Supporting Information

Significance and Mechanism of Androgen Receptor (AR) Overexpression and AR-mTOR Crosstalk in Hepatocellular Carcinoma

Hong Zhang, Xiaoxing Li, Yang Yang, Yanjie Zhang, Hui-Yun Wang and X.F. Steven Zheng

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Table S1. Univariate and multivariate Cox regression analysis of AR and survival in HCC patients

Variables	Univariate analysis		p value	Multivariate analysis		p value
	HR	95% CI		HR	95% CI	
Age (≥ 60y vs.< 60y)	1.999	0.989-4.040	0.054			
Gender (Female vs. Male)	0.796	0.331-1.911	0.609			
Serum AFP (≥ 400 vs.< 400 ng/ml)	2.188	1.044-4.582	0.559			
HBV DNA Copy (≥ 1000 vs.< 1000)	1.893	0.976-3.669	0.059			
Serum HBsAg (positive vs. negative)	0.726	0.257-2.048	0.545			
Tumor Size (≥ 5 cm vs.< 5 cm)	1.805	0.941-3.461	0.075			
Tumor Number (> 1 vs. 1)	1.647	0.635-4.270	0.305			
Tumor grade (III-IV vs I-II)	2.333	1.222-4.456	**0.010**	1.582	0.933-2.681	**0.019**
TNM Stage (III vs. I-II)	3.614	1.564-8.351	**0.003**	2.575	1.102-6.014	**0.029**
AR Expression (High vs. Low)	3.031	1.272-7.225	**0.012**	2.534	1.101-5.829	**0.028**

NOTE: The bold type represents *P* values smaller than 0.05.HBsAg, hepatitis B surface antigen; AFP, α-fetoprotein; CI, confidence interval; HR, hazard ratio; AR, androgen receptor; HBV, hepatitis B virus; HCC, hepatocellular carcinoma.

Table S2. Significantly Altered Key AR Target Genes by AR Overexpression in HCC

Gene	Control Group	AR Group	Fold Change (AR/Control)
ACSL3	0.048833	0.251739	5.1551
ALDH1A3	0.005277	0.013322	2.5245
APPBP2	0.006452	0.042986	6.6622
AR	0.00262	0.521233	198.9139
CENPN	0.059705	0.273573	4.5821
CYP2U1	0.002378	0.005962	2.5071
DBI	0.424548	1.189207	2.8011
EAF2	0.000692	0.006045	8.7301
ELL2	0.004227	0.037421	8.852
ENDOD1	0.004112	0.009753	2.3718
ERRFI1	0.015777	0.074842	4.7437
FAM105A	0.000168	0.001501	8.9135
FKBP5	0.008281	0.036147	4.3651
HERC3	0.000747	0.004072	5.449
HPGD	0.000259	0.001874	7.2401
LIFR	0.000299	0.00282	9.4218
LRRFIP2	0.012639	0.036906	2.9201
MME	0.000979	0.007867	8.0333
NKX3-1	0.002114	0.005119	2.4217
ORM1	0.000033	0.000135	4.0446
PAK1IP1	0.010409	0.051119	4.9109
PIAS1	0.010554	0.047039	4.4568
PIK3R3	0.002188	0.005226	2.3883
PPAP2A	0.009125	0.033262	3.6452
RAB4A	0.034055	0.102238	3.0022
REL	0.001395	0.009099	6.5251
RHOU	0.002395	0.009355	3.9068
SGK1	0.038848	0.089622	2.307
SLC26A2	0.006773	0.017579	2.5955
SMS	0.152196	0.476319	3.1296
SNAI2	0.000147	0.000581	3.9614
STK39	0.03477	0.084202	2.4217
TIPARP	0.014219	0.05872	4.1296
TPD52	0.009125	0.03125	3.4248
VAPA	0.080996	0.351111	4.3349
ZBTB10	0.001266	0.009753	7.7061
ZNF189	0.002989	0.015093	5.049
B2M	0.141023	0.707107	5.0141
HPRT1	0.020532	0.068869	3.3543
ACKR3	0.000055	0.000016	0.2944

ELK1	0.014518	0.002123	0.1462
GUCY1A3	0.000033	0.00001	0.2924
IGFBP5	0.056094	0.011518	0.2053
KLK2	0.000033	0.00001	0.2924
KLK3	0.000033	0.00001	0.2924
KLK4	0.000033	0.00001	0.2924
KRT8	0.020249	0.008201	0.405
MAF	0.000319	0.000075	0.2359
MAP7D1	0.094995	0.041521	0.4371
NFKB2	0.021109	0.007922	0.3753
PGC	0.000033	0.000015	0.4432
RELA	0.06906	0.02797	0.405
SLC45A3	0.001434	0.000523	0.365
SRF	0.037266	0.012007	0.3222
STEAP4	0.000033	0.00001	0.2924
TMPRSS2	0.000033	0.00001	0.2924
VIPR1	0.000055	0.000014	0.2599

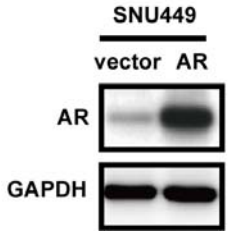

Fig. S1. AR overexpression in HCC cