Linc00342 serves as a diagnostic and prognostic biomarker in Kidney Renal Clear Cell Carcinoma:A Comprehensive Study Based on Data mining, Clinical Analysis, and in vitro Validation

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Abstract

Background: Kidney renal clear cell carcinoma (KIRC), as the most common type of renal cancer, has a high mortality and recurrence rate due to the fact that many patients are in advanced stage at the time of consultation. Finding a biological marker for early-stage KIRC has become a top priority. Recently, accumulating studies have shown that lncRNA can serve as a target for diagnosis and prognosis of malignancy. However, the involvement and mechanism of linc00342 has never been researched in KIRC. The aim of this study was to investigate the diagnostic and prognostic value of linc00342 in KIRC, and to explore the effects of linc00342 on the biological functions of KIRC cells.

Methods: We downloaded the linc00342 expression data and clinical information of KIRC from the TCGA database and constructed a prognostic prediction model. *In vitro,* the effect of knocking down linc00342 on KIRC cell proliferation, apoptosis, metastasis, and invasion was measured by colony-formation assay, flow cytometric analysis, wound-healing assay and Transwell assay, respectively.

Results: Our nomogram predictive model suggested linc00342 can serve as an independent prognostic factor for KIRC. GO functional analysis and KEGG pathway analysis showed linc00342 was involved in various biological functions of KIRC, and experiments *in vitro* verified this. *In vitro,* linc00342 was overexpressed in KIRC cells as shown by RT-qPCR. Moreover, we found that linc00342 can inhibit cell apoptosis and promote cell proliferation, invasion, and migration.

Conclusions: Our study is the first to examine the diagnostic and prognostic value of linc00342 in KIRC and provides new ideas for the treatment of KIRC.

Background

Renal cell carcinoma (RCC), a common aggressive urinary cancer, has a high incidence worldwide, accounting for about 3% of adult malignancies[1]. The three common pathological subtypes of RCC are kidney renal clear cell carcinoma (KIRC), kidney renal papillary cell carcinoma (KIRP), and kidney chromophobe (KICH). Among them, KIRC is the most common subtype at 70–75% of cases, followed by KIRP at 10–16% and KICH at 5% of cases[2]. Effective treatments of KIRC include surgery, radiofrequency ablation, and targeted drug therapy such as sunitinib and pazopanib[3, 4]. However, most KIRC patients are in the advanced stage and have distant metastasis at the time of diagnosis, leading to their poor prognosis and high recurrence rate. Finelli et al [5] found that the recurrence rate of RCC after surgery is approximately 25%. The poor prognosis and high recurrence of RCC makes it urgent to discover potential early-stage biomarkers in order to improve overall survival (OS).

As a type of non-protein-coding RNAs with length over 200 nucleotides [6, 7], aberrantly expressed lncRNA is insinuated to be tightly linked with diagnosis and prognosis of numerous malignancies, including KIRC. Apart from chromatin modification[8, 9], lncRNA also perform functions in transcription and post-transcription by interacting with DNA, mRNAs, and proteins[10, 11]. Another competing endogenous RNA (ceRNA) mechanism hypothesis is that lncRNAs regulate the expression of target genes by interacting

with miRNA [12–15]. Mounting evidence has indicated lncRNA can be used as diagnostic and prognostic indicator for cancers [16–18]. Recently, research about mechanism of lncRNAs in KIRC has been a hotspot. LncRNA MALAT1 was reported to modify progression of KIRC by regulating miR-194-5p/ACVR2B axis and may be used as a novel therapeutic target in KIRC [19]. LINC00997 is associated with metastasis in KIRC [20]. LncRNA SARCC is confirmed to control tumorigenesis via altering the androgen receptor miRNA 143-3p signals [21]. As one of non-protein-coding genes, linc00342 has been verified to perform oncogenic functions in lung cancer [22, 23] and infantile hemangioma [24], however, the significance of linc00342 in KIRC is still unconfirmed. Our research is the first to explore the difference of linc00342 expression between KIRC and kidney normal tissues, and to study the correlation between linc00342 and prognosis through biological and clinical data analysis, coupled with experimental verification, which may provide us with new ideas into the treatments of KIRC.

Methods

Analysis of linc00342 expression in KIRC based on GEPIA and the Cancer Genome Atlas (TCGA) database

To investigate the expression of linc00342 in diverse malignancies, we input linc00342 into the GEPIA database (http://gepia.cancer-pku.cn/). |Log2FC| = 1 and *P*-value = 0.01 was referred as cutoff. Then linc00342 gene expression data and clinical information of KIRC patients were obtained from the TCGA database (https://gdc.cancer.gov/) for further study.

Construction of predictive nomogram model

Univariate and multivariate Cox regression analysis was performed to discover independent prognostic factors of KIRC. A value of $P < 0.05$ was considered to be significant. The factors included linc00342 expression, age, gender, and TMN stages, all obtained from TCGA database. A nomogram predictive model was applied to study the significance of linc00342 expression in KIRC based on the multivariate Cox regression analysis.

GO function and KEGG pathway analysis

To elaborate the potential functions of linc00342 in the tumorigenesis of KIRC, we perform Gene Ontology (GO) function annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis using the David database (https://david-d.ncifcrf.gov/). $P < 0.05$ was considered as significant. The results of GO functions (including biological processes, cellular components, and molecular functions) were visualized using R packages “ggplot2 and GOplot”. The relationships between related genes and both GO functions and KEGG pathways were shown using Cytoscape (version: 3.7.2).

Protein-protein interaction (PPI) network and Pearson’s correlation analysis

In order to investigate the relationship among linc00342 related genes, we put the related genes into the string database (https://string-db.org/). 0.900 was set as interaction score in the network. Finally, we used Cytoscape (version: 3.7.2) for hub gene analysis. In Cytoscape, MCC was used as calculation

method and cytoHubba plugin was used to obtain the first 10 hub genes. The Starbase database (http://starbase.sysu.edu.cn/) was used to evaluate the correlation between linc00342 and hub genes.

Cell culture and transfection

OSRC2 and ACHN KIRC cell lines and HK-2 normal kidney cell lines were provided by Peking Union Medical College Hospital. RPMI-1640, 10% fetal bovine serum, 1% double antibiotic (100 X penicillin-streptomycin) and MEM, purchased from GIBCO (Waltham, MA, USA), were used to prepare cell culture medium. The linc00324 shRNA1,2,3 plasmids were introduced into OSRC2 and ACHN cells, and the shRNA with the highest silencing effect was selected for subsequent functional experiments. The transfected cells were cultured in an incubator with 5% CO_2 at 37 °C.

Real-time quantitative polymerase chain reaction (RT-qPCR)

Trizol (Vazyme, Nanjing, China) was added into the cell culture medium to extract RNA. The transcription level of linc00324 was detected by qPCR. The primers for qPCR were TCCACAGACACTACCCAAAGC linc00324-F, GCAGTTCACTCTGCTGCTTC LINC00324-R; TCATTGACCTCAACTACATGG GAPDH-F, TCGCTCCTGGAAGATGGTG GAPDH-R. The relative expression of linc00342 was calculated by the formula: relative expression = 2^ (GAPDH Ct-target gene Ct). Three repeated experiments were carried out for each group, and the average and variance were used to plot the final result.

Colony-formation assay

The OSRC2 and ACHN KIRC cells were cultured in the incubator at 37℃ with 5% CO_2 for 7 days. Then crystal violet dye (Solarbio, Beijing, China) was added to each well for staining. After removing crystal violet, we rinsed 3 times and took pictures for analysis.

Flow cytometric analysis

The resuspended cells were centrifugedfollowing the manufacturer’s protocol, then Annexin v-FITC and propidium iodide (PI) (Cwbiotech, Beijing, China) staining solution were added for double-staining. Annexin V-FITC fluoresces green and propidium iodide (PI) fluoresces red. Red fluorescence was detected by flow cytometry at the excitation wavelength of 488 nm.

Wound-healing assay

The cells were grown in 6-well plates to 80% confluence. A 200-μl pipette tip was used to remove cells along a straight line drawn through the center of the culture plate. PBS was used to remove non-adherent cells, then cultured at a room temperature with 5% CO_2. We took photos at 0 and 24 hrs for further study.

Transwell assay

200 μl of cell suspension (4×10^4 cells) was added to the upper part of the Transwell plate and 700 μl complete medium (Gibco, Waltham, MA, USA) was added to the lower chamber of Transwell culture plate, then incubated for 24 hrs at an 37℃ atmosphere of 5% CO_2. The cells were rinsed three times with water after 30 min of crystal violet staining.\

Statistical analyses

IBM SPSS Statistics 22(IBM, USA) and GraphPad Prism 8 (GraphPad Software Inc., USA) were used to conduct statistical analyses. The Kaplan–Meier method was applied for analysis of overall survival (OS). Univariate and multivariate Cox regressions were performed independent prognostic factors analysis. Results are considered significant when $P < 0.05$ (*), $P < 0.01$ (**) or $P < 0.001$ (***).

Results

Aberrant expression of linc00342 in various tumors

Significant expression differences between the tumor group and the normal group were observed in the adrenocortical carcinoma (ACC), breast invasive carcinoma (BRCA), cholangiocarcinoma (CHOL), colon adenocarcinoma (COAD), lymphoid neoplasm diffuse large B-cell lymphoma (DLBC), glioblastoma multiforme (GBM), kidney renal clear cell carcinoma (KIRC), acute myeloid leukemia (LAML), lung adenocarcinoma (LUAD), lung squamous cell carcinoma (LUSC), ovarian serous cystadenocarcinoma (OV), pancreatic adenocarcinoma (PAAD), pheochromocytoma and paraganglioma (PCPG), prostate adenocarcinoma (PRAD), rectum adenocarcinoma (READ), skin cutaneous melanoma (SKCM), stomach adenocarcinoma (STAD), testicular germ cell tumors (TGCT), thyroid carcinoma (THCA), thymoma (THYM), uterine corpus endometrial carcinoma (UCEC), uterine carcinosarcoma (UCS) in GEPIA dataset. High expression in tumor relative to normal tissue was found in CHOL, DLBC, KIRC, LAML, PAAD, PCPG and THYM; low expression in tumor relative to normal tissue was found in ACC, BRCA, COAD, GBM, LUAD, LUSC, OV, PRAD, READ, SKCM, STAD, TGCT, THCA, UCEG and UCS. (Fig. 1A, 1B, 1C, $p < 0.05$). Meanwhile, we downloaded the gene expression and clinical information (including survival time and survival state) of linc00342 in KIRC from the TCGA database. The expression of linc00342 in KIRC was significantly higher than in normal tissues (Fig. 1D, $p < 0.001$), and in the receiver operating characteristic (ROC) curve, the area under the curve (AUC) was greater than 0.5, indicating that linc00342 has significant diagnostic value in KIRC (Fig. 1E, AUC = 0.889, 95%CI, 0.860–0.919). The results of survival analysis suggested that the 5-year survival rate of patients with high expression of linc00342 in KIRC was significantly lower than that of patients with low linc00342 expression (Fig. 1F, $P < 0.05$).

The prognostic role of linc00342 in KIRC

In order to better study the importance of linc00342 in the prognosis of KIRC, we conducted univariate and multivariate Cox analysis on the clinical data from KIRC patients (including linc00342 gene expression, age, gender, clinical stage), and constructed a nomogram prognosis prediction model according to the multivariate Cox analysis. Univariate analysis results suggested that linc00342 gene expression and clinical stage (TMN) might be independent factors for KIRC prognosis (Fig. 2A, $p < 0.01$), and multivariate analysis illustrated that linc00342 expression level was an independent factor for KIRC prognosis (Fig. 2B, $p < 0.01$). The nomogram prediction model results suggested that KIRC patients with the higher linc00342 expression had poorer 3-year and 5-year OS (Fig. 2C). We then plotted validation curves to evaluate the prediction model. The blue line was not far from the diagonal, suggesting the predicted probabilities were consistent with the observed proportions (Fig. 2D).

GO annotation and KEGG pathway enrichment analysis

Linc00342-related genes obtained from the MEM dataset (https://biit.cs.ut.ee/mem/) were used to conduct functional analysis through the David database (https://david-d.ncifcrf.gov/). Enriched GO terms and their target genes and *P* values are shown in Fig. 3A, 3B, including cell cycle, cell cycle arrest, M phase, cell division, mRNA metabolic process, RNA splicing, RNA processing, DNA repair, negative regulation of gene expression, and nuclear division, all of which may be associated with the tumorigenesis of KIRC ($p < 0.05$). KEGG pathway analysis demonstrated that linc00342 related genes were involved in a variety of significant cancer-associated pathways, such as MAPK signaling pathway, mTOR signaling pathway, insulin signaling pathway, NOD-like receptor signaling pathway, Wnt signaling pathway and GnRH signaling pathway ($p < 0.05$). The detailed data of KEGG analysis are listed in Table. A network was plotted by Cytoscape to present the relationship between the GO terms, KEGG terms and their correlated genes. (Fig. 3C, 3D)

Protein-protein interaction (PPI) network and Pearson's correlation analysis

In the PPI network (Fig. 4), nodes represent proteins, edges represent protein-protein associations, and line color indicates the type of interaction. Through correlation analysis based on the Starbase database, CCAR1 (r = 0.578, *P* = 6.20e-49), HNRNPA1 (r = 0.089, *P* = 4.07e-02), HNRNPA2B1 (r = 0.649, *P* = 3.31e-65), HNRNPD (r = 0.410, *P* = 4.62e-23), HNRNPL (r = 0.422, *P* = 1.62e-24), RBM5 (r = 0.805, *P* = 5.46e-123), SRSF5 (r = 0.698, *P* = 2.57e-79), SRSF7 (r = 0.542, *P* = 3.92e-42), SRSF11 (r = 0.795, *P* = 8.14e-118), and WDR33 (r = 0.411, *P* = 4.e-02) (Fig. 5A-5J) all have significant relationships with linc00342.

shRNA with the highest knockdown efficiency in KIRC cells was selected

RT-qPCR analysis showed linc00342 was remarkably upregulated in OSRC2 and ACHN KIRC cells compared with normal kidney cells (Fig. 6A, $P < 0.05$). In order to investigate the potential mechanism of linc00342 in tumorigenesis of KIRC, linc00342 shRNA#1, #2, #3 were transfected into OSRC2 and ACHN KIRC cells to knock down the expression of linc00342. ShRNA#2 had the highest knockdown efficiency (Fig. 6B) and was used for further studies.

Depletion of linc00342 retards proliferation in KIRC cells.

