

Cell cycle arrest and senescence in P53-wild type renal carcinoma by enhancer RNA - P53-bound enhancer regions 2(p53BER2) through p53-dependent pathway

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Research

Keywords: Enhancer RNA, p53BER2, TP53, renal cell cancer, nutlin-3

Posted Date: April 16th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-22612/v1>

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Abstract

Background

P53 is a classic tumor suppressor, but its role in kidney cancer remains unclear. In our study, we tried to explain the role of p53 in kidney cancer through p53 enhancer RNA-related pathway.

Methods

qPCR and luciferase reporters were used to detect the expression of p53-related enhancer RNA. Nutlin3 and artificial "microRNA" were used to induce and inhibit the expression of p53 enhancer RNA, respectively. Cycle analysis and β -galactosidase assay were used to explore whether P53-bound enhancer regions 2(p53BER2) plays a role in the cell cycle and senescence response of p53-wild type (WT) renal cancer cells. The function of p53BER2 was further analyzed in vivo by nude mice. RNA sequencing was used to identify the potential target of p53BER2.

Results

The results showed that P53BER2 expression was down-regulated in renal cancer tissues and cell lines and could specifically express in p53-WT renal cancer cell lines. Knockdown p53BER2 could reverse nutlin-3-induced cytotoxic effect in p53-WT cell lines. Further, downregulation of p53BER2 could reverse nutlin-3-induced G1 arrest and senescence in p53-WT cell lines. What is more, knockdown of p53BER2 showed a resistance to nutlin-3 treatment in Vivo. Additionally, we found BRCA2 could be regulated by p53BER2 in vitro and vivo, which suggested BRCA2 might mediate the function of p53BER2 in RCC.

Conclusions

The p53-associated enhancer RNA-p53BER2 mediates the cell cycle and senescence of p53 in p53-WT renal cancer cells. This further provides a novel approach and insight for the RCC and p53 research in renal cancer.

Background

Kidney cancer is one of the most common tumors of the urinary system¹. According to GLOBAN guideline 2018, there were 403,262 new renal cancer cases and 175,098 death cases for renal cancer worldwide in 2018¹. In U.S., corresponding number of above cases in 2014 were 63,290 and 13.860². In past decade, the incidence of kidney cancer has been steadily rising. For that, the reasons deep within it need to be further explored except for improvements in cancer screening and diagnostic technologies. Additionally, chemotherapy and radiotherapy usually function well in epithelial cancers, but it seems relatively

resistant in renal cancer³. Interestingly, one of most famous pathways that causing therapy resistance to epithelial cancers is inactivation of p53 signaling pathway⁴.

The p53 gene is the most common mutant gene in human cancers, which mutations occur in more than 50% of tumors. In the case of tumors carrying the wild-type p53 gene, changes in other p53 pathway components are believed to be responsible for its inactivation⁵. Tp53, encoded by p53 gene, is a transcription factor that can regulated expression of multiple target gene to mediate the diverse cellular responses, such as apoptosis, cell cycle arrest and DNA repair⁶. Although p53 plays a very important in tumor, its' function in renal cancer seems a little controversial. Some studies suggested that p53 expression may be up-regulated in renal cancer and related to poor outcome^{7,8}. But others found that re-activating p53 could repress the proliferation of renal cancer cell^{9,10}. The accurate function of p53 in renal cancer need to be further explored.

Enhancer RNAs (eRNAs) is a kind of novel long non-coding RNA, which is encoded by some enhancer sites¹¹. Under some special situation, RNA polymerase II (RNAPII) will bind to specific enhancer sites to produce eRNAs¹². It was believed that the expression of eRNAs related with the mRNA level of surrounding protein-coding genes¹³. What is more, recent studies have revealed that eRNAs play an important role in cancers^{14,15}. For instance, 17 β -oestradiol (E2)- bound oestrogen receptor α (ER- α) related eRNAs could induce the expression of target coding genes by increasing the binding of specific enhancer-promoter looping with ER- α .¹⁴ Recently, it was found that p53 could regulate the noncoding genomic regions to produce eRNAs as well¹⁶. P53-bound enhancer regions (p53BERs) produce eRNAs, which are required for efficient transcriptional of target genes to exert p53's function¹⁷. Since the function of p53 in renal cancer cannot be fully explained from protein's perspective¹⁸, we aimed to figure out whether eRNAs play an important role in p53-pathway in renal cancer.

In this study, we included some p53-related eRNAs and tested it in our clinical renal cancer sample. We demonstrated that p53BER2 is extremely low in renal cancer compared with normal kidney tissue. Further, we show the p53BER2 is a functional eRNA involved in regulation of cell cycle and senescence in renal cancer cell. Therefore, our results suggested that p53BER2 mediates the cell cycle and senescence of p53 in p53-WT renal cancer cells. This further provides a new research approach for the molecular mechanism of renal cancer.

Materials And Methods

Patients and clinical renal carcinoma samples collections

A total of 84 fresh kidney cancer samples and pair-matched normal adjacent renal tissue were collected in Peking university first hospital from 2015 to 2019. All samples were stored in RNA-later Stabilization Solution (Invitrogen, US) before extraction and were transferred to liquid nitrogen immediately. The main feature of included patients was shown in Supplementary table 1. All the patients have signed informed

consent after understanding of the process consequences of the study and the project was approved by the Medical Ethics Committee of Peking University First Hospital (Beijing, China).

Cell lines and transfection and infection

RCC cell lines Caki-1, 786-O, ACHN, OSRC-2 and the normal tubular epithelial cell line HK-2 was purchased from the American Type Culture Collection (ATCC, Manassas, VA). HK-2 cells were cultured in DMEM/F12 medium containing 10% fetal bovine serum (HyClone Laboratories Inc., Logan, UT), 786-O and OSRC-2 were cultured in RPMI-1640 (HyClone, Logan, UT) medium. Plus add 10% GibcoTM FBS (Life Technologies, Grand Island, NY). caki-1 and ACHN were cultured in DMEM (HyClone, Logan, UT) medium plus 10% GibcoTM FBS (Life Technologies, Grand Island, NY). All cells were incubated at 37 ° C in a standard humidified incubator containing 5% CO₂ and 95% O₂. The MDM2 inhibitor nutlin-3 (Selleck S1061, US) was added according to different cell and experimental conditions.

All the vector and siRNA were bought from Beijing Syngentech Co.,Ltd. For transfection, RCC cell lines were seeded on 6-well plates at 3 × 10⁵ cells/well (approximately 90–95% confluency). Cells were transfected the following day with Lipofectamine 3000 transfection reagent (L3000008, Thermo Fisher Scientific). Cells were harvested 48 hours after transfection. For construction of stable cell lines, 293T packaging cell lines were used for lentiviral amplification. Lentiviral infection was carried out as previously described¹⁹. Briefly, viruses were collected at 48 h and 72 h post-transfection. After passing through 0.45-µm filters, viruses were used to infect target cells in the presence of 8 µg/mL polybrene. Subsequently, target cell lines underwent appropriate antibiotic selection.

RNA isolation and quantitative real time polymerase chain reaction (qRT-PCR)

Extraction of total RNA from cells using TRIzol reagent (Invitrogen, USA) according to manufacturer's instruction. Reverse transcription total RNA was performed using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA). RT-qPCR was performed using a standard SYBR Green PCR kit (Thermo Fisher Scientific, USA) on an ABI 7500 PCR System (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Relative expression levels were calculated using the 2- $\Delta\Delta C_t$ method. The transcription level of GAPDH serves as an internal reference. Supplementary Table 2 lists all siRNA, artificial microRNA vector and specific primer sequences.

