

Long noncoding RNA UPK1A-AS1 indicates poor prognosis of hepatocellular carcinoma and promotes cell proliferation through interacting with EZH2

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Abstract

Background

Dysregulations of lncRNA are responsible for cancer initiation and development, positioning lncRNAs as not only biomarkers but also promising therapeutic targets for cancer treatment. Growing number of lncRNAs have been reported in HCC but their functional and mechanistic roles remain unclear.

Methods

Gene Set Enrichment Analysis was used to investigate the molecular mechanism of lncRNA UPK1A antisense RNA 1 (UPK1A-AS1). CCK-8 assay, EdU assay, flow cytometry, western blot, and xenograft assay were used to confirm the role of UPK1A-AS1 in proliferation of HCC cells both *in vitro* and *in vivo*. Bioinformatics analysis and qRT-PCR were performed to explore the interplay between UPK1A-AS1 and Enhancer of Zeste Homologue 2 (EZH2). RNA immunoprecipitation, RNA-pull down assay, western blot, qRT-PCR, and were conducted to confirm the interaction between UPK1A-AS1 and EZH2. Finally, the expression level and prognosis value of UPK1A-AS1 in HCC were analyzed using RNA-seq data from TCGA datasets.

Results

We showed that UPK1A-AS1, a newly identified lncRNA, promoted cellular proliferation and tumor growth by accelerating cell cycle progression. Cell cycle related genes including CyclinD1, CDK2, CDK4, CCNB1 and CCNB2 were significantly upregulated in HCC cells with UPK1A-AS1 overexpression. Furthermore, overexpression of UPK1A-AS1 could protect HCC cells from cis-platinum toxicity. Mechanistically, UPK1A-AS1 interacted with EZH2 to mediate its nuclear translocation and reinforce its binding to SUZ12, leading to the increasing trimethylation of H27K3. Targeting EZH2 with specific siRNA impaired UPK1A-AS1-mediated upregulation of proliferation and cell cycle progression related genes. Moreover, UPK1A-AS1 was significantly upregulated in HCC, and upregulation of UPK1A-AS1 predicted poor prognosis for patients with HCC.

Conclusions

Our study reveals that UPK1A-AS1 promotes HCC development by accelerating cell cycle progression in an EZH2-dependent manner, suggesting that UPK1A-AS1 possesses substantial potential as a novel biomarker for HCC prognosis and therapy.

Background

Hepatocellular carcinoma (HCC) is the sixth most common malignancies, accounting for the fourth leading cause of cancer-related mortality worldwide[1]. Surgical resection, radiofrequency ablation, liver transplantation, chemotherapy, molecular-targeted therapy, as well as immunotherapy have been applied in HCC treatment[2, 3]. However, survival rate still remains unsatisfactory in patients with HCC because of lacking typical clinical feature and specific indicator in the early stage of HCC[4]. Therefore, there is an urgent need to discover and develop more effective biomarkers and targets for better diagnosis, prognosis and treatment of HCC.

With thousands of non-coding RNA being identified and annotated, academicians have come to realize the great importance of non-coding RNA, which makes up for no less than 90% of human genome[5]. Non-coding RNA can be classified as microRNAs and long non-coding RNAs (lncRNAs) based on their size[6]. lncRNAs are a class of transcripts more than 200 nucleotides (nt) in length with no or limited protein-coding potential[7]. Recently, the importance of lncRNAs in tumorigenesis has gradually come to light[8]. lncRNAs have been recognized as key regulators involved in many biological processes rather than by-products of RNA polymerase II transcription or genomic noises[9]. Accumulating evidences demonstrated that lncRNAs take part in the regulation of carcinogenesis through multiple pathways, including transcription modulation, post-transcription modulation, epigenetic modification, RNA decay and so on[10]. lncRNAs have also been regarded as potential biomarkers and therapeutic targets for cancers, including HCC[11]. For instance, lncRNA MCM3AP-AS1, an oncogenic lncRNA which is highly expressed in HCC, promotes the growth of HCC via targeting miR-194-5p/FOXA1 axis[12]. lncRNA TUG1 is overexpressed in HCC and promotes proliferation by epigenetically silencing KLF2[13]. DILC represses self-renewal of cancer stem cell through inhibiting autocrine IL-6/STAT3 axis[14]. On the contrary, our previously finding showed that MIR22HG, a highly conserved lncRNA, was downregulated and predicted poor prognosis in patients with HCC[15]. These findings indicate that lncRNAs are critically involved in the development and progression of HCC and may serve as biomarkers for HCC diagnosis and prognosis.

UPK1A antisense RNA 1 (UPK1A-AS1) is a newly discovered lncRNA with little information about its functional role and clinical significance in cancers. It is reported that UPK1A-AS1 was downregulation in esophageal squamous cell carcinoma (ESCC), and it suppressed proliferation, migration, and invasion of ESCC cells by sponging microRNA-1248[16]. To date, no study has reported the biological role and clinical importance of UPK1A-AS1 in HCC. Here, we determined the functional role of UPK1A-AS1 in HCC progression and uncovered the underlying molecular mechanism. Our results showed that UPK1A-AS1 was overexpressed in HCC and upregulation of UPK1A-AS1 predicted poor prognosis for HCC patients. Functionally, UPK1A-AS1 promoted proliferation by accelerating G1/S transition of HCC cells. UPK1A-AS1 exerted its oncogenic activities via binding with EZH2 to mediate its nuclear translocation and reinforce its binding to SUZ12. Our results uncovered the critical role of UPK1A-AS1 in HCC progression, and UPK1A-AS1 might serve as a potential biomarker for HCC diagnosis and prognosis.

Methods

Cell lines and cell culture

The MHCC-97H and SK-Hep-1 cell lines were provided by the Cell Bank of Type Culture Collection (CBTCC, Chinese Academy of Science, Shanghai, China). Cells were maintained in DMEM with 10% fetal bovine serum (Gibco, USA), and cultured in a humidified incubator containing 5% CO₂ at 37 °C.

Small interference RNAs (siRNA) and lentivirus transduction

The siRNAs used in the current study were designed and synthesized by Ribobio Technology (Guangzhou, China). The transfection of siRNAs was performed according to the recommendation using Lipofectamine RNAiMAX Reagent (Invitrogen, USA). For lentivirus UPK1A-AS1 overexpression and knockdown, full-length UPK1A-AS1 was inserted in GV438, and two hairpin precursors specifically targeting UPK1A-AS1 were cloned in GV112. SK-Hep-1 and MHCC-97H cells were infected with lentivirus at a multiplicity of infection of 20 using polybrene (GeneChem, Shanghai, China). The sequences of siRNAs and target sequences of shRNAs were listed in supplementary Table 1.

Gene Sets Enrichment Analysis (GSEA)

GSEA was carried out using the GSEA program provided by Broad Institute (<http://www.broadinstitute.org/gsea/index.jsp>) to examine the gene sets or signatures that were associated with UPK1A-AS1 or EZH2 in HCC samples from the TCGA dataset. RNA-seq data of HCC samples were downloaded from The Cancer Genomic Atlas project (TCGA) followed by GSEA analysis. An ordered list of all genes was generated according to their correlation with UPK1A-AS1 or EZH2 and predefined gene sets or signatures receive an enrichment score and *P* value.

RNA extraction and quantitative polymerase chain reaction (qRT-PCR)

Total RNA was isolated from HCC cells and tumor tissues by TRIzol reagent (Invitrogen, USA) following the manufacturer's instructions, and a total of 500 ng RNA was reverse transcribed into cDNA by cDNA Reverse Transcription Kit (Takara, Japan). Quantitative PCR was performed by SYBR Green PCR kit (Takara, Japan). β -actin was considered as internal control. Primer sequences in the present study were shown in supplementary Table 1.

Cell proliferation assay

Cell proliferation assay was monitored with Cell counting Kit (CCK)-8 Kit and Cell-Light EdU Apollo 567 in Vitro Imaging Kit (Ribobio Technology, Guangzhou, China). A total of 2,000 cells were seeded in 96-well plates with indication treatments, and the CCK-8 proliferation assay was detected according to the instructions. EdU dye assay was tested as manufacturer's recommendations.

Cell cycle analysis and apoptosis assay

Cell cycle distribution was tested by flow cytometry on a FACScan (Beckman Instruments, USA). Cell apoptosis was detected with Annexin V-FITC kit (Keygen Biotech, China).

