncRNAs, such as MINCR [27] and IDH1-AS1 [28]. In the present study, we found three E-box motifs on linc02042 promoter, thus speculated that linc02042 might be also regulated by c-Myc. By performing a series of assays, we confirmed above hypothesis, in which c-Myc was able to directly bind to the E-box motif proximal to TSS on linc02042 promoter and increase linc02042 expression. This positive feedback loop between linc02042 and c-Myc magnifies the pro-tumor effect of linc02042 in ESCC.

Conclusion

Taken together, our study for the first time describes the important biological functions of linc02042 in ESCC, and meanwhile provides a novel biomarker and therapeutic target for patients with ESCC. Whether linc02042 also plays the fundamental role in other malignancies is worth further investigation.

Abbreviations

lncRNA: Long non-coding RNA; ESCC: Esophageal squamous cell carcinoma; miRNAs: microRNAs; FISH: Fluorescence in situ hybridization; CCK-8: Cell Counting Kit-8; RIP: RNA immunoprecipitation; ChIP: Chromatin immunoprecipitation; UTR: Untranslated region.

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None.

Authors' contributions

WY designed this study and drafted the manuscript; JD, GZ, HQ conducted the experiments and analyzed the results. HY provided technical guidance. All authors read and approved the final manuscript.

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Availability of data and materials

Please contact authors for data request.

Ethics approval and consent to participate

This study was performed in accordance with institutional ethical guidelines and was approved by the Ethics Committee of Henan Provincial Chest Hospital. Informed written consent was obtained from each participants.

Consent for publication

All authors approved publication of the manuscript.

Competing interests

The authors report no conflicts of interest in this work.

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