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Original Article

Downregulated antisense lncRNA *ENTPD3-AS1* contributes to the development of lung adenocarcinoma

Hung-Hsing Chiang[1,2], Chai-Tung Ong[3,4], Chao-Yuan Chang[6], Kuan-Li Wu[4,7,8,9], Yu-Yuan Wu[7], Jia-Chen Lai[4], Tzu-Yen Shen[5], Jen-Yu Hung[7,8,9,10], Hsiao-Chen Lee[11], Ying-Ming Tsai[4,7,8,9], Ya-Ling Hsu[4,9]

[1]Division of Thoracic Surgery, Department of Surgery, Kaohsiung Medical University Hospital, Kaohsiung Medical University, Kaohsiung 807, Taiwan; [2]Ph.D. Program in Environmental and Occupational Medicine, Kaohsiung Medical University, Kaohsiung 807, Taiwan; [3]Department of Fragrance and Cosmetic Science, College of Pharmacy, Kaohsiung Medical University, Kaohsiung 807, Taiwan; [4]Graduate Institute of Medicine, College of Medicine, Kaohsiung Medical University, Kaohsiung 807, Taiwan; [5]School of Medicine, College of Medicine, Taipei Medical University, Taipei 110, Taiwan; [6]Department of Anatomy, Kaohsiung Medical University, Kaohsiung 807, Taiwan; [7]School of Medicine, College of Medicine, Kaohsiung Medical University, Kaohsiung 807, Taiwan; [8]Division of Pulmonary and Critical Care Medicine, Kaohsiung Medical University Hospital, Kaohsiung Medical University, Kaohsiung 807, Taiwan; [9]Drug Development and Value Creation Research Center, Kaohsiung Medical University, Kaohsiung 807, Taiwan; [10]Internal Medicine, Kaoshiung Municipal Ta-Tung Hospital, Kaohsiung Medical University Hospital, Kaohsiung Medical University, Kaohsiung 807, Taiwan; [11]Division of Plastic Surgery, Department of Surgery, Kaohsiung Medical University Hospital, Kaohsiung Medical University, Kaohsiung 807, Taiwan

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Abstract: The poor outcome of patients with lung adenocarcinoma (LUAD) highlights the importance to identify novel effective prognostic markers and therapeutic targets. Long noncoding RNAs (lncRNAs) have generally been considered to serve important roles in tumorigenesis and the development of various types of cancer, including LUAD. Here, we aimed to investigate the role of *ENTPD3-AS1 (ENTPD3 Antisense RNA 1)* in LUAD and to explore its potential mechanisms by performing comprehensive bioinformatic analyses. The regulatory effect of *ENTPD3-AS1* on the expression of *NR3C1* was validated by siRNA-based silencing. The effect of miR-421 on the modulation of *NR3C1* was determined by miRNA mimics and inhibitors transfection. *ENTPD3-AS1* was expressed at lower levels in tumor parts and negatively correlated with unfavorable prognosis in LUAD patients. It exerted functions as a tumor suppressor gene by competitively binding to oncomir, miR-421, thereby attenuating *NR3C1* expression. Transfection of lung cancer A549 cells with miR-421 mimics decreased the expression of *NR3C1*. Transfection of lung cancer A549 cells with miR-421 inhibitors increased the expression of *NR3C1* with lower cellular functions as proliferation and migration via epithelial-mesenchymal transition. In addition, inhibition of *ENTPD3-AS1* by siRNA transfection decreased the levels of *NR3C1*, supporting the *ENTPD3-AS1*/miR-421/NR3C1 cascade. Moreover, the bioinformatic analysis also showed that *ENTPD3-AS1* could interact with the RNA-binding proteins (RBPs), CELF2 and QKI, consequently regulating RNA expression and processing. Taken together, we identified that *ENTPD3-AS1* and its indirect target *NR3C1* can act as novel biomarkers for determining the prognosis of patients with LUAD, and further study is required.

Keywords: CELF2, ENTPD3-AS1, lung adenocarcinoma, miR-421, NR3C1, QKI

Introduction

Lung cancer, one of the high-mortality cancers, accounts for 1 in 5 of all cancer death worldwide [1]. Traditionally, lung cancer could be classified into small cell and non-small cell lung cancer (NSCLC), which could be further divided into adenocarcinoma (LUAD), squamous cell carcinoma, and large cell carcinoma. The prognosis of advanced-stage LUAD is still not satisfactory. The median overall survival of patients with advanced-stage NSCLC is approximately 12 months with conventional chemotherapy and radiotherapy. Although immunotherapy has significantly improved outcomes, a durable response occurs among less than 30% of patients [2, 3]. The complexity of initiation, promotion and progression in lung cancer remains

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unsolved despite the elucidated molecular events by advanced techniques. More factors other than oncogenes or tumor suppressor genes play roles in mediating lung cancer pathogenesis. For these critical conditions, more effort should be paid to untangle the underlying mechanisms of lung cancer development.

Noncoding RNAs (ncRNAs), especially microRNAs (miRNAs) and long noncoding RNAs (lncRNAs), contribute to various biological processes in cancer tumorigenesis and progression [4-6]. LncRNAs can be divided into sense, antisense, intronic, bidirectional, and intergenic forms according to their relationship with adjacent protein-coding genes [7, 8]. LncRNAs have been demonstrated to involve in various complex mechanisms for regulating the biogenetic process. Antisense lncRNAs are transcribed from the complementary strand of protein-coding DNA, and approximately 30% of annotated human genes have an antisense component that highly impacts the gene expression profiles [9]. Recently, antisense lncRNA has been reported that abnormal expressions are involved in various pathological processes including angiogenesis [10], particularly in several types of cancers [11-13]. *MCM3AP-AS1* promotes lung cancer progression by targeting miR-195-5p, which suppresses oncogene *EIF3* expression [14]. *OIP5-AS1* increases *ITGA6* or *SMAD3* expressions by sponging miR-143-3p, resulting in promoting cell proliferation and invasion in cervical cancer and lung cancer [15, 16]. *LMCD1 antisense RNA 1* exerts pro-tumorigenic function through sponging miR-1287-5p to elevate *GLI2* expression in thyroid cancer [17]. However, the relationship between antisense lncRNAs and the progression of LUAD remained largely unclear.

Comprehensive bioinformatics and systemic biological analysis can facilitate the understanding of the pathogenesis of LUAD and the identification of potential prognostic biomarkers and actionable targets. Numerous transcriptomic analyses and datasets of lung cancer were built. Nevertheless, compared with coding genes and miRNAs, the specific lncRNAs associated with the development of lung cancer remain unknown. The present study was designed to explore *ENTPD3-AS1* expression (located at 40,313,801 to 40,457,209 on chromosome 3) and its prognostic value in LUAD. Our results showed that *ENTPD3-AS1* functioned as a tumor suppressor gene in LUAD by sponging miR-421 and CELF2/QKI-interacted axis, then regulated cell migration and epithelial-mesenchymal transition (EMT). Our findings may be used to further investigate the characteristics of LUAD and are useful in determining the prognosis and therapy of patients with LUAD.

Materials and methods

Cell culture

Human LUAD CL1-5 (RRID: CVCL_D521) cells were kindly provided by Dr. Pan-Chyr Yang of National Taiwan University. A549 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in F-12K Medium supplemented with fetal bovine serum (FBS, 10%), penicillin (100 U/mL) and streptomycin (100 μg/mL) (Thermo Fisher Scientific, Boston, MA, USA). CL1-5 cells were cultured in RPMI 1640 supplemented with 10% of FBS and 1% of penicillin-streptomycin (Lonza). A549 cells were authenticated by a short tandem repeat (Promega, Madison, WI, USA) and detected to be negative for mycoplasma contamination by the MycoAlert™ mycoplasma detection kit (Lonza, Switzerland).

Data collection

The gene expression of LUAD was extracted from samples of The Cancer Genome Atlas (TCGA) (available online: https://portal.gdc.cancer.gov, accessed on March 13, 2023). The criterion in the analysis was a p-value <0.05 which was calculated using the University of Alabama at Birmingham CANcer (UALCAN) data analysis portal (available online: http://ualcan.path.uab.edu, accessed on March 13, 2023), the Encyclopedia of RNA Interactomes (ENCORI) (available online: https://starbase.sysu.edu.cn/index.php, accessed on March 10, 2023), or TNM plotter (available online: https://tnmplot.com/analysis/, accessed on March 10, 2023).

Bulk RNA sequencing (RNA-seq)

The pairs of adjacent non-tumor lungs and tumors were harvested from the Division of Thoracic Surgery and Division of Pulmonary and Critical Care Medicine, Kaohsiung Medical

University Hospital, Kaohsiung, Taiwan (KMUH-IRB-20180023; KMUH-IRB-20200038). The bulk RNA-seq for the 10 pairs of LUAD and adjacent normal tissue was carried out by the Welgene biotechnology company (Taipei, Taiwan). The criteria for differentially expressed mRNA by bulk RNA-seq were fold change >2 and fragments per kilobase million (FPKM) >0.3.

Survival analysis of ENTPD3-AS1, miR-421, NR3C1, CELF2, QKI of LUAD

The survival analyses of the various genes in LUAD were evaluated using the Kaplan Meier (KM) plotter (available online: http://kmplot.com/analysis/, accessed on March 13, 2023). The hazard ratios (95% confidence intervals) were calculated using the Cox proportional model.

Screening for targets of miR-421

The target genes of miR-421 were predicted by miRWalk (available online: http://mirwalk.umm.uni-heidelberg.de/, accessed on April 7, 2023), which searched the target genes based on the intersection of miRDB and miRTarbase. A target score >95% was set as the cutoff criterion.

Interaction of miRNAs and RNA-binding protein (RBP) with ENTPD3-AS1

The miRNA or RBP interaction analyses with lncRNA (miRNA-lncRNA, and RBP-lncRNA) were executed using the ENCORI online database. The potential interactive RBPs with *ENTPD3-AS1* were searched using RBP-lncRNA module, with a selection criterion of the stringency equal to, or more than 1, in the crosslinking immunoprecipitation (CLIP) database.

Functional analysis and the gene set variation analysis (GSVA) of gene sets

To investigate the role of NR3C1, the LUAD patients of TCGA were divided into *NR3C1* or miR-421 high- and low-expressed groups according to the highest and lowest quartiles, and gene set enrichment analysis (GSEA) was conducted to assess the enrichment of datasets between high- and low-target gene groups. False discovery rate (FDR) <0.05 and nominal *p*-value <0.05 were set as the cutoff criteria. The gene set "c2.cp.kegg.v6.2.symbols.gmt" was chosen as the reference gene set. The criteria in gene extraction were Pearsons correlation coefficient (CC) >0.3 and *p*-value <0.05, which was calculated using the UALCAN. The GSVA score of LUAD was calculated using gene set cancer analysis (GSCA) (available online: http://bioinfo.life.hust.edu.cn/GSCA/#/, accessed on March 13, 2023).

Transfection of miR-421 mimics/inhibitors and ENTPD3-AS1 siRNA

Knockdown of *ENTPD3-AS1* in CL1-5 and A549 cells were performed using SMARTpool Lincode siRNA (target siRNA for sequencing; GUAAACUUUGAUAGCUA, UGGACGGGUCGAUAGUUU, UCAGAUGGAUCUCGAUUU and GUGUAGCGAGACCACA: 20 nM) (Dharmacon, La-fayette, CO). The knockdown efficacy of *ENTPD3-AS1* siRNA was determined by qRT-PCR, as described below. A549 and CL1-5 cells were also transfected with control (mimics or inhibitors), miR-421 mimics (200 nM), miR-421 inhibitors (20 nM) using DharmaFECT reagent, and the levels of NR3C1 were determined by qRT-PCR or Immunoblot after 48 hours transfection.

Quantitative real-time polymerase chain reaction (qRT-PCR)

The total RNA of CL1-5 and A549 cells were isolated using the TRIzol Reagent (Life Technologies, Carlsbad, CA, USA), and cDNA was reverse transcribed using reverse transcriptase kits (Takara, Shiga, Japan). The levels of *NR3C1* or *ENTPD3-AS1* RNA were detected using real-time analysis with SYBR Green on a QuantStudio 5 machine (Thermo Scientific, CA, USA). The relative expression levels of the *ENTPD3-AS1* or *NR3C1* mRNA were normalized to glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). The relative standard method ($2^{-\Delta\Delta Ct}$) was used to calculate relative RNA expression. The following primers were used: *ENTPD3-AS1* (forward, 5'-AGGCAACATGAGGATGACGT-3' and reverse, 3'-ACCCTGAAGGCCTCTGGAT-5'), NR3C1 (forward, 5'-ACCTGCTGTGTTTTGCTCCT-3' and reverse, 3'-CTCTTGGCTCTTCAGACCGT-5') and GAPDH (forward, 5'-TTCACCACCATGGAGAAGGC-3' and reverse, 5'-GGCATGGACTGTGGTCATGA-3').

Immunoblot and immunohistochemical staining

The total protein was isolated from A549 cells using the radio-immunoprecipitation

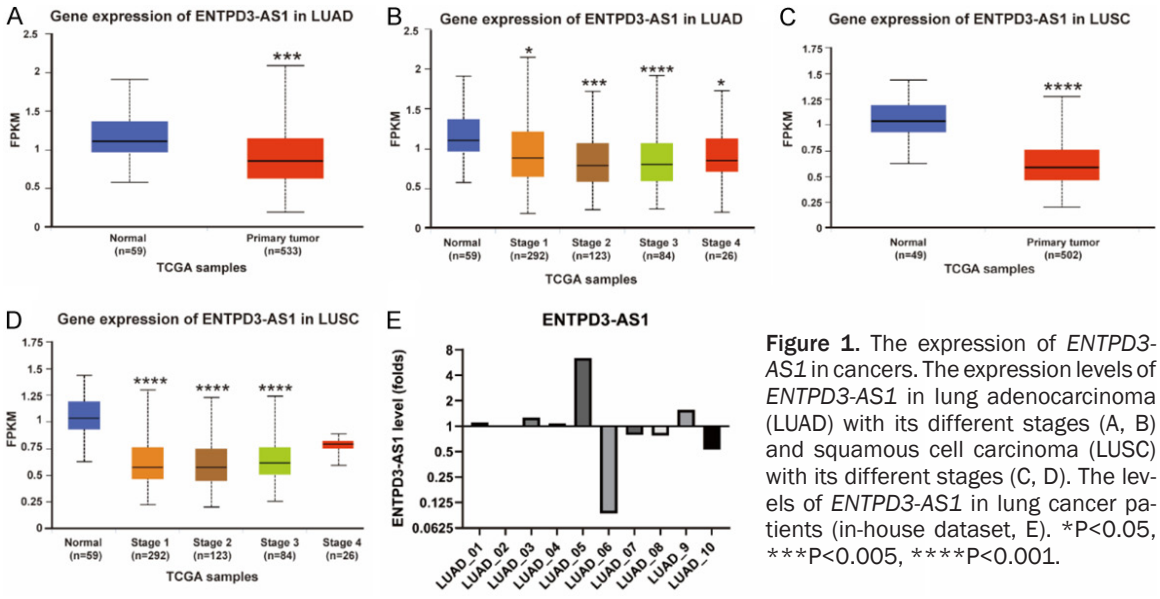


Figure 1. The expression of *ENTPD3-AS1* in cancers. The expression levels of *ENTPD3-AS1* in lung adenocarcinoma (LUAD) with its different stages (A, B) and squamous cell carcinoma (LUSC) with its different stages (C, D). The levels of *ENTPD3-AS1* in lung cancer patients (in-house dataset, E). *P<0.05, ***P<0.005, ****P<0.001.

assay (RIPA) (EMD Millipore, Billerica, MA, USA) supplemented by a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). Equal total protein was denatured and separated by a sodium dodecyl-sulfate polyacrylamide gel electrophoresis. Proteins in the gel were transferred onto polyvinylidene difluoride membranes (EMD Millipore) and then probed with anti-NR3C1 (catalog#12041, Cell Signaling Technology, Carlsbad, CA, USA) or anti-GAPDH (catalog#MAB374, EMD Millipore) primary antibodies, followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies (Cell-Signaling Technology, Danvers, MA, USA). The signal of the specific protein was detected using a chemiluminescence kit (EMD Millipore). The expressions of NR3C1 of normal and tumor sections by IHC were demonstrated using anti-NR3C1 antibodies (dilution 1:200, catalog#12041, Cell Signaling Technology). All of the sectioned tissues were counterstained with hematoxylin.

Statistical analysis

Spearman's correlation was applied for the analysis of the correlation. Most analyses were conducted using GraphPad Prism 9.0. Logistic regression, univariate, and multivariate analyses were used to assess the influence of clinical variables on patient survival. *p*-value less than 0.05 were considered statistically significant.

Results

Downregulated ENTPD3-AS1 in patients with lung cancer

We first analyzed *ENTPD3-AS1* expression in the two different types of lung cancer, adenocarcinoma (LUAD) and squamous cell carcinoma (LUSC), respectively. As shown in **Figure 1A-D**, *ENTPD3-AS1* expressions were lower in both LUAD and LUSC in the cancer genome atlas (TCGA) database composed from the UALCAN. However, the expression levels were lower in higher stages but not stage-dependent in both LUAD and LUSC (**Figure 1A-D**). Also, *ENTPD3-AS1* levels were significantly decreased in 10 cancer types other than lung cancer including breast, colon, cholangiocarcinoma, head and neck squamous cell carcinoma, kidney chromophobe, sarcoma and thyroid carcinoma on the TCGA cohort (Supplementary Figure 1) compared with these in normal tissues. Moreover, 4 out of 10 patients from in-house LUAD cohort had lower levels of *ENTPD3-AS1* (**Figure 1E**) with the clinicopathologic characteristics (Supplementary Table 1). These data showed that *ENTPD3-AS1* may play a tumor suppressor role in various cancers.

Loss of function in ENTPD3-AS1 led to aggressive cellular behaviors contributing poor survival outcomes in patients with LUAD

Next, we investigated the correlation between *ENTPD3-AS1* levels and clinical outcomes in

LUAD and LUSC in TCGA database. The results indicated that high *ENTPD3-AS1* expression was associated with better overall survival (OS) and relapse-free survival (RFS) in LUAD (**Figure 2A**). However, *ENTPD3-AS1* was not correlated with OS and RFS in LUSC (**Figure 2B**). To elucidate the plausible mechanisms of affecting survival in LUAD, *ENTPD3-AS1* siRNA knockdown to learn several cellular behaviors such as proliferation, wound-healing assay and Transwell migratory assay were performed. After *ENTPD3-AS1* knockdown, enhanced proliferation was found in A549 cancer cell line (**Figure 2C** and Supplementary Figure 2). The migratory abilities also elevated while *ENTPD3-AS1* was inhibition in both CL1-5 and A549 cells (**Figure 2D** and **2E**). Epithelial-mesenchymal transition (EMT) has been known to mediate aggressive cellular behaviors in cancer which lead to poor survival in lung adenocarcinoma patients. The results indicated attenuation of a epithelial marker (E-cadherin) and accentuation of mesenchymal markers (N-cadherin, Vimentin, α-SMA) after *ENTPD3-AS1* knokdown (**Figure 2F**). These data suggested decreased expression of *ENTPD3-AS1* might promote cancer progession and shorten survival in patients with LUAD.

Lower levels of miR-421 regulatd by ENTPD3-AS1 conferred longer survival in LUAD

Antisense lncRNAs have been reported to serve as competing-endogenous RNAs (ceRNAs), which interact with microRNA (miRNAs) to further regulate the translation of the specific target mRNA. We thus predicted the interactions of *ENTPD3-AS1* with miRNAs using the ENCORI website. The results showed that eleven miRNAs (hsa-miR-2278, hsa-miR-6504-5p, hsa-miR-208a-3p, hsa-miR-3118, hsa-miR-519e-5p, hsa-miR-124-3p, hsa-miR-1323, hsa-miR-421, hsa-miR-6582-3p-, hsa-miR-155-5p and hsa-miR-1247-5p) were complementary to *ENTPD3-AS1* (Supplementary Table 2) and only miR-421 were upregulated (1.64-fold, $P = 1.54\times10^{-14}$) and associated with OS of LUAD in TCGA cohort, as assessed by UALCAN and KM plotter website (**Figure 3A** and **3B**). The miR-421 expression levels were higher in tumor parts compared with normal parts, advanced stages and lymph node metastasis of LUAD (**Figure 3C-E**). But the expression levels of miR-421 was not cancer stages nor nodal metastasis status dependent in LUAD (**Figure 3D** and **3E**). Higher level of miR-421 was found in 5 out of 8 LUAD in our in-house corhort (**Figure 3F**). GSEA analysis revealed that upregulated miR-421 was associated with cancer proliferation, undifferentiated and poor survival rate of lung cancer patients (**Figure 3G-I**). Our results show that miR-421 regulated by ENTPD3-AS1 may be an oncomir in LUAD.

Higher levels of NR3C1 regulated by miR-421 favored a better survival

We further assessed the potential targets of miR-421 predicted by miRWalk (possibility >95% and predicted by both miRDB and miRTarbase). According to the screening by bioinformatics, targets of miR-421 included 8 protein-coding genes, *DFFB, DDX3X, RAB26, RALGAPB, DFFB, PRCC, SIRT3,* and *NR3C1*. The expression of *NR3C1* was downregulated in tumor parts in LUAD, cancer stages, and nodal metastasis status but not stage nor nodal metastatic status dependent (**Figure 4A** and **4B**). The expression of *NR3C1* was negatively correlated with OS, RFS and first progression (FP) of LUAD (**Figure 4C** and Supplementary Figure 3A). miR-421 also have high-binding affinity on the 3'UTR of *NR3C1* (Supplementary Figure 3B). In addition, the level of NR3C1 protein decreased in LUAD of CPTAC cohort (**Figure 4D**). Moreover, LUAD patients had lower expression of NR3C1 at mRNA levels in our cohort (Supplementary Figure 3C). Also, immunohistochemial staining of NR3C1 in tissues from lung adenocarcinoma revealed lower expression levels of NR3C1 compared with adjacent normal tissues (pneumocytes) (**Figure 4E**). Most importantly, *in vitro* results showed that transfection of miR-421 mimics decreased the expression of NR3C1 (**Figure 4F**), whereas transfection of miR-421 inhibitors increased the expression of NR3C1 in both CL1-5 and A549 LUAD cell lines (**Figure 4G**). Several cellular behaviors scuh as proliferation and wound healing assay performed to elucidate the possible explanation of favorable survival after tranfection of miR-421 inhibitors of CL1-5 and A549. Knockdown of NR3C1 by miR-421 inhibitors caused decrease proliferation of cancer cells (**Figure 4H**) and retarded cell migration (**Figure 4I** and **4J**, Supplementary Figure 3D). Moreover, an attenuation of mesenchymal markers (N-cadherin, Vimentin, α-SMA) and enhancement of epithe-

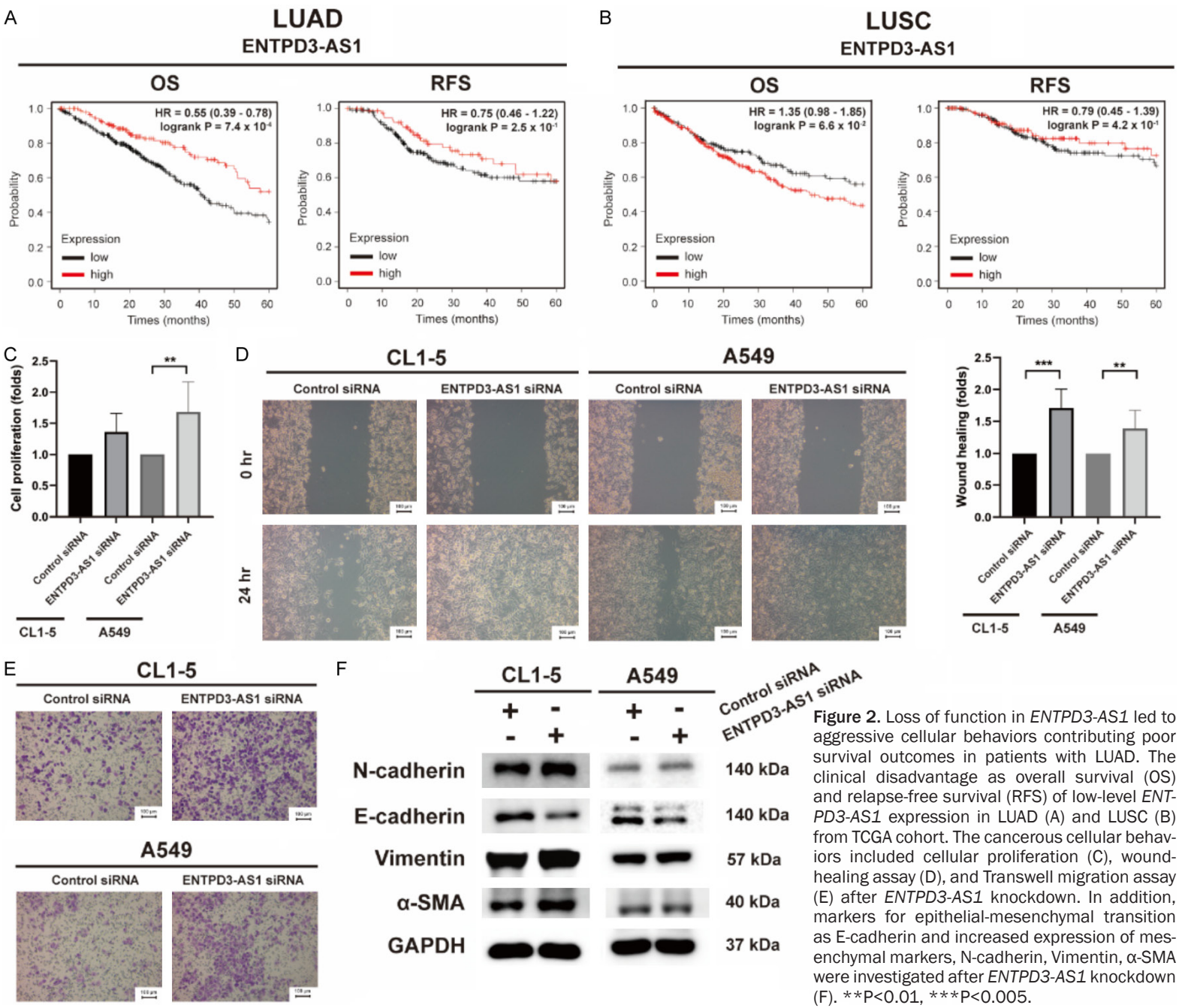


Figure 2. Loss of function in *ENTPD3-AS1* led to aggressive cellular behaviors contributing poor survival outcomes in patients with LUAD. The clinical disadvantage as overall survival (OS) and relapse-free survival (RFS) of low-level *ENTPD3-AS1* expression in LUAD (A) and LUSC (B) from TCGA cohort. The cancerous cellular behaviors included cellular proliferation (C), wound-healing assay (D), and Transwell migration assay (E) after *ENTPD3-AS1* knockdown. In addition, markers for epithelial-mesenchymal transition as E-cadherin and increased expression of mesenchymal markers, N-cadherin, Vimentin, α-SMA were investigated after *ENTPD3-AS1* knockdown (F). **P<0.01, ***P<0.005.

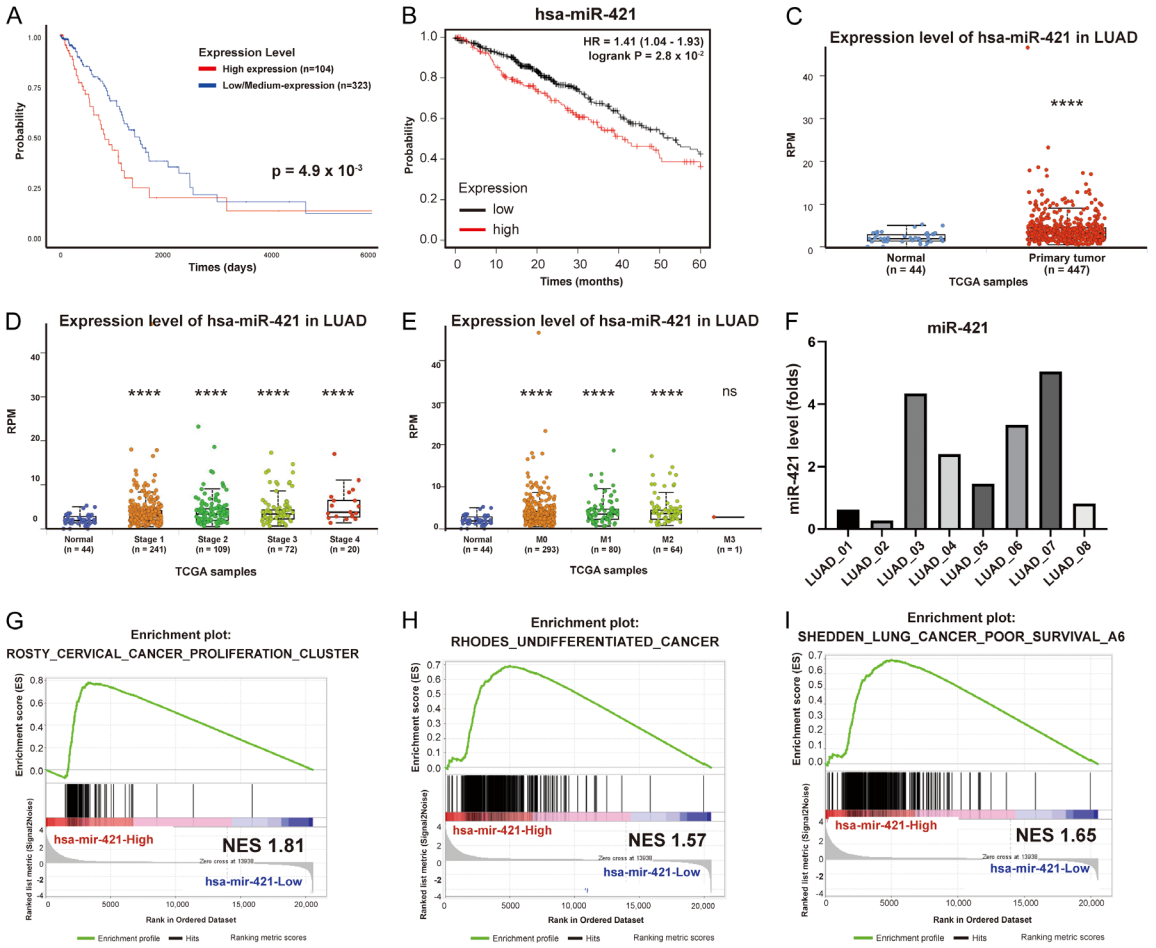


Figure 3. miR-421 function regulated by *ENTPD3-AS1* affected survival in LUAD. Competing-endogenous RNAs affect the expression of miRNAs. The analysis of the impact of miR-421 on overall survival from UALCAN (A) and K-M plotter from TCGA cohort (B) in LUAD. The expression of miR-421 in LUAD based on tumor (C), stages (D), and lymph node metastasis (E). The expression levels of miR-421 from the in-house cohort (F). The influence of miR-421 on cancer proliferation (G), undifferentiated (H), and lung cancer poor survival (I) via GSEA. ****P<0.001; ns, not significant.

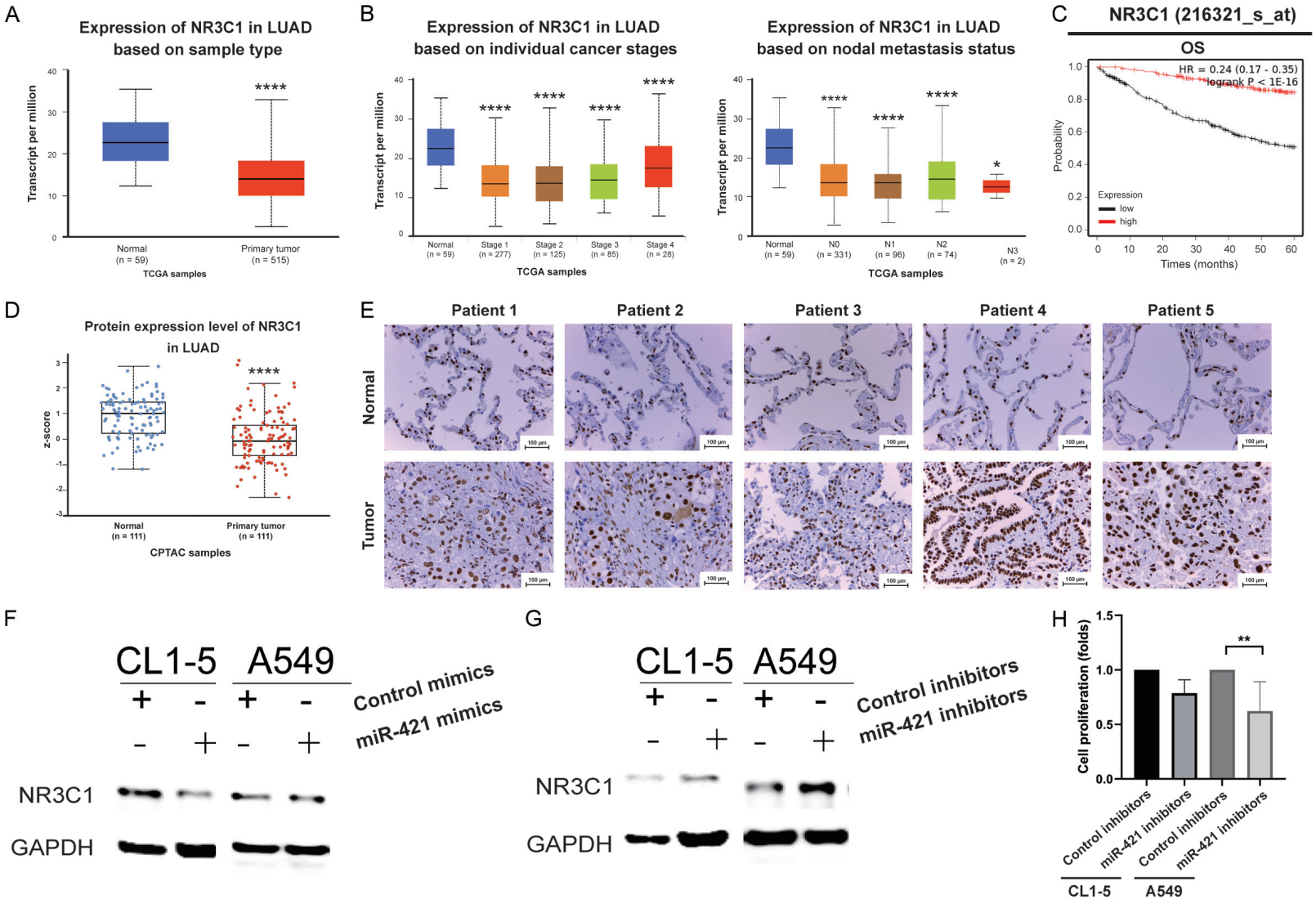
lial marker (E-cadherin) was observed after miR-421 inhibitors transfection (**Figure 4K**). Our data suggests *NR3C1* is the regulatory target of miR-421 and favors a survival benefit.

The loop of ENTPD3-AS1/NR3C1

To identify whether *NR3C1* expression was correlated with LUAD development, we extracted the gene sets with a positive correlation of *NR3C1* in LUAD and assessed by Gene Set Cancer Analysis (GSCA) website. The GSVA score (log2FC = -0.17, P = 6.25×10^{-25}) of the top 200 gene set (R>0.45, P<0.05) was lower in tumors than that in normal lung tissue (**Figure 5A**). GSEA showed that decreased levels of *NR3C1* in lung cancer was associated with the knockdown of KRAS (**Figure 5B**), suggesting the function of *NR3C1* was associated with the inhibition of KRAS-mediated oncogenic cascade. The OS benefit of higher levels of *ENTPD3-AS1* was attenuated while *NR3C1* expression was lower (**Figure 5C**). Inhibition of *ENTPD3-AS1* by siRNA transfection decreased the expression of NR3C1 protein showing *ENTPD3-AS1* as the upstream regulator of *NR3C1* (**Figure 5D**). The results suggest ENTPD3-AS1 synergizes with NR3C1 which confers a poor surivival in lower levels of expression.

Interaction of ENTPD3-AS1 with RNA-binding proteins (RBPs), CELF2 and QKI

RBPs are known to be abnormally expressed in cancer and regulate the function of lncRNAs [18]. To better understand the potential regula-


A
Expression of NR3C1 in LUAD based on sample type
Transcript per million
****
Normal (n = 59)
Primary tumor (n = 515)
TCGA samples
B
Expression of NR3C1 in LUAD based on individual cancer stages
Transcript per million
Normal (n = 59)
Stage 1 (n = 277)
Stage 2 (n = 125)
Stage 3 (n = 85)
Stage 4 (n = 28)
TCGA samples
Expression of NR3C1 in LUAD based on nodal metastasis status
Normal (n = 59)
N0 (n = 331)
N1 (n = 96)
N2 (n = 74)
N3 (n = 2)
TCGA samples
C
NR3C1 (216321_s_at)
OS
HR = 0.24 (0.17 - 0.35)
logrank P < 1E-16
Probability
Expression
low
high
Times (months)
D
Protein expression level of NR3C1 in LUAD
Z-score
Normal (n = 111)
Primary tumor (n = 111)
CPTAC samples
E
Patient 1
Patient 2
Patient 3
Patient 4
Patient 5
Normal
Tumor
F
CL1-5
A549
Control mimics
miR-421 mimics
NR3C1
GAPDH
G
CL1-5
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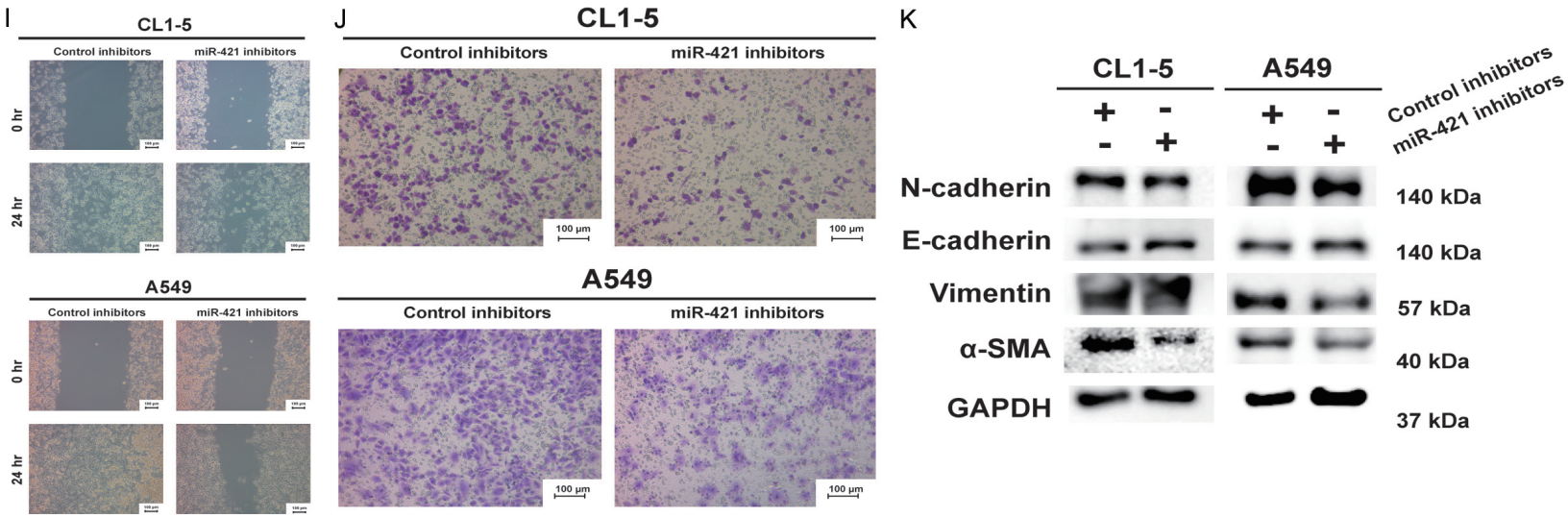

Figure 4. Higher expressions of *NR3C1*, the target of miR-421, favored longer survival in LUAD. The expression of NR3C1 at a mRNA level in tumor parts (A), cancer stages and nodal metastasis status (B). The survival significance of *NR3C1* was expressed as OS (C). The expression level of NR3C1 protein in lung adenocarcinoma cohort (CPTAC) (D). The expression of NR3C1 protein from 5 lung adenocarcinoma patients (E). miR-421 mimics decreased the expression of *NR3C1* (F). miR-421 inhibitors increased the expression of *NR3C1* (G). The cellular behaviors, proliferation (H), wound-healing assay (I), and transwell migratory assay (J) performed to evaluate the effects after NR3C1 restoration by miR-421 inhibitors. Mesenchymal-epithelial transition as increased E-cadherin and decreased expression of mesenchymal markers, N-cadherin, Vimentin, α-SMA were investigated after miR-421 inhibtors (K). OS, overall survival. *P<0.05, ****P<0.001.

