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Original Paper

LncRNA TP73-AS1 Promotes Cell Proliferation and Inhibits Cell Apoptosis in Clear Cell Renal Cell Carcinoma Through Repressing KISS1 Expression and Inactivation of PI3K/Akt/mTOR Signaling Pathway

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Key Words
Clear cell Renal cell carcinoma • LncRNA TP73-AS1 • KISS1 • PI3K/Akt/mTOR

Abstract
Background/Aims: Emerging evidence suggests that long non-coding RNAs (lncRNAs) play a vital regulatory role in the pathogenesis and progression of renal cell carcinoma (RCC). We aim to determine lncRNA profiles in clear cell RCC (ccRCC) and investigate key lncRNAs involved in ccRCC tumorigenesis and progression. ***Methods:*** RNA sequencing technique and qPCR were used to determine the candidate lncRNAs in ccRCC tissues. The correlations between lncRNA P73 antisense RNA 1T (TP73-AS1) levels and survival outcomes were analyzed to elucidate its clinical significance. The underlying mechanisms of TP73-AS1 in ccRCC were analyzed through *in vitro* functional assays. ***Results:*** We found TP73-AS1 was upregulated in 40 ccRCC tissues compared with adjacent normal renal tissues and increased TP73-AS1 was correlated to aggressive clinicopathologic features and unfavorable prognosis. Knockdown of TP73-AS1 suppressed cell proliferation, invasion and induced cell apoptosis. We also identified KISS-1 metastasis-suppressor (KISS1) was significantly upregulated in TP73-AS1 knockdown cells. Further, we revealed that TP73-AS1 suppressed KISS1 expression through the interaction with Enhancer of zeste homolog 2 (EZH2) and the specific binding to KISS1 gene promoter region. Knockdown of KISS1 partly reversed TP73-AS1 knockdown-induced inhibition of cell proliferation and promotion of apoptosis. We further determined that TP73-AS1 knockdown activated PI3K/Akt/mTOR signaling pathway, while overexpression of TP73-AS1 induced inhibition of PI3K/Akt/mTOR pathway and these effects could be partly abolished by overexpression of KISS1. ***Conclusion:*** In conclusion, we identified that TP73-AS1 as an oncogenic lncRNA in the development of ccRCC and a potential target for human renal carcinoma treatment.

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Introduction

Renal cell carcinoma (RCC) accounts for 3–5% of all malignant diseases in adults and is the tenth most common cancer in women and the sixth in men [1]. The mortality of RCC patients appears to be increasing each year, resulting in frequent studies on biological detections and treatments [2]. Among which, clear cell RCC (ccRCC) is the most common subtype of all renal cancers. ccRCC is refractory to traditional radiotherapy and chemotherapy, which makes its clinical management a thorny problem [3, 4]. Despite various studies that have identified that many genetic and epigenetic changes are associated with the development and progression of ccRCC, the molecular mechanism of renal cancer pathogenesis is still elusive [5]. Better understanding of molecular events and mechanisms involved in the carcinogenesis of ccRCC may provide effective therapeutic targets.

Long non-coding RNAs (lncRNAs) are most commonly defined as RNA transcript of more than 200 nucleotides (nt) and located in nuclear or cytosolic fractions with no protein-coding capacity [6]. Although thousands of these molecules have been identified, few have been assigned a biological function. Those that have been characterized have been found to be involved in a broad spectrum of processes such as proliferation, invasion, apoptosis and regulation of mRNA splicing [7-9]. Due to the advances of high-throughout technology, numerous lncRNAs have been detected and profiled and many of them participated in RCC progression. For example, lncRNA PVT1 was shown to be upregulated in ccRCC, which was associated with poor outcome [10] due to its promotion of cell proliferation and invasion by competitively binding with miR-200 family through regulating the expression of metastasis related genes [11]. Similarly, Xu et al. identified that increased lncRNA PANDAR expression correlated with an advanced TNM stage and with lymph node involvement and distant metastasis in ccRCC and PANDAR downregulation induced cell cycle arrest and apoptosis in RCC cells [5]. Using microarray analysis, Li et al. identified MRCCAT1 was significantly upregulated in metastatic ccRCC tissues compared with that in non-metastatic tissues and then validated it could promote ccRCC metastasis via inhibiting NPR3 and activating p38-MAPK signaling [12]. Although multiple lncRNAs have been reported to modulate tumor development and metastases, the functional roles of lncRNAs in ccRCC remain largely unknown and more research is needed to clarify the role of other lncRNAs.

In the present study, we firstly report that lncRNA P73 antisense RNA 1T (TP73-AS1), located on chromosome 1p36, was more highly expressed in ccRCC tissues than in adjacent normal renal tissues through RNA sequencing. Increased TP73-AS1 expression was associated with shorter survival. Then we found downregulation of TP73-AS1 could result in significant suppression of proliferation and invasion and promotion of apoptosis. Our data further demonstrated that TP73-AS1 promoted cell viability and suppressed apoptosis by suppressing KiSS-1 metastasis-suppressor (KISS1) expression via interaction with EZH2 and subsequent inhibition of PI3K/Akt/mTOR pathway.

Materials and Methods

Tissues samples and RNA sequencing

Forty paired of ccRCC tissues samples from patients who underwent surgery at the Department of Urology, Peking Union Medical College Hospital, were collected. Two independent genitourinary pathologists confirmed diagnoses. No treatment had been conducted on these patients before surgery. All tissues were frozen in liquid nitrogen immediately after surgery and stored at −80 °C. The clinicopathological characteristics of the enrolled patients were collected. Tumor classification and staging were assigned in accordance with the 2009 TNM system. Overall survival (OS) was defined as the interval between the dates of surgery and death or date of last contact. Disease free survival (DFS) was defined as the time between the initial resection of the kidney tumor and the occurrence of recurrence or metastasis. Among which, 6 paired samples were randomly chosen for RNA sequencing. The data were compared to determine differentially expressed lncRNAs. The differentially expressed lncRNAs were identified with the Bioconductor R package

limma [13]. The P value was adjusted for multiple testing using the false discovery rated (FDR) method (FDR<0.05), and |log2 fold change|⩾2.0 and adjusted P value < 0.05 were considered significant. Written informed consent was gotten from each patient and the procedure was approved by the Ethics Committee of Peking Union Medical College Hospital Hospital.

Cell culture

Renal cancer cell lines (including A498, Caki1 and Caki2) and normal kidney epithelial cells (HK2) were purchased from the Type Culture Collection (Shanghai, China). ACHN and 786O renal cancer cell lines were preserved in our lab previously. All cell lines were maintained at 37ºC in a humidified incubator with 5% CO2 in RPMI1640 medium (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (Gibco).

