A Novel Micropeptide Encoded by Y-Linked LINC00278 Links Cigarette Smoking and AR Signaling in Male Esophageal Squamous Cell Carcinoma

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ABSTRACT

Long noncoding RNAs (lncRNA) have been shown to play critical roles in many diseases, including esophageal squamous cell carcinoma (ESCC). Recent studies have reported that some lncRNA encode functional micropeptides. However, the association between ESCC and micropeptides encoded by lncRNA remains largely unknown. In this study, we characterized a Y-linked lncRNA, LINC00278, which was downregulated in male ESCC. LINC00278 encoded a Yin Yang 1 (YY1)-binding micropeptide, designated YY1BM. YY1BM was involved in the ESCC progression and inhibited the interaction between YY1 and androgen receptor (AR), which in turn decreased expression of eEF2K through the AR signaling pathway. Downregulation of YY1BM significantly upregulated eEF2K expression and inhibited apoptosis, thus conferring ESCC cells more adaptive to nutrient deprivation. Cigarette smoking decreased m6A modification of LINC00278 and YY1BM translation. In conclusion, these results provide a novel mechanistic link between cigarette smoking and AR signaling in male ESCC.

Significance: Posttranscriptional modification of a micropeptide-encoding lncRNA is negatively impacted by cigarette smoking, disrupting negative regulation of the AR signaling pathway in male ESCC.

Introduction

Esophageal squamous cell carcinoma (ESCC) is two to four times more common in men than in women worldwide (1). Previous studies suggest that several male-specific factors contribute to such gender disparity, including cigarette smoking and sexual hormone. A survey in 2010 indicated that 52.9% of Chinese men while only 2.4% of Chinese women were current smokers (2, 3). Expression of androgen receptors has been reported in ESCC as well as association with prognosis (4, 5). However, the exact underlying molecular mechanisms in male ESCC progression remain largely unknown.

A recent study identified a tumor suppressor gene on Y chromosome for male breast cancer (6), suggesting that genetic material encoded by Y chromosome could be involved in male-dominant tumors. Long noncoding RNAs (lncRNA) are defined as RNA transcripts longer than 200nt that lack protein-coding potential (7, 8). LncRNAs act as master regulators for gene expression, thus play an important role in many biological functions and diseases, including cancer (9). However, no study so far has reported on the involvement of Y-linked lncRNAs in ESCC.

Recent computational and genome-wide studies have demonstrated that hundreds of functional micropeptides (<100 amino acids) are embedded in lncRNAs. For example, myomixer is an 84-amino acid micropeptide encoded by a lncRNA that controls the critical steps in myofiber formation during muscle development (10); myoregulin is identified as a skeletal muscle-specific IncRNA, which regulates muscle performance by impeding Ca2+ uptake into the sarcoplasmic reticulum (SR; ref. 11). It is still unclear whether micropeptides play a key role in tumor development, although a recent study has identified a micropeptide encoded by HOXB-AS3 IncRNA that suppresses colon cancer growth (12).

N6-methyladenosine (m6A) is the most abundant posttranscriptional modification on eukaryotic mRNAs and IncRNAs (13). Recent studies...
show that m’A modification is dynamic and reversible in cells, whose level is regulated by m’A methyltransferases (also called “writers”: METTL3, METTL14, etc.) and m’A demethylases (also called “erasers”: FTO, ALKBH5, etc.). m’A regulates gene expression through m’A binding proteins (also called “readers”: YTHDF1, YTHDF2, YTHDF3, etc.; refs. 14, 15). These m’A-associated proteins play critical roles to regulate the metabolism and functions of m’A-modified mRNAs and lncRNAs (15).

In this work, we identified a micropeptide encoded by a Y-linked lncRNA, LINC00278, which is downregulated in male ESCC. The expression of this micropeptide was downregulated by cigarette smoking in ESCC through erasing m’A modification. It specifically bound to Yin Yang 1 (YY1) and blocked the interaction between YY1 and androgen receptor (AR), therefore named YY1-blocking micropeptide (YY1BM). YY1BM downregulated eEF2K expression through AR signaling pathway and induced apoptosis in ESCC under nutrient deprivation (ND). Furthermore, YY1BM also acts as a potential anticancer micropeptide for ESCC.

Materials and Methods

Human study subjects

A total of 281 pairs of fresh-frozen ESCC and adjacent noncancerous tissue samples were obtained from patients in Eastern China who underwent tylectomies at the Affiliate Hospitals of Soochow University (Suzhou cohort; Suzhou, China). Another 288 pairs of fresh-frozen ESCC tissues were collected from patients in Southern China at the Cancer Hospitals affiliated with Guangzhou Medical University (Guangzhou cohort; Guangzhou, China). None of the patients received anticancer treatment before surgery, including chemotherapy or radiotherapy. The Medical Ethics Committees of Soochow University (Suzhou, China) and Guangzhou Medical College (Guangdong, China) approved this study. The clinical characteristics of patients in this study are listed in Supplementary Table S1.

Statistical analysis

The data analysis was performed using the SPSS 19.0 software for Windows. The statistical significance between datasets was expressed as P values, and P < 0.05 was considered statistically significant. Survival curves were obtained using the Kaplan–Meier method and compared using the log-rank test. Multivariable Cox regression analysis was performed using the R package “survival.” Paired or unpaired Student t test and Pearson correlation coefficients were used for various types of data comparison. Mediation analysis was conducted using the procedure described by Baron and Kenny (16) and a P < 0.05 was considered significant.

Animals and cell cultures

Male nude mice of 6–8 weeks of age were purchased from the Shanghai Laboratory Animal Center at the Chinese Academy of Sciences (Shanghai, China). All animal studies were conducted with the approval of Soochow University Institutional Animal Care and Use Committee and were performed in accordance with established guidelines.

All cell lines were purchased from Procell Life Science&Technology Co., Ltd. These cell lines were all characterized by DNA finger printing analysis and passaged less than 6 months in this study. DMEM, RPMI1640, and FBS were purchased from Invitrogen. Eca-109, TE-1, and KYSE-30 cells were grown in RPMI1640 with 10% FBS; Het-1A and 293T cells were grown in DMEM with 10% FBS. All cell lines were grown in penicillin/streptomycin containing medium, at 37°C in a humidified atmosphere with 5% CO₂. In addition, cells were treated with 1 nmol/L R1881 (methyltrienolone) to activate AR signaling pathway. To inhibit specific signaling pathways, cells were pretreated with vehicle (DMSO) or 10 μmol/L A-484954 (EMD Millipore) for 1 hour at 37°C prior to the experiments.

Transplantation of human ESCC tissues

Primary viable human ESCC samples were obtained from surgical ESCC specimens (n = 50) at the Affiliate Hospitals of Soochow University (Suzhou, China). During surgery, fresh tumor tissue was collected in transport medium, [RPMI1640 medium supplemented with penicillin/streptomycin (100 U/mL; 100 μg/mL), fungizone (1 μg/mL), and gentamicin (50 μg/mL; all from Life Technologies)] and implanted in mice within 4 hours. In parallel, primary tumor tissue fragments were also fresh-frozen and formalin-fixed for further analyses. Before implantation, tumor tissue was rinsed in PBS supplemented with penicillin/streptomycin and fungizone. Each tumor specimen was cut into three small fragments (1.5 mm × 1.5 mm) and grafted subcutaneously into NCG mice. The NCG mice were anesthetized by intraperitoneal of pentobarbital (10 mg/mL) at a dose of 65 mg/kg.

Microarray data analysis

To identify male ESCC-associated lncRNAs, differential gene expression analysis was performed on gene expression profiles of 179 pairs of ESCC and matched adjacent normal tissues, and the tissues were separated in male and female groups. Differential gene expression analysis was performed by the R package “limma.” The probe that adjusted P-value (P adj) < 0.01 and the absolute value of log₂-fold change (abs.logFC)>1 were defined as differentially expressed probes. The differentially expressed probes were subsequently annotated by mapping onto the genomic coordinates of lncRNAs derived from GENCODE.

Chromatin immunoprecipitation–sequencing data analysis

Chromatin immunoprecipitation (ChiP)–sequencing (ChiP-seq) data were obtained from the GEO database. ChiP-seq reads were aligned to the hg19 by Bowtie2 with default parameters; the mapped reads of ChiP-seq were preprocessed by Samtools and then submitted to MACS2 for peaks calling. The peaks were annotated by the R package “ChIPseeker” and visualized by IGV software. Finally, genes that contained peaks at <800 bp upstream of transcriptional start sites (TSS) to +200 bp downstream of TSS region were defined as genes regulated by corresponding transcription factors.

Overall survival analysis

Using the median expression level of LINC00278 among ESCC tissues, we separated patients with ESCC into two different groups: patients with high LINC00278 expression (relative expression level>median expression level); and patients with low LINC00278 expression (relative expression level<median expression level), in both the Suzhou cohort (discovery set, 281 patients) and Guangzhou cohort (validation set, 288 patients). Furthermore, Kaplan–Meier survival curves and log-rank tests were performed between the high LINC00278 group and the low LINC00278 group.

RNA extraction and qRT-PCR

Total RNA was isolated from ESCC tissues and corresponding adjacent nonneoplastic tissues using the RNA Isolater Total RNA Extraction Reagent (Vazyme). The purity and concentration of RNA were determined by the ratio of absorbance at 260 nm (A260) and 280 nm (A280) using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). The RNA was considered pure and suitable for downstream experiments when A260/A280 was within the range
Chinese brand cigarette (12 mg tar/cigarette) by a vacuum machine previously (20). Briefly, Preparation of cigarette smoke condensate and YY1BM transcripts were quantified by qRT-PCR. We performed polysome profiling to measure the translation of LINC00278 and YY1BM transcripts, as shown in Supplementary Fig. S1E.

**DNA methylation analysis**

DNA methylation analysis was performed as reported previously (17). Briefly, we designed primers of the CpG islands in the promoter region of \( \text{ALKBH5} \) gene using MethPrimer. After robustly digesting 22 nL of the cleavage reaction onto the silicon matrix preloaded chips (SpectroCHIP; Sequenom), the mass spectra were collected using a MassARRAY Compact MALDI-TOF (Sequenom). The methylation ratios were generated by EpiTYPER software (version 1.0; Sequenom). Mass spectrometry analysis was carried out at the AIMS Scientific Co., Ltd. (Shanghai, China) in the positive-ion mode with an automated data-dependent MS/MS analysis. Mass spectrometry analysis was performed using a Thermo Fisher LTQ Orbitrap ETD mass spectrometer. Briefly, the samples were loaded onto an high-performance liquid chromatography (HPLC) chromatography system named Thermo Fisher Easy-nLC 1000 equipped with a C18 column (1.8 mm, 0.15 µm) at a flow rate of 300 nL/minute. Mass spectrometry analysis was carried out at the AIMS Scientific Co., Ltd. (Shanghai, China) in the positive-ion mode with an automated data-dependent MS/MS analysis.

**Mass spectrometry analysis**

The samples were analyzed on Thermo Fisher LTQ Orbitrap ETD mass spectrometer. Briefly, the samples were loaded onto an high-performance liquid chromatography (HPLC) chromatography system named Thermo Fisher Easy-nLC 1000 equipped with a C18 column (1.8 mm, 0.15 × 1.0 mm). Solvent A contained 0.1% formic acid and solvent B contained 100% acetonitrile. The elution gradient was from 4% to 18% in solvent A for 182 minutes and 18% to 90% in solvent B for 13 minutes at a flow rate of 300 nL/minute. Mass spectrometry analysis was carried out at the AIMS Scientific Co., Ltd. (Shanghai, China) in the positive-ion mode with an automated data-dependent MS/MS analysis.

**Production of YY1BM knockout and FLAG knockin cells**

The gRNA sequence designed specifically for the open reading frame (ORF) of YY1BM start codon inserted to the Cas9/gRNA (puro-GFP) vector (VK001-02, ViewSolid BioTech) was 5’-GACTCCAGG-CATGCTATCAGG-3’. The donor oligo was purchased from Cyagen Biosciences Inc. (Suzhou, China). The constructed targeting vector and donor oligo were subsequently transfected into the cells using Lipofectamine 3000 (Thermo Fisher Scientific), after which, the cells were cultured under puromycin drug selection (2 µg/mL) for 48 hours. Viable clones were grown to a larger size and picked up for Western blot analysis or sequencing. The schematic diagram and the sequence of YY1BM-KO cells were present in Supplementary Fig. S1A. The levels of \( \text{LINC00278} \) transcription and YY1BM translation were present in Supplementary Fig. S1B-S1D.

**Preparation of cigarette smoke condensate**

Cigarette smoke condensate (CSC) was prepared as reported previously (20). Briefly, cigarette smoke was collected from a popular Chinese brand cigarette (12 mg tar/cigarette) by a vacuum machine into a container and frozen with liquid nitrogen. CSC was dissolved in DMSO at a concentration of 235 mg/mL, and aliquots were stored at –80°C until use.
lentiviral expression vector pLVX-IREs-neo (Clontech Laboratories Inc.). To produce lentivirus containing full-length LIN00278 or YY1BM targeting sequence, 293T cells were cotransfected with the vector described above and the lentiviral vector packaging system using Lipofectamine 3000. Infectious lentiviruses were collected at 48 and 72 hours after transfection and filtered through 0.45-μm filters. These lentiviruses were, respectively, designated as LIN00278 overexpression or YY1BM overexpression. We used an empty plenti-pLVX-IRES-neo vector to generate negative control lentiviruses. Recombinant lentiviruses were concentrated by centrifugation. The virus-containing pellet was dissolved in DMEM, and aliquots were stored at −80°C until use. Cells were infected with the concentrated virus in the presence of polybrene (Sigma-Aldrich). The supernatant was replaced with complete culture medium after 24 hours, followed by selection with 800 μg/ml G418, and the expression of LIN00278 and YY1BM in infected cells was verified by qRT-PCR.

Micropeptide synthesis

The micropeptides used in ESCC cell treatment and intratumoral injection were synthesized from ChinaPeptides Co., Ltd. The micropeptides were purified by HPLC, and the sequence and structure were confirmed by mass spectrometry. The peptides were ≥95% pure and kept at 100 mg/ml stock solution at −20°C.

Data sharing statement

Microarray data are available at the NCBI Gene Expression Omnibus (geo) repository with accession number GSE53625. The ribosome profiling data are available at the GEO repository with accession number GSE61742. The ChIP-seq data are available at the GEO repository with accession numbers GSE32465 and GSE62472.

Results

Identification of ESCC-associated IncRNA LIN00278

To identify male ESCC-associated IncRNAs, we analyzed differentially expressed IncRNAs in IncRNA expression profiles of 179 pairs of ESCC and matched adjacent normal tissues, which were separated into male and female groups (21). In total, 3,401 differentially expressed genes were screened from the male group (146 patients) and 3,284 differentially expressed genes were screened from the female group (33 patients). In addition, we filtered 254 differentially expressed IncRNAs in the male group and 244 differentially expressed IncRNAs in the female group (Fig. 1A). Among these, we found 51 differentially expressed IncRNAs were present in the male group, but not in the female group, and 3 of them were mapped to Y chromosome (Fig. 1B–D).

Next, we measured the expression of these 3 Y-linked IncRNAs by qRT-PCR in 281 pairs of male ESCC tissue samples from an Eastern Chinese population (Suzhou cohort). Only LIN00278 was significantly downregulated in ESCC tissues when compared with adjacent normal tissues (P < 0.001; Fig. 1E; Supplementary Fig. S2A and S2B). We further validated the downregulation of LIN00278 in ESCC tissues using an independent 288 pairs of male ESCC samples from a Southern Chinese population (Guangzhou cohort; P < 0.001; Fig. 1E).

We also determined whether LIN00278 expression was associated with overall survival (OS) among male patients with ESCC. Using the median expression level of LIN00278 among ESCC tissues, we separated patients with ESCC into two different groups: patients with high LIN00278 expression (relative expression level≥median expression level); and patients with low LIN00278 expression (relative expression level< median expression level), in both the Suzhou cohort (discovery set, 281 patients) and Guangzhou cohort (validation set, 288 patients). Using the log-rank test and Kaplan–Meier survival curves, we showed that patients with low LIN00278 expression had significantly shorter OS than patients with high LIN00278 expression in both the discovery set [median survival time (MST): 29 vs. 36 months, log-rank P = 0.0004, HR = 1.848] and the validation set (MST: 27 vs. 39 months, log-rank P < 0.0001, HR = 1.850; Fig. 1F and G). Multivariable Cox regression analysis also indicated that low LIN00278 expression was associated with shorter OS (Fig. 1F and G).

Because cigarette smoking has been associated with poor OS, we determined whether LIN00278 expression and cigarette smoking acted synergistically in ESCC. We showed that patients with low LIN00278 expression and who were current smokers had worst OS in both the discovery set (MST: 28 vs. 40, log-rank P < 0.0001, HR = 2.818) and the validation set (MST: 25 vs. 41, log-rank P < 0.0001, HR = 2.613; Supplementary Fig. S2C and S2D). Using Multivariable Cox regression analysis, we also confirmed that smoking was associated with shorter OS in patients with ESCC (Fig. 1F and G; Supplementary Fig. S2C and S2D).

Biological characterization of LIN00278

LIN00278 locus is located on the short arm of Y chromosome. It spans from 3,002,887 to 3,200,509 and is comprised of four exons. LIN00278 transcript is 537 bp long and Northern blot analysis has confirmed the expected size of LIN00278 transcript in total RNA from two pairs of male ESCC samples (Supplementary Fig. S2E). Both nuclear/cytoplasmic fractionation experiment and confocal microscopy analysis of FISH showed that LIN00278 is a cytoplasmic RNA (Supplementary Fig. S2F and S2G).

LIN00278 encodes a micropeptide

Because recent studies suggested that many IncRNAs could encode functional micropeptides (<100 amino-acids), we determined whether LIN00278 encoded any micropeptides. We found that LIN00278 could potentially encode four small ORFs (sORF; Fig. 2A). We subsequently cloned each sORF with an in-frame FLAG epitope tag at the C terminus and transfected it into male ESCC cell line TE-1. Western blot analysis indicated that LIN00278-sORF1 generated a micropeptide (Fig. 2B). This sORF is located on Y chromosome from 3,003,090 to 3,003,155, inside the ribosome occupancy peak in the transcribed region (Supplementary Fig. S2C and S2D). Among these, we found 51 differentially expressed IncRNAs were present in the male group, but not in the female group, and 3 of them were mapped to Y chromosome (Fig. 1B–D).

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We next determined whether an in-frame ATG codon of LIN00278-sORF1 could promote the initiation of translation. We fused GFPmut ORF (in which the initiation codon ATGGTG has mutated to ATGGTT) and FLAG-tag to the C terminus of LIN00278-sORF1 to construct expression plasmids and transfected these plasmids into ESCC cells. After 24 hours, we observed substantial expression of LIN00278-sORF1-GFP fusion protein in the transfected cells (Supplementary Fig. S2H). Meanwhile, LIN00278-sORF1-FLAG was observed in transfected cells using anti-FLAG Western blot analysis (Supplementary Fig. S2I). Our data indicated that LIN00278-sORF1 could produce micropeptide and the initiation codon of LIN00278-sORF1 could be utilized effectively to drive the expression of the fusion protein.
LINC00278-sORF1 was endogenously expressed and downregulated in male ESCC

To determine LINC00278-sORF1 expression, we generated a rabbit polyclonal antibody (anti-LINC00278-sORF1). To confirm the specificity of anti-LINC00278-sORF1, we respectively performed Western blot analysis to identify LINC00278-sORF1, LINC00278-sORF1-GFP, and LINC00278-sORF1-FLAG in ESCC cells (Supplementary Fig. S2H and S2I). We also performed polysome profiling in the cell lysate of Het-1A (a nonneoplastic squamous esophageal epithelial cell line), TE-1, and KYSE-30. The mRNA-protein particles (mRNP) were separated into three groups: nonribosome (mRNPs without any ribosome), 40S–80S (mRNPs associated with ribosome but not being translated) and polysome (mRNPs being actively translated). The presence of LINC00278 was quantitated in polysome fraction via qRT-PCR (Supplementary Fig. S2J and S2K).

To further confirm the existent of endogenous LINC00278-sORF1 micropeptide, we inserted a C-terminal FLAG-tag at the 3' end of the ORF of LINC00278-sORF1 (FLAG-KI) and detected endogenous LINC00278-sORF1 by Western blot analysis in Het-1A cell line, which expresses a higher level of LINC00278 and LINC00278-sORF1 (Fig. 2D; Supplementary Fig. S2L). In addition, we showed that the LINC00278-sORF1 translation-blocking antisense oligo could block the expression of LINC00278-sORF1 micropeptide (Supplementary Fig. S2M).

Because of the limited number of tumor cells obtained from ESCC tissues, we determined LINC00278-sORF1 translation and expression in male ESCC patient-derived xenograft (PDX) models using polysome profiling and Western blot analysis. A total of 50 ESCC PDXs were generated for the experiment. Tumor cells were harvested and lysed from ESCC PDXs. Our data indicated that LINC00278 was endogenously expressed and downregulated in male ESCC tissues (Fig. 2E). In addition, the level of LINC00278 in polysome fraction was positively correlated with the transcription level of LINC00278 in the tissues (Fig. 2F).
LINC00278-sORF1 knockout promoted ESCC tumor growth

We next generated LINC00278-sORF1-knockout cell lines and determined the effect of LINC00278-sORF1-knockout on tumor growth using mouse ESCC xenograft models. We showed that tumor growth from LINC00278-sORF1-knockout ESCC cells was significantly higher than that from wild-type (WT) ESCC cells (Fig. 2F). To investigate whether the transcript of LINC00278 is functional in ESCC tumor growth, we knocked down LINC00278 in WT and LINC00278-sORF1-knockout ESCC cells. We showed that LINC00278-knockdown promoted tumor growth in WT ESCC cells, but not in LINC00278-sORF1-knockdown ESCC cells (Fig. 2G and H).

Furthermore, we showed that reintroducing either full-length-LINC00278 (full-LINC00278-FLAG) or LINC00278-sORF1 (LINC00278-sORF1-FLAG) into the LINC00278-sORF1-knockout could reverse tumor growth (Fig. 2I). In addition, we also showed that reintroducing LINC00278-sORF1 (LINC00278-sORF1-FLAG) into the LINC00278-knockdown cells could reverse tumor growth (Supplementary Fig. S2N). Interestingly, overexpression of full-length LINC00278...
demonstrated stronger suppression of tumor growth than LINC00278-sORF1 in LINC00278-sORF1-knockout ESCC cells (Fig. 2I). Furthermore, we detected higher LINC00278-sORF1 micropeptide expression by full-length LINC00278-FLAG than by LINC00278-sORF1-FLAG (Fig. 2J). We also found that the RNA stability of full-length LINC00278 and LINC00278-sORF1 were not significantly different (Supplementary Fig. S2O). Our results suggest that LINC00278-sORF1 had the main effect on ESCC tumor growth while the untranslated region of LINC00278 augmented such effect.

m^A modification of LINC00278 promoted LINC00278-sORF1 translation

Because m^A modification is the most prevalent posttranscriptional modification of mRNA and IncRNA, and it regulates translation (13), we determined whether m^A modification of LINC00278 regulated LINC00278-sORF1 translation. Using m^A-specific RNA immunoprecipitation, we showed that LINC00278 contained m^A modification in both Het-1A and ESCC cell lines (Fig. 3A).

Next, we identified three m^A modification sequence motifs in the untranslated region of LINC00278 using a computation software called SRAMP (24; Supplementary Fig. S3A). To determine which m^A sequence motif was modified and facilitating LINC00278-sORF1 translation, we generated full-length LINC00278-FLAG and LINC00278-sORF1-FLAG WT construct as well as constructs with each m^A sequence motif mutated (mut1-LINC00278-FLAG, mut2-LINC00278-FLAG, and mut3-LINC00278-FLAG), and transfected into Eca-109 and KYSE-150 cells (which are female ESCC cell lines that do not have endogenous LINC00278 transcript and LINC00278-sORF1 micropeptide, Supplementary Table S3). The results showed that only mut3-LINC00278-FLAG significantly reduced LINC00278 m^A level compared with full-length LINC00278-FLAG expression, which was approximately equal to LINC00278-sORF1-FLAG (Fig. 3B). This was consistent with the lower LINC00278-sORF1 protein level produced by mut3-LINC00278-FLAG expression (Fig. 3C). Furthermore, we expressed mut3-LINC00278 and LINC00278-sORF1 in LINC00278-sORF1 ESCC cells and implanted them to generate xenograft models. The results showed that mut3-LINC00278 and LINC00278-sORF1 have no significant difference in tumor growth inhibition (Fig. 3D). To prove the GAACU motif mutated in mut3-LINC00278-FLAG was endogenously m^A modified in LINC00278, we used a Morpholino antisense oligo that specifically blocked m^A modification to this motif in Het-1A cell line. We showed that both the levels of LINC00278 m^A modification and LINC00278-sORF1 were decreased (Fig. 3E and F). Our results indicated that only the GAACU motif was m^A modified in LINC00278.

Finally, we conducted a mediation analysis to determine whether m^A modification was the mediator of the LINC00278 transcript level and LINC00278-sORF1 micropeptide level in PDXs. First, we investigated the relationships between LINC00278 expression and LINC00278-sORF1 expression using linear regression. Second, we analyzed the relationship between each LINC00278 expression and m^A modification by linear regression. Third, we examined the relationship between m^A modification and LINC00278-sORF1 expression using linear regression. Fourth, we included both the LINC00278 expression and m^A modification in the model examining associations with LINC00278-sORF1 expression to evaluate mediation. As shown in Supplementary Fig. S3B, the total effect of the LINC00278 transcription (X) on the LINC00278-sORF1 translation (Y) was statistically significant (Y = cX + e; c = 0.675; SE, 0.106; R = 0.675; P < 0.001); and the m^A modification of LINC00278 (M) had a partial mediation effect on the relationship between LINC00278 transcription and LINC00278-sORF1 translation (M = aX + e; a = 0.796; SE, 0.0875; R = 0.796; P < 0.001; Y = cX + bM + e; c = 0.396, P = 0.024; b = 0.351, P = 0.044).

Identification of regulators of m^A modification in LINC00278

We next investigated which proteins were involved in LINC00278 m^A modification. First, we determined which known m^A “reader” proteins were bound to m^A modified LINC00278 using RNA pull-down and Western blot analysis (25). As shown in Fig. 3G, we showed that only YTHDF1 could be pulled down by m^A modified LINC00278. Using electrophoretic mobility shift assay (EMSA) and RNA immunoprecipitation (RIP), we further confirmed that YTHDF1 interacted with m^A modified LINC00278 (Fig. 3H and I). Finally, we showed that YTHDF1 knockdown significantly downregulated LINC00278-sORF1 translation without changing the LINC00278 m^A modification level (Supplementary Fig. S3C).

Next, we knocked down each known m^A “writer” and “eraser” protein and determined its effect on LINC00278 expression, LINC00278 m^A modification level, and LINC00278-sORF1 translation. We showed that METTL3, METTL14, and WTAP knockdown significantly reduced LINC00278 m^A modification level and LINC00278-sORF1 translation, while ALKBH5 knockdown significantly increased LINC00278 m^A modification level and LINC00278-sORF1 translation. Furthermore, the YTHDF1 knockdown did not affect the LINC00278 m^A modification level and LINC00278-sORF1 translation (Supplementary Fig. S3C). None of these proteins affected the expression of LINC00278 (Supplementary Fig. S3C).

Our data suggest that METTL3, METTL14, and WTAP acted as “writers,” ALKBH5 acted as “eraser,” and YTHDF1 acted as “reader” for LINC00278 m^A modification.

Cigarette smoking modulates LINC00278-sORF1 translation

Because cigarette smoking actuated synergetically with low LINC00278 expression to confer worse prognosis in patients with ESCC, we investigated whether cigarette smoking affected LINC00278-sORF1 translation. When we divided patients with ESCC into smoking and nonsmoking groups, we did not detect significant difference in LINC00278 expression level between the two groups (Supplementary Fig. S3D). However, LINC00278 m^A modification and LINC00278-sORF1 translation levels were significantly lower in the smoking group than in the nonsmoking group (Fig. 3J and K).

To determine how cigarette smoking affected m^A modification, we exposed Het-1A cells to CSC and measured the expression level of m^A regulators. CSC treatment only increased the level of ALKBH5 protein (Fig. 3L). Meanwhile, CSC treatment decreased the level of LINC00278-sORF1 micropeptide but did not affect LINC00278 expression (Fig. 3L and M).

To investigate the mechanism of how CSC upregulated the expression of ALKBH5, we analyzed ALKBH5 gene promoter CpG island methylation using the massArray DNA methylation analysis. We found that ALKBH5 CpG island was hypomethylated in CSC-treated cells compared with DMSO mock-treated cells (Fig. 3N). Finally, we showed that ALKBH5 knockdown completely abolished the effect of CSC treatment on LINC00278 m^A modification and LINC00278-sORF1 translation (Fig. 3O).