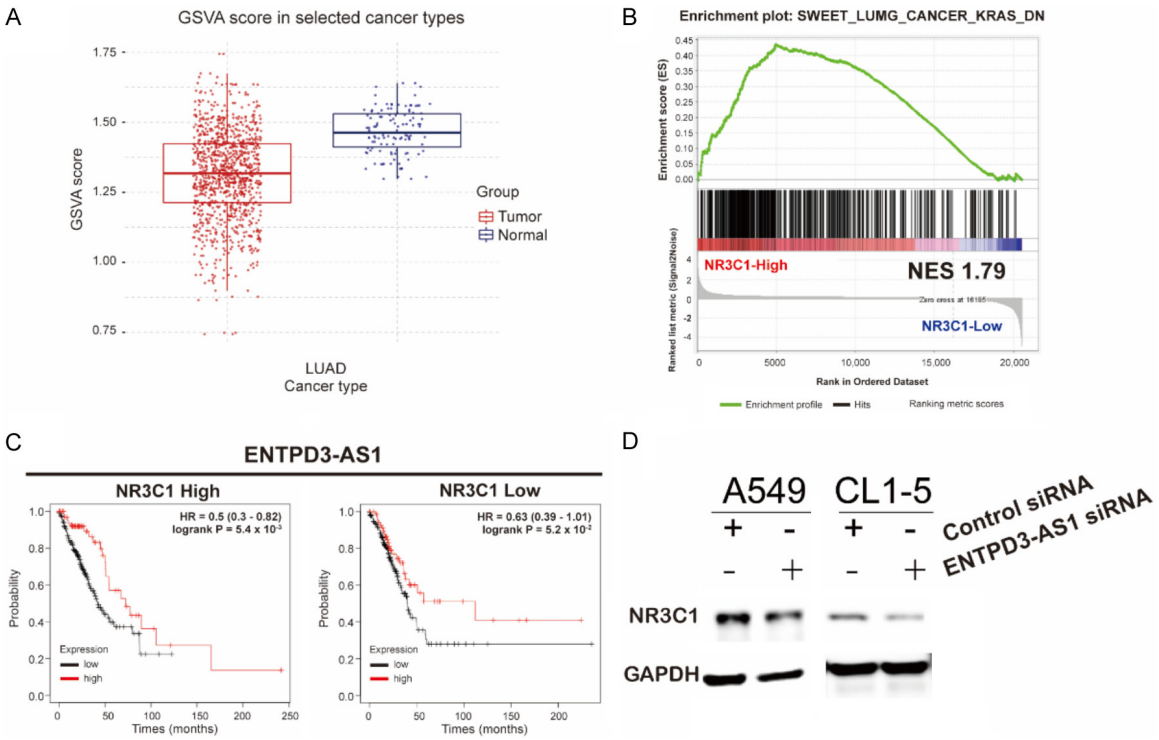

Figure 5. The interaction between *ENTPD3-AS1/NR3C1*. NR3C1 expression plays a role in mediating LUAD. The top 200 genes correlated with *NR3C1* underwent GSVA analysis (A). GSEA analysis revealed *NR3C1* was associated with suppression genes by KRAS mutation (B). The cross-analysis for OS of *ENTPD3-AS1* was performed in lung cancer patients with high or low levels of *NR3C1* (C). Knockdown of *ENTPD3-AS1* by siRNA transfection decreased NR3C1 protein expression in A549 and CL1-5 (D).

tory mechanisms of *ENTPD3-AS1* in LUAD, we examined the expression of RBPs, which interacts with *ENTPD3-AS1* in LUAD. We used the ENCORI database to explore possible RBPs for *ENTPD3-AS1*. The results showed that 109 RBPs might modulate *ENTPD3-AS1* function. A total of 62 RBPs were upregulated and 25 RBPs were downregulated in LUAD. Among them, CELF2, QKI, and AUH expressions were reduced (**Table 1**; **Figure 6A-C**). To identify the RBP which most probably regulates the *ENTPD3-AS1*, we conducted a cross-analysis of *ENTPD3-AS1* with these three RBPs on OS. The OS benefit of *ENTPD3-AS1* was lost while CELF2 and QKI expression was lower (**Figure 6D** and **6E**). In contrast, the effect of *ENTPD3-AS1* only slightly, or insignificantly, affected the OS of LUAD patients with lower expressions of *AUH* (**Figure 6F**). Reduced CELF2 and QKI expression were also associated with shorter OS in patients with LUAD (**Figure 6G** and **6H**). These results favor that CELF2 and QKI could be RBPs of *ENTPD3-AS1*, contributing to the development of LUAD.

Discussion

For poor clinical outcomes, improvement of the prognosis of LUAD patients through discovering novel diagnostic biomarkers and druggable targets is still required. The main finding of this study is that *ENTPD3-AS1* expression is reduced in LUAD patients, and high expression of *ENTPD3-AS1* is positively correlated with the prognosis of patients with LUAD. This is the first comprehensive evaluation of *ENTPD3-AS1* that implicates its potential role as a tumor suppressor in LUAD.

LncRNAs can serve as competing endogenous RNA (ceRNA) to regulate gene expression by competitively binding miRNAs [19]. *ENTPD3-AS1* has been reported to be associated with prostate cancer susceptibility [20]. In contrast, *ENTPD3-AS1* has also been regarded as a tumor suppressor in renal cell carcinoma by inhibiting miR-155/HIF axis [21]. The current study identified the reduced expression of *ENTPD3-AS1* in LUAD patients, who have poor

Table 1. The RBPs interacted with ENTPD3-AS1

Genes	Fold change	*P* value
ADAR	1.384	$<1\times10^{-12}$
AIFM1	1.339	1.62×10^{-12}
AUH	0.829	1.46×10^{-3}
BCCIP	1.452	$<1\times10^{-12}$
BUD13	0.979	4.25×10^{-2}
CX10LF2	0.371	1.62×10^{-12}
CNBP	0.896	3.40×10^{-3}
CPSF6	1.414	1.62×10^{-12}
CSTF2T	0.916	8.05×10^{-1}
DDX3X	0.763	3.00×10^{-6}
DDX42	1.305	1.62×10^{-12}
DDX54	1.376	$<1\times10^{-12}$
DGCR8	1.217	2.33×10^{-9}
DHX9	1.211	$<1\times10^{-12}$
DICX10R1	0.759	1.30×10^{-3}
DKC1	2.077	1.62×10^{-12}
X10IF4A3	1.605	$<1\times10^{-12}$
X10IF4G2	0.995	8.52×10^{-3}
X10LAVL1	1.194	4.44×10^{-16}
X10WSR1	1.059	5.24×10^{-4}
FAM120A	1.260	1.62×10^{-12}
FBL	1.433	$<1\times10^{-12}$
FMR1	0.929	4.22×10^{-2}
FTO	0.763	3.86×10^{-5}
FUS	1.189	1.63×10^{-12}
FXR1	1.169	$<1\times10^{-12}$
FXR2	0.717	8.81×10^{-8}
GNL3	2.279	$<1\times10^{-12}$
GTF2F1	0.998	8.89×10^{-2}
HNRNPA1	1.179	$<1\times10^{-12}$
HNRNPA2B1	1.303	$<1\times10^{-12}$
HNRNPC	1.581	$<1\times10^{-12}$
HNRNPD	1.202	$<1\times10^{-12}$
HNRNPK	1.176	1.65×10^{-12}
HNRNPM	1.006	1.53×10^{-2}
HNRNPU	1.305	4.26×10^{-13}
HNRNPUL1	1.043	1.33×10^{-6}
IGF2BP1	4.400	8.41×10^{-11}
IGF2BP2	1.083	1.64×10^{-12}
IGF2BP3	6.199	1.62×10^{-12}
ILF3	1.277	1.62×10^{-12}
KHDRBS1	1.218	$<1\times10^{-12}$
KHDRBS2	0.063	1.62×10^{-12}
KHDRBS3	0.742	9.04×10^{-2}
KHSRP	1.171	1.76×10^{-12}
LARP4B	0.834	9.77×10^{-1}
LARP7	0.936	4.91×10^{-1}
LIN28	NA	NA
LIN28A	NA	NA
LIN28B	NA	NA
LSM11	1.132	8.41×10^{-5}
MBNL2	0.635	1.22×10^{-12}
MOV10	1.506	$<1\times10^{-12}$
MSI1	0.769	$<1\times10^{-12}$
MSI2	2.981	$<1\times10^{-12}$
NONO	1.515	1.62×10^{-12}
NOP56	1.668	$<1\times10^{-12}$
NOP58	1.782	$<1\times10^{-12}$
NPM1	1.607	$<1\times10^{-12}$
NUMA1	1.043	2.58×10^{-2}
PCBP2	1.011	1.94×10^{-5}
PRPF8	0.793	9.28×10^{-3}
PTBP1	1.331	1.62×10^{-12}
PUM2	1.026	8.22×10^{-3}
QKI	0.434	1.62×10^{-12}
RANGAP1	1.497	1.62×10^{-12}
RBFOX2	NA	NA
RBM10	1.221	9.06×10^{-10}
RBM22	0.927	2.38×10^{-1}
RBM47	1.178	7.19×10^{-10}
SAFB2	0.994	2.65×10^{-1}
SF3A3	1.119	1.62×10^{-12}
SF3B4	1.637	1.62×10^{-12}
SLBP	1.529	$<1\times10^{-12}$
SLTM	0.887	9.73×10^{-1}
SMNDC1	1.157	1.65×10^{-12}
SND1	1.465	1.62×10^{-12}
SRSF1	NA	NA
SRSF10	NA	NA
SRSF3	NA	NA
SRSF7	NA	NA
SRSF9	NA	NA
TAF15	1.135	3.52×10^{-8}
TARDBP	1.144	2.07×10^{-8}
TIA1	1.537	1.63×10^{-12}
TIAL1	1.415	1.62×10^{-12}
TNRC6A	1.135	1.03×10^{-4}
TRA2A	1.112	4.17×10^{-4}
TROVX102	1.215	4.79×10^{-10}
U2AF1	1.094	5.21×10^{-7}
U2AF2	1.300	1.62×10^{-12}
UPF1	1.099	1.83×10^{-6}
XRN2	1.125	3.98×10^{-11}
YTHDC1	0.944	9.90×10^{-1}
YTHDF1	1.346	$<1\times10^{-12}$
YWHAG	1.416	1.62×10^{-12}
ZC3H7B	0.790	3.18×10^{-3}

NA, not availablx10, thx10 gx10nx10 not includx10d in LUAD of TCGA cohort.