Colony-formation assays revealed decreased cell proliferation in OSRC2 and ACHN KIRC cells transfected with the linc00342 shRNA#2, compared with control group with scrambled shRNA (Fig. 7, $P < 0.01$). The results indicated that downregulation of linc00342 could inhibit the proliferative properties of KIRC cells.

Silencing linc00342 accelerates cell apoptosis and arrests cell cycle in G0/G1

On the basis of the results of flow cytometry analysis, we found that along with the knockdown of linc00342 in OSRC2 and ACHN KIRC cells, the cell apoptotic rate in linc00342-shRNA-KIRC cells was elevated compared with linc00342-scramble-KIRC cells. (Fig. 8A, $P < 0.05$). Furthermore, the results showed linc00342-silencing KIRC cells were arrested in G0/G1. (Fig. 8B, $P < 0.05$)

Knocking down linc00342 exhibited significantly reduced migration and invasion ability

By comparing the results of transfection with linc00342-scramble and linc00342-shRNA, it was revealed that after knocking down linc00342, the post-healing width of the scratches as a percentage of the original scratch width was significantly lower than in the control group, indicating linc00342 can promote the migration ability of OSRC2 and ACHN KIRC cell lines. (Fig. 9A, $P < 0.05$) Furthermore, similar findings were obtained via a Transwell assay, in which the density of KIRC cells transfected with the linc00342-shRNA was remarkably decreased compared with controls. (Fig. 9B, $P < 0.001$) Accordingly, we drew the conclusion that linc00342 had the properties that promoted migration and invasion in KIRC.

Discussion

Kidney cancer, the most common malignant tumor of renal parenchyma, is a malignancy originating from the urinary tubule epithelial system. Siegel et al. reported there were 65,349 new KIRC cases and 14,970 deaths due to KIRC in the United States in 2018[25]. Surgery and radiotherapy are of no avail to patients with late-stage KIRC, leading to high mortality and recurrence rates. There is thus an urgent need to find early-stage diagnostic markers to improve OS of KIRC patients. As indicated by numerous publications, lncRNA, which is featured by non-protein-coding, presently is involved in tumorigenesis and progression of various cancers. Recently, advances in bioinformatics tools bring improvements in analysis, facilitating diagnosis and treatment for enormous cancers. TCGA, a large-scale genomics-based database with the expression data and clinical information on 33 cancer types, provides a molecular basis for understanding the pathogenesis of tumors. Nomograms derived from statistical models are used to improve cancer prognostication by integrating diverse clinical variables [26].

In our study, we first observed aberrant expression of linc00342 in a majority of tumors based on the GEPIA database. After that, we used the gene expression and clinical data downloaded from TCGA to construct a nomogram prediction model. Analysis of that TCGA data showed that the expression of linc00342 in KIRC tissues was significantly higher than that of normal paracancerous tissues ($p < 0.001$), and the ROC curve suggested that linc00342 had diagnostic significance for KIRC (AUC = 0.889, 95%CI, 0.860–0.919). A nomogram based on multivariate analysis indicated that the overexpressed linc00342 level was responsible for the poor OS of KIRC, which is consistent with the results of the survival curve. Together, the bioinformatics analysis suggested that linc00342 may be related to the diagnosis and prognosis of KIRC. *In vitro*, we found that linc00342 was indeed higher in KIRC cells than in the normal control group. Subsequently, in order to understand the pathogenesis of linc00342 in KIRC, we used the David database to conduct functional analysis of linc00342 related genes. As showed by GO annotation analysis, linc00342 was related with cell cycle, cell cycle arrest, M phase, cell division, mRNA metabolic process, RNA splicing, RNA processing, DNA repair, negative regulation of gene expression, and nuclear division, indicating that linc00342 may play carcinogenic roles by affecting the cell cycle. The *in vitro* experiments in our study verified the hypothesis. Thus, we found that depletion of linc00342 retarded cell proliferation, accelerated cell apoptosis, and significantly reduced migration and invasion ability in KIRC cells. KEGG analysis showed linc00342-related genes were enriched in several important signaling pathways – MAPK, mTOR, insulin, NOD-like receptor, Wnt, and GnRH – that are reported to play key roles in many malignancies [27–32]. The effect of these KEGG pathways on the progress of KICC was not

explored in our current study. Undoubtedly, experiments *in vivo* and *in vitro* are still needed to validate this point.

Conclusions

In sum, our study provides the first evidence that linc00342 is overexpressed in KIRC tissues and cells and has a significant prognostic value for KIRC patients. Our study indicates linc00342 suppresses apoptosis and promotes proliferation, migration, and invasion in KIRC cells. With further verification, linc00342 may be a potential therapeutic target for KIRC patients.

List Of Abbreviations

RCC, Renal cell carcinoma

KIRC, kidney renal clear cell carcinoma

KIRP, kidney renal papillary cell carcinoma

KICH, kidney chromophobe

OS, overall survival

ceRNA, competing endogenous RNA

TCGA, The Cancer Genome Atlas

GO, Gene Ontology

KEGG, Kyoto Encyclopedia of Genes and Genomes

PPI, Protein-protein interaction

RT-qPCR, Real-time quantitative polymerase chain reaction

PI, propidium iodide

ACC, adrenocortical carcinoma

BRCA, breast invasive carcinoma

CHOL, cholangiocarcinoma

COAD, colon adenocarcinoma

DLBC, lymphoid neoplasm diffuse large B-cell lymphoma

GBM, glioblastoma multiforme

KIRC, kidney renal clear cell carcinoma

LAML, acute myeloid leukemia

LUAD, lung adenocarcinoma

LUSC, lung squamous cell carcinoma

OV, ovarian serous cystadenocarcinoma

PAAD, pancreatic adenocarcinoma

PCPG, pheochromocytoma and paraganglioma

PRAD, prostate adenocarcinoma

READ, rectum adenocarcinoma

SKCM, skin cutaneous melanoma

STAD, stomach adenocarcinoma

TGCT, testicular germ cell tumors

THCA, thyroid carcinoma

THYM, thymoma

UCEC, uterine corpus endometrial carcinoma

UCS, uterine carcinosarcoma

ROC, receiver operating characteristic

AUC, area under the curve

Declarations

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Consent to publish

Not applicable.

Authors' contributions

DG wrote the article, HP administrated the whole research, DG, GYZ, HP conducted functional assays and experiment, DG made statistical analysis. All authors have read and approved the manuscript.

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Availability of data and materials

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Disclosure

The authors report no conflicts of interest in this work.