Western blot

Protein were separated on an SDS-polyacrylamide gel followed by transferring onto polyvinylidene fluoride membranes (Bio-Rad, USA). The membranes were blocked with 5% BSA for 50 minutes at room temperature before incubated with primary antibody in 4 °C overnight. The membranes were incubated with secondary antibody conjugated to horseradish peroxidase, followed by signal detection by enhanced chemiluminescence Western blotting substrate (Bio-Rad, USA). The primary antibodies used here were listed in supplementary Table 2.

Xenograft assay

Four-week-old male nude mice were subcutaneously injected with 1×10^7 UPK1A-AS1-overexpressing or negative control MHCC-97H cells. Tumor diameters was measured every other day and tumor volumes was calculated as $(\text{length} \times \text{width}^2)/2$. The mice were sacrificed before tumor removal at 4 weeks after injection. All procedures for animal study were approved by the Animal Used and Care Committee of Nanfang Hospital, Southern Medical University (Guangzhou, China).

Functional Enrichment Analysis

A total of 500 genes positively correlated with EZH2 in HCC samples from The Cancer Genome Atlas (TCGA) dataset were subjected to Functional Enrichment Analysis using online software Metascape (<https://metascape.org/>). Only terms with $P < 0.01$, minimum count of 3 and enrichment factor greater than 1.5 were identified as significant.

RNA immunoprecipitation (RIP)

RIP assay was performed using Magna RIP RNA-binding protein immunoprecipitation kit (Millipore, USA) following the manufacturer's recommendations. Cells were lysed with lysis buffer and cell lysates was immunoprecipitated with anti-EZH2 and IgG antibodies. Immunoprecipitated RNA were extracted, purified and reverse transcribed to cDNA. The transcribed cDNA was subjected to qRT-PCR using UPK1A-AS1 specific primers. The primer sequences used for UPK1A-AS1 amplification were listed in supplementary Table 1.

RNA pull-down assay

RNA pull-down assay was carried out by Magnetic RNA-Protein Pull-Down Kit (Pierce, USA) following the manual. Full length of UPK1A-AS1 and antisense were yield using RiboMAX Large Scale RNA Production Systems (Promega, USA). Biotin labeled UPK1A-AS1 and antisense were bound to the beads and incubated with the whole cell protein lysates for immunoprecipitation. The beads were washed before eluted with SDS-PAGE loading buffer. Sample eluted from beads was subjected to western blot analysis.

Immunofluorescence (IF)

MHCC-97H cell with UPK1A-AS1 overexpression were seeded on coverslips for IF staining. Briefly, cells were washed with PBS, fixed with 4% paraformaldehyde, permeabilized with 0.25% Triton-X-100, and blocked with 5% BSA at room temperature. Cells on the coverslips were incubated with anti-EZH2 (1:100) in 4 °C overnight, followed by incubation with Cy3-conjugated CA goat antibodies against rabbit IgG (Santa Cruz Biotechnology, USA) for 1 h at room temperature. The cells were further counterstained with 4, 6-diamidino-2-phenylindole (Invitrogen) before imaging with microscope (Carl Zeiss LSM880, Germany)

Statistical methods

All statistical analysis was carried out by the SPSS statistical software version 22 (Abbott Laboratories, USA). Student's t-test and one-way ANOVA were carried out for statistical analysis when appropriate. Kaplan-Meier and log-rank test was used for survival analysis. A *P*-value < 0.05 (two-tailed) was statistically significant.

Results

Upregulation of UPK1A-AS1 promotes proliferation of HCC cells

To investigate the molecular mechanism of UPK1A-AS1, we conducted GSEA of TCGA cohort and found that high UPK1A-AS1-expressing groups were enriched for cell cycle-related gene sets (Figure 1A), suggesting UPK1A-AS1 may hold a function on cell proliferation. To confirm its function on cell proliferation, lentivirus with full length of UPK1A-AS1 or negative control were introduced into HCC cells and the proliferation rate of HCC cells was examined. UPK1A-AS1 was successfully overexpressed in HCC cells, and upregulation of UPK1A-AS1 significantly promoted HCC cell proliferation, as detected by CCK-8 assay (Figure 1B-C). Since upregulation of UPK1A-AS1 correlated with cell cycle-related gene sets, we further determine whether UPK1A-AS1 could affect HCC cell cycle progression. We then performed EdU dye assay to examine the ratio change of cells entering the S phase. The results showed that more UPK1A-AS1-overexpressing cells entered S phase than its control cells (Figure 1D-G). Taken together, overexpression of UPK1A-AS1 could promote HCC proliferation.

UPK1A-AS1 downregulation inhibits HCC cell proliferation

To further confirm the regulatory function of UPK1A-AS1 on cell proliferation, we knocked down UPK1A-AS1 expression in HCC cells using siRNAs and shRNAs. The knocked down efficiency was verified using qRT-PCR. CCK-8 assays showed that downregulation of UPK1A-AS1 visibly inhibited HCC cell proliferation (Figure 2A-D). Locked nucleic acids (LNAs) specific targeting UPK1A-AS1 were introduced into HCC cells to further verified the effect of UPK1A-AS1 downregulation on HCC proliferation. Consistently, downregulation of UPK1A-AS1 by LNAs also impaired HCC proliferation (Figure 2E). Moreover, cells in LNAs treatment groups that entered S phase were significantly less than its control groups (Figure 2 F-G). In summary, knocked down of UPK1A-AS1 inhibits HCC cell proliferation.

UPK1A-AS1 accelerates G1/S transition of HCC cells

It is well accepted that rapid cell cycle progression accounts for cancer proliferation. Above results showed that upregulation of UPK1A-AS1 correlated with cell cycle-related gene sets and UPK1A-AS1 promotes cell proliferation. This led us to hypothesize that UPK1A-AS1 might regulate cell cycle progression. To this end, we carried out flow cytometry analysis to detect cell cycle distributions in HCC cells after UPK1A-AS1 overexpression or downregulation. The results showed that HCC cells with UPK1A-AS1 overexpression had a decreased rate of G1 phase cells and increased rate of S phase cells (Figure 3A-D). Consistently, CyclinD1, one of the most important modulators in G1/S transition, was highly expressed in cells with UPK1A-AS1 overexpression (Figure 3E). On the contrary, si-UPK1A-AS1 resulted in an evident cell cycle arrest at the G1/G0 phase (Supplementary Figure 1A-B), and CyclinD1 was visibly decreased in cells with UPK1A-AS1 downregulation (Supplementary Figure 1D).

We also explored the effect of UPK1A-AS1 on apoptosis and drug resistance. Lower levels of apoptosis were founded in UPK1A-AS1-overexpressing cells, indicating that overexpression of UPK1A-AS1 could protect HCC cells from cis-platinum toxicity (Figure 3F-G). Consistently, the expression level of well-defined apoptosis markers, including cleaved caspase3 and cleaved PARP, were obviously decreased in UPK1A-AS1-overexpressing cells after cis-platinum exposure. This suggests that UPK1A-AS1 may boost the resistance to chemotherapy with cis-platinum in HCC cells. In conclusion, upregulation of UPK1A-AS1 accelerates G1/S transition of HCC cells.

UPK1A-AS1 promotes tumor growth *in vivo*

Based on the results of UPK1A-AS1 *in vitro* assay, we speculated that UPK1A-AS1 may take an important part in tumor growth *in vivo*. HCC cells with stable UPK1A-AS1-overexpressing or negative control were then subcutaneously injected into nude mice. The tumors formed in UPK1A-AS1-overexpressing group grew faster than those in negative control group. The tumor weight and volume were significantly higher in UPK1A-AS1-overexpressing group than in negative control group (Figure 4A-C, Supplementary Figure 2). UPK1A-AS1 was remarkably overexpressed in UPK1A-AS1-overexpressing group, as detected by qRT-PCR (Figure 4D). In addition, the positive rate of proliferation marker Ki-67, was obviously increased in tumor with UPK1A-AS1-overexpressing (Figure 4E). Collectively, UPK1A-AS1 boosts tumor growth *in vivo*.

UPK1A-AS1 correlates with EZH2-mediated cell cycle progression

To dissect the molecular mechanism involved in HCC progression through UPK1A-AS1, GSEA was carried out with the HCC tumor samples in TCGA dataset. GSEA results suggested that high expression of UPK1A-AS1 was correlated with EZH2 targets (Figure 5A). Interestingly, GSEA outputs showed that high expression of EZH2 was positively correlated with cell cycle gene sets (Figure 5B). Additionally, the functions of EZH2 and its correlated genes in HCC were predicted by analyzing GO and KEGG in Metascape. The top 20 Go enrichment items also suggested that EZH2 was associated with cell cycle. It has been long recognized that EZH2 has a crucial role in regulating cancer cell proliferation[17]. Consistently with previous studies, downregulation of EZH2 with siRNA significantly inhibited HCC cell

proliferation. CyclinD1, CDK2, CDK4, which accelerates cell cycle progression, were reported to be downstream targets of EZH2[18]. Not surprisingly, these genes were significantly downregulated after EZH2 silencing in HCC cells (Figure 5F). We also found that CCNB1 and CCNB2 were significantly decreased after EZH2 silencing in HCC cells (Figure 5F). These results suggested that CyclinD1, CDK2, CDK4, CCNB1, and CCNB2 were direct targets of EZH2 in HCC. We further investigated the correlation between EZH2 and its targets from TCGA dataset. Strong positive correlations between EZH2 and CyclinD1, CDK2, CDK4, CCNB1, and CCNB2 were found in HCC samples (Supplementary Figure 3A). These results suggested that EZH2 promoted HCC proliferation by regulating cell cycle-related genes.

Given that UPK1A-AS1 correlated with EZH2 target, and both regulated HCC proliferation, we speculated that UPK1A-AS1 boosted HCC cell progression by regulating those cell cycle-related EZH2 targets. As expected, upregulation of UPK1A-AS1 significantly increased the expression of EZH2 targets, including CyclinD1, CDK2, CDK4, CCNB1 and CCNB2. Furthermore, positive correlations between UPK1A-AS1 and CDK2, CDK4, CCNB1, and CCNB2, but not CyclinD1 were found in HCC samples (Supplementary Figure 3B), indicating that UPK1A-AS1 regulated CyclinD1 in a more complicated way. In short, these results indicated that UPK1A-AS1 regulates EZH2-mediated cell cycle progression.

UPK1A-AS1 interacts with EZH2

To further investigate the molecular mechanisms by which UPK1A-AS1 contributes to the progression of HCC, we examined the subcellular distribution of UPK1A-AS1 in HCC cells by fractionation assays. UPK1A-AS1 located in both nucleus and cytosol in HCC cells, indicating that UPK1A-AS1 could function as a modulator of gene transcription (Figure 6A). It is reported that one-fifth of human lncRNAs identified physically interacted with polycomb repressive complex 2 (PRC2), consisting of EZH2, SUZ12, and EED, with EZH2 as a crucial component of PRC2[19]. Our results that UPK1A-AS1 regulates EZH2-mediated cell cycle progression triggered us to come up with an assumption that UPK1A-AS1 may interact with and bind to EZH2. To test our hypothesis, RNA immunoprecipitation assay (RIP) against EZH2 was performed. RIP assay showed that UPK1A-AS1 was significantly enriched with the EZH2 antibody compared with negative control (IgG) in HCC cells (Figure 6B-C). To further confirm our assumption, the interaction of UPK1A-AS1 with EZH2 was determined using RNA pull-down assay. The results showed that biotin-labeled UPK1A-AS1, but not antisense, exhibited the ability to harbor EZH2 protein (Figure 6D). These results demonstrated that UPK1A-AS1 could physically interact with EZH2.

We next wondered if UPK1A-AS1 had an impact on the expression level of EZH2. Western blot showed that neither overexpression nor downregulation of UPK1A-AS1 altered the expression of EZH2 (Figure 6E). Moreover, no significant correlation was found between UPK1A-AS1 and EZH2 expression level (Figure 6F). These results demonstrated that UPK1A-AS1 interacted with EZH2 without changing the expression of EZH2. Surprisingly, overexpression of UPK1A-AS1 increased the trimethylation of H27K3 which was caused by PRC2 activation. On the contrary, silencing UPK1A-AS1 led to obvious reduction of trimethylation on H27K3 (Figure 6E), suggesting that interaction between UPK1A-AS1 and EZH2 led to PRC2 activation.

It has been reported that lncRNA physically interacts with and binds to proteins to alter their subcellular distribution[20]. Fractionation assays showed that overexpression of UPK1A-AS1 decreased the cytoplasmic expression of EZH2, but increased the expression level of EZH2 in the nucleus (Figure 6G). Immunofluorescence experiment also confirmed that overexpression of UPK1A-AS1 induced translocation of EZH2 from the cytoplasm to the nucleus (Figure 6H). EZH2, SUZ12 and EED form complex in the nucleus for PRC2 activation. An increased interaction between EZH2 and SUZ12 was found after UPK1A-AS1 overexpression (Figure 6I). In brief, UPK1A-AS1 interacted with EZH2 and mediated its nucleus translocation, reinforced its binding to SUZ12, leading to the increased trimethylation of H27K3.

UPK1A-AS1 functions through EZH2

To explore whether EZH2 mediated the regulative effect of UPK1A-AS1 on HCC cell proliferation, we co-transfected EZH2 siRNA and UPK1A-AS1 vectors into HCC cells and analyzed the expression of EZH2 targets related to cell cycle. Overexpression of UPK1A-AS1 increased the expression of CCND1, CDK2, CDK4, CCNB1 and CCNB2. Downregulation of EZH2 eliminated upregulation of these genes caused by UPK1A-AS1 overexpression (Figure 7A-B). Consistent with results of qRT-PCR, EdU assay showed that more UPK1A-AS1-overexpressing cells entered S phase than its control cells. The increase of S phase ratio by UPK1A-AS1 overexpression was reversed in part by silencing EZH2 (Figure 7C-D). Taken together, targeting EZH2 with specific siRNA impaired UPK1A-AS1-mediated upregulation of proliferation and cell cycle progression related genes.

High expression of UPK1A-AS1 predicts poor prognosis for patients with HCC

UPK1A-AS1 is a newly identified lncRNA and little is known about its clinical implication in cancers. Analysis from genotype-Tissue Expression (GTEx) benign tissue RNA-seq revealed that UPK1A-AS1 was highly expressed in the bladder, but scarcely in other tissues (Supplementary Figure 4A). However, data from TCGA datasets showed UPK1A-AS1 was relatively induced in some kinds of cancers, including HCC (Supplementary Figure 4B), indicating its important role in development and progression of malignancies.

To explicit the clinical implication of UPK1A-AS1 in HCC, UPK1A-AS1 expression level in HCC was analyzed with RNA-seq data from TCGA dataset. UPK1A-AS1 was highly expressed in HCC (Figure 8A). To eliminate the possibility that the significant difference between HCC tissues and non-tumor tissues was caused by imbalanced of sample size, paired HCC and corresponding non-tumor samples was reanalyzed. The results convinced that UPK1A-AS1 was significantly overexpressed in HCC (Figure 8B). Moreover, high expression of UPK1A-AS1 positively correlated with tumor stage of HCC (Figure 8C). Survival analysis showed that patients with high expression of UPK1A-AS1 exhibited worse overall survival (OS) as compared with those with low UPK1A-AS1 expression group (Figure 8D). Because UPK1A-AS1 expression correlated with HCC stage, we reanalyzed the data from subgroups. Patients with high UPK1A-AS1 level of UPK1A-AS1 presented shorter OS than those with low UPK1A-AS1 expression, though the difference did not reach statistical significance (Figure 8E-F). Vascular invasion is a sign of poor prognosis for patients with HCC. Survival analysis showed that in vascular invasion group, patients

with high UPK1A-AS1 level of UPK1A-AS1 suffered poorer OS. Due to limitation in sample size, the difference did not reach statistical significance (Figure 8F). Since infection of hepatitis virus and alcohol abuse were risk factors for HCC, we also clarified correlation between UPK1A-AS1 expression level and prognosis in patients with HCC risk factor exposure. It is shown that patients with high UPK1A-AS1 expression suffered shortened OS in patients with HCC risk factors (Figure 8G). Furthermore, univariate Cox regression analysis demonstrated that the OS risk of patients with HCC was significantly associated with upregulation of UPK1A-AS1 (Table 1).

We also explored the clinical significance of EZH2 in cancer. Data from TCGA datasets showed that EZH2 was highly expressed in various cancers, including HCC (Supplementary Figure 5A). Overexpression of EZH2 predicted poor prognosis in various cancer, suggesting its oncogenic role in tumorigenesis (Supplementary Figure 5B). A series of HCC datasets from Gene Expression Omnibus (GEO) confirmed that EZH2 was highly expressed in HCC (Supplementary Figure 5C). Moreover, high expression of EZH2 correlated with development and progression of HCC (Supplementary Figure 5 D-G). Survival analysis showed that EZH2 predicted poor prognosis for patients with HCC (Supplementary Figure 6A, 6C). Nonetheless, in patients undergoing sorafenib treatment, EZH2 was a factor obviously effecting their survival (Supplementary Figure 6B). Furthermore, high expression of UPK1A-AS1 indicated poor prognosis in patients with vascular invasion (Supplementary Figure 6D). UPK1A-AS1 was also potent in clarifying prognosis in patients with hepatitis virus and alcohol consumption (Supplementary Figure 6 E-F). Our results showed that UPK1A-AS1 functioned through EZH2, at least by part. Consistently, patients with simultaneous high UPK1A-AS1 and EZH2 expression also exhibited shorter OS. Collectively, UPK1A-AS1 was significantly upregulated in HCC, and upregulation of UPK1A-AS1 predicted poor prognosis for patients with HCC.