Vector construction and cell transfection

Stably over-expressing cell lines were identified by real-time PCR and stable cell lines expressing the empty pcDNA3.1 plasmid (Invitrogen, Carlsbad, CA, USA) was used as controls. Briefly, cDNA encoding TP73-AS1 or KISS1 was PCR-amplified and was inserted at the EcoR I and Xho I site of the plasmid pcDNA3.1. The transfection of the expression vector pcDNA3.1-TP73-AS1 or pcDNA3.1^{+}-KISS1 to the RCC cell lines was performed by a lipofection method using Lipofectamine 2000 (Invitrogen, MA, USA) according to the manufacturer's instructions. SiRNAs specifically targeting TP73-AS1, EZH2 and KISS1 were synthesized by GenePharma (Shanghai, China), Transfections were performed by a lipofection method using Lipofectamine 2000 (Invitrogen). All the experiments of gene knockdown using were performed after transfected for 24 h.

RNA reverse transcription and quantitative real-time PCR (qPCR)

Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized from extracted total RNA using the SuperScript VILO cDNA Synthesis Kit (Invitrogen) according to the manufacturer's instructions and amplified with Sybr Green PCR mastermix (Applied Biosystems, Foster City, CA, USA) on an ABI PRISM 7500 Sequence Detection System (Applied Biosystems). The PCR primers used were as follows: TP73-AS1 forward, 5'- CCGGTTTTCCAGTTCTTGCAC-3' and reverse, 5'-ATTGACATGTTAGTGCCCTT-3'; KISS1 forward, 5'- CCAGATCAACCTGCCATGT -3' and reverse, 5'-GCCTCACAGGGAAACTTCATGC-3'; GAPDH forward, 5'-CTGGGCTACACTGAGCACC-3' and reverse, 5'-AAGTGGTCGTTGAGGGCAATG-3'. The $2^{-\Delta\Delta Ct}$ method was used to determine the relative expression of genes.

Cell proliferation assay

Cell growth was quantified by Cell Counting Kit-8 (CCK-8, Beyotime, Jiangsu, China). Briefly, cells from the different transfection groups were plated at 1×10^3 cells per well in 96-well plates grown for 24 h. At different time point, 10 μl of CCK-8 reagent was added to each well and the optical density was measured at 450 nm using the SpectraMax Plus 384 Microplate Reader (Molecular Devices, San Francisco, CA, USA).

Cell invasion assay

Transwell assay was performed in 24-well plates with Transwell chamber (8.0-μm pore size polycarbonate filter). After various treatment, 1×10^4 cells in 100 μl serum-free medium were added into the upper chamber with Matrigel (BD Biosciences, Bedfold, MA, USA). RP1640 medium (500 μl) containing 10% FBS was added into the lower chamber. After 48 h, cells on the upper chamber were softly removed, fixed with 4% paraformaldehyde for 10 min and stained with 0.1% crystal violet, and counted using light microscopy (Sony, Tokyo, Japan). The experiments were repeated three times.

Flow cytometric analysis

The Annexin V-FITC/PI apoptosis detection kit (BD Biosciences, San Jose, CA, USA) were used to evaluate the apoptosis rates of RCC cells with and without the genetic modification of TP73-AS1. In brief, cells were trypsinized and rinsed with PBS to achieve the final concentration of 1×10^6/ml, resuspended in 500 μl buffer containing 5 μl Annexin V-FITC, 5 μl PI and incubated in the dark for 15 min at room temperature. The apoptosis rate was assayed by flow cytometry (FACSCalibur, Bio-Rad, Hercules, CA, USA). For cell cycle distribution analysis, Alexa Fluor™ 488 Click-iT™ Plus EdU Flow Cytometry Assay Kits (Cat. no. C10632, invitrogen) was used. After treatment as described previously, the cells were harvested after incubated with Click-iT™ EdU for another 2 h. Subsequently, cells were fixed with cold 70% ethanol for 15 min and

permeabilized with 1X Click-iT™ saponin. Next, Click-iT™ Plus reaction cocktail was added and incubated for 30 min at room temperature and propidium iodide (PI, DNA stainer) were added with incubated for 30 min in the dark room. At last, cell cycle were analyzed by a Attune™ NxT Acoustic Focusing Flow Cytometer (ThermoFisher Scientific, Waltham, MA).

Human Tumor Metastasis PCR Array

Total RNA was extracted from A498 cells transfected with si-TP73-AS1 or si-NC and was then detected by Human Tumor Metastasis PCR Array (PAHS-028A; SuperArray Biosciences, Maryland, USA) according to the manufacturer's instructions. The PCR array contained 84 genes known to be involved in tumor metastasis. The fold-change in the expression of metastasis-related genes was determined via the ΔΔCt method that normalized to GAPDH. Fold changes and P values were calculated using Student's t test. $P < 0.05$ with a fold change greater than 2.0 was considered to indicate significant dysregulation.

Chromatin immunoprecipitation (ChIP)

ChIP was conducted using the EZ ChIP Chromatin Immunoprecipitation Kit (Millipore, Bedford, MA, USA). Briefly, cross-linked chromatin was sonicated into 200-1000 bp fragments. The chromatin located on the promoter of TP73-AS1 was immunoprecipitated by using anti-EZH2 antibody (ab186006, Cambridge, MA, USA) anti-H3K27me3 (Millipore) and anti-RNA Pol II antibodies. An isotype-matched IgG was served as the negative control. qPCR was then performed to detect the relative enrichment of the TP73-AS1 promoter with the sequences 5'-CGGCGTAGGACTAGGTGCT-3' (forward) and 5'-GGTCGTCCGCTCCCGTGAC-3' (reverse).

RNA immunoprecipitation (RIP)

RIP assay was carried out by using a Magna RIP RNA Binding Protein Immunoprecipitation Kit (Millipore) according to the manufacturer's instructions as previously described [14]. Antibodies for RIP assays against EZH2 and control IgG were purchased from Abcam.

Western blot

After treatment, cells were harvested protein concentration was determined using the bicinchoninic acid (BCA). Subsequently, 30 μg protein were separated by 10-12% SDS-PAGE gel and transferred to PVDF membranes. The membranes were then blocked by 5% skim milk for 1 h at room temperature and incubated with primary antibodies overnight at 4˚C. The primary antibodies were KISS1 (ab19028, 1:1000; abcam), Akt (#9772, 1:1000), phospho-AKT (#9271, 1:1000), mTOR (#2983, 1:1000), phospho-mTOR (#2971, 1:1000; all from Cell Signaling Technology) and β-actin antibody (MAB8929, 1:1000; R&D systems). Then, blots were incubated with HRP-conjugated anti-rabbit antibodies (1:5000; Santa Cruz, CA, USA) for 90 min at room temperature. Detection was performed using a Odyssey Scanning system (LI-COR Inc., Lincoln, NE, USA).

RNA pull-down assays

The biotin-labeled full length lncRNA TP73-AS1 RNA were *in vitro* transcribed using the Pierce RNA 3′ End Desthiobiotinylation Kit (Thermo scientific) and T7 RNA polymerase (Thermo scientific) that were then purified using the RNeasy Mini Kit (Qiagen) and treated with RNase-free DNase I (Qiagen). Positive (U6), negative (no RNA), and biotinylated RNAs were mixed and incubated with A498 cell lysates. Magnetic beads were then added to each binding reaction, followed by incubation for 30 min at room temperature with agitation. Then, the beads were collected with washing buffer and the eluted proteins were assigned to western blot analysis.

Statistical analysis

Data were expressed as mean ±standard deviation (SD). Statistical analyses were performed using Student's t-test. TP73-AS1 expression was classified as high or low according to the median, and associations between TP73-AS1 status and clinicopathological variables were analyzed with Fisher's exact test or Pearson's $\chi 2$ test. Analysis of OS and DFS followed the Kaplan-Meier method and comparisons were made using Log-rank tests. Statistical analyses were performed with SPSS 19.0 software package (SPSS, Chicago, IL, USA) and P value < 0.05 was considered significant.

Results

Upregulated TP73-AS1 in renal carcinoma tissues indicates a poor outcome

Expression levels of lincRNAs in ccRCC tissues (n=40) and matched normal tissues were analyzed using RNA-seq. a total of 367 differential genes were identified in tumor samples compared with adjacent normal tissues as depicted in the volcano plot (Fig. 1A) and 50 up-regulated and 50 down-regulated lncRNAs were shown in the heat map (Fig. 1B). Top 5 up- and down-regulated lncRNAs in CRC tissues were listed in Table 1. Among them, lncRNA TP73-AS1 was the most upregulated lincRNA, which was then validated by quantitative real-time PCR (qRT-PCR) (Fig. 2A). To determine the association between TP73-AS1 and the clinicopathological features of ccRCC, patients were assigned to TP73-AS1 high group and low group according to the median value. As illustrated in Table 2, there was no significant association of TP73-AS1 levels with age, gender and tumor site, whereas TP73-AS1 levels were closely correlated with tumor metastasis (P=0.045), tumor size (P=0.036) and TNM stage (P=0.010). To reveal the prognostic significance of TP73-AS1 in subjects with ccRCC, DFS and OS curves were plotted according to TP73-AS1 levels and analyzed by the Kaplan–Meier method and log-rank test. The mean DFS was 22.85 months for patients with high TP73-AS1 expression and 37.31 months for low TP73-AS1 expression (Fig. 2B). The mean OS time for cases with high TP73-AS1 expression was 35.88 months, but 44.11 months for low TP73-AS1 expression (Fig. 2C). The results suggested that increased TP73-AS1 levels were associated with ccRCC prognosis.

TP73-AS1 promotes cell proliferation and invasion but inhibits apoptosis

We then performed qRT-PCR to determine the expression of TP73-AS1 in five RCC cell lines and normal kidney cell line HK-2, and the results showed that its level was significantly increased in all the five RCC cells compared with normal cells (Fig. 3A). A498 and 786O, which owned the highest levels of TP73-AS1 expression, were chosen for the loss- or gain-of function experiments to determine the role of TP73-AS1 in ccRCC cells. As shown in Fig. 3B, we overexpressed TP73-AS1 to investigate its role on cell proliferation, cell cycle analysis, invasion and apoptosis. Interesting, CCK8 assay showed that overexpression of TP73-AS1 markedly promoted cell proliferation rate (Fig. 3C). We then identified that TP73-AS1 significantly increased the percent

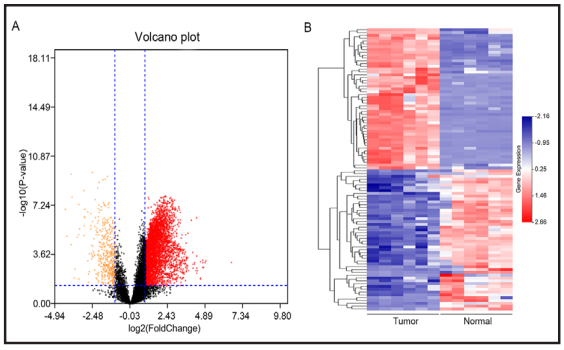

Fig. 1. LncRNA TP73-AS1 is upregulated in clear cell renal cell carcinoma (ccRCC). (A) A volcano plot showed the ectopic lincRNAs identified in ccRCC tissues and adjacent normal tissues by RNA sequencing. (B) A heat map showed the most 50 upregulated and 50 downregulated LncRNAs in ccRCC tissues and adjacent normal tissues. The blue bar means low and red part means high.

Table 1. Top 5 up- and down-regulated lncRNAs in clear cell renal cell carcinoma tissues

Gene ID	Gene Name	Log2 FC	Adjust P value	Regulation	FDR
ENSG00000227372.5	TP73-AS1	-9.802741762	2.44E-11	Down	1.46E-115
ENSG00000227094.1	RP11-565P22.2	-6.359841483	4.12E−46	Down	1.13E−39
ENSG00000224358.1	RP11-466F5.8	-6.261405457	1.82E−15	Down	2.29E−38
NR_073383	-	-6.023784964	0	Down	0
ENSG00000245614.2	DDX11-AS1	-5.908023409	1.49E−138	Down	1.57E−150
ENSG00000258017.1	RP11-386G11.10	6.920076741	0	UP	0
NM_002273	-	6.228226754	5.56E−23	UP	3.98E−128
ENSG00000257509.1	RP11-762I7.4	5.707550353	0	UP	0
TCONS_l2_00006264	-	5.663628249	2.32E−35	UP	2.26E−31
ENSG00000197503.4	LINC00477	5.529878106	6.76E-5	UP	1.48E−32

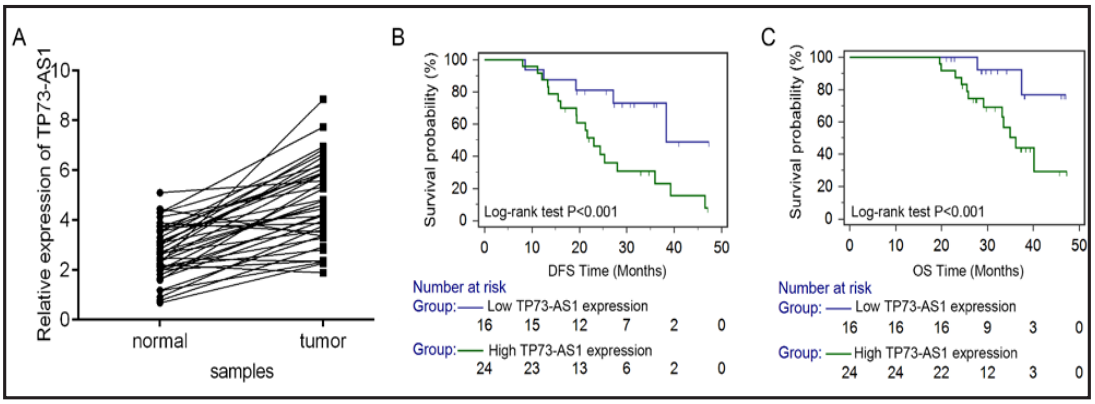

Fig. 2. High TP73-AS1 expression indicates poor outcome in clear cell renal cell carcinoma (ccRCC). (A) RT-qPCR validated that lncRNA TP73-AS1 was the dramatically upregulated in ccRCC tissues compared with adjacent normal tissues. Kaplan-Meier survival curve analysis showed that higher expression of TP73-AS1 was associated with shorter (B) disease-free survival (DFS) and (C) overall survival (OS) of patients with ccRCC.

of cell population in G1/G0 and S phase, but decreased the cell number in G2/M phase in both A498 and 786O cells, as illustrated in Fig. 3D. Transwell assay showed TP73-AS1 overexpression increased cell invasive ability (Fig. 3E) both in above two cells. On the contrary, FACS apoptosis assay revealed that overexpression of TP73-AS1 dramatically inhibited cell apoptosis (Fig. 3F). Consistently, TUNEL assay showed TP73-AS1 overexpression inhibited nuclear apoptosis of A498 cells (Fig. 3G). In parallel, the roles of TP73-AS1 knockdown in A498 and 786-O cells were also investigated. Three independent TP73-AS1-specific siRNAs were designed and transfected into A498 and 786-O cells to reduce TP73-AS1 expression (Fig. 4A). CCK8 assay indicated that knockdown of TP73-AS1 suppresses cell proliferation of A498 and 786-O cells (Fig. 4B). Cell-cycle analysis found TP73-AS1 knockdown increased the percent of cell population in G2/M phase and decreased the cell number in G1/G0 and S phase of cell cycle (Fig. 4C). Transwell assay suggested that cell invasion ability was weakened by TP73-AS1 silencing in A498 and 786-O cells (Fig. 4D). Moreover, Fig. 4E showed that the number of early and late apoptotic cells post-siRNA-transfection were significantly higher than those cells transfected with si-NC. TP73-AS1 knockdown also clearly induced apoptosis in A498 cell as indicated by the TUNEL staining assay (Fig. 4F). Together, these data indicated that TP73-AS1 played critical role in biofunction of ccRCC cells.

Table 2. Correlation between the clinicopathological features and expression of TP73-AS1

Clinical Characteristics	TP73-AS1 expression		P value
	Low(n=16)	High(n=24)	
Age(years)			0.468
<60	8	16	
≥60	8	8	
Gender			0.942
Male	11	18	
Female	5	6	
Tumor size			0.036
<5 cm	14	12	
≥5 cm	2	12	
Laterality			0.560
Left	7	14	
Right	9	10	
Tumor stage			0.010
I/II	14	10	
III/IV	2	14	
Status			0.045
Primary tumors	12	9	
Metastatic tumors	4	15	

TP73-AS1 directly interacted with EZH2 to repress KISS1 expression in ccRCC cells

It is reported that lncRNAs may exert their effects by functioning as a bridge to recruit EZH2, thereby leading to the gene methylation and chromatin modifications [15, 16]. To determine whether TP73-AS1 regulates target genes using a similar mechanism, we performed RNA immunoprecipitation (RIP) assays which showed a significant enrichment of TP73-AS1 with EZH2 antibody when compared with the non-specific IgG antibody in both

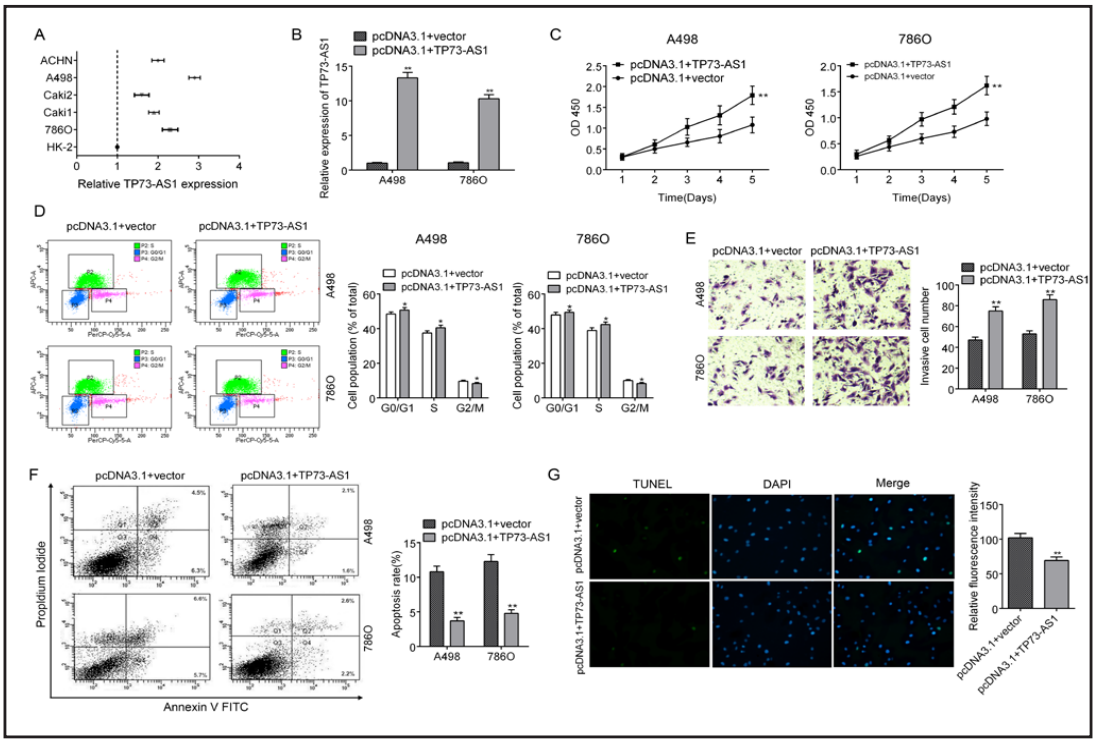

Fig. 3. Overexpression of TP73-AS1 promotes cell proliferation, invasion and inhibits apoptosis. (A) The expression level of TP73-AS1 was upregulated in all ccRCC cell lines including ACHN, A498, Caki2, Caki1 and 786O, compared with HK-2 cell. (B) The expression of TP73-AS1 was marked increased in A498 and 786O cells after transfected with pcDNA3.1+ TP73-AS1. (C) CCK8 assay showed TP73-AS1 overexpression promoted cell vitality both in A498 and 786O cells. (D) Cell cycle arrest analysis showed that overexpression of TP73-AS1 increased cell numbers in G0/G1 and S phase and decreased cell numbers in G2/M phase of cell cycle. (E) Transwell assay indicated that TP73-AS1 overexpression promoted cell invasion in A498 and 786O cells. (F) Flow cytometry revealed that TP73-AS1 overexpression repressed cell apoptosis in A498 and 786O cells. (G) TUNEL assay showed that TP73-AS1 overexpression repressed cell apoptosis in A498 cells. *, P<0.05; **, P<0.01.

A498 and 786-O cells. However, no enrichment of β-actin was confirmed in A498 cells (Fig. 5A). RNA pulldown assay further confirmed the interaction between TP73-AS1 and EZH2 (Fig. 5B). Subsequently, to identify the potential mechanisms of the role of TP73-AS1 on cell biological activity, metastasis-related genes that deregulated between A498 cells transfected with si-TP73-AS1 and si-NC were determined using a human tumor metastasis PCR array. As shown in Fig. 5C, several metastasis-related genes were significantly differentially expressed in cells with or without TP73-AS1 knockdown. The expression levels of CHD4, ITGB3, TCF20, KISS1, MMP7 and KRAS were further verified via qRT-PCR (Fig. 5D). We found KISS1 was one of the most upregulated genes, which was selected for further analysis. Therefore, the level of KISS1 in ccRCC tissues were then tested. As expected, the KISS1 expression was significantly reduced in tumor tissues compared with normal ones (Fig. 5E). Subsequent correlation analysis indicated that TP73-AS1 was negatively correlated with KISS1 in ccRCC (r=-0.53, P<0.01, Fig. 5F). Then we tested the ability of TP73-AS1 to regulate the expression of KISS1. The mRNA and protein levels of KISS1 were significantly elevated in TP73-AS1 knockdown cells compared with the control cells, as detected by qRT-PCR (Fig. 5G) and western blot (Fig. 5H). QRT-PCR and western blot analysis showed that EZH2 inhibition upregulated the mRNA and protein expression levels of KISS1 (Fig. 5I and 5J). In addition, ChIP suggested that EZH2 bounds the promoter region of KISS1, while knockdown of TP73-AS1 reduced the histone

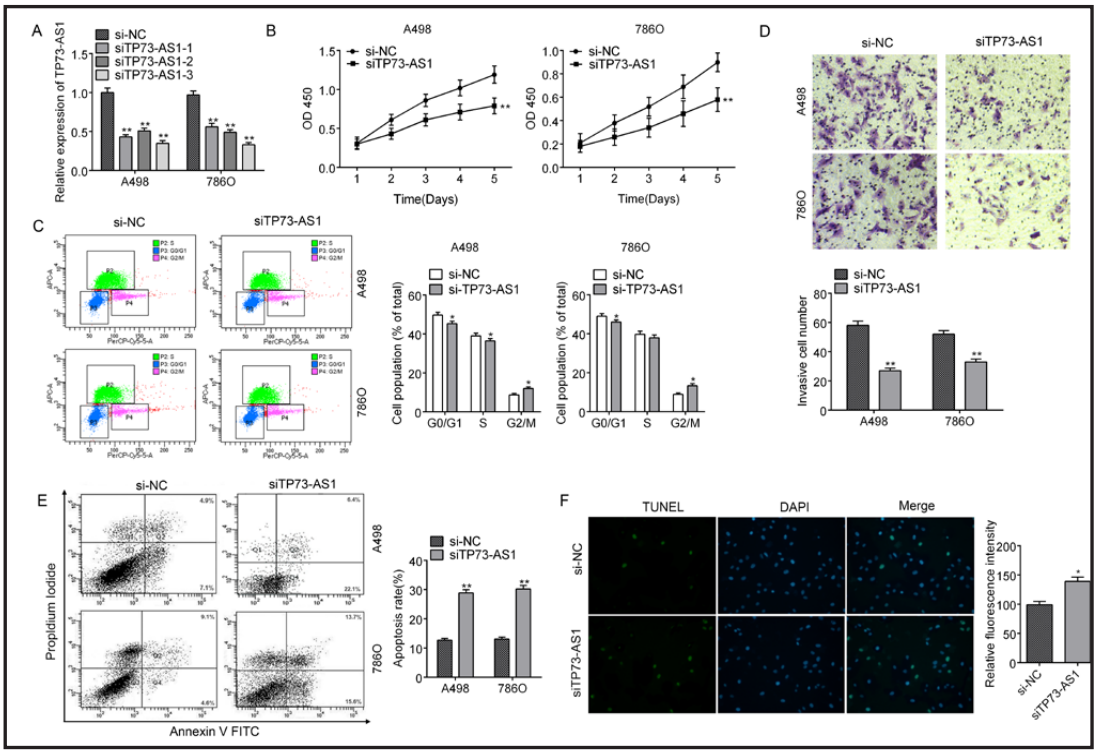

Fig. 4. TP73-AS1 knockdown inhibits cell proliferation, invasion and promotes apoptosis (A) Knockdown of TP73-AS1 using three designed specific siRNAs. (B) CCK8 assay revealed TP73-AS1 knockdown inhibited cell proliferation both in A498 and 786O cells. (C) Cell cycle arrest analysis showed that TP73-AS1 knockdown increased cell numbers in G2/M phase arrest and decreased cell numbers in G0/G1 and S phase of cell cycle. (D) Transwell assay indicated that TP73-AS1 knockdown repressed cell invasion. (E) Flow cytometry showed that TP73-AS1 knockdown induced cell apoptosis in A498 and 786O cells. (F) TUNEL assay showed that TP73-AS1 knockdown induced cell apoptosis in A498 cells. *, P<0.05; **, P<0.01.

H3 lysine 27 trimethylation (H3K27me3) occupancy of the KISS1 promoter locus (Fig. 5K). Collectively, these results demonstrated that TP73-AS1 suppressed KISS1 expression through the interaction with EZH2 and the specific binding to KISS1 gene promoter region.

TP73-AS1 functions through its binding to KISS1

As protein is the executor of gene function, we then aimed to determine whether KISS1 is required for the role of TP73-AS1 in ccRCC cells. We thus performed siRNAs to knockdown KISS1 in A498 cells (Fig. 6A) and found si-RNA1 was the most effective vehicles, which was therefore chosen for subsequent analysis. Notably, depletion of KISS1 reversed the inhibition of cell proliferation and migration caused by TP73-AS1 knockdown (Fig. 6B and 6C). In addition, TUNEL assay revealed that co-transfected with si-TP73-AS1 and si-KISS1 dismissed the effects of si-TP73-AS1 on cell apoptosis in A498 cells (Fig. 6D). These results confirmed that TP73-AS1 regulates cell proliferation and apoptosis partly through the downregulation of KISS1 expression.