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Figures

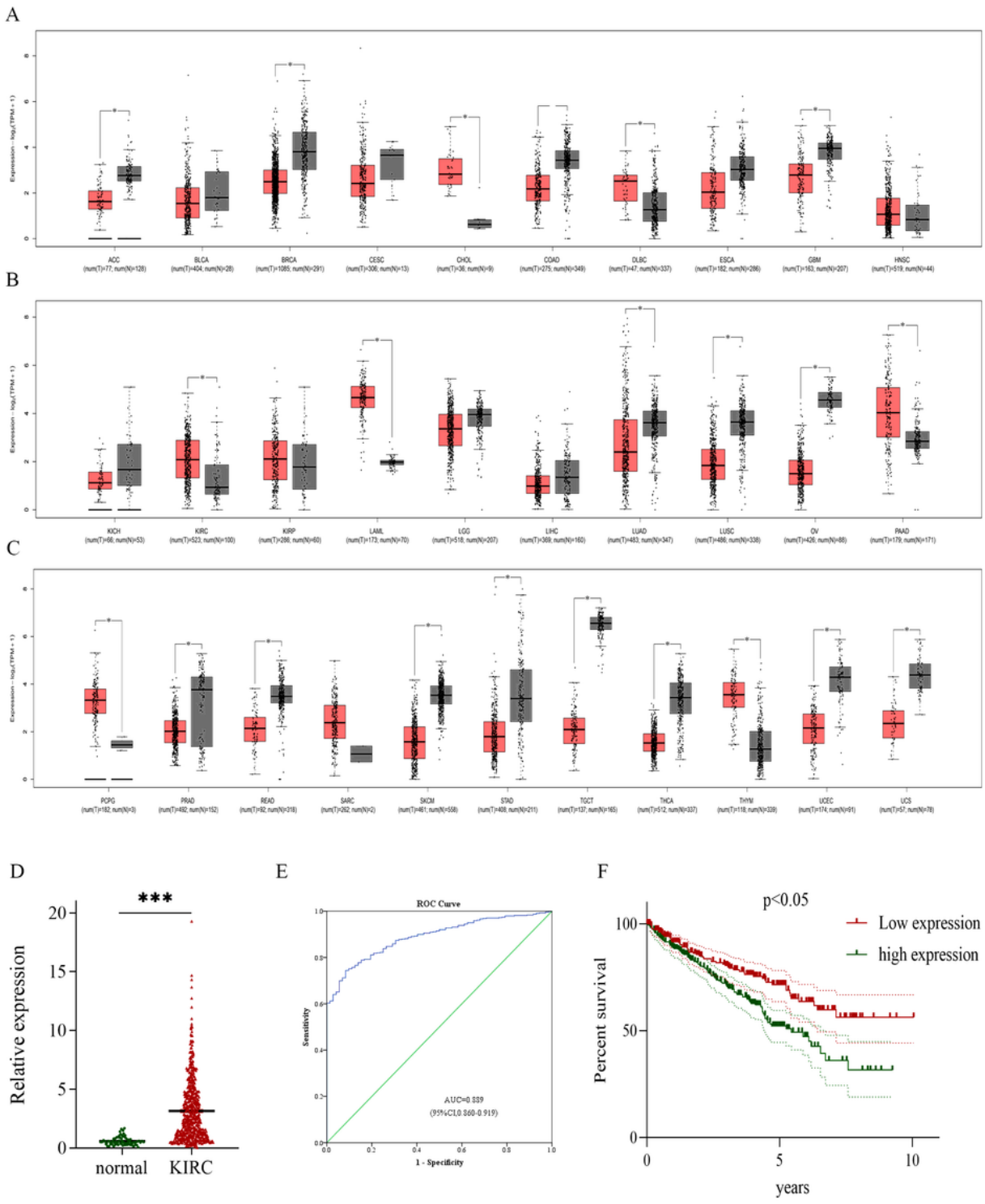

Figure 1

Expression data and clinical information of linc00342 in various cancers (A-C) Expression data of linc00342 in different cancers based on GEPIA database. (D) Relative expression of linc00342 in KIRC tissues and normal tissues based on the TCGA database. Expression of linc00342 was significantly upregulated in KIRC tissues compared to normal tissues. (E) Linc00342 has notable significance in prognosis of KIRC patients. (F) KIRC patients with high expression of linc00342 have poorer OS.

Abbreviations: TCGA, The Cancer Genome Atlas; KIRC, Kidney renal clear cell carcinoma; OS, overall survival

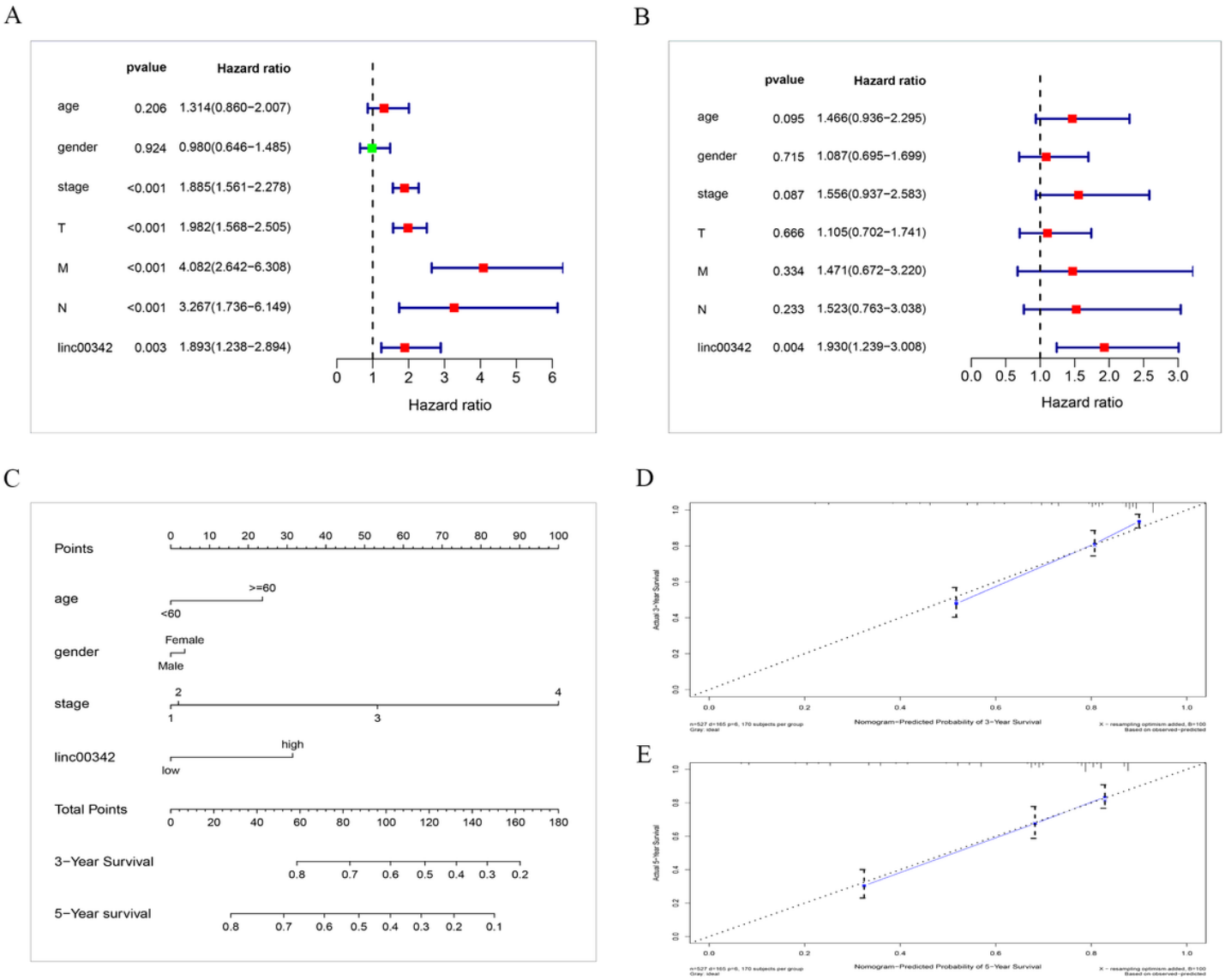

Figure 2

Construction of the nomogram prognostic model (A) Univariate analysis results suggested linc00342 gene expression and clinical stage (TMN) might be independent factors for KIRC prognosis (P < 0.001). (B) Multivariate analysis illustrated linc00342 expression level was an independent factor for KIRC prognosis (P < 0.001). (C) Nomogram prediction model results suggested KIRC patients with higher linc00342 expression had poorer 3-year and 5-year overall survival (OS). (D, E) Validation curve suggests the predicted probabilities were consistent with the observed proportions. Abbreviations: KIRC, Kidney renal clear cell carcinoma