Discussion

Despite the profound advances made in HCC therapeutic strategies, the long-term prognosis of HCC patients remains poor as a result of limited understanding of the underlying mechanisms of tumor initiation and development[21]. Dysregulation of lncRNAs has been recognized to be involved in the onset and progression of malignancies, suggesting their clinical potential as biomarkers for diagnosis and prognosis, as well as therapeutic targets. Here, we demonstrated that UPK1A-AS1 was highly expressed in HCC, and high expression of UPK1A-AS1 predicted poor prognosis in patients with HCC. Biological experiments showed that UPK1A-AS1 promoted proliferation and tumor growth by accelerating G1/S transition of HCC cells. Furthermore, we also found that overexpression of UPK1A-AS1 could protect HCC cells against cis-platinum toxicity, suggesting that UPK1A-AS1 may promote the resistance to chemotherapy in HCC cells. Our findings suggested that UPK1A-AS1 may serve as a novel prognostic biomarker and potential therapeutic targets for HCC.

UPK1A-AS1 is a newly identified lncRNA with little information about its functional role and clinical significance in cancers. UPK1A-AS1, downregulated in ESCC, inhibited the proliferation, migration, and invasion of ESCC cells by serving as a miRNA decoy[16]. On the contrary, our finding showed that UPK1A-

AS1 was upregulated in HCC, and overexpression of UPK1A-AS1 promoted proliferation by regulation of cell cycle progression. It is well accepted that lncRNA constantly acts in a more tissue-specific or disease-specific manner[22]. RNA-seq data from GTEx revealed that UPK1A-AS1 was highly expressed in bladder, and modestly expressed in esophagus, cervix, and vagina, but hardly expressed in other tissues (Supplementary Fig. 4A), indicating the expression of UPK1A-AS1 was tissue-specific, and the biological role of UPK1A-AS1 may vary depending on organic context. Tissue-specific or disease-specific context of UPK1A-AS1 may account for the distinct roles of UPK1A-AS1 in ESCC and HCC.

EZH2 serves as the enzymatic core subunit of PRC2, a complex that has the ability to methylate lysine 27 of histone H3 and facilitates chromatin remodeling and transcriptional silencing[23]. Growing number of evidences have implicated EZH2 in the progression of a variety of human malignancies[24]. Consistently, our findings also confirmed that EZH2 was highly expressed in various cancer type, including HCC[25]. Here, we found that high level of EZH2 correlated with the development and progression of HCC. Upregulation of EZH2 predicted poor prognosis in patients with HCC. Moreover, in patients undergoing sorafenib treatment, EZH2 was a factor obviously effecting their survival, indicating that the expression level of EZH2 may distinguish patients who would benefit from sorafenib treatment. It has been reported that the subcellular localization of EZH2 correlates with mechanism of EZH2 oncogenic activity. EZH2 present in the cytoplasm may participate in actin polymerization to influence tumor dissemination[26]. However, EZH2, SUZ12, and EED form a complex in nucleus and transcriptionally regulate gene expression[27]. The association of EZH2, SUZ12, and EED is responsible for PRC2 activation. Here, we found that UPK1A-AS1 induced translocation of EZH2 from the cytoplasm to the nucleus. Furthermore, UPK1A-AS1 increased the interaction between EZH2 and SUZ12, promoted the methylation of lysine 27 in histone H3, indicating that UPK1A-AS1 contributes to the formation and activation of PRC complex. EZH2 mediated PRC2 activation contributes to the transcriptionally silencing of tumor suppress genes, leading to the activation of NOTCH[28], JAK-STAT[29], or β -catenin signaling pathways[30], and upregulation of cell cycle genes, like CDK2, CDK4, CyclinD1, et. al. However, UPK1A-AS1 overexpression did not change the expression of p-STAT3 (data not shown) and β -catenin (Fig. 6G), while si-EZH2 abolished the upregulation of CDK2, CDK4, and CyclinD1 caused by UPK1A-AS1, suggesting that UPK1A-AS1 upregulated the aforementioned genes via EZH2, but not EZH2-mediated JAK-STAT, and β -catenin signaling activation. Growing evidence have manifested that EZH2 can function through a PRC2-independent to facilitate transcriptional activation rather than repression[31–33]. EZH2 could directly bind to the promoter regions of CyclinD1 and promote its transcriptional activation[34]. Whether UPK1A-AS1-mediated upregulation of CDK2, CDK4, and CyclinD1 was EZH2-dependent transcriptional activation or not still requires further investigation.

Conclusions

Taken together, our findings uncovered the biological function and underlying mechanism of a newly identified lncRNA, UPK1A-AS1, which promotes HCC progression by accelerating cell cycle G1/S transition in an EZH2-dependent manner. Moreover, UPK1A-AS1 was highly expressed in HCC, and high

expression of UPK1A-AS1 predicts poor prognosis in patients with HCC, suggesting that UPK1A-AS1 may serve as a potential biomarker for HCC prognosis and therapy.

Abbreviations

CCK-8: Cell counting Kit; CBTCC: Cell Bank of Type Culture Collection; EZH2: Zeste Homologue 2; ESCC: esophageal squamous cell carcinoma; GEO: Gene Expression Omnibus; GSEA: Gene Sets Enrichment Analysis; GTEX: genotype-Tissue Expression; HCC: hepatocellular carcinoma; IF: Immunofluorescence; lncRNA: long non-coding RNA; LNA: Locked nucleic acid; nt: nucleotide; OS: overall survival; qRT-PCR: RNA extraction and quantitative polymerase chain reaction; RRC2: polycomb repressive complex 2; RIP: RNA immunoprecipitation; siRNA: small interference RNA; TCGA: The Cancer Genome Atlas; UPK1A-AS1: UPK1A antisense RNA 1.

Declarations

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Authors' contributions

Conception and design of the study were provided by Dong-Yan Zhang and Qing-Can Sun. The experiments were performed by Dong-Yan Zhang, Qing-Can Sun, Yang Song, Xue-Jing Zou, Wen-Wen Li, and Ze-Qin Guo. Analysis of data: Dong-Yan Zhang, Li Liu and De-Hua Wu. Dong-Yan Zhang wrote the paper, Li Liu and De-Hua Wu coordinated and conceived the study. Dong-Yan Zhang and Qing-Can Sun was responsible for its revision.

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Availability of data and materials

The cancer genome atlas program data was downloaded from National Cancer Institute (https://www.cancer.gov/about-nci/organization/ccg/research/structural_genomics/tcga).

Gene expression data (GSE10143, GSE14520, GSE22058, GSE54236, GSE64041) were downloaded from Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo>).

[ncbi.nlm.nih.gov/geo](http://www.ncbi.nlm.nih.gov/geo)).

Ethics approval and consent to participate

All experiments were in accordance with the ethical standards of the ethics committee of the Nanfang Hospital, Southern Medical University.

Consent for publication

Not applicable.

Competing interests

The authors have declared that no competing interest exists.

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Table

Table 1. Univariate and multivariate analyses of OS in TCGA cohort by Cox regression analysis

Variables	Univariate analysis			Multivariate analysis		
	HR	CI (95%)	P value	HR	CI (95%)	P value
Age(years)	1.502	0.821-2.748	0.285			
Gender	1.364	0.742-2.506	0.185			
Race	1.165	0.690-1.966	0.556			
Histologic grade	1.687	1.023-2.783	0.124			
HCC risk factor	0.472	0.263-0.848	0.015*	0.639	0.410-0.995	0.048*
Stage	2.552	1.584-4.112	0.000*	2.214	1.408-3.481	0.001*
Vascular invasion	0.561	0.336-0.936	0.120			
AFP	0.560	0.304-1.035	0.704			
New tumor event after initial treatment	1.035	0.628-1.707	0.631			
UPK1A-AS1	2.043	1.299-3.397	0.036*	1.704	1.100-2.640	0.017*

Abbreviations: HBV, hepatitis B virus; HCV, hepatitis C virus; CI, confidence interval; HR, hazard ratio; *The values had statistically significant differences.