TP73-AS1 regulates cell vitality and apoptosis through inactivation of PI3K/Akt/mTOR signaling pathway

It has been reported that KISS1 activate mTOR pathway to be involved into cell function [17], we thus investigate whether TP73-AS1 could inhibit mTOR pathway to promote cell vitality and inhibit apoptosis. As expected, western blot experiments showed that knockdown of TP73-AS1 promoted the levels of AKT and mTOR phosphorylation, but, no change of total AKT and mTOR protein levels were found (Fig. 7A and 7B). We found that

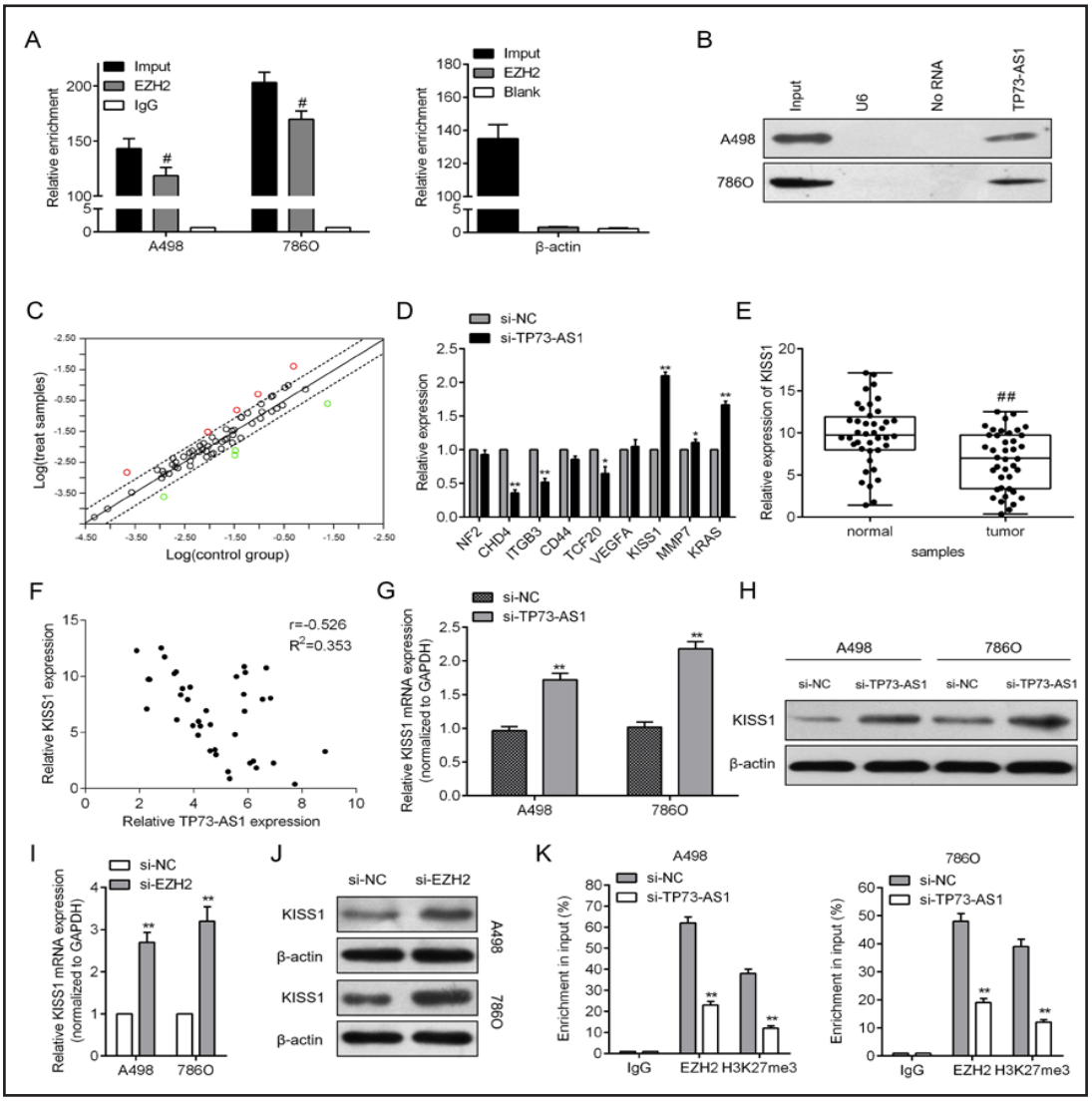

Fig. 5. TP73-AS1 represses KISS1 transcription via binding with EZH2 in ccRCC cells. (A) RIP experiments were performed in A498, 786O cells and the corprecipitated RNA was subjected to qPCR for TP73-AS1. Expression levels of TP73-AS1 RNA were as fold enrichment in EZH2 relative to IgG immunoprecipitates (left). RIP experiments showed that no enrichment of β-actin was confirmed in A498 cells (right). (B) RNA pulldown and western blotting assays revealed biotinylated TP73-AS1 could bind to EZH2. (C) Metastasis-related genes that were deregulated between A498-si-NC and A498-si TP73-AS1 were determined using a human tumor metastasis PCR array. (D) CHD4, ITGB3, TCF20, KISS1, MMP7 and KRAS were further verified through qPCR in A498-si NC and A498-si TP73-AS1 cells. (D) Relative expression levels of KISS1 mRNA in 20 paired ccRCC tissues. (F) Correlation of TP73-AS1 and KISS1 at RNA levels in 40 ccRCC tumor samples. r=−0.526, P<0.01 by Pearson correlation analysis. TP73-AS1 knockdown induced upregulation of KISS1 expression at (G) mRNA and (H) protein levels. The expression of KISS1 in EZH2 knockdown A498 and 786O cells at (I) mRNA and (J) protein levels. (K) ChIP-qPCR of EZH2 occupancy and H3K27me3 binding in the KISS1 promoter in A498 and 786O cells treated with si-TP73-AS1 or si-NC, IgG was used as a negative control. *, P<0.05; **, P<0.01 vs. si-NC; # P<0.05 vs. input; ## P<0.05 vs. Normal.

the levels of phosphorylated AKT/AKT and phosphorylated mTOR/mTOR were decreased in TP73-AS1 overexpressed cells, which could be abolished by KISS1 overexpression (Fig. 7C-E). In addition, CCK8 assay revealed that TP73-AS1 knockdown inhibited cell viability could be perturbed by LY294002 (50 μmol/L, a PI3K inhibitor) or CCI-779 (20 μmol/L, a mTOR