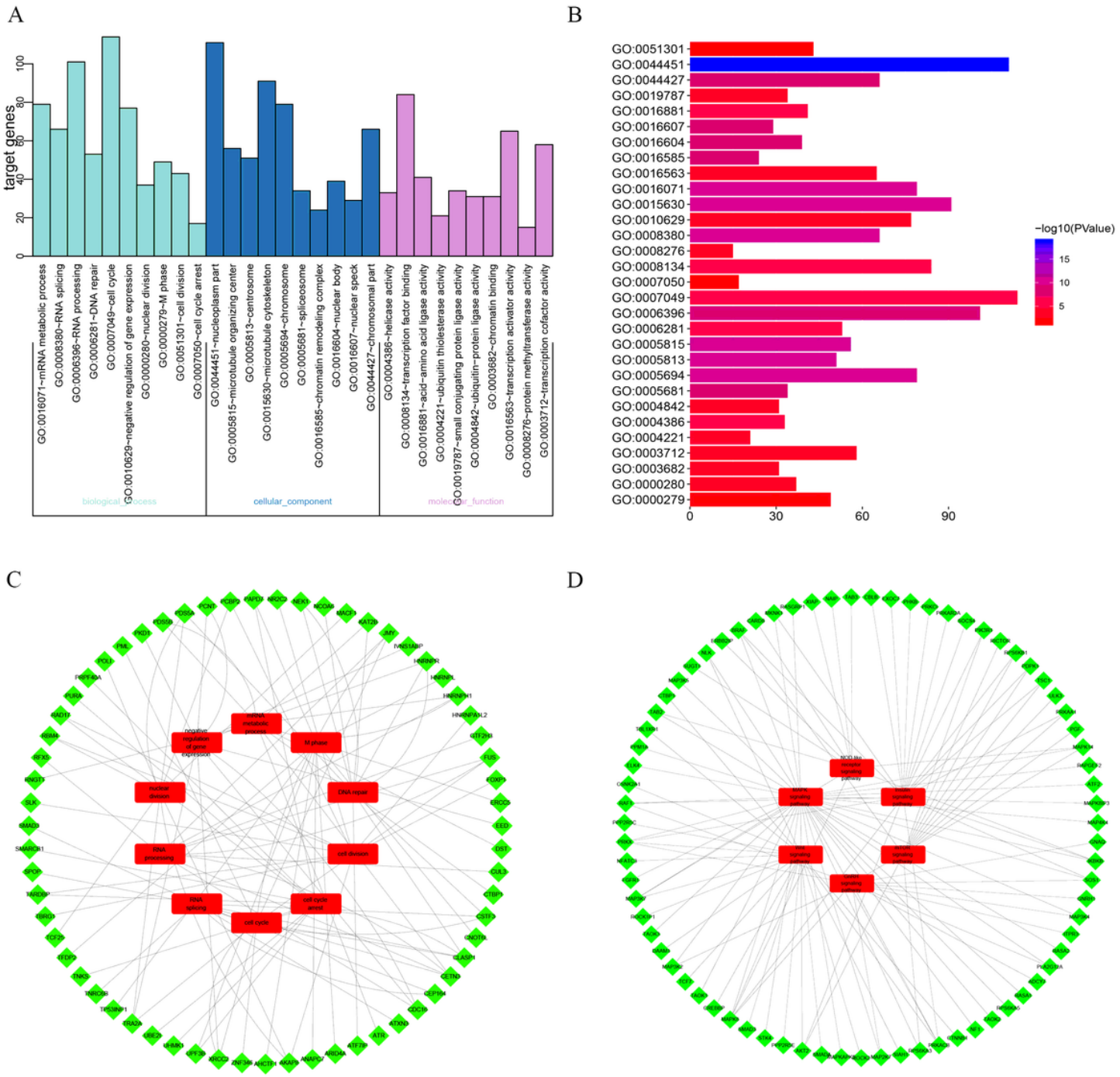

Figure 3

GO annotation and KEGG pathway enrichment analysis (A, B) Enriched GO terms and their target genes and P Values are visualized using R packages “ggplot2 and GOplot”. (C, D) The relationship between GO functions, KEGG pathways and related genes were shown by using Cytoscape respectively. Abbreviations: GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes

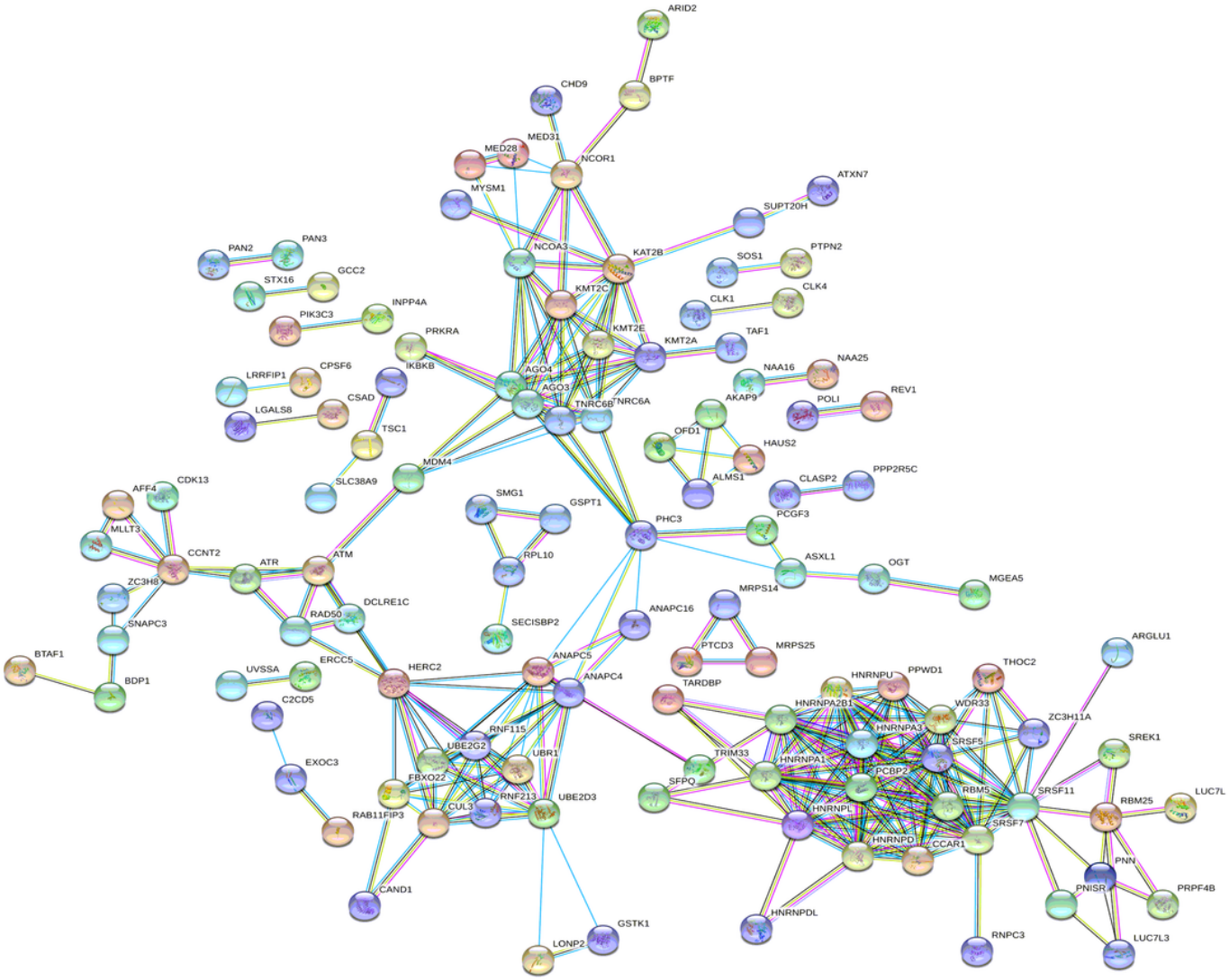

Figure 4

PPI network of linc00342 related genes Abbreviations: PPI, protein-protein interaction

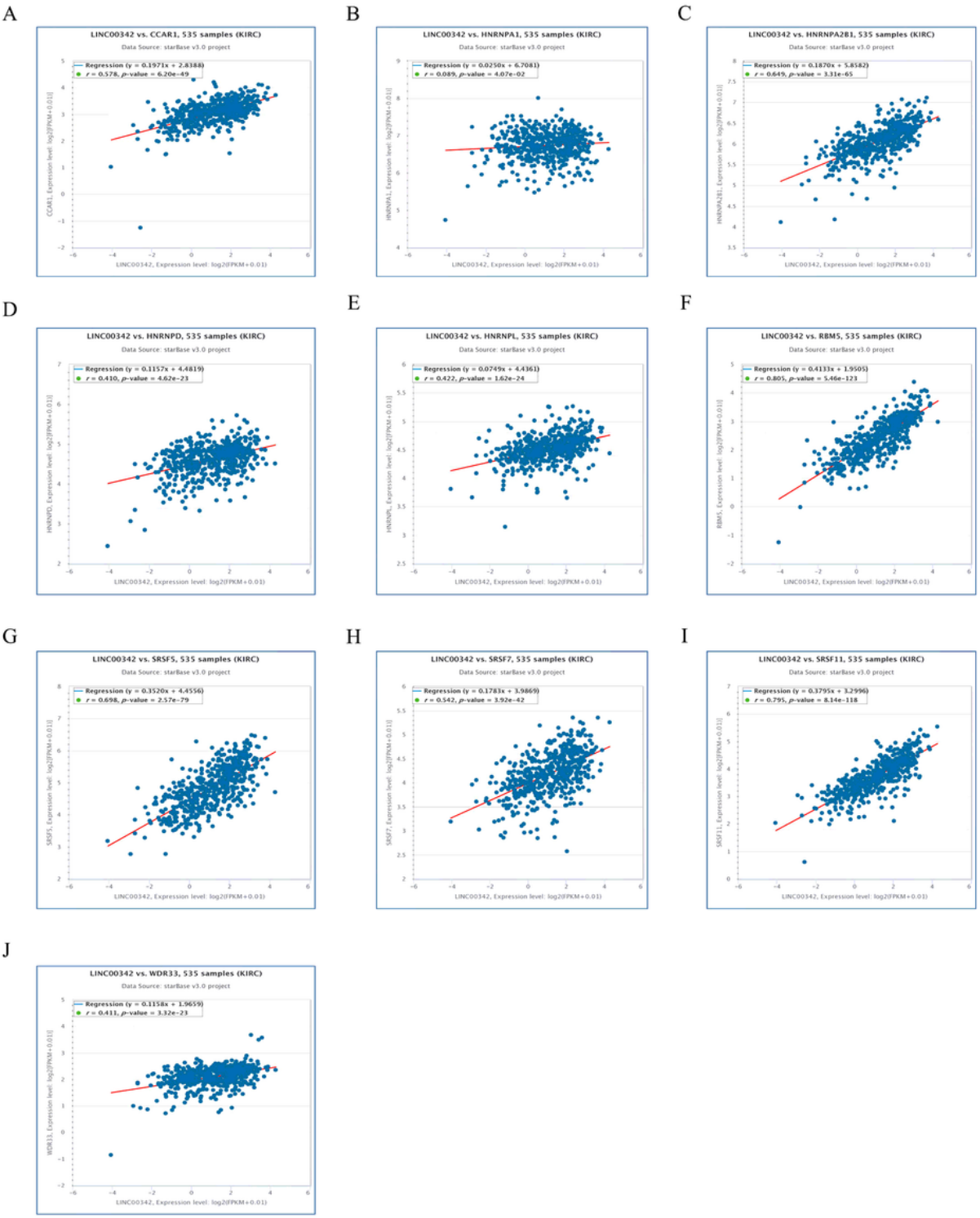

Figure 5

Pearson's correlation analysis based on the Starbase database between linc00342 and hub genes There is great significance in the relationships between linc00342 and its related genes. (A) CCAR1; (B) HNRNPA1; (C) HNRNPA2B1; (D) HNRNPD; (E) HNRNPL; (F) RBM5; (G) SRSF5; (H) SRSF7; (I) SRSF11; (J) WDR33.