Figures

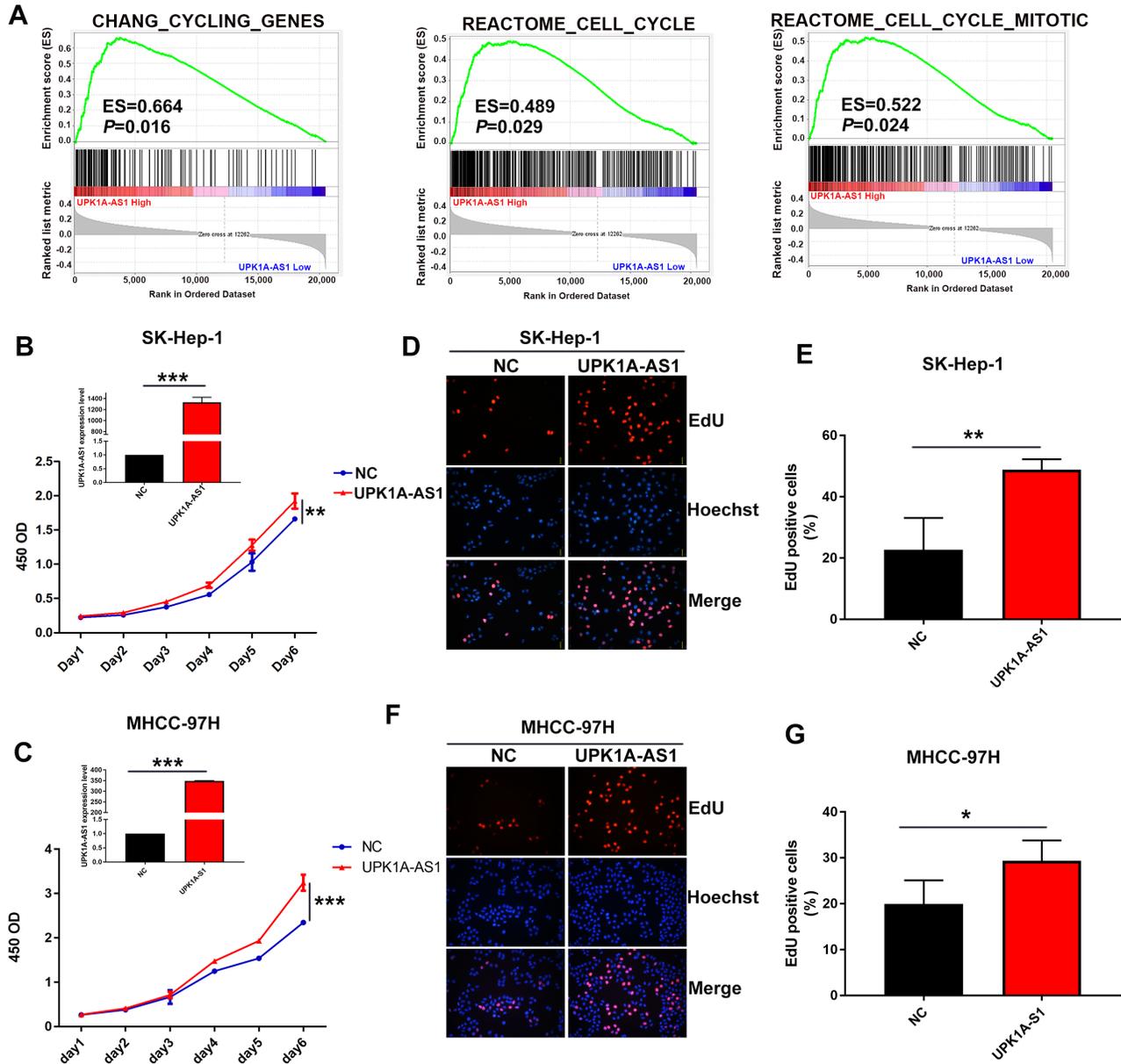


Figure 1

Upregulation of UPK1A-AS1 promotes proliferation in HCC cells. A. Results of Gene Set Enrichment Analysis (GSEA) were plotted to visualize the correlation between UPK1A-AS1 and cell cycle gene signatures in HCC sample from TCGA dataset ($P < 0.05$). B-C. CCK-8 assay was performed to determine the effect of UPK1A-AS1 overexpression on proliferation in SK-Hep-1 (B) and MHCC-97H (C) cells (** $P < 0.01$, *** $P < 0.001$). D-G. Overexpression of UPK1A-AS1 in SK-Hep-1 (D-E) and MHCC-97H (F-G) cells promoted more cells into S phase than negative control as detected by EdU assay (* $P < 0.05$, ** $P < 0.01$).

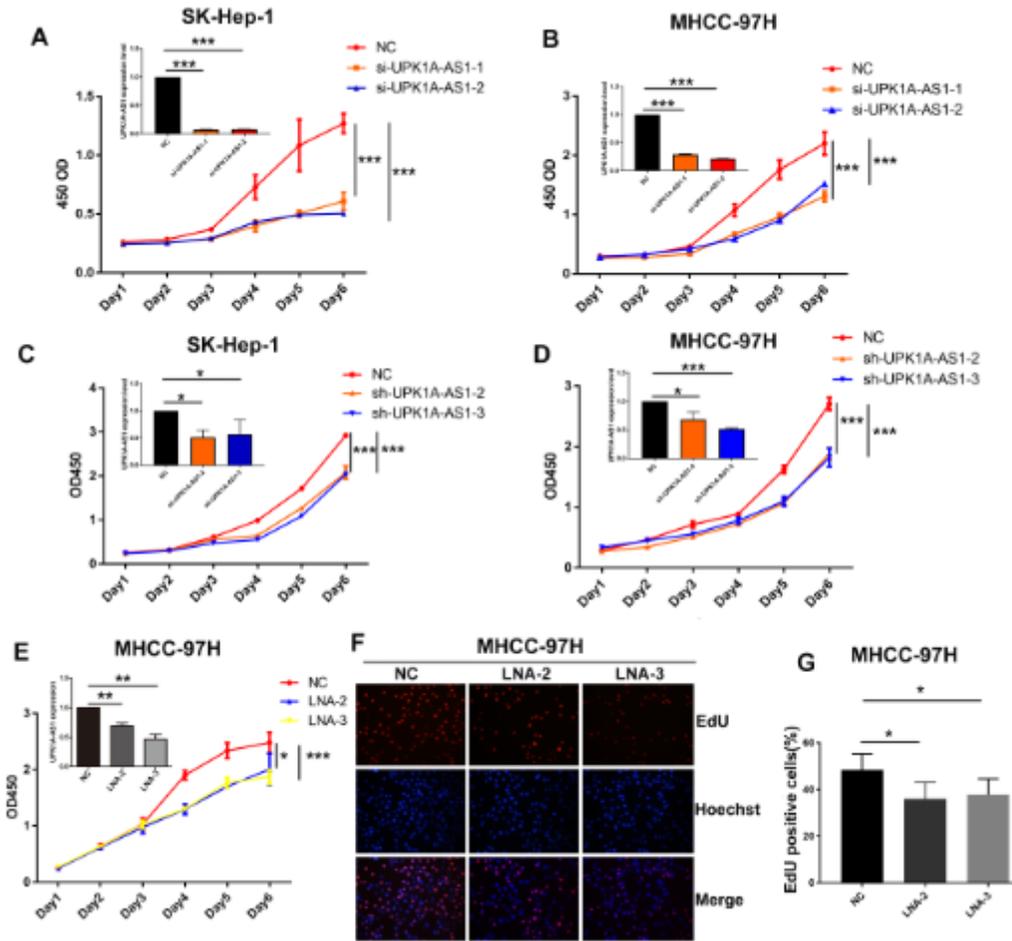


Figure 2

Knocked down of UPK1A-AS1 inhibits proliferation in HCC cells. A. CCK-8 assay was performed to determine the effect of si-UPK1A-AS1 on proliferation in SK-Hep-1 (B) and MHCC-97H (C) cells (*** $P < 0.001$). C-D. Proliferation curve of HCC cells transfected with sh-UPK1A-AS1 (* $P < 0.05$, *** $P < 0.001$). E. Representative CCK-8 analysis of cell viability after knocked down of UPK1A-AS1 by LNA in MHCC-97H cells (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). (F-G). EdU assay showed that downregulation of UPK1A-AS1 decreased ratio of S phase cells in MHCC-97H (* $P < 0.05$).