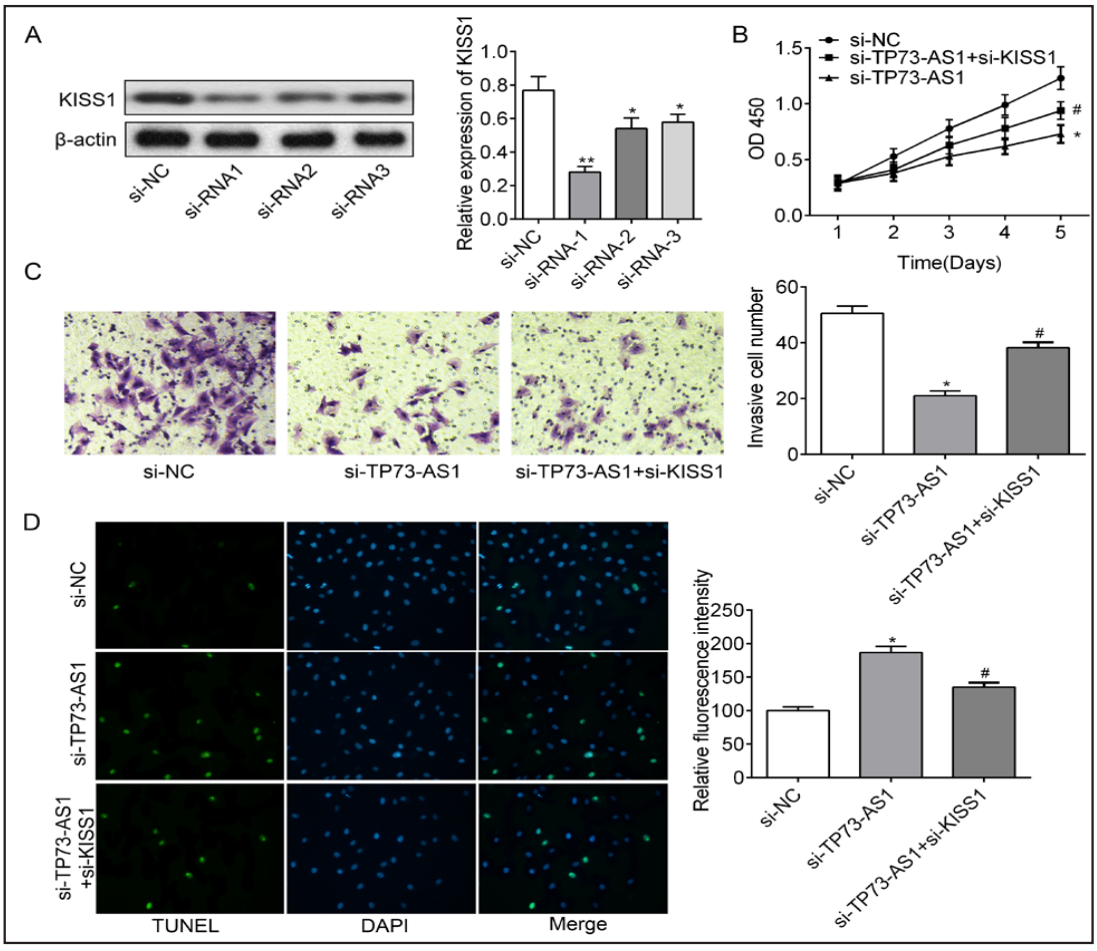

Fig. 6. KISS1 knockdown could partly reverse the oncogenic function of TP73-AS1. (A) Western blot assays showed the levels of KISS1 in A498 cells transfected with specific KISS1 siRNAs. (B) CCK8 assays revealed that TP73-AS1 knockdown induced inhibition of cell proliferation was partly abolished by depletion of KISS1. (C) Transwell assays showed that knockdown of KISS1 significantly reversed inhibitory effects on cell invasion induced by TP73-AS1 knockdown. (D) TP73-AS1 knockdown induced cell apoptotic levels were reversed by KISS1 knockdown as detected by TUNEL assay. *, P<0.05 vs. si-NC; #, P<0.05 vs. si-TP73-AS1.

inhibitor) treatment (Fig. 7F). Apoptosis assay also indicated that treatment of si-TP73-AS1 alone effectively promoted cell apoptosis, while, treatment of si-TP73-AS1 with LY294002 or CCI-779 could partly reverse this promotive effect (Fig. 7G). These results suggested that TP73-AS1 regulates cell vitality and apoptosis dependent on PI3K/Akt/mTOR pathway.

Discussion

Although studies have reported the important role of lncRNAs in RCC pathogenesis [18-20], the role and mechanism of lncRNA TP73-AS1 in ccRCC has not been identified before. TP73-AS1 was first reported to be down-regulated in glioma and may possess the capacity to modulate apoptosis [21]. Thereafter, it was reported that TP73-AS1 was generally upregulated in esophageal cancer tissues and was strongly associated with malignant level in clinical samples. Besides, TP73-AS1 knockdown could enhance the chemosensitivity of esophageal cancer cells to 5-FU and cisplatin [22]. These opposite changes indicated its heterogeneity in different cancer. Recently, Li et al. identified that high TP73-AS1 expression was associated with aggressive clinicopathological features and poorer outcome in patients

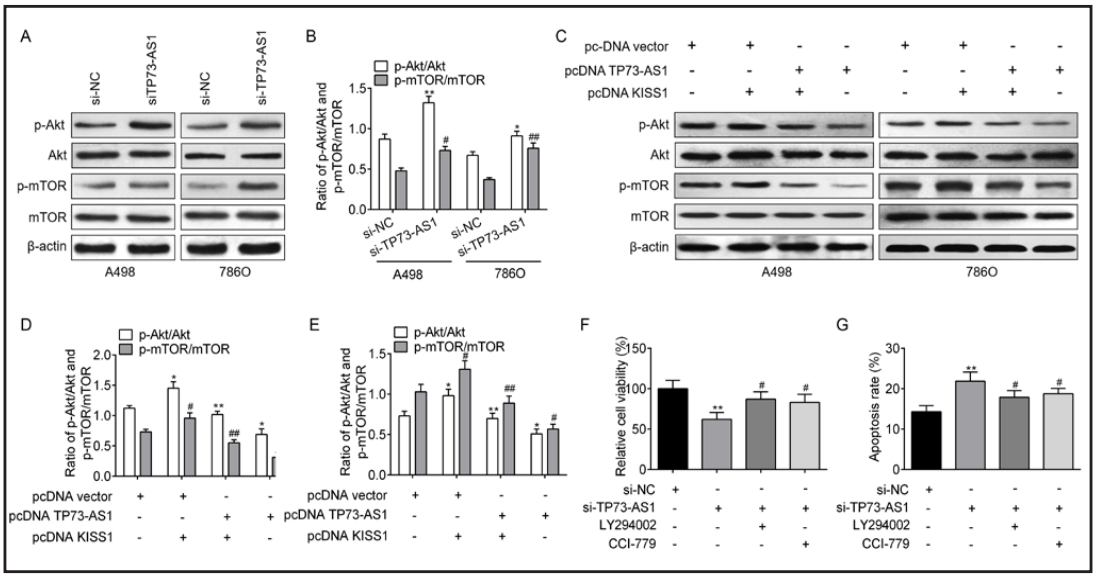

Fig. 7. TP73-AS1 promotes cell proliferation and inhibits apoptosis through inactivating PI3K/Akt/mTOR signaling pathway. (A-B) Western blot analysis showed that TP73-AS1 knockdown promoted phosphorylated Akt and phosphorylated mTOR expression, however, no change of total Akt and mTOR protein levels were identified. (C-E) overexpression of TP73-AS1 repressed phosphorylated Akt and phosphorylated mTOR expression, while overexpression of KISS1 partly reversed these trends. (F) CCK8 assay showed that TP73-AS1 knockdown induced inhibition of cell proliferation was alleviated by treatment with PI3K inhibitor (LY294002) or mTOR inhibitor (CCI-779). (G) Flow cytometry showed that TP73-AS1 knockdown induced apoptosis was reversed by LY294002 or CCI-779 treatment. *, P<0.05, **, P<0.05 vs. si-NC; #, P<0.05, ##, P<0.05vs. si-TP73-AS1.

with hepatocellular carcinoma and found that TP73-AS1 could promote cell proliferation through HMGB1/RAGE regulation [23]. And similarly, Zhang et al. revealed that TP73-AS1 promoted the brain glioma growth and invasion through acting as a competing endogenous RNA to promote HMGB1 expression by sponging microRNA-142 [24]. However, the expression level of TP73-AS1 in RCC is unclear.