Western blotting and Immunofluorescence

The method of extracting total protein from cultured cells and specimens was to use RIPA buffer (Applygen, China) according to the manufacturer's instructions. SDS-PAGE and Western blot were performed according to standard protocols. Immunoreactive bands were visualized by Pierce Fast Western Blot Kit (Thermo Fisher, USA) using a SYNGENE G: BOX imaging system (Frederick).

For immunofluorescence, we seeded stable RCC cell in 24-well plates. 4% formaldehyde were used to fix the cells (approximately 70–90% confluency) for 15 min at room temperature. PBS wash 3 times for

5 min each. Block specimen in Blocking Buffer for 60 min. Aspirate blocking solution, apply diluted primary antibody. Incubate overnight at 4 °C. Rinse three times in PBS for 5 min each. Incubate specimen in fluorochrome-conjugated secondary antibody diluted in Antibody Dilution Buffer for 1–2 hour at room temperature in the dark. Rinse three times in PBS for 5 min each. Coverslip slides with DAPI for 5 min. The images were captured by Confocal microscope (Leica, Heidelberg, Germany). All of above solution were included in Immunofluorescence Application Solutions Kit (Cell signaling technology, #12727, USA)

The antibody conjugated to the primary antibody and the secondary HRP is described in Supplementary table 2.

Dual- luciferase reporter assay

To directly figure out the interaction between p53 and p53BER2, a dual-luciferase reporter assay was performed using p53BER2 constructs (Fig. 2C). The p53BER2 reporter contained copies of p53BER2-binding sites, minimal promoter and dual-reporter vector (Beijing Syngentech CO., Ltd, China). cDNAs of the binding sites of p53BER2 are shown in supplementary table 2. Cells were seeded into six-well plates (5×10^5 per well) and transfected with p53BER2 reporter vectors. Luciferase activity was measured using the dual luciferase assay system (Promega, Madison, WI, USA) as per the manufacturer's instructions at 48 hours after transfection. Firefly luciferase activities were normalized to Renilla luciferase activities. All assays were performed in duplicate, and all data shown are representative of at least two independent experiments.

RNA florescent in situ hybridization

FISH assay was performed using Ribo™ Fluorescent in Situ Hybridization Kit (Ribobio Company, China). P53BER2, 18S and U6 probes were designed and synthesized by Ribobio Company and labeled with Cy3 fluorescent dye. RNA FISH were performed using fluorescent in situ hybridization kit (RiboBio) following the manufacturer's instructions. Fluorescence detection was performed with a confocal laser-scanning microscope (Leica, Heidelberg, Germany). 18S and U6 were used as cytoplasm and nuclear reference.

In vitro cell proliferation assay

Cell proliferation was determined using Cell Counting Kit-8 (TransGen Biotech, China) according to the manufacturer's instructions. Briefly, 5×10^3 cells/well were seeded in a 96-well flat-bottomed plate, and grown at 37 °C for 24 h, then transfected with corresponding vector, or incubated with nutlin-3. Finally, the absorbance was finally determined at a wavelength of 450 nm using a microplate reader (Bio-Rad, Hercules, CA, USA). Experiments were repeated at least three times.

Cell proliferation was also determined by Ethynyl-2- deoxyuridine incorporation assay using an EdU Apollo DNA in vitro kit (RIBOBIO, Guangzhou, China) following the manufacturer's instructions. Briefly, after transfected with corresponding vector cells were incubated with 100 µl of 50 µM EdU per well for 2 h at 37 °C, respectively. Finally, the cells were visualized under a fluorescence microscopy. Experiments were repeated at least three times.

In vivo tumor growth

RCC cell-derived xenograft (CDX) models were established as previously described²⁰. Briefly, 5×10^6 stable RCC cells were injected into the axilla of 5-weeks BALB/c nude mice (Vital River Laboratory Animal Technology Co., Ltd.). When the length of tumor size reached over 2 mm, the volume size would be collected twice per week. When tumors reached a mass of approximately 50 mm³, mice were treated with Nutlin3 (20 mg/kg) every two days. Tumor samples were resected when the length of tumor size reach 2 cm.

Flow cytometry

Cell apoptosis was assayed by staining with Annexin V-APC and PI (KeyGEN BioTECH) following manufacturer's instructions and detected by a flow cytometer (FACSCalibur, Becton Dickinson, New Jersey, USA).

Cell cycle assay

For the cell cycle assay, DATS (Diallyl trisulfide)-treated cells were harvested, washed twice in phosphate-buffered saline (PBS) and fixed in 75% cold alcohol overnight at 4 °C. After washing in cold PBS three times, cells were incubated with 1 × PI/RNase staining buffer for 15 min in the dark at room temperature. Samples were then analyzed for their DNA content using flow cytometry. All assays were performed in duplicate, and all data shown are representative of at least two independent experiments.

Senescence β-Galactosidase Staining assay

Cells after 72 hr of culture in each group were seeded into a six-well plate, adjusted to a cell density of ~50% using RPMI 1640 medium. After cell attachment, the culture medium was removed and the cells were washed once with PBS. In accordance with the instructions of senescence β-galactosidase Staining kit (Beyotime, China), SA-β-gal (1 mL) was added into cells for incubation overnight at 37 °C. Positive cells presented blue. Five fields were randomly selected and observed under an optical microscope to calculate the number of positive cells.

High- throughput cDNA sequencing (RNA-Seq)

Total RNA was extracted from two RCC cell lines (OSRC2 and ACHN) treated with recombinant lentivirus overexpressing amicroRNA-p53BER2 or a negative control and quantitated using a NanoDrop-1000 spectrophotometer, and the integrity was subsequently assessed with an Agilent 2100Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). RNA-sequencing was performed on an Illumina HiseqX10. Experiments were repeated three times. For detection of differentially expressed genes (DEGs), a fold change ≥ 2 or $\leq 1/2$ and a false discovery rate (FDR) < 0.01 were set as the screening criteria. P-values ≤ 0.05 were considered statistically significant. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis and Gene ontology pathway analysis was used to clarify the physiological functions and signaling pathways related to the DEGs in RCC cell lines.

Statistical analysis

Each experiment was performed in triplicate. All quantitative data are expressed as mean \pm standard deviation (SD). Statistical analyses were performed using SPSS8.0 software (IBM Corp., Armonk, NY, USA). Statistical significance was tested by Student's t-test, Chi square test or ANOVA. $P < 0.05$ was considered to be statistically significant.

Results

P53BER2 expression is down-regulated in renal cancer tissues and cell lines

As previously reported, we selected p53-associated enhancer RNA (p53BER2 and p53BER4) for our study. First, we examined the expression of p53BERs in seven renal cell carcinoma cell lines and the renal epithelial cell line (HK2), and found that the expression level of p53BER2 was down-regulated in all renal cancer cell lines, relative to the normal renal epithelial cell line (HK2) (Fig. 1A). However, there was no significant difference in p53BER2 levels between renal cell carcinoma and normal renal epithelial cell lines (Fig. 1A). Further, we examined the expression level of p53BER2 in 84 paired kidney cancer tissues and found that the expression level of p53BER2 in kidney cancer was much lower than that in normal kidney tissues (Fig. 1C). Among them, p53BER2 was expressed in 79% (67/84) of renal cancer tissues relative to normal tissues (Fig. 1B). More importantly, we found that p53BER2 predicts a higher tissue grade ($p = 0.007$) in 83 patients (1 with loss of pathology information), accompanied by a trend toward higher-level T staging (Table 1). Detailed patient clinical information and related analysis can be found in Table 1 and supplementary table1.

Table 1

Association between p53BER2 expression level and clinical features of RCC patients.

Variable	Patients	P53BER2 (-ΔCT/median)		χ^2	P-value
		Low(n = 42)	High(n = 42)		
Age(years), mean \pm SD	54.73 \pm 12.24	57.05 \pm 12.18	52.48 \pm 11.86		
Age(years), n (%)				0.764	0.382
≥ 55	45	20	24		
< 55	39	22	18		
Gender, n (%)				0.223	0.637
Male	59	28	30		
Female	25	14	12		
T stage, n (%)				4.402	0.111
T1a	41	18	23		
T1b + T2	29	14	15		
T3	13	10	3		
Fuhrman grade, n (%)				9.973	0.007
G1	29	8	21		
G2	43	26	17		
G3 + G4	11	8	3		
RCC: renal cell carcinoma, CT: cycle threshold, Fuhrman nuclear grade.					

P53BER2 could express specifically in p53-WT renal cancer cell lines

Since p53BER2 is an enhancer RNA mediated by wild-type p53, we wonder whether p53BER2 is mediated in wild-type p53 in renal cancer cells. First, as shown in Fig. 1A, we could find that p53BER2 expression in p53 mutant cells (786-O) is the lowest, consistent with our conjecture. To further understand the relationship between p53BER2 and wild-type p53 protein, we used the p53 protein activator nutlin3a to treat p53 wild-type and p53 mutant kidney cell lines. Western Blot showed that nutlin3 was effective in inducing p53 expression (Fig. 2A (786 and ACHN30-20-15-0uM)). qPCR results indicated that nutlin3a can induce p53BER2 expression in p53 wild-type cells (OSRC-2, ACHN, CAKI-2), but does not induce p53 mutant cell expression (Fig. 2B). It has been reported in the literature that wild-type p53 can bind to

p53BER2 to enhance promoter expression. Using this principle, we designed the p53BER2 reporter to further investigate whether P53 initiates the promoter by direct binding to p53BER2. First, we combined the p53BER2-specific sequence with the minimal-promoter to form a "p53 promoter" that specifically recognizes the wild-type p53 protein. Further, we used the GAL4-UAS system to enhance the promoter efficiency and use dual luciferase as a dual luciferase. Report the gene and finally form the "p53BER2 reporter" (Fig. 2C). We transfected the "p53 reporter" and "control" reporters into the HK2, 293T and 786-O cell lines, respectively, and we found that the p53 reporter can effectively detect wild-type p53 expression (Fig. 2D). Further, we transferred the reporter into p53 wild-type cells treated with nutlin3, and found that p53 wild-type cells can express higher dual luciferase intensity with increasing nutlin3 concentration (Fig. 2E), suggesting that the "p53BER2 reporter" can be effectively. The expression of the p53 wild-type renal cancer cell line p53BER2 was reported and the results were consistent with the qPCR results. These results demonstrate that wild-type P53 can bind p53BER2 specific sequence to promote transcription of p53BER2, thus confirming our conjecture.

P53BER2 could mediate p53-related function in p53-WT renal cancer cell lines

Knockdown P53BER2 could reverse nutlin-3-induced cytotoxic effect in p53-WT cell lines

In order to explore whether p53BER2 affects p53-related functions. We constructed siRNA to knock down p53BER2, because it has been reported that siRNA knockdown of eRNAs is not efficient²¹, so we also added artificial microRNA for verification. Interestingly, we found that amicroRNA can effectively knock down p53BER2 expression), but siRNA could not knock down p53BER2 (Fig. 1D), so in the subsequent experiments we constructed the corresponding lentiviral vector using amicroRNA, and further we constructed the p53BER2 knockdown stable cell lines of OSRC-2 and ACHN-786-O by lentivirus, and The knockdown effect was examined (Fig. 1E) and it was found that amicroRNA can knock down p53BER2 in the presence of nutlin3 and in the absence of nutlin3. In order to examine the effect of p53BER2 on cell proliferation activity, cck8 assay first showed that nutlin3 can effectively inhibit the proliferation of p53 wild-type renal cell carcinoma cell line (Supplementary Fig. 1A). Interestingly, we found that knockdown of p53BER2 partially reverses nutlin3-mediated the cytotoxic activity (Fig. 3A), accompanied by an elevated IC50 value (Supplementary Fig. 1B). We used EDU assay to further validates this phenomenon in Fig. 3B, Fig. 3C. To explore why p53BER2 mediates changes in cell proliferation activity, we first hypothesized that p53BER2 affects proliferative activity by mediating cell apoptosis. Using PI/FITC flow cytometry, we found that nutlin3 can induce apoptosis in p53 wild-type renal cell carcinoma cells (Fig. 3D and Fig. 3E), but knocking down p53BER2 does not reverse nutlin3-mediated apoptotic response in ACHN (Fig. 3D) and OSRC-2 (Supplementary Fig. 2).

Knockdown p53BER2 could reverse nutlin-3-induced G1 arrest and senescence in p53-WT cell lines

Previous literature reports that p53 can mediate cell cycle responses²². Therefore, we further analyzed whether p53BER2 affects cell phenotype by mediating changes in cell cycle. Flow cytometry revealed that nutlin3 can cause the p53-WT renal cancer cell line G1-arrest, but has no effect on p53-MUT type renal cell carcinoma (Supplementary Fig. 3), which is consistent with previous research results. Interestingly, knockdown of p53BER2 reversed the nutlin3-mediated cell cycle arrest p53-WT renal cancer cell line (Fig. 4A). To confirm this finding, we first evaluated cellular entry into mitosis using phospho-H3 (ser10) staining. Cells stable expression amicroRNA targeting p53BER2 showed a significant decrease of phospho-H3 (ser10) compared with cells stable expressing NC vector after nutlin-3 treatment (Supplementary Fig. 4).

It is well known that p53 can also mediate cell senescence, so we further analyzed the effect of p53BER2 on cell senescence. We analyzed the senescence response of kidney cancer cell lines induced by nutlin3 by β -galactosidase assay. The results showed that nutlin3 can effectively induce senescence reaction in p53-WT renal cancer cells (Supplementary Fig. 5). At the same time, as we expected, knocking down p53BER2 effectively reversed the number of β -galactosidase-positive cells mediated by nutlin3, compared to the normal control group (Fig. 4B).

In conclusion, p53 can mediate the cell cycle and senescence response of p53-WT renal cancer cell line through p53BER2, thus affecting the development of renal cancer cell lines.