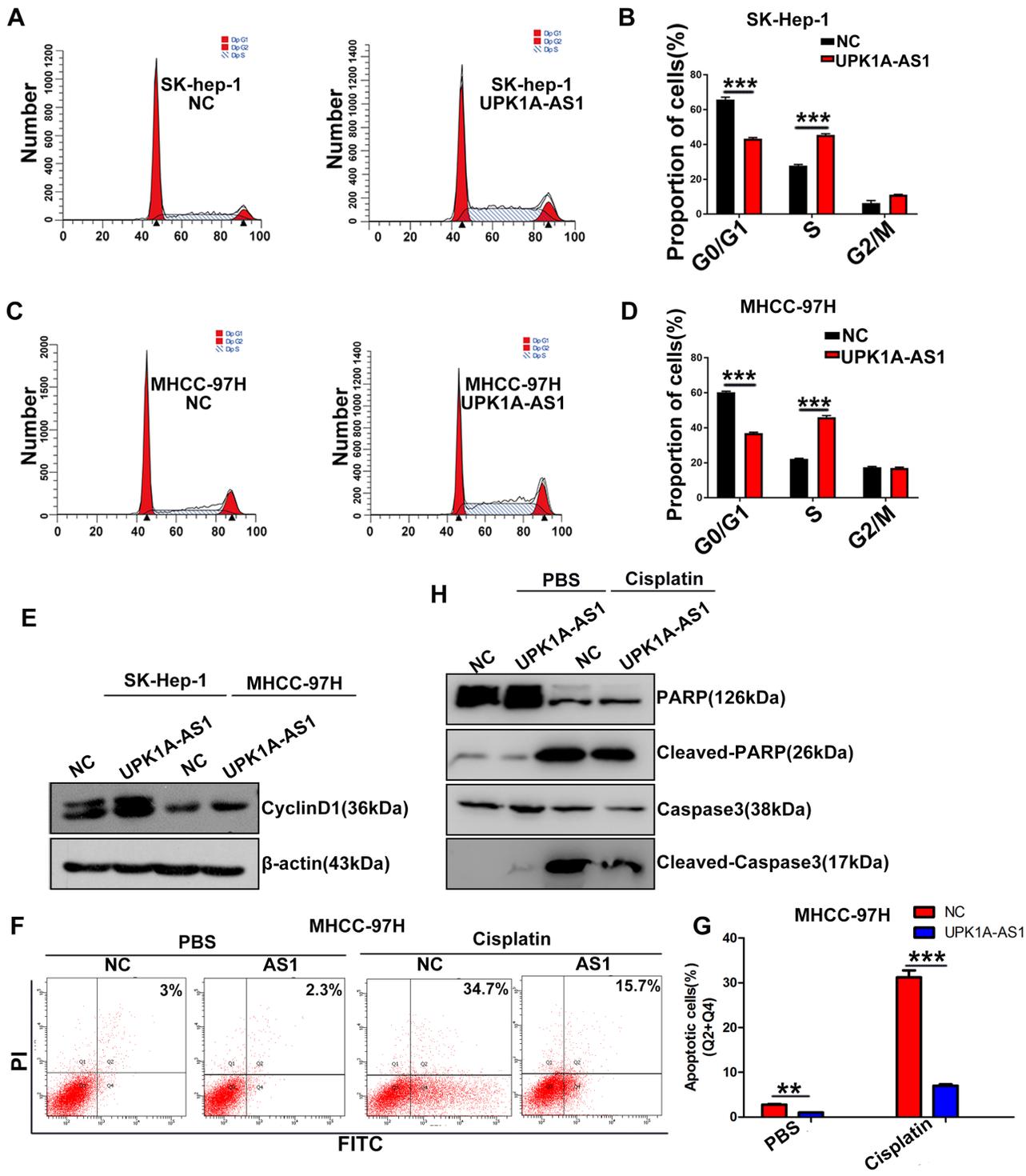


Figure 3

UPK1A-AS1 promotes G1/S transition of HCC cells. A-D. UPK1A-AS1 increased the ration of cells in S phase in comparison with negative control in SK-Hep-1 (A-B) and MHCC-97H (C-D) cells (***P* < 0.001). Error bars represent the mean ± S.D. of three independent experiments. E. UPK1A-AS1 increased expression of CyclinD1 in HCC cells by western blot. F. UPK1A-AS1-overexpressing cells treated with cisplatin (40 μM) for 24h. The cells stained with Annexin V-FITC and PI were subjected to FACS analysis.

Error bars represent the mean \pm S.D. of triplicate experiments (**P < 0.01, ***P < 0.001). H. The expression of caspase-3 and PARP were analyzed using western blot in UPK1A-AS1-overexpressing MHCC-97H cells treated with cis-platinum.

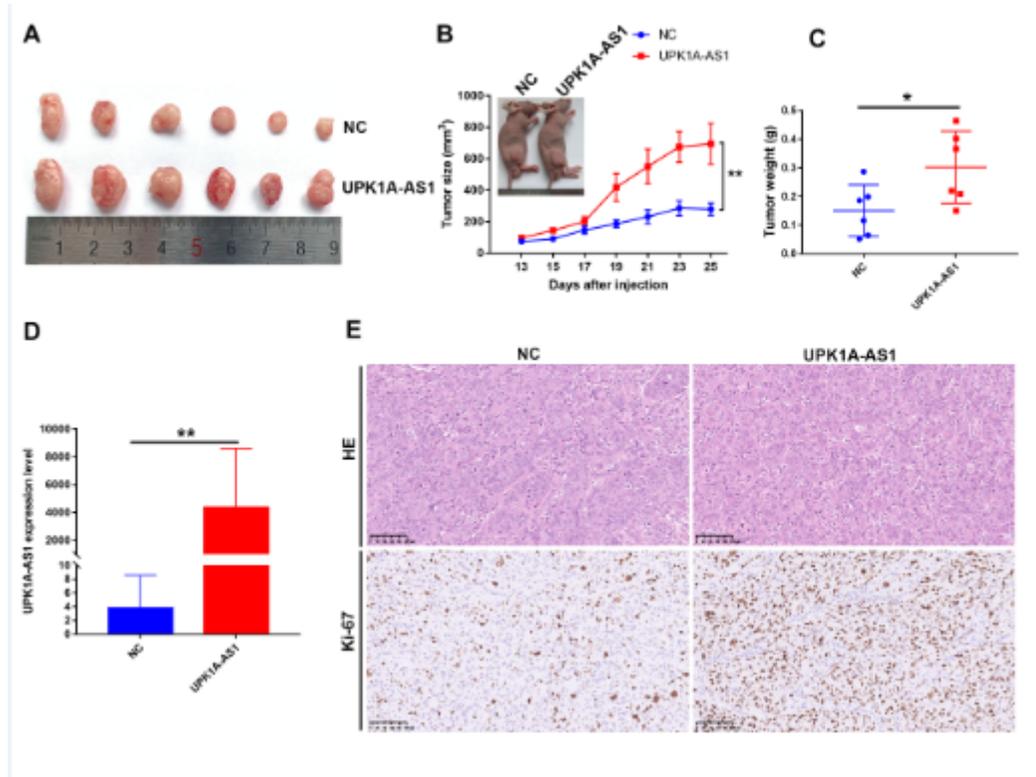


Figure 4

Overexpression of UPK1A-AS1 boosts tumor growth in HCC. A-C. Overexpression of UPK1A-AS1 in MHCC-97H cells promoted tumor growth in vivo. Tumor burden and tumor weight in cell with UPK1A-AS1 overexpression was visibly higher than those of control cells (*P < 0.05, **P < 0.01). D. Expression of UPK1A-AS1 in subcutaneous tumor was determined by qRT-PCR (**P < 0.01). E. Sections of xenograft tumors stained with hematoxylin and eosin (H&E), Ki-67 staining was performed for further determination of the effect of UPK1A-AS1 on cell proliferation.