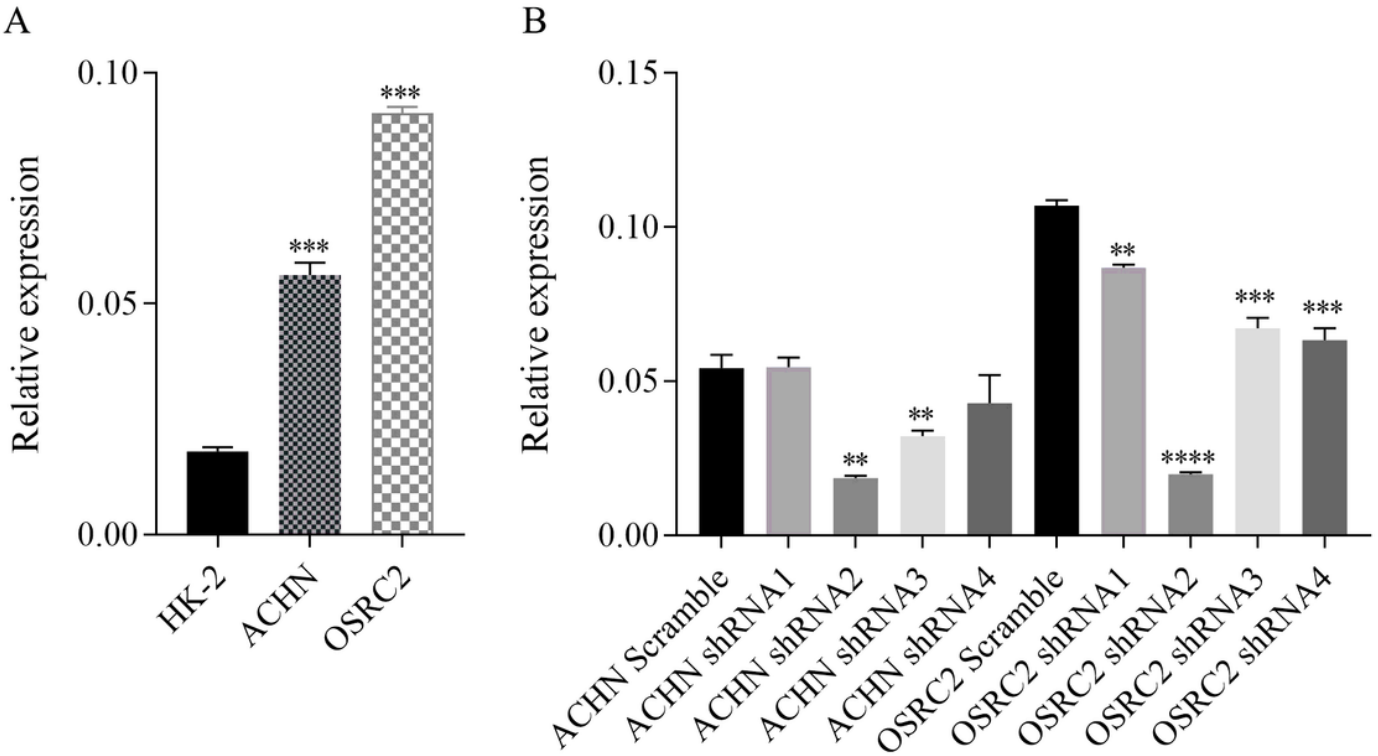

Figure 6

High expression of linc00342 in KIRC cells RT-qPCR was used to detect expression of linc00342 in KIRC cells. (A) Linc00342 was highly expressed in OSRC2 and ACHN KIRC cells compared with normal kidney cells. (B) shRNA#2 had the highest knockdown efficiency. Abbreviations: KIRC, Kidney renal clear cell carcinoma; RT-qPCR, real-time quantitative polymerase chain reaction

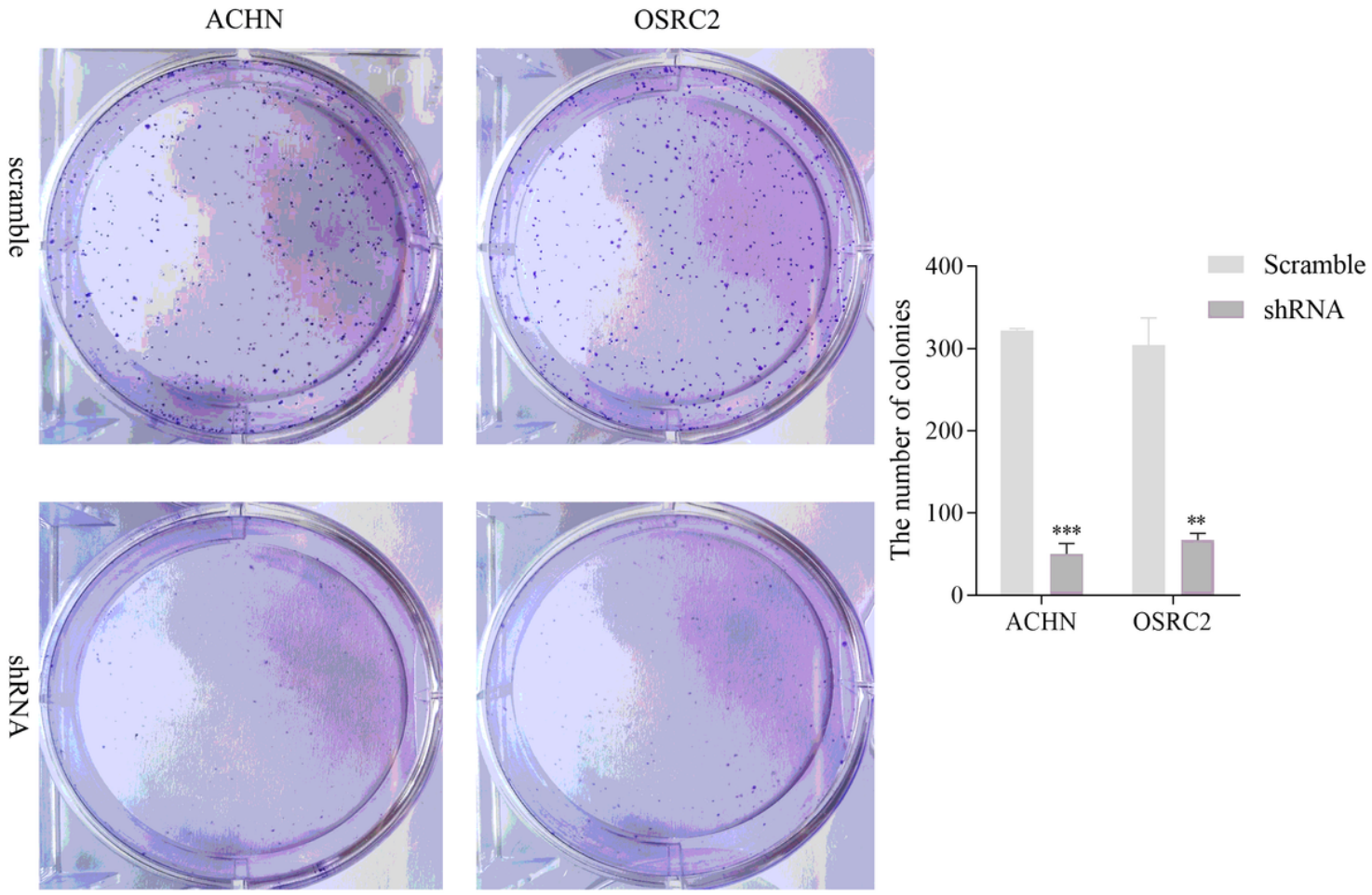

Figure 7

The effect of linc00342 on KIRC cell proliferation Cell proliferation in OSRC2 and ACHN KIRC cells were analyzed by colony-formation assays. The results indicated that downregulation of linc00342 could inhibit the proliferative properties of KIRC cells. Abbreviations: KIRC, Kidney renal clear cell carcinoma