Taken together, our data suggest that LINC00278 downregulation promoted ESCC progression. LINC00278 encodes a micropeptide, whose expression was modulated by LINC00278 m^A modification. Finally, LINC00278 m^A modification was regulated by cigarette smoking via ALKBH5 hypomethylation.
Role of Micropeptide Encoded by IncRNA in Male ESCC

LINC00278-sORF1 blocks the interaction between YY1 and AR

To determine the function of LINC00278-sORF1 in ESCC progression, we first investigated the LINC00278-sORF1 interacting proteins by coimmunoprecipitation and mass spectrometry analysis in TE-1 and KYSE-30 cells. We sought for the proteins that could be immunoprecipitated by LINC00278-sORF1-FLAG fusion protein but not by IgG in both TE-1 and KYSE-30 cells. As shown in Fig. 4A, we identified YY1 as the potential LINC00278-sORF1 binding protein. We validated that YY1 could be immunoprecipitated by LINC00278-sORF1-FLAG fusion protein (Fig. 4B). Furthermore, we cotransfected YY1-HA and LINC00278-sORF1-FLAG into TE-1 cells and performed coimmunoprecipitation using anti-HA. Western blot analysis showed that YY1-HA and LINC00278-sORF1-FLAG proteins were immunoprecipitated (Fig. 4B). Finally, we showed that endogenous LINC00278-sORF1 in TE-1 cells could be immunoprecipitated using anti-YY1 antibody (Fig. 4B). We concluded that LINC00278-sORF1 bound to YY1 and we named the micropeptide YY1BM.

YY1 is a ubiquitous and multifunctional transcriptional factor that plays a regulatory role in tumorigenesis, including ESCC (26, 27). YY1 truncation experiment indicated that YY1BM bound to YY1 C-terminal domain (331–414 amino acid), where it has been documented that bound to AR (28; Fig. 4C). Given that YY1 is a transcriptional coactivator of AR in prostate cancer (28), we tested whether it was also true in ESCC (Fig. 4D). Finally, we showed that the interaction between YY1 and AR was downregulated by LINC00278 overexpression and upregulated by YY1BM knockout in both TE-1 and KYSE-30 cell lines (Fig. 4E). Our data suggested that YY1BM was blocking the interaction between YY1 and AR.

YY1 promotes AR-regulated eEF2K transcription

We first examined whether YY1 and AR occupied the same genomic locations by reanalyzing publically available ChIP-seq data on YY1 (29) and AR (30). We found that YY1 bound to 2,865 gene promoters, while AR bound to 312 gene promoters, respectively. Taken together, 33 genes were common in genes regulated by YY1 and AR (33 genes). We found that expression of eEF2K was decreased in LINC00278

Figure 3.

m^A modification of LINC00278 promoted YY1BM translation, which was reduced by cigarette smoking. A, [m^A]LINC00278 or [m^A]MALAT1 was detected by immunoprecipitation with antibody against m^A, followed by qRT-PCR analysis in individual cells (mean ± SD; n = 3). MALAT1 was used as a positive control. B, m^A level of LINC00278 in female ESCC cells that were transfected with indicated plasmids (mean ± SD; n = 3). C, LINC00278-sORF1-FLAG fusion protein levels in female ESCC cells that were transfected with indicated plasmids. D, Tumor growth in xenograft mice subcutaneously implanted with ESCC cells that were transfected with indicated constructs (mean ± SD; n = 5). E, m^A level of LINC00278 in Het-1A cells that were transfected with Morpholino antisense oligo, which was specifically blocking the m^A motif of LINC00278. F, LINC00278-sORF1 micropeptide level in Het-1A cells that were transfected with Morpholino antisense oligo, which was specifically blocking the m^A motif of LINC00278. G, The interaction between [m^A]LINC00278 and YTHDF1 was detected by RNA pulldown assays. H, The interaction between [m^A]LINC00278 and YTHDF1 was detected by EMSA assays. I, RIP assays indicated that YTHDF1 interacts with LINC00278. PNPLA2 was used as a positive control. J, Significant [m^A]LINC00278 level difference in male ESCC tissues from smokers (n = 34) and nonsmokers (n = 16; mean ± SD). K, Significant LINC00278-sORF1 level difference in male ESCC tissues from smokers (n = 34) and nonsmokers (n = 16; mean ± SD). L, METTL3, METTL14, WTAP, ALKBH5, YTHDF1, and LINC00278-sORF1 levels in Het-1A cells were detected by Western blotting after treatment with CSC (100 μg/mL) or DMSO as solvent control. M, The relative levels of LINC00278, [m^A]LINC00278, and LINC00278-sORF1 in Het-1A cells that were treated with CSC (100 μg/mL) or DMSO as solvent control for 48 hours (mean ± SD; n = 3). N, Amplicon size and place of CpG sites in the amplicon. Methylation profile of CpG sites for the ALKBH5 gene. The color of the circles is related to the percentage of methylation in each CpG site. Boxes indicate the different methylation patterns between CSC (100 μg/mL; 48 hours) or DMSO-treated Het-1A cells. O, ALKBH5-knockdown abolished effect of CSC (100 μg/mL; 48 hours) treatment on levels [m^A]LINC00278 and LINC00278-sORF1 in Het-1A cells. Mean ± SD; n = 3. **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.
upregulated cells, but increased in YY1BM knockout cells (Fig. 4H). These expression changes were abolished by YY1 siRNAs treatment (Supplementary Fig. S4A), indicated that eEF2K expression was regulated by YY1BM via modulated interaction between YY1 and AR.

To identify YY1 and AR binding sites in the eEF2K promoter, we first analyzed the ChIP-seq data and identified overlapping YY1 and AR peaks surrounding the eEF2K TSS (Fig. 4I). Next, we analyzed the binding sites of YY1 and AR in the promoter of eEF2K using PROMO and JASPAR. The results suggested that AR and YY1 potentially co-binding to the −200 to 100 region of eEF2K promoter. We subsequently carried out ChIP experiments to fine map YY1 and AR bound to the −200 to 100 region of eEF2K promoter (Fig. 4).
Furthermore, we generated an eEF2K promoter luciferase reporter plasmid (pGL3-eEF2K) and an eEF2K mutant promoter luciferase reporter plasmid that deleted the −200 to 100 region (pGL3-eEF2K-mut) to identify eEF2K transcription regulators. The pGL3-eEF2K luciferase activity was decreased in cells overexpression of full-length LINC00278 and increased in cells with YY1BM knockout, whereas pGL3-eEF2K-mut abolished these difference (Fig. 4K). Furthermore, the pGL3-eEF2K luciferase activity was not significantly different when cells were treated with YY1 siRNAs, confirming the involvement of YY1 in AR-regulated eEF2K expression (Supplementary Fig. S4B).