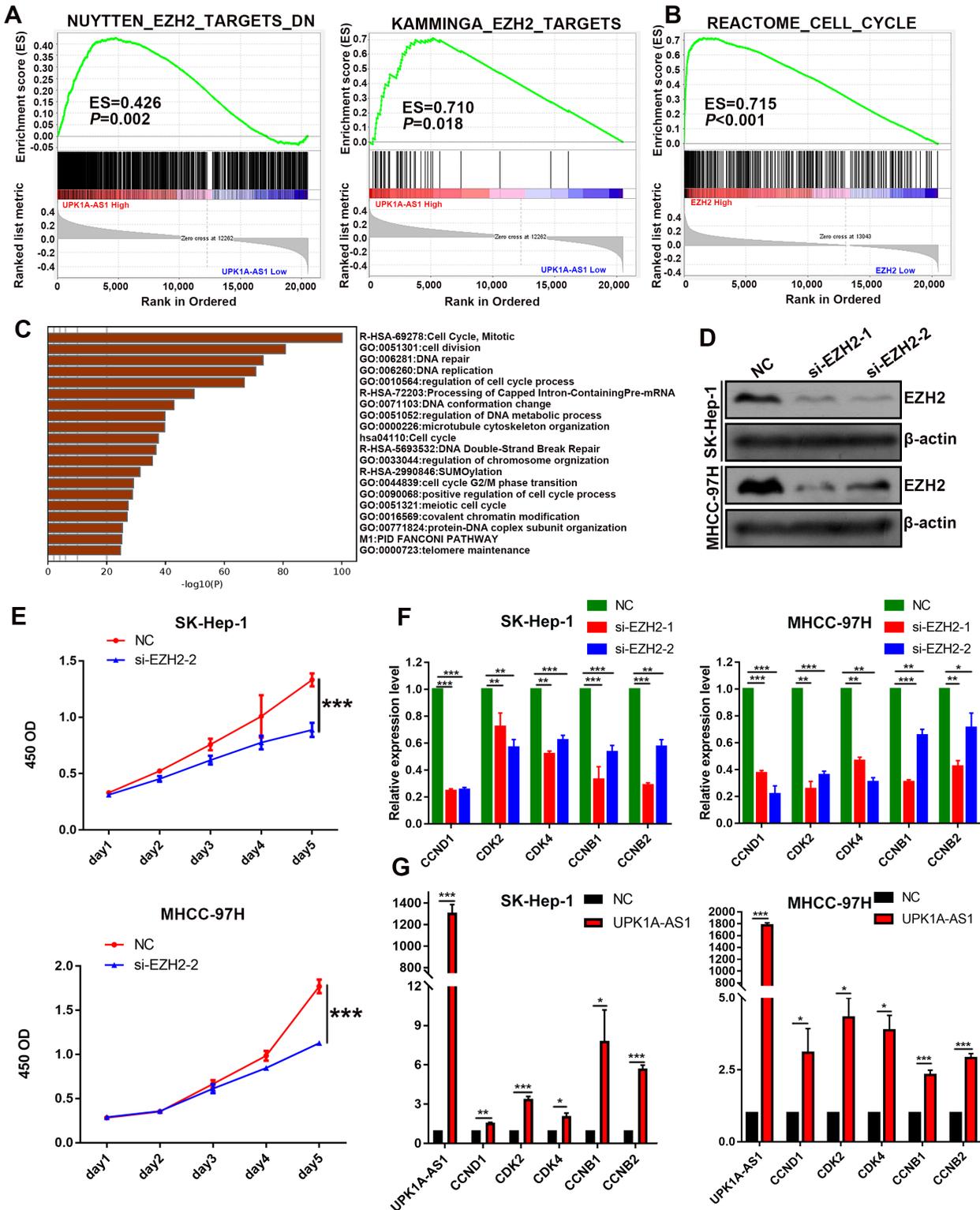


Figure 5

UPK1A-AS1 correlates with EZH2 targets. A. GSEA analysis plot indicated a significant correlation between UPK1A-AS1 and EZH2 targets ($P < 0.05$). B. Results of GSEA were plotted to visualize the correlation between EZH2 and cell cycle gene signatures in HCC sample from TCGA dataset ($P < 0.05$). C. Heatmap of Gene Ontology (GO) enriched terms colored by P-values. D. Knockdown efficiency of si-EZH2 in SK-Hep-1 and MHCC-97H cells was measured by western blot. E. Growth curve of HCC cells transfected

with si-EZH2 (** $P < 0.001$). F. Expression of the indicated genes after si-EZH2 was measured using qRT-PCR (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). G. Overexpression of UPK1A-AS1 increased the expression of the indicated genes measured by qRT-PCR (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

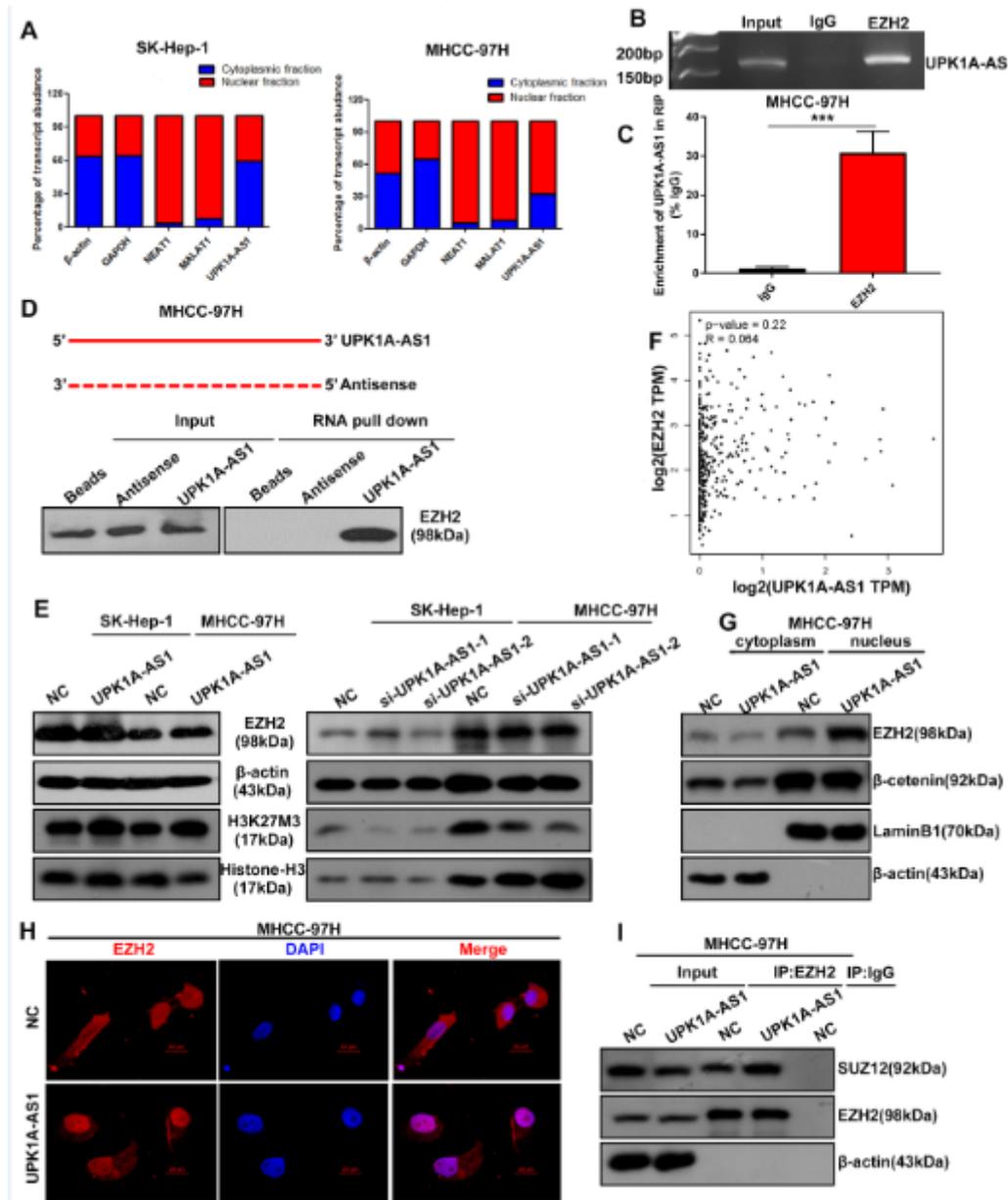


Figure 6

UPK1A-AS1 interacts with EZH2. A. UPK1A-AS1 expression level in the cytoplasm or nucleus of SK-Hep-1 and MHCC-97H cells. β -Actin was used as a cytosol marker, U6 was used as a nuclear marker, both NEAT1 and MALAT1 were nucleus located lncRNAs. B-C. RIP assay was performed in MHCC-97H cells and the co-precipitated RNA was subjected to qRT-PCR for UPK1A-AS1 (** $P < 0.001$). D. RNA pull-down assay was carried out to confirm the association between UPK1A-AS1 and EZH2. E. Effect of UPK1A-AS1 overexpression on expression level of EZH2 and H3K27M3 was measured by western blot. F. Correlation between UPK1A-AS1 and EZH2 was analyzed in HCC samples from TCGA dataset. G. EZH2 expression level in the cytoplasm or nucleus of MHCC-97H cells. β -Actin was used as a cytosol marker, LaminB1

served as a nuclear marker. H. Translocation of EZH2 from the cytoplasm to the nucleus was detected by immunofluorescence assay. I. Immunoprecipitation assay identified the increased interaction between EZH2 and SUZ12 in UPK1A-AS1-overexpressing MHCC-97H cells. β -Actin was used as negative control.