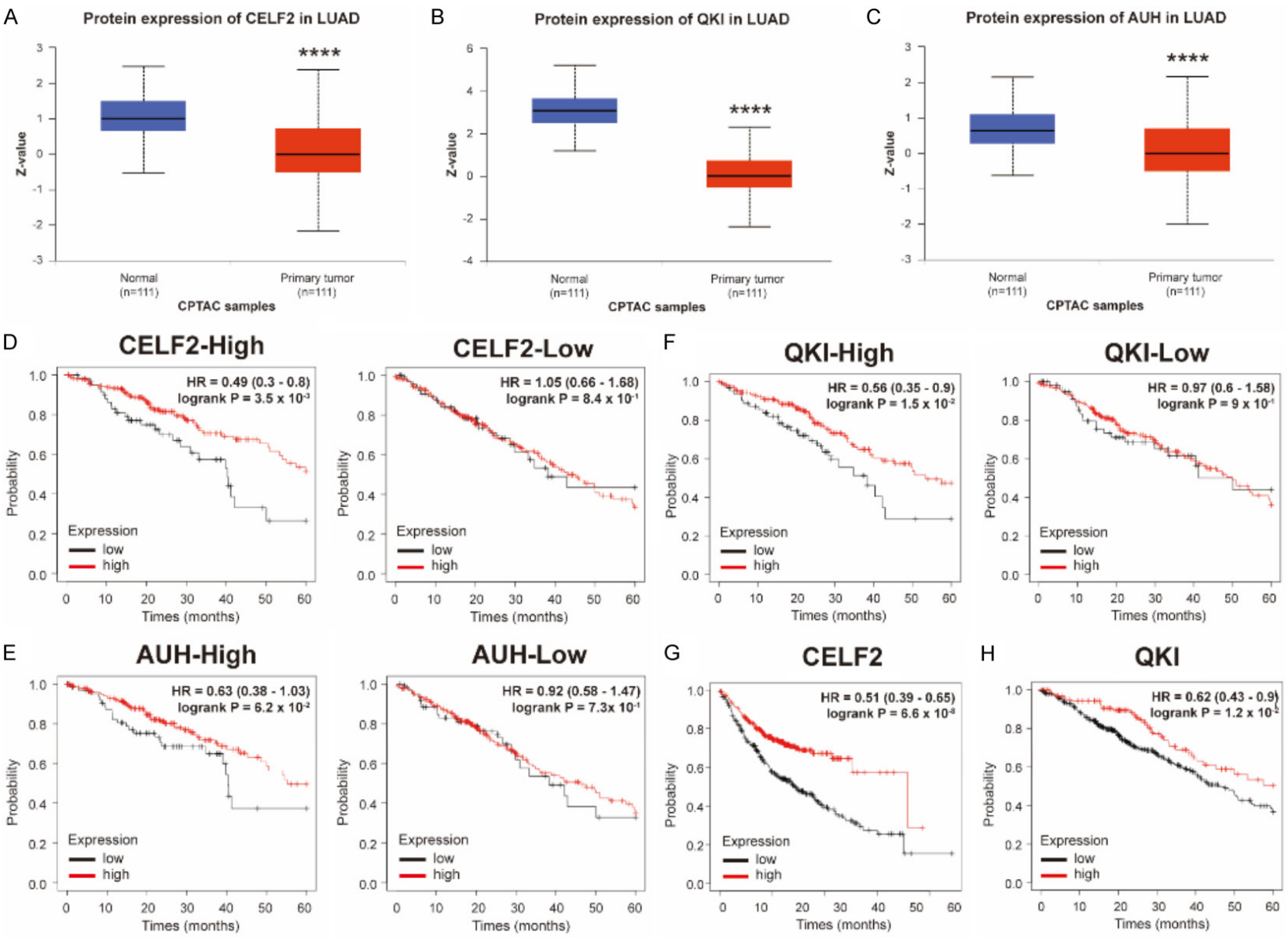

Figure 6. RNA-binding proteins, CELF2 and QKI, interacted with *ENTPD3-AS1*. The expressions of different RBPs, *CELF2* (A), *QKI* (B), and *AUH* (C) in LUAD patients. The cross-analysis for OS of *ENTPD3-AS1* in LUAD patients as conditions of high or low levels of *CELF2* (D), *QKI* (E), or *AUH* (F). The OS of *CELF2* (G) and *QKI* (H) in LUAD patients. ****P<0.001. OS, overall survival.

clinical outcomes, supporting that *ENTPD3-AS1* could act as a promising prognostic predictor in LUAD. miR-421, which is an oncomir associated with various cancers, including pancreatic cancer, cholangiocarcinoma, and LUAD [22-24], is sponged by *ENTPD3-AS1*. Indeed, the knockdown of *ENTPD3-AS1* or miR-421 inhibitors decreased cell proliferation, enhanced cell migration and epithelial phenotype transition in A549 cells, supporting the findings that *ENTPD3-AS1* inhibited the oncogenic function of miR-421 in LUAD. However, the direct interaction of *ENTPD3-AS1*-miR-421 requires further experimental studies.

The gene, *NR3C1,* encodes for a glucocorticoid receptor (GR), which is a nuclear receptor (NR) and transcription factor that is involved in cell proliferation, metabolism, and homeostasis, and also confers resistance to androgen receptor-targeted therapy [25-27]. The role of *NR3C1* is controversial in oncology. *NR3C1* expression is upregulated in breast cancer, and the inhibition of *NR3C1* decreases the migration and invasion of breast cancer cells [28]. In contrast, *NR3C1* is greatly downregulated in colon cancer, and miR-19b strengthens oxaliplatin resistance and colon cancer malignant progression by targeting *NR3C1* [29]. Caratti et al. revealed that GR, the product of *NR3C1* gene, was present in cytoplasmic KRAS-containing complexes and inhibited the activation of wild-type and oncogenic KRAS in mouse embryonic fibroblasts and human lung cancer A549 cells [30]. Our results found that decreased NR3C1 expression at both mRNA and protein levels has been observed in LUAD patients and correlated with an adverse clinical outcome, including OS, PPS, and FP, in LUAD. *In vitro* study demonstrated the expression of *NR3C1* was regulated by miR-421, supported by miR-421 mimics repressed NR3C1 protein expression. Functional enrichment analysis showed that the upregulated *NR3C1* was associated with KRAS knockdown, consistent with Caratti's study [30]. Also, enhanced NR3C1 protein level when adding miR-421 inhibitors induced cancer cell death, less migratory ability and mesenchymal-epithelial transition. All of the results above indicate that *NR3C1* has a tumor suppressive role on LUAD and is regulated by *ENTPD3-AS1*/miR421 negative loop.

LncRNAs can specifically bind to RBPs and influence the biological functions of RBPs. Conversely, certain specific RBPs can bind to lncRNAs to influence the function of lncRNAs on the regulation of downstream gene expression [27]. RBPs are involved in multiple post-transcriptional processes, including RNA splicing, localization, polyadenylation, modification, stability, and translation [31, 32]. Therefore, functional dysregulation of RBPs have been increasingly identified as critical regulators of cancer. CELF2, one of the members of shuttling nucleocytoplasmic RNA-binding proteins, is able to control most stages of mRNA processing, such as alternative splicing, RNA editing, deadenylation, and the control of RNA stability and translation [33]. CELF2 has been reported as a tumor suppressor in lung cancer. CELF3 is the RBP of hedgehog-interacting protein antisense RNA 1 (HHIP-AS1), which inhibits LUAD progression by increasing HHIP stability, resulting in the suppression of cell growth and migration [34]. Circular RNAs (circRNAs), LIFR, also increases CELF2 expression by sponging miR-429 and then decreased lung cancer progression [35]. In contrast, CELF3, the role of RBP, QKI, in lung cancer is controversial. Circ-SHPRH inhibits the cadmium-induced transformation of human bronchial epithelial cells via sponging miR-224-5p to regulate QKI expression [36]. In contrast, increase QKI expression by G protein-coupled estrogen receptor/NOTCH1 signaling promotes cell growth of non-small cell lung cancer cells [37]. The present study revealed that CELF2 and QKI could be RBPs of *ENTPD3-AS1* due to the loss of *ENTPD3-AS1* impact on OS in the patients with lower levels of CELF2 and QKI. Reduced expression levels of CELF2 and QKI were found in LUAD patients with adverse outcomes and are related to a poor OS. However, research on the direct interaction of *ENTPD3-AS1* with CELF2 or QKI is still limited, and further exploration of the associated regulatory mechanism is required.