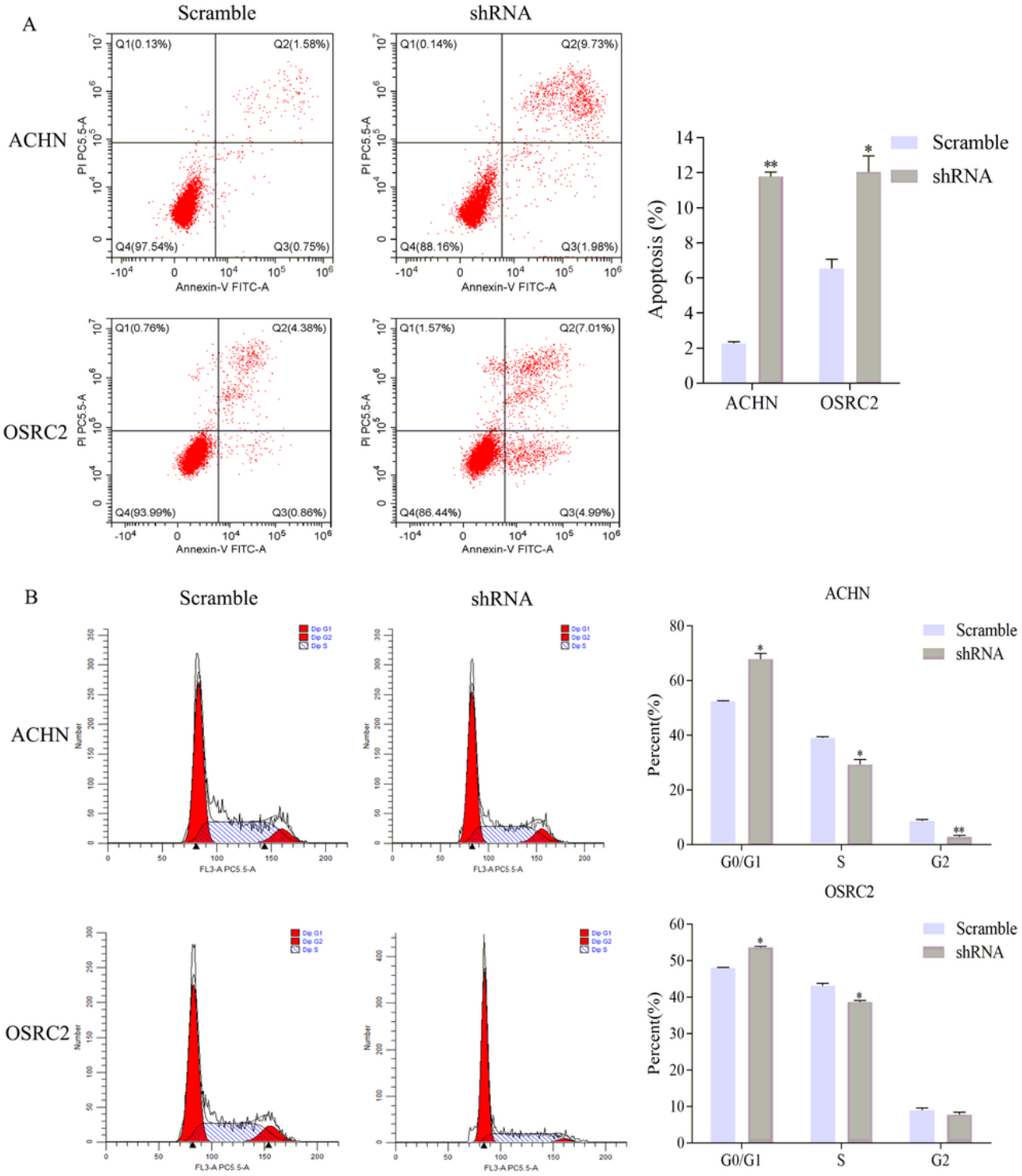

Figure 8

The impact of linc00342 on KIRC cell cycle Flow cytometry analysis was used to appraise the effect of linc00342 on KIRC cell cycle. (A) Silencing linc00342 accelerates cell apoptosis compared with linc00342-scramble-KIRC cells. (B) linc00342-silencing KIRC cells were arrested in G0/G1. Abbreviations: KIRC, Kidney renal clear cell carcinoma

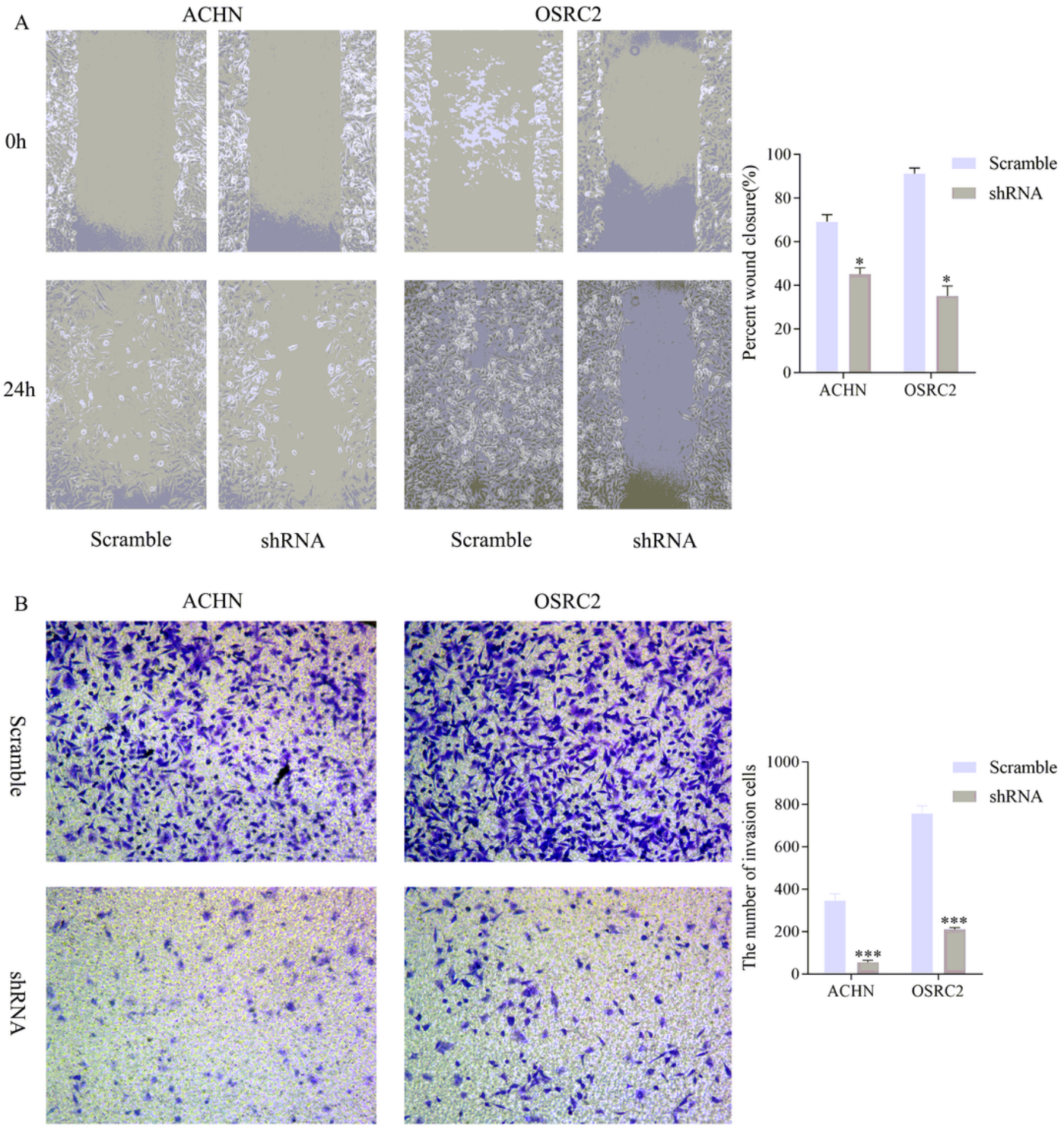

Figure 9

The effect of linc00342 on KIRC migration and invasion Wound-healing and Transwell assays were used to analyze the effect of linc00342 on KIRC migration and invasion, respectively. (A) Linc00342 can promote migration of OSRC2 and ACHN KIRC cell lines. (B)Linc00342 has properties that promote invasion in KIRC cells. Abbreviations: KIRC, Kidney renal clear cell carcinoma