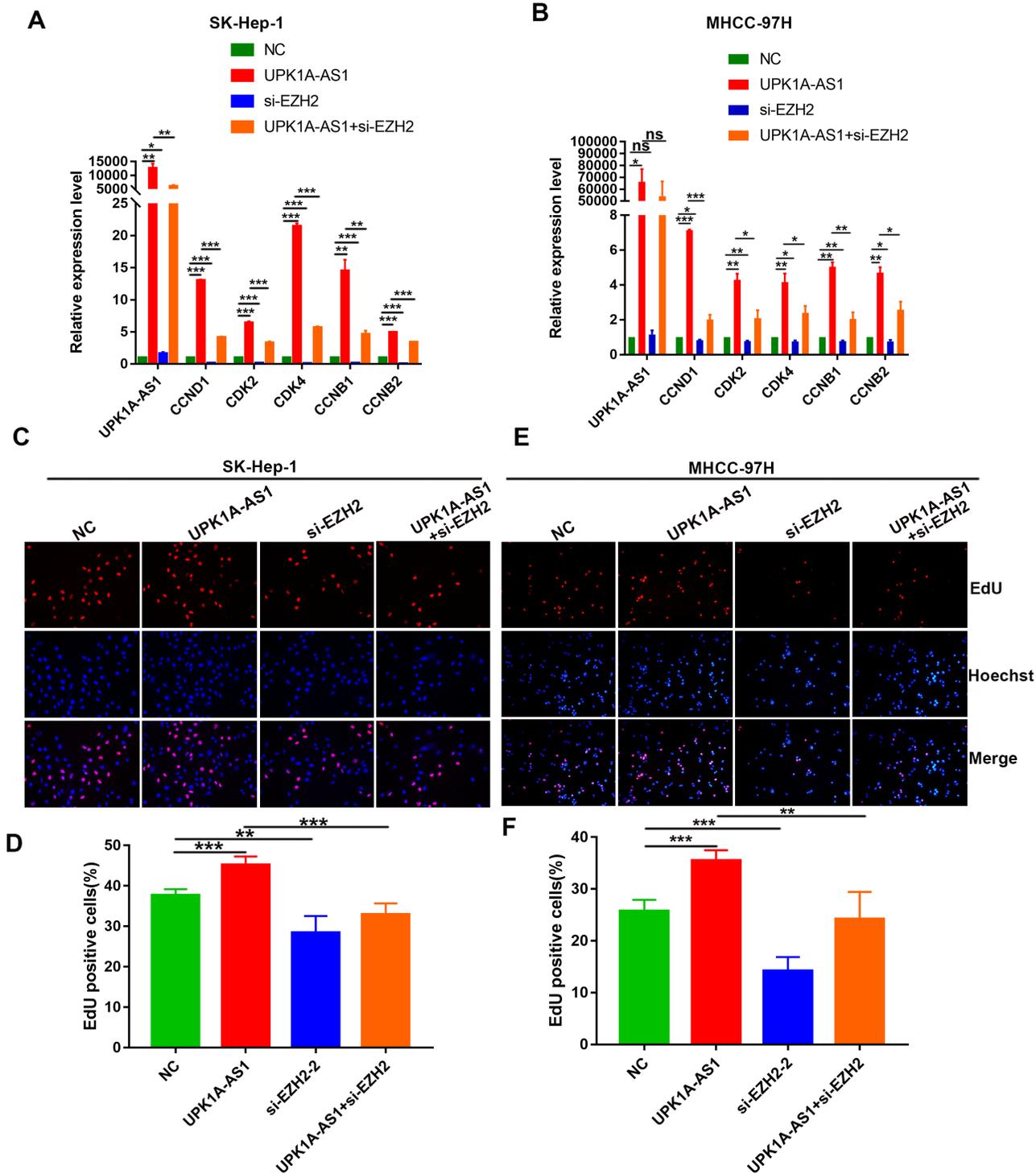


Figure 7

UPK1A-AS1 functions through EZH2. A-B. Expression of the indicated genes were monitored by qRT-PCR after co-transfected UPK1A-AS1 vectors and si-EZH2 into SK-Hep-1 (A) and MHCC-97H (B) cells (*P <

0.05, **P < 0.01, ***P < 0.001). C-F. Effect of co-transfected UPK1A-AS1 vectors and si-EZH2 on SK-Hep-1 (C-D) and MHCC-97H (E-F) cells cell proliferation as measured by EdU assay (**P < 0.01, ***P < 0.001).

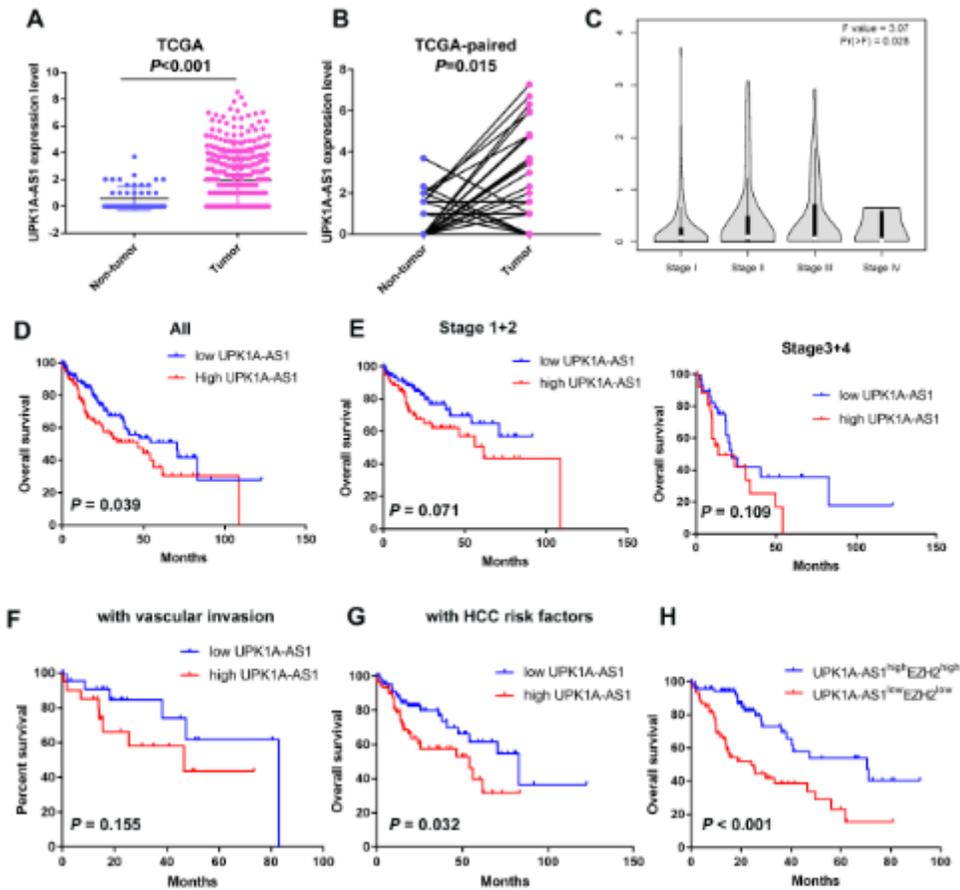


Figure 8

Upregulation of UPK1A-AS1 correlates with poor prognosis in patients with HCC. A. Expression level of UPK1A-AS1 in HCC from TCGA dataset ($P < 0.001$). B. The expression of UPK1A-AS1 from 50 paired HCC samples and adjacent non-tumor liver tissues from TCGA dataset ($P < 0.05$). C. Correlation between UPK1A-AS1 and tumor stage in HCC patients (GEPHA). D. Kaplan-Meier analysis of overall survival in the TCGA dataset on the basis of UPK1A-AS1 expression ($P < 0.05$). E-G. Kaplan-Meier analysis showed the correlation of UPK1A-AS1 and survival in indicated groups in HCC from TCGA dataset. H. Kaplan-Meier analysis of overall survival in the TCGA dataset based on UPK1A-AS1 and EZH2 expression levels ($P < 0.001$).

Supplementary Files

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