P53BER2 positively regulates BRCA2 expression

Since the subcellular localization of lncRNA is associated closely with its biological function. We performed RNA-fish experiment to find the location of P53ber2 in renal cancer. As expect, the results showed most of p53BER2 was localized in the nucleus (Fig. 5A). Furthermore, we performed RNA sequencing by using OSRC-2-KD, ACHN-KD and corresponding control cells. We found that many differential genes were enriched in mitosis and cell cycle-related pathways (Fig. 5B). Furthermore, we found that most of the genes enriched in cell cycle-related pathways showed a downregulated trend (Fig. 5C). Therefore, we hypothesized that p53BER2 affects the cell cycle by affecting cell cycle-related genes. We further overlapped the differential genes of the two cell lines and the differential genes related to the cell cycle pathway. This resulted in a list of 4 potential downstream genes (Fig. 5D). We verified the mRNA levels of several genes in cell lines and found that downregulating p53BER2 caused BRCA2 down-regulation (Fig. 5E), while others do not (Supplementary Fig. 6). According to previous studies, BRCA2 affects the cell-cycle process from G1 to S / G2 by affecting DNA repair. The loss of BRCA2 can lead to G1 arrest of cells. Therefore, the above results suggest that p53BER2 might mediate the G1 arrest of renal cancer cells through the BRCA2 pathway.

Downregulation of p53BER2 show a resistance to nutlin-3 treatment in cell-derived xenografts (CDX)

To understand the function of p53BER2 in vivo, the CDX model was established to compare the tumorigenic ability of the p53BER2 knockdown cell line and the control knockdown cell line. As shown in Fig. 6A, we can find that OSRC-2, which stably knocks down p53BER2, has stronger tumorigenic ability than OSRC-2 of the control group. The P53BER2 knockdown group had a faster tumorigenic capacity relative to the control group (Fig. 6B). At the same time, the tumor weight of the p53BER2 knockdown group was slightly higher than that of the control group ($P < 0.05$) (Fig. 6C). Further, we injected intraperitoneal injection of p53BER2-KD group and NC group with nutlin-3, and the results showed that the p53BER2 knockdown group was small. Xenograft formed by RCC infected with p53BER2-KD were significantly resistant to nutlin-3 mediated anti-tumor effect compared to control mice (Fig. 6D), with faster growth rates (Fig. 6E) and larger tumors (Fig. 6F). Moreover, BRCA2 expression in mice subcutaneous xenograft showed a consistence with the expression with p53BER2 (Fig. 6G), which suggested BRCA2 could also be regulated by p53BER2 in vivo.

Discussion

In this study, we found that p53BER2 expression was down-regulated in renal cancer tissues and cell lines and could specifically express in p53-WT renal cancer cell lines. Knockdown p53BER2 could reverse nutlin-3-induced cytotoxic effect in p53-WT cell lines. Further, downregulation of p53BER2 could reverse nutlin-3-induced G1 arrest and senescence in p53-WT cell lines. What is more, knockdown of p53BER2 showed a resistance to nutlin-3 treatment in vivo. Additionally, we found BRCA2 could be regulated by p53BER2 in vitro and vivo, which suggested BRCA2 might mediate the function of p53BER2 in RCC. Therefore, our study revealed that involvement of eRNAs in p53 pathway could also function in RCC, not just byproducts of gene expression.

P53 research has caught much attention in oncology, including kidney cancer. Although VHL is the most important mutation in kidney cancer, p53 still plays an important role in the development of kidney cancer²³. Some studies indicate that p53 is highly expressed in kidney cancer tissues, indicating that p53 may play a cancer-promoting effect in kidney cancer^{7,24}. However, through the MDM2 inhibitor method, studies have found that p53 activation can effectively inhibit the proliferation of renal cancer cell lines^{9,25}. This suggests that p53 also plays an important role in kidney cancer, and new related signaling pathways may need to be explored. Recently, studies have found that as a transcription factor, p53 can not only activate the transcription of coding genes, but also the transcription of non-coding genes^{17,26}. Among them, the most interesting is the application of eRNAs in p53-pathway. TP53 has been proven to target some enhancer sequences and induce the corresponding eRNAs expression, further activating the expression of adjacent genes¹⁷. More importantly, these eRNAs were proven to be functional in cell physiology. Therefore, we wanted to figure out whether p53-related eRNAs also play a role in kidney cancer. Our results indicate that p53BER2 expression is significantly down-regulated in renal cancer

clinical specimens. Further in vivo and in vitro experiments found that p53BER2 can effectively mediate p53's functions in the cell cycle and aging. The above shows that p53-related RNA can play a key role in the occurrence and development of renal cancer.

At present, there are literatures showing that p53-related enhancer RNA can effectively regulate the expression of downstream genes^{17,27}. In addition to being regulated by a chromosomal loop, p53-associated enhancer RNA can also be regulated by long non-coding RNA²⁷. In our research, by RNA sequencing, we found that knockdown of p53BER2 can cause down-regulation of cell cycle-related downstream genes. Among them, through experimental verification, we found that knocking down p53BER2 can repress BRCA2 expression level. Furthermore, this result was verified in nude mouse experiments. BRCA2 is a tumor suppressor, which plays important part in cell physiology by promoting DNA replication and DNA double-strand breaks (DSBs)²⁸. Studies show that the loss of BRCA2 can trigger a significant reduction in the replication fork process and affect DNA replication^{29,30}. At this time, in tumor cells, replication stress and DNA damage tolerance will allow the cells to survive the loss of BRCA2 and further stimulate the cell's tumorigenesis potential^{31,32}. Therefore, patients who carry BRCA2 germline mutations are much susceptible to breast cancer and ovarian cancers³³⁻³⁵. Consistent with these studies, we found a positive correlation between P53BER2 and BRCA2, suggesting that p53BER2 may mediate cell cycle-related phenotypic effects through BRCA2(Fig. 6H).

The highlight of our work was that we found p53ber2 can function in the RCC, which is the first time to discussion about eRNAs in RCC. Compared with traditional coding-gene related analysis, we paid attention to p53 relative eRNAs and tried to figure out its function in RCC, which partly explained the function of p53 in RCC. Additionally, MDM2 inhibitor- nutlin3 can effectively repress the proliferation of RCC in vitro and vivo and, therefore, might be able to serve as an anti-tumor reagent potentially in p53-WT RCC patient. However, there are a few limitations that need to be addressed. Firstly, although we found a correlation between p53BER2 and BRCA2, the specific mechanism between them still unknown, which require further explore. Secondly, the complete sequence of p53BER2 still cannot be captured, which might need some advanced technique to make it come true, such as global nuclear run-on sequencing (GRO-seq). At last, the incidence of p53 mutation in RCC is less than the other tumors, but p53BER2 still maintain a low expression level, which might be caused by some “wrong” p53 in RCC. The relationship between p53BER2 and “wrong” p53 would be interesting and worth to dig out. Even so, we still believe our study provided a novel approach in RCC and p53 related study.

Conclusion

Enhancer, under some conditions, could transcript some specific RNA, which can interact with other genes and regulate it. In our study, we showed that enhancer RNA- p53BER2 could partly mediated the function of p53 in p53-WT RCC, in vitro and vivo. Moreover, we found tumor suppressor- BRCA2 have a positive correlation with p53BER2, which needed to further discussion and explore. Although it is still unknown for

how p53BER2 works in RCC mechanically, our study found an interesting association in RCC and provided a novel way to study RCC and p53 research.

Declarations

Acknowledgements

The study was supported by the National Natural Science Foundation of China (No. 81572506 and 81872081), and the Fundamental Research Funds for the Central Universities (No. BMU2018JI002).

Authors' contributors

XHB, MKF, ZKN, LL and YWP conducted the specific experiments. XHB and MKF analyzed the clinical data. XHB and ZKN drafted the manuscript, ZJC and CL provided clinical expertise. GK, GYQ and CL designed and supervised the project, established the workflow of the analysis. All authors have read and agreed to the final version of the manuscript.

Funding

This study was funded by the National Natural Science Foundation of China (No. 81572506 and 81872081), and the Fundamental Research Funds for the Central Universities (No. BMU2018JI002).

Availability of data and materials

All data are fully available without restriction.

Competing interests

The authors declare that they have no competing interest.

Consent for publication

Not applicable

Ethics approval and consent to participate

The project was approved by the Medical Ethics Committee of Peking University First Hospital (Beijing, China). Informed consent was acquired from the subjects after understanding of the process and possible consequences of the study.

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Additional Files

Supplementary figure 1. Cell viability of RCC cell lines with nutlin3 treatment. A: The cell viability curves of RCC cell lines with nutlin3 treatment. B: IC50 value of different RCC cell lines infected p53BER2-KD

virus and vectors after nutlin-3 treatment.

Supplementary figure 2. The apoptotic rate of different RCC cell lines or stable cell lines after nutlin-3 treatment.

Supplementary figure 3. The proportion of cell cycle of different RCC cell after nutlin-3 treatment.

Supplementary figure 4. H3ser10 staining with immunofluorescence showed the process of cell cycle in different RCC cell lines or stable cell lines after nutlin-3 treatment.

Supplementary figure 5. The rate of senescence cell in different RCC cell lines after nutlin-3 treatment.

Supplementary figure 6. Expression level of potential target genes in ACHN cell lines infected p53BER2-KD or vectors lentivirus.

Supplementary table. The detailed information was shown in the file- “supplementary table”.

Figures

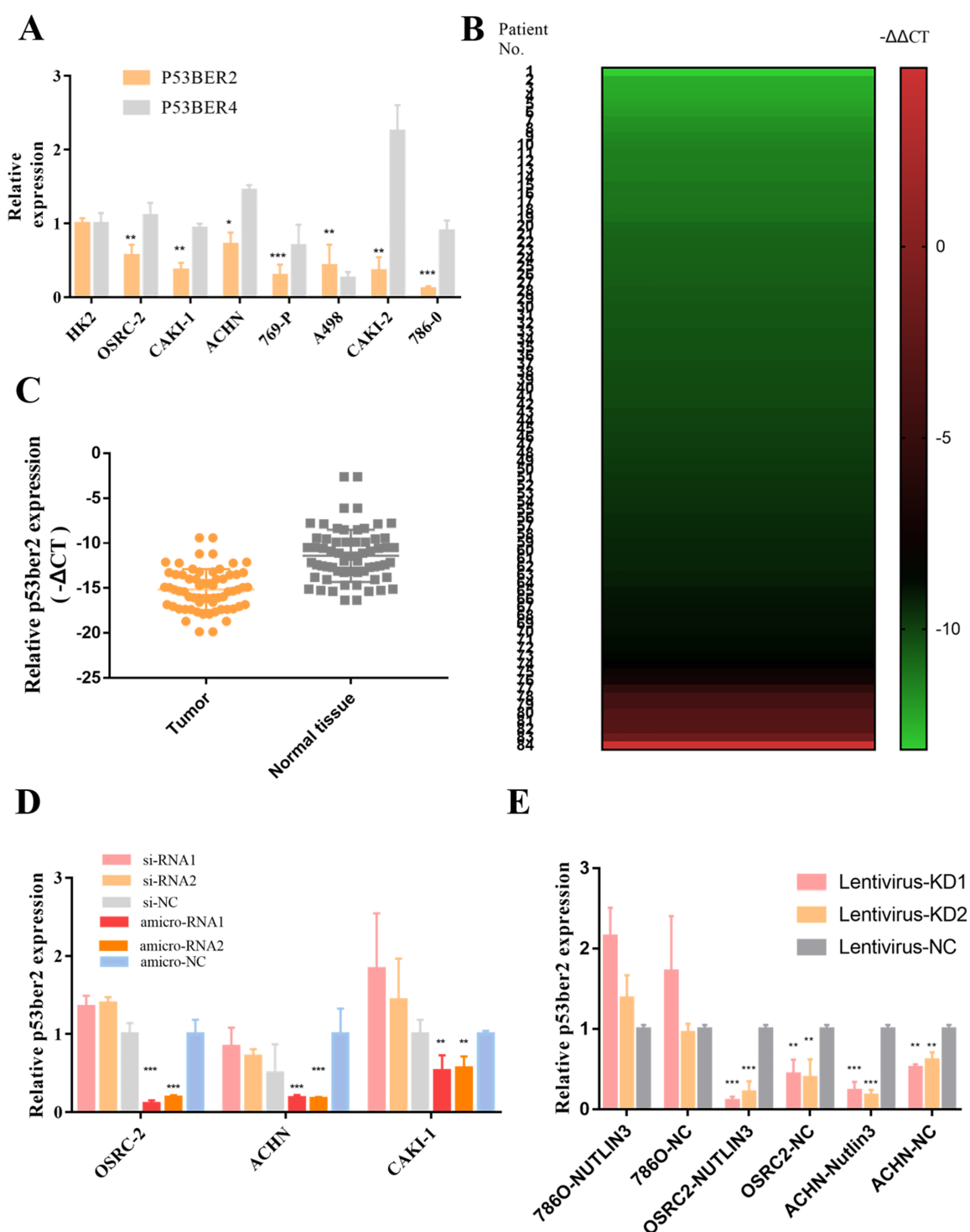


Figure 1

Clinical information and p53-related eRNAs expression level in RCC tumor samples and cell lines. A: P53-related eRNAs expression level in RCC cell lines. B: The heatmap about the expression level of p53BER2 in 84 RCC tumor samples. C: The expression of p53BER2 in RCC tumor samples and normal tissues. D: The relative p53BER2 expression level in different RCC cell lines after transfecting siRNA or amicroRNA. E: The relative p53BER2 expression level in different RCC cell lines after infecting lentivirus. Results are

shown as mean \pm SD. ***P < 0.001 compared with control. ** P < 0.01 compared with control. * P < 0.05 compared with control.

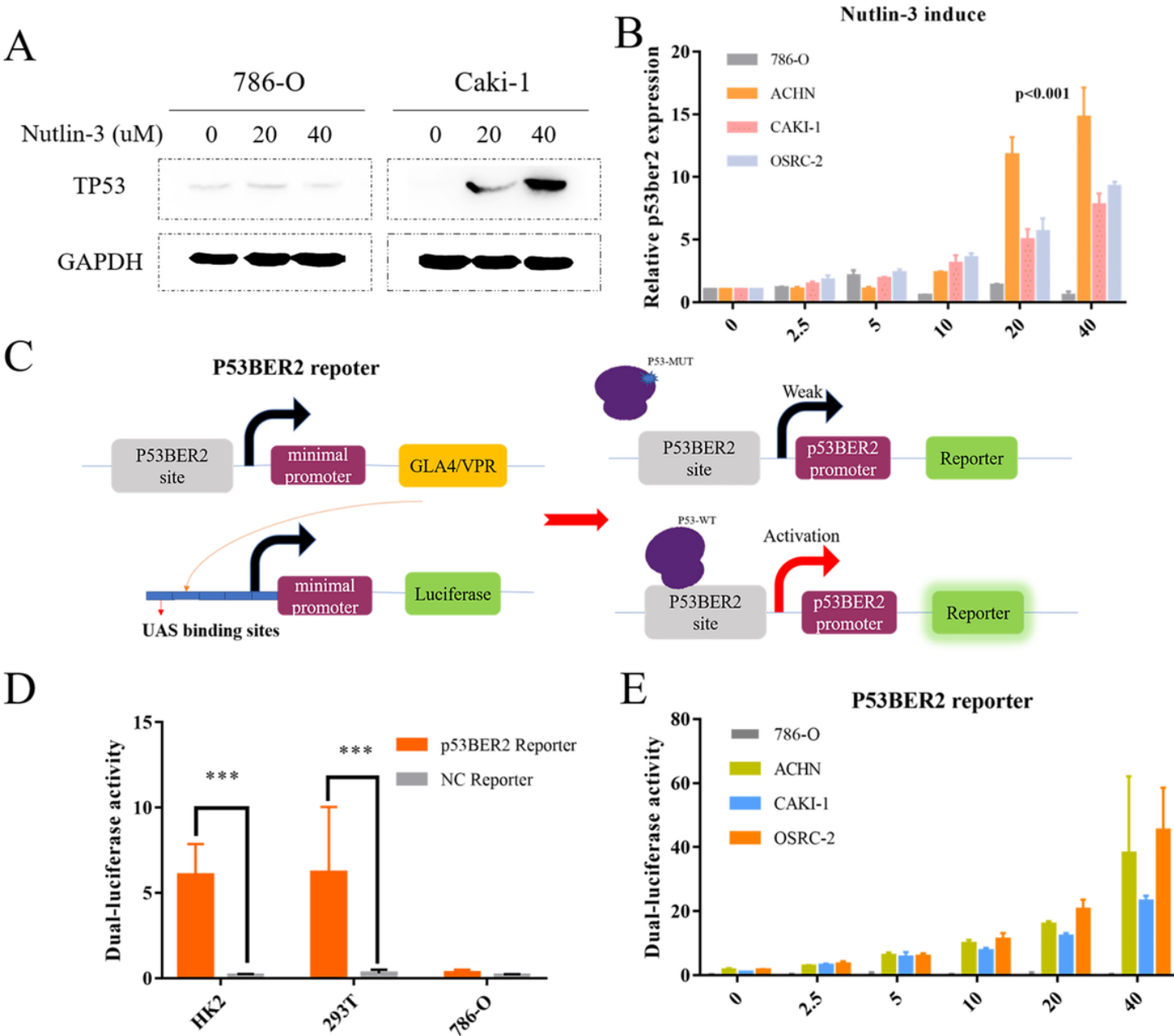


Figure 3

P53BER2 could be induced by MDM2 inhibitor- nutlin3 in p53-WT RCC cell lines. A: TP53 could be induced by nutlin3 in p53-WT RCC cell lines. B: P53BER2 could be induced by nutlin3 in p53-WT RCC cell lines. C: The design and construction of the p53BER2 reporter. D: The verification of p53BER2 Reporter in p53-WT and p53-MUT cell lines. E: The dual-luciferase assay showed WT-p53 could induce p53BER2 by binding the p53BER2 promoter. Results are shown as mean \pm SD. ***P < 0.001 compared with control. ** P < 0.01 compared with control. * P < 0.05 compared with control.

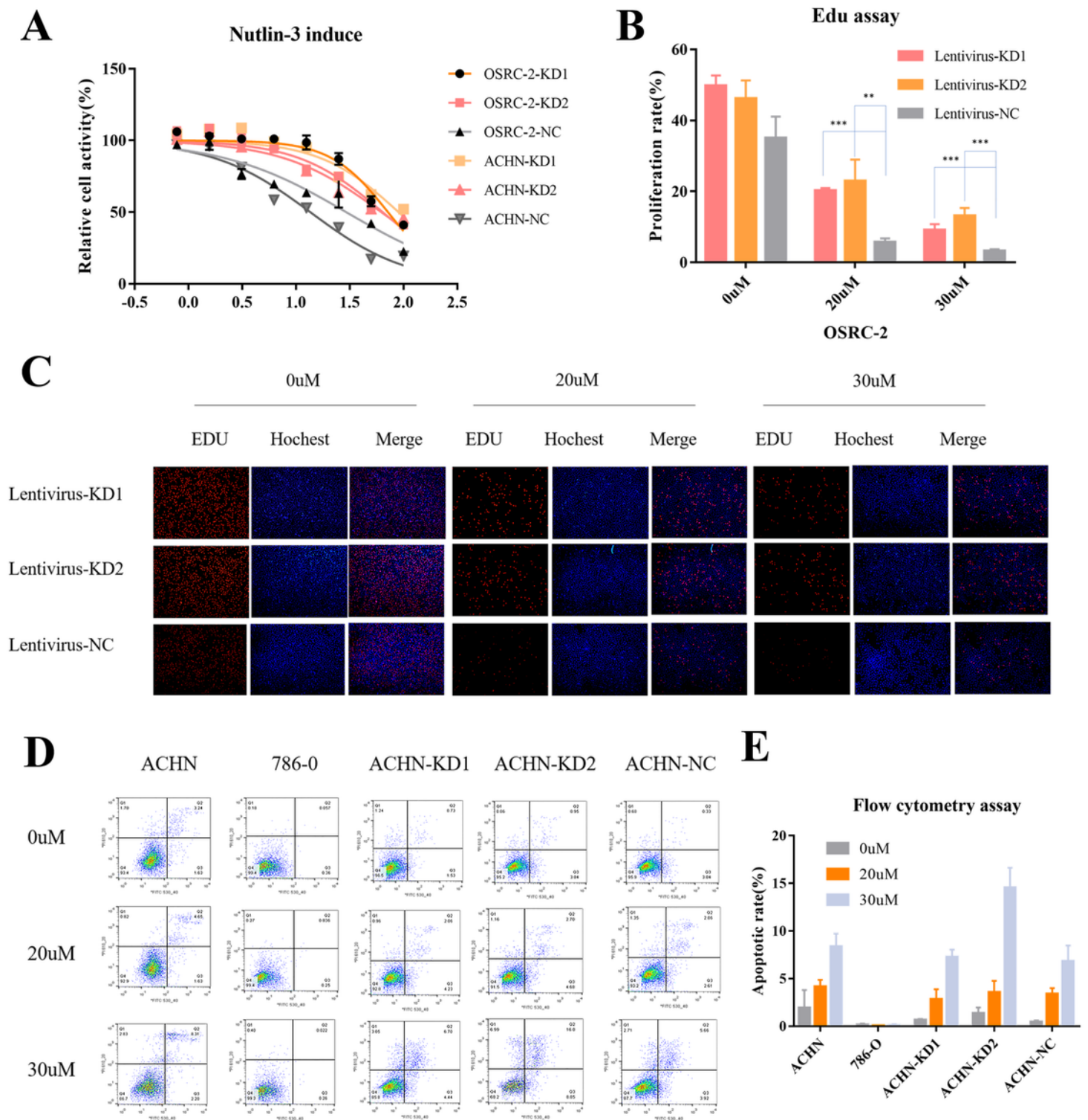


Figure 5

P53BER2 could mediate p53-related function in p53-WT renal cancer cell lines. A: The relative cell activity of different stable RCC cell lines after nutlin-3 treatment. B and C: The proliferation rate of OSRC-2 stable cell lines infected amicroRNA or vector after nutlin-3 treatment by EDU assay. D and E: The apoptotic rate of different RCC cell lines or stable cell lines after nulin-3 treatment. Results are shown as mean \pm SD.