Therefore, our results reveal a novel mechanism in which the *ENTPD3-AS1*/miR-421/*NR3C1* and *ENTPD3-AS1/CELF2* or *QKI* axis are involved in LUAD development, suggesting that lncRNA *ENTPD3-AS1* could be a potential biomarker and therapeutic target in LUAD.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Ying-Ming Tsai, Graduate Institute of Medicine, College of Medicine, Kaohsiung Medical University, Kaohsiung 807, Taiwan. E-mail: yingming@kmu.edu.tw

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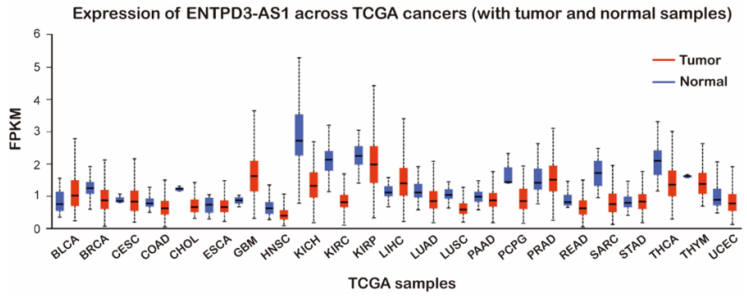
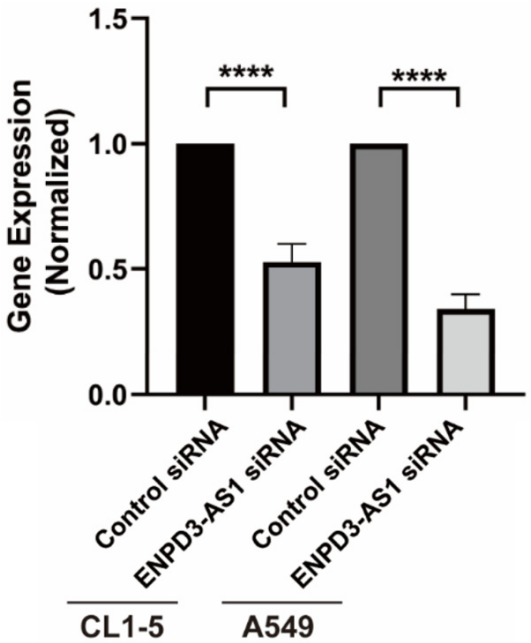


Supplementary Figure 1. The levels of *ENTPD3-AS1* in different types of cancer. Data were extracted from the UALCAN website.

Supplementary Table 1. The clinicopathologic characteristics

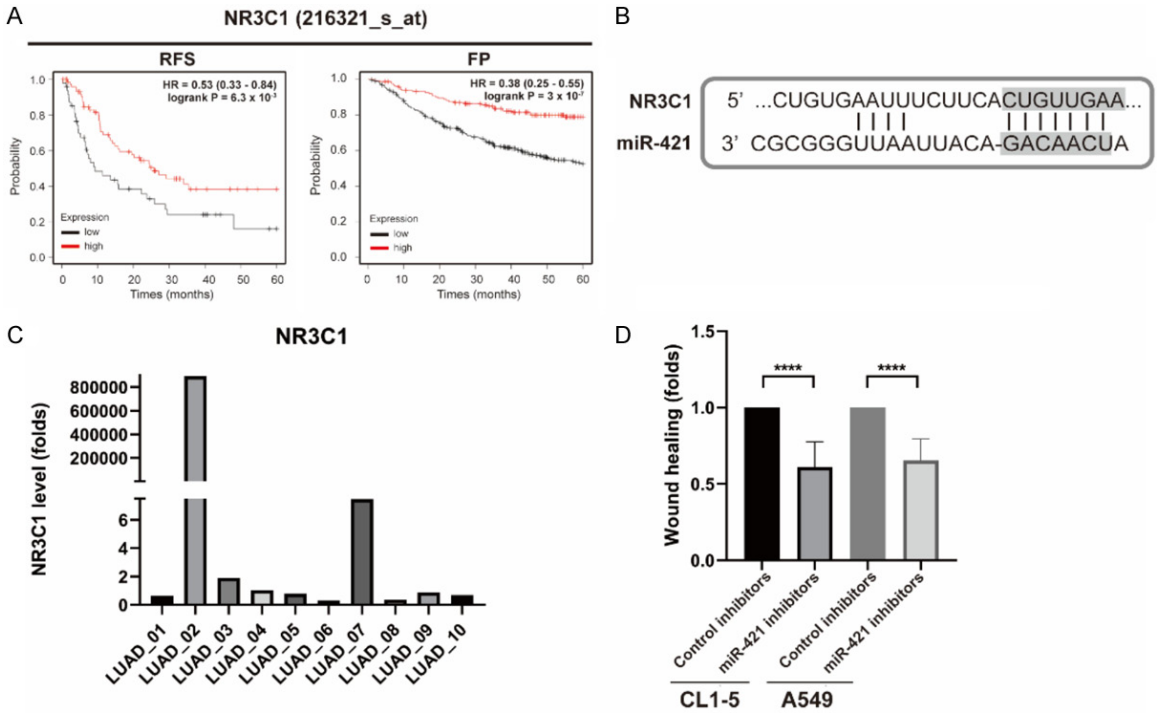
Case No.	Category	Age	Gender	Pathologic Findings	Stage (TNM)
1	RNAseq	46	F	Adenocarcinoma grade 2	1 (T1N0M0)
2		50	F	Adenocarcinoma grade 1	1A1 (T1aN0M0)
3		58	F	Adenocarcinoma grade 2	1A1 (T1aN0M0)
4		63	M	Adenocarcinoma grade 2	1B (2aN0M0)
5		64	F	Adenocarcinoma grade 2	1A1 (T1aN0M0)
6		48	M	Adenocarcinoma grade 2	1A1 (T1aN0M0)
7		82	M	Adenocarcinoma grade 2	4 (T1bN2M1b)
8		62	F	Adenocarcinoma grade 3	4 (T1bN0M1b)
9		75	M	Adenocarcinoma grade 2	2B (T3N0M0)
10		65	F	Adenocarcinoma grade 3	3A (T2aN2M0)
11	Immunohistochemical staining	55	M	Adenocarcinoma grade 3	4B (T3N2M1c)
12		70	M	Adenocarcinoma grade 3	2B (T3N0M0)
13		70	M	Adenocarcinoma grade 3	3A (T2aN2M0)
14		60	F	Adenocarcinoma grade 2	1A2 (T1bN0M0)
15		59	M	Adenocarcinoma grade 2	1B (T2aN0M0)



Supplementary Figure 2. The knockdown effeacy of ENTPD3-AS1 siRNA.

Supplementary Table 2. The targets of ENTPD3-AS1

miRNAid	miRNAname	merClass	miRseq
MIMAT0011778	hsa-miR-2278	7mer-m8	ggUCCGUUGUGUGUGACGAGAg
MIMAT0025464	hsa-miR-6504-5p	8mer	gacguaaugucguGUCGGUCu
MIMAT0000241	hsa-miR-208a-3p	7mer-m8	ugUUCGAAAAACGAGCAGAAUa
MIMAT0014980	hsa-miR-3118	7mer-m8	ucuuaaaagUAUUAC-GUCAGUGu
MIMAT0002828	hsa-miR-519e-5p	8mer	cuuucacgagggaaAACCUCUu
MIMAT0000422	hsa-miR-124-3p	8mer	ccguaAGUGGCGCACGGAAu
MIMAT0005795	hsa-miR-1323	8mer	ucuuUUACGGGGAGUCAAAACu
MIMAT0003339	hsa-miR-421	7mer-m8	cgcggGUUAAUUACAGACAACUa
MIMAT0027605	hsa-miR-6852-3p	7mer-m8	gacucCUU-GUCUCCUGu
MIMAT0000646	hsa-miR-155-5p	7mer-m8	uggggauaGUGCUAAUCGUAAUu
MIMAT0005899	hsa-miR-1247-5p	7mer-m8	aggcccCUGCUUGCCCUGCCCa



Supplementary Figure 3. Survival advantage of NR3C1 in LUAD. A. Lower NR3C1 associated with poor RFS and FP. B. The binding affinity between miR-421 and *NR3C1*. C. The expression of NR3C1 mRNA in in-house cohort. D. The quantification data of wound-healing analysis. PPS, post progression survival; FP, first progression.