We firstly identified that TP73-AS1 was upregulated in ccRCC tissues and cell lines using RNA sequencing technique. We also identified that high TP73-AS1 expression was closely correlated to the aggressive clinicopathologic features and unfavorable prognosis of ccRCC patients. Based on the previous findings, we explored the role of TP73-AS1 on cell proliferation, migration and apoptosis *in vitro* and tried to uncover its underlying mechanisms. Interestingly, our data suggested that overexpression of TP73-AS1 promotes cell proliferation and invasion but inhibits apoptosis in A498 and 786O cells. On the contrary, TP73-AS1 knockdown achieved the opposite effects on cell proliferation, cell cycle, invasion and apoptosis. lncRNAs play roles in regulating a wide range of biological processes, such as cell differentiation, proliferation, apoptosis, and migration [20, 25, 26]. TP73-AS1 was associated with cell proliferation and tumor progression. TP73-AS1 knockdown suppressed cancer cell proliferation and induced apoptosis in esophageal squamous cell carcinoma [22] and knockdown of TP73-AS1 also induced apoptosis in glioblastoma cells [27]. In ccRCC, we also identified that TP73-AS1 induced cell proliferation, migration and inhibits cell apoptosis. The control of cell cycle regulation through targeting cell death signaling pathways, plays a significant role in the biological process [28]. Thus, cell cycle regulation analyses of TP73-AS1 established that the cell cycle arrest in G0/G1 and S phase was more prominent than the G2/M phase, thus to promote cell proliferation and inhibit apoptosis. In addition, we unexpectedly found that the cell necrosis was at some extent increased when overexpression of TP73-AS1 while knockdown of it lead to the opposite result (data not shown). Although the role of other lncRNAs on cell necrosis have been reported [29], whether TP73-AS1 could

control the necrotic pathway and contribute to the survival of ccRCC cells still needs to be defined. Previously studies have indicated that lncRNAs participate in the pathogenesis of cancers via various mechanisms of downstream gene regulation, such as epigenetic, post-transcriptional, and transcriptional regulation [30, 31]. Moreover, the pseudogenes can recruit the histone modification protein Enhancer of zeste homolog 2 (EZH2), functions as a histone methyltransferase that specifically induces histone H3 lysine 27 trimethylation (H3K27me3), to target a gene promoter, thereby regulating their transcription [32, 33]. In this study, following RIP and RNA pulldown assays, we found that TP73-AS1 could directly bind EZH2 in ccRCC cells. Take a step further, we sought to determine the underlying regulatory mechanisms by which TP73-AS1 exerts its function. In TP73-AS1 knockdown cells, tumor apoptotic related gene KISS1, which was also located on chromosome 1, showed significantly upregulation. Our further experiments revealed that knockdown of EZH2 also upregulated KISS1 expression, while ChIP assays showed that TP73-AS1 could recruit EZH2 to the KISS1 promoter region and repress its transcription by mediating H3K27me3. These results suggested that TP73-AS1 epigenetically silence the transcription of downstream target gene KISS1 by recruiting and binding to EZH2.

KISS1 is one of the major metastasis suppressors that is inactivated in the majority of cancer types. Studies found KISS1 silencing increased cancer invasion and metastasis in breast cancer [34], bladder cancer [35] and renal cancer [36]. Lack of KISS1 expression was significantly associated with rapid tumor progression and activation of KISS1 decreased the motility and invasive capacity of tumor cells [37]. Consistently, we showed that KISS1 knockdown partly reversed the effects of knockdown of TP73-AS1 in A498 cells. Another study showed activation of KISS1/KISS1R signaling decreased the motility and invasive capacity of 786O cells [38]. But, differently, we identified that mTOR signaling, may be served as another way that mediated the role of KISS1 in cell function in ccRCC cells. Activation of the PI3K/Akt/mTOR pathway protects various cell types from apoptosis that is induced by the withdrawal of survival factors [39, 40]. We found that knockdown of TP73-AS1 significantly activated mTOR signaling, whereas overexpression of TP73-AS1 induced inhibition of mTOR pathway while overexpressed KISS1 partly abolished this inhibition effects. Subsequent pathway inhibitors validated that mTOR pathway may be involved in the role of TP73-AS1.

We are aware of the potential limitations in this study. Firstly, as the limited clinical samples, the clinical significance of TP73-AS1 should be further validated in our future studies. Secondly, although we identified that PI3K/Akt/mTOR pathway may be another way involved in the KISS1-mediated cell function, the key molecules remain to be thoroughly clarified. Moreover, *in vivo* studies are required to further validate the cell function of TP73-AS1 in the development and progression of ccRCC.

In summary, our study for the first time determined that TP73-AS1 is up-regulated in ccRCC tissues and cells and it overexpression is associated with poor outcome in patients. TP73-AS1 directly binds to KISS1 via interacting with EZH2 and influences biological behaviors of ccRCC cells by through inactivation of PI3K/Akt/mTOR pathway (Fig. 8), suggesting that TP73-AS1 plays vital role in the development of ccRCC. This study provides new insights into the roles and mechanisms of TP-AS1 in ccRCC and may offer a new therapeutic target for patients with ccRCC.

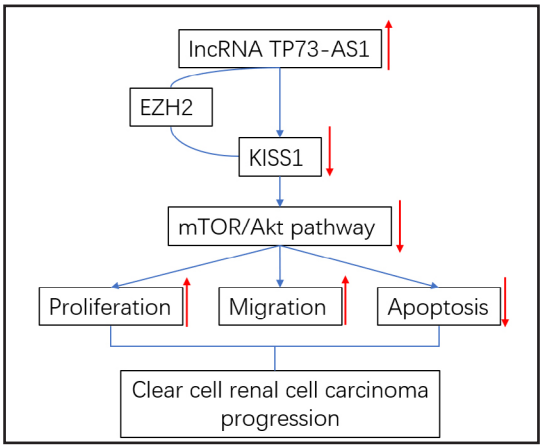

Fig. 8. A schematic diagram showing the role of lncRNA TP73-AS1 in the progression of clear cell renal cell carcinoma

Disclosure Statement

All authors declare that there is no Disclosure Statements.

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