***P < 0.001 compared with control. ** P < 0.01 compared with control. * P < 0.05 compared with control.

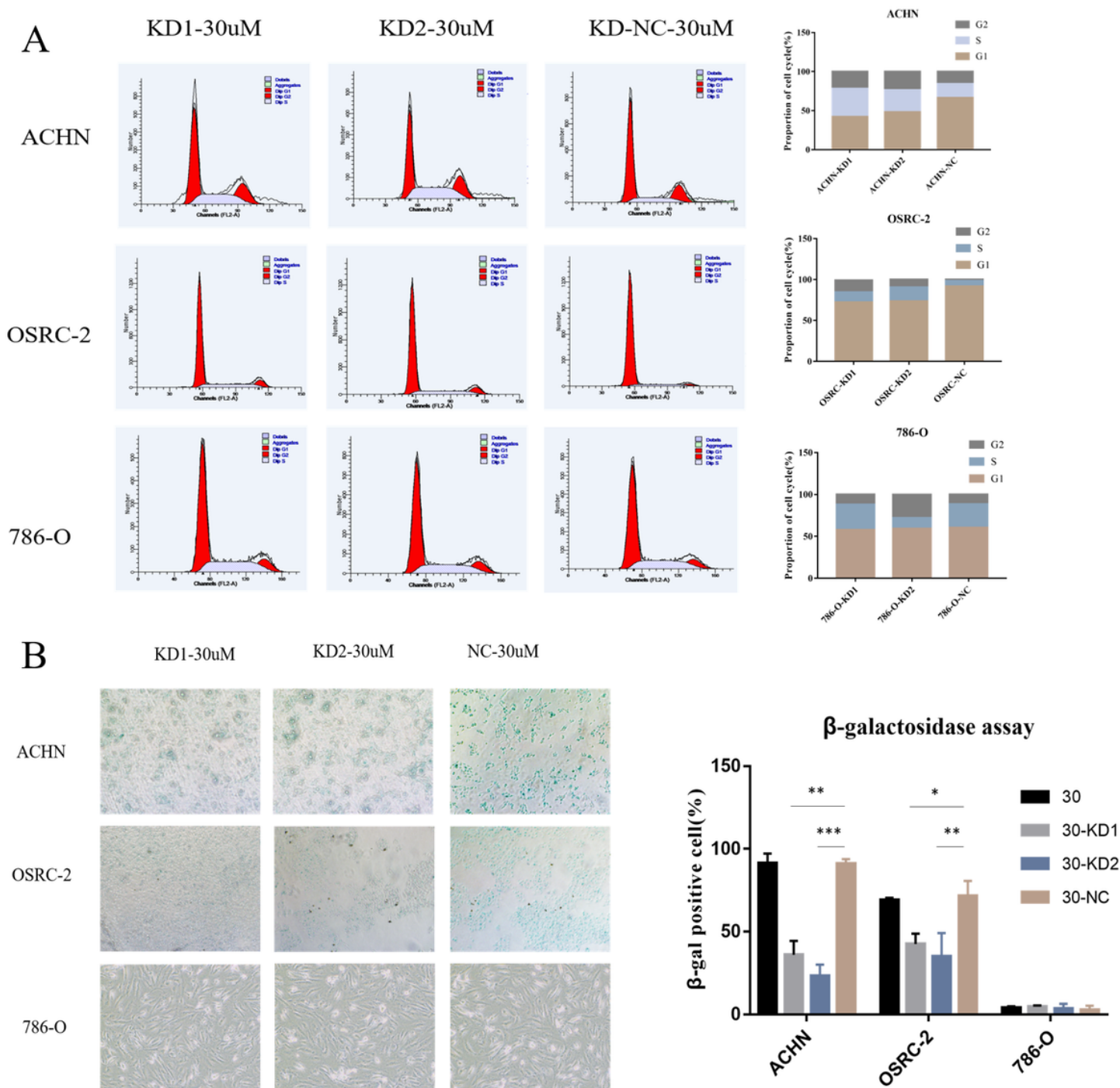


Figure 7

P53BER2 could mediate cell cycle arrest and senescence in p53-WT renal cancer cell lines. A: The proportion of cell cycle of different RCC cell lines infected p53BER2-KD virus and vectors after nutlin-3 treatment. B: The rate of senescence cell in different RCC cell lines infected p53BER2-KD virus and vectors after nutlin-3 treatment. Results are shown as mean \pm SD. ***P < 0.001 compared with control. **P < 0.01 compared with control. *P < 0.05 compared with control.