Finally, we determined the levels of testosterone and eEF2K expression in patients with ESCC by Electrochemiluminescence immunoassay and qRT-PCR. We found that the testosterone level was positively correlated with eEF2K expression level in males, but not in females (Supplementary Fig. S4C). To further confirm the involvement of AR signaling pathway, we showed that YY1BM overexpression did not affect the tumor growth in female mice (Supplementary Fig. S4D), indicating that YY1BM is indeed involved in male ESCC progression via AR signaling pathway.

**YY1BM decreases survival of ESCC cells under ND through eEF2K signaling pathway**

Because eEF2K confers cell survival under acute severe ND by inhibiting eEF2 activity and translation elongation (31), we determined whether YY1BM regulated ESCC cell survival under ND. Compared with WT cells, YY1BM knockout cells showed increased survival under ND (Supplementary Fig. S4E). This increased survival was abolished by A-484954 treatment, a known small-molecule eEF2K inhibitor that could decrease the phospho-eEF2 level in ESCC cells (Supplementary Fig. S4E).

We also analyzed apoptosis of ESCC cells under ND, using flow cytometry for Annexin V staining (Supplementary Fig. S4F and S4G) and Western blot analysis of caspase-3 cleavage (Supplementary Fig. S4H and S4I). We showed that YY1BM knockout reduced apoptosis in ESCC cells under ND, which was abolished by A-484954 treatment. Whereas overexpression of full-length LINC00278 induced apoptosis under ND, which was also abolished by A-484954 treatment. Meanwhile, the expression of eEF2K was upregulated by YY1BM knockout and downregulated by LINC00278 overexpression under ND, which was also abolished by treatment with A-484954 (Supplementary Fig. S4H and S4I). Finally, we found that eEF2 phosphorylation was increased in YY1BM knockout cells and decreased in LINC00278 overexpressed cells under ND, which was also abolished by A-484954 treatment (Supplementary Fig. S4H and S4I).

Interestingly, we found that A-484954 treatment under ND reduced the speed of YY1BM translation decrease (Supplementary Fig. S4I), suggesting the presence of a positive-feedback loop between the eEF2K/eEF2 axis and YY1BM.

These data suggested that YY1BM inactivated the AR-regulated transcription of eEF2K under ND, thereby enhancing translation elongation and resulting in ESCC cell apoptosis.

**Low YY1BM expression is associated with reduced apoptosis in ESCC xenografts and tissues**

We then sought to explore the relationship between YY1BM expression and ESCC apoptosis in xenograft model. YY1BM knockdown xenografts showed higher expression of eEF2K and lower expression of cleaved caspase-3 by IHC analysis (Fig. 5A and B), consistent with reduced apoptosis by Tunel staining (Fig. 5C). Moreover, we also found that expression of eEF2K and cleaved caspase 3 were not changed in LINC00278 overexpression xenografts when we implanted female ESCC cells into female mice (Fig. 5D).

When we correlated eEF2K and caspase-3 expression (≥30%, strong staining; <30%, weak staining) with YY1BM expression in 50 ESCC tissues, we found that expression of YY1BM was inversely correlated with eEF2K expression, but positively correlated with cleaved caspase 3 (Fig. 5E and F).

**YY1BM is a potential anticancer micropeptide**

Because several anticancer peptides have been reported, we investigated whether YY1BM is a novel anticancer micropeptide. We first tested the cytotoxicity of YY1BM in ESCC cells. As shown in Fig. 6A and B, we found that YY1BM was cytotoxic to TE-1 and KYSE-30 cells, while scrambled YY1BM (svYY1BM) control micropeptide was not. To probe the anticancer effect of YY1BM in vivo, we injected YY1BM intratumorally into ESCC tumors grafted in nude mice and analyzed the survival time. We found YY1BM injection significantly improved the survival rate of male mice, but not female mice (Fig. 6C and D). Furthermore, IHC analysis revealed a higher apoptosis rate and lower eEF2K expression in male mice, but not female mice (Fig. 6C and D), suggesting that YY1BM intratumoral injection downregulated the expression of eEF2K and induced apoptosis, ultimately improved male mouse survival.

**Discussion**

 Globally, ESCC is a male-dominant malignancy. Both sex hormone and lifestyle factors, such as cigarette smoking, contribute to this gender disparity. In this study, we discovered a 21-amino-acid micropeptide (YY1BM) encoded by Y-linked IncRNA LINC00278. The translation of YY1BM was modulated by cigarette smoking–mediated LINC00278 m6A modification. YY1BM blocked YY1 binding to AR to activate the expression of eEF2K, which is a key regulator for male ESCC progression.

Through mining a large cohort of ESCC IncRNA profiling data, we discovered that LINC00278 might play a critical role in male-specific ESCC progression. LINC00278 is a 537 bp transcript, located on Y chromosome and previously annotated as a noncoding RNA. It has been reported that IncRNAs are involved in ESCC progression, such as Linc-POU3F3 promotes methylation of POU3F3 by interacting with EZH2 in ESCC (17). Interestingly, our data indicated that YY1BM, instead of the LINC00278 transcript, plays a major role in ESCC progression. We also found that the m⁶A modification motif of LINC00278 is close to the stop codon of YY1BM, consistent with its role in the regulation of YY1BM translation (15). m⁶A is the major reversible posttranscription modification in RNAs (32, 33), involved in RNA stability (34) and protein production (15). m⁶A modification changes have been linked to various disease processes, including tumorigenesis. It has been shown that the physiologic functions of m⁶A modification mainly depend on the “reader” proteins that bind to the m⁶A modification motif. YTHDF1, a member of YTH family that has been reported to facilitate protein synthesis by interacting with translation machinery (15), is the “reader” for m⁶A modification of LINC00278. Based on previously reported studies, we speculate that YTHDF1 binds to m⁶A modified LINC00278 to recruit the translation machinery, therefore promote the translation efficiency of YY1BM. This is consistent with our mediation analysis showing that m⁶A modified LINC00278 has an incomplete mediating effect on the relation between LINC00278 and YY1BM expression levels in ESCC tissues. Our data showed that CSC treatment leads to ALKBH5 promoter hypomethylation and increased expression of ALKBH5.

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ALKBH5 is an m6A demethylase that acts as the “eraser” protein of m6A modified LINC00278, which leads to a decreased level of m6A modified LINC00278, and in turn reduced YY1BM expression through YTHDF1. In China, current smokers are significantly more prevalent in the male population than in the female population. Cigarette smoking is a key factor in ESCC carcinogenesis. In this study, we found that cigarette smoking contributes to poor prognosis in ESCC in part by regulating LINC00278 translation through m6A modification. In summary, we conclude that m6A modification of LINC00278 modulates YY1BM translation and results partially in the sex bias of ESCC.

Recently, several lncRNA-encoded micropeptides have been identified and reported to play crucial roles in a variety of physiologic processes. MLN, a micropeptide encoded by a skeletal muscle-specific lncRNA, has been shown to interact directly with SERCA and impede Ca\textsuperscript{2+} uptake into the SR, thereby regulating muscle performance (11). Expression of another lncRNA-encoded micropeptide Myomixer, together with Myomaker, controls the critical step in myofiber formation during muscle development (10). Furthermore, lncRNA HOXB-AS3 encodes a conserved 53 amino-acid micropeptide, which suppresses colon cancer growth by regulating the pyruvate kinase M (PKM) splicing and suppressing glucose metabolism reprogramming (12).

YY1BM is also identified as a novel micropeptide encoded by Y-linked LINC00278. It interacts with YY1 and blocks its interaction with AR. YY1 is a zinc finger protein belonging to the GLI-Kruppel family that can activate or inactivate gene expression depending on interacting partners, promoter context, and chromatin structure (35). YY1 is known to be overexpressed in various cancers, including ESCC (27). Moreover, YY1 acts as a coactivator of several transcription factors that play important roles in carcinogenesis, such as P53, GATA-4, and AR (28, 36). Sex hormone, especially androgen, has been documented to be associated with ESCC progression (37). AR promotes ESCC cell invasion and proliferation via matrix metalloproteinase 2 (38). AR and IL6 form a reciprocal regulatory circuit to sustain STAT3 oncogenic signaling in ESCC (39). High-level AR expression in ESCC predicts poor clinical outcome in tobacco-using patients with ESCC (39), suggesting another mechanism of how smoking contributes to poor prognosis in patients with ESCC.

**Figure 5.** YY1BM induced apoptosis of ESCC cells. 
A, Cleaved caspase-3 and eEF2K immunostaining in xenograft tumors of LINC00278 OE, YY1BM KO, and respective control TE-1 cells. B, Cleaved caspase-3 and eEF2K immunostaining in xenograft tumors of LINC00278 OE, YY1BM KO, and respective control KYSE-30 cells. C, TUNEL staining in xenograft tumors of LINC00278 OE, YY1BM KO, and respective control ESCC cells. D, Cleaved caspase-3 and eEF2K immunostaining in xenograft tumors of female LINC00278 OE and control ESCC cells that were implanted into female mice. E, Left, eEF2K immunostaining in male ESCC samples. Right, the relative level of YY1BM in eEF2K strong and weak samples. F, Left, cleaved caspase-3 immunostaining in male ESCC samples. Right, the relative level of YY1BM in cleaved caspase-3 strong and weak samples. *, *P < 0.05; ***, P < 0.001.
data are consistent with these findings by showing that YY1BM modulates the transcription activity of YY1 and AR, which directly coregulate the expression of eEF2K. eEF2K is a conserved mediator of the cellular response to ND (31). Activated eEF2K phosphorylates and inactivates eEF2, thereby blocking the translation elongation of mRNAs (40). eEF2K reduces cancer cell apoptosis and promotes cancer cell survival under ND (31). In this study, we demonstrated that YY1BM can regulate the eEF2K/eEF2 axis via inhibiting the transcriptional activity of YY1 and AR. Furthermore, inactivation of eEF2 could in turn block the translation of YY1BM, leading to the formation of LINC00278-YY1BM-YY1-AR-eEF2K-eEF2 cycle, which is probably one of the underlying molecular mechanisms for micropeptide encoded by IncRNA to induce tumorigenesis and progression of male ESCC.

Because YY1BM has such a critical role in male ESCC, we determined whether YY1BM is a potential anticancer micropeptide. Similar to PNC-27 targeting HDM-2 in the membrane to kill cancer cells (41), we showed that YY1BM is a potent anticancer micropeptide in ESCC.

In summary, we found that the Y-linked IncRNA LINC00278 encodes a micropeptide termed YY1BM. YY1BM suppresses the transcription of eEF2K by blocking the interaction between YY1 and AR, thereby promoting the activity of eEF2 and resulting in apoptosis of ESCC. LINC00278 has a classical mA modification motif close to the stop codon of YY1BM, which interacts with YTHDF1 and facilitates the translation of YY1BM. Cigarette smoking increases ALKBH5 expression and reduces mA modification of LINC00278, thereby inhibiting the translation of YY1BM and induces the ESCC progression. Interestingly, exogenous YY1BM has anticancer potential. In conclusion, our study reveals that LINC00278 and its product YY1BM are at the intersection of hormones, lifestyle factors, and genetics in male ESCC progression, highlighting the fact that LINC00278 and YY1BM could serve as potential prognostic biomarkers and therapeutic targets for male ESCC.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.
Authors' Contributions

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Wu, L. Zhang, J. Deng, S. Zhang, J. Lu, Y. Zhou

Writing, review, and/or revision of the manuscript: S. Wu, L. Zhang, J. Lu, Y. Zhou

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Study supervision: Y. Zhou

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