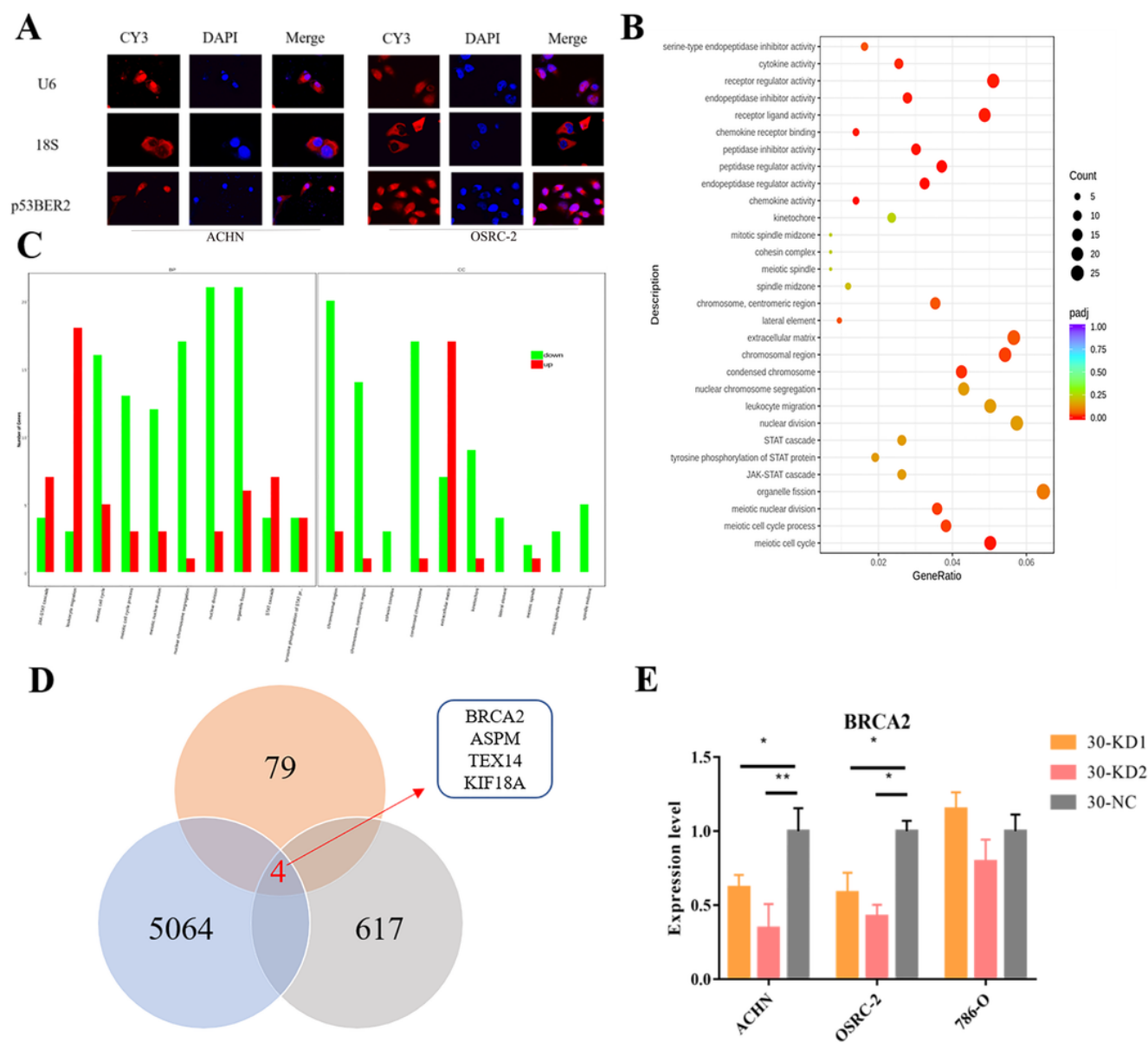


Figure 9

P53BER2 positively regulates BRCA2 expression. A. The location of p53BER2 in RCC cell lines with confocal image. B. The Go terms enriched analysis of different biological process in p53-KD and NC RCC line by RNA-sequencing. C. The overall expression level of several biological process related gene between p53-KD and NC RCC line from RNA-sequencing data. D. Overlap of the differential genes of the two cell lines and the differential genes related to the cell cycle pathway. E. BRCA2 expression level of different RCC cell lines infected p53BER2-KD virus and vectors after nutlin-3 treatment. Results are shown as mean \pm SD. *** $P < 0.001$ compared with control. ** $P < 0.01$ compared with control. * $P < 0.05$ compared with control.

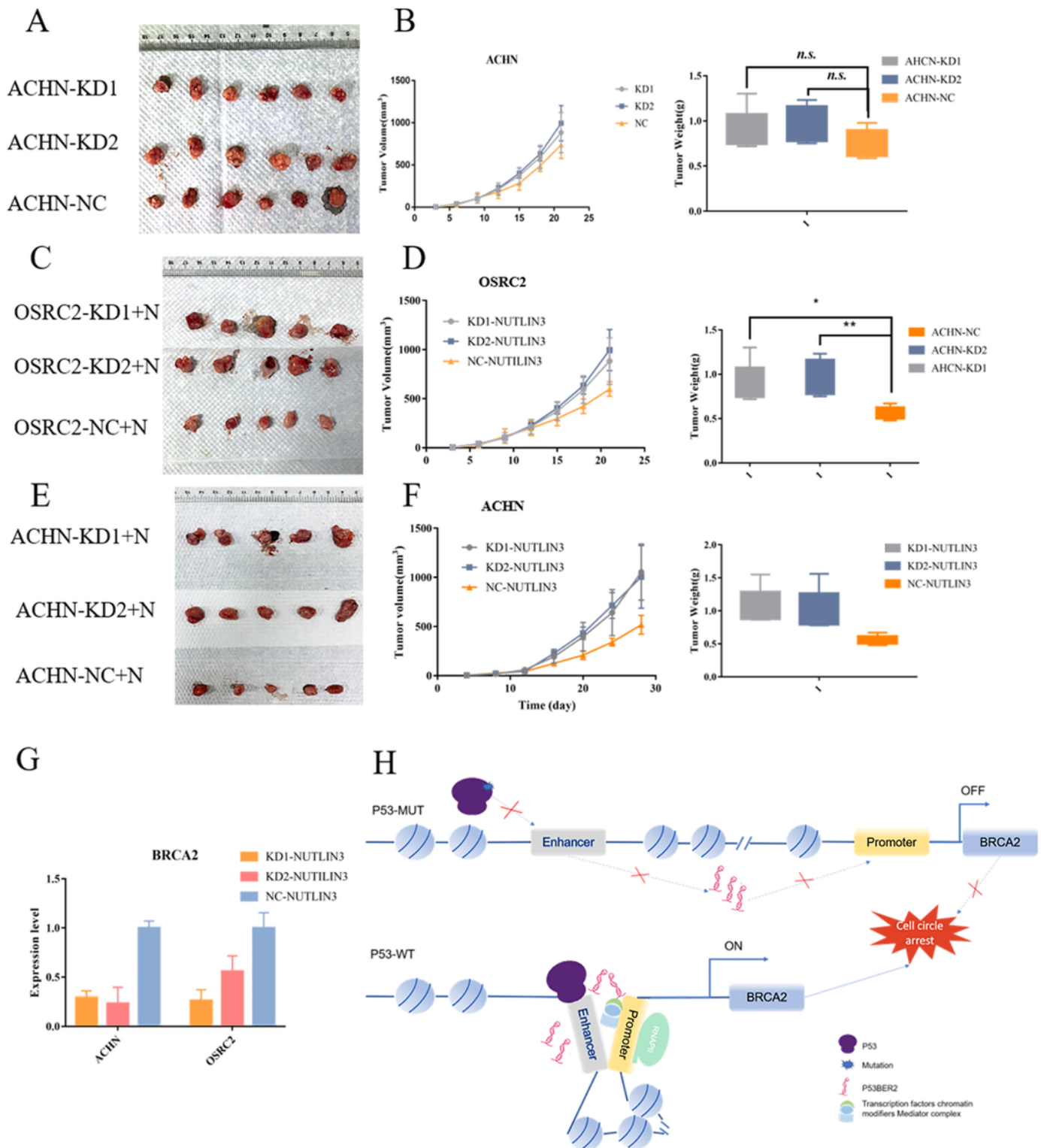


Figure 11

Downregulation of P53BER2 show a resistance to nutlin-3 treatment in cell-derived xenografts (CDXs). A: Image of 18 xenografts formed by ACHN-KD1, KD2 and Control. B: Growth curves of mice subcutaneous xenografts derived from ACHN-KD1, KD2 and Control cells (Left panel). Tumor weights were statistical analyzed (Right panel). C: A: Image of 15 xenografts formed by OSRC-2-KD1, KD2 and Control with nutlin-3 treatment. D: Growth curves of mice subcutaneous xenografts derived from OSRC-2-KD1, KD2 and

Control cells with nutlin-3 treatment. (Left panel). Tumor weights were statistical analyzed (Right panel). E: Image of 15 xenografts formed by ACHN-KD1, KD2 and Control with nutlin-3 treatment. F: Growth curves of mice subcutaneous xenografts derived from ACHN-KD1, KD2 and Control cells with nutlin-3 treatment. (Left panel). Tumor weights were statistical analyzed (Right panel). G: BRCA2 expression in mice subcutaneous xenograft. Results are shown as mean \pm SD. ***P < 0.001 compared with control. ** P < 0.01 compared with control. * P < 0.05 compared with control. H: Schematic model of P53BER2-dependent regulation of P53 activity in p53-MUT or p53-WT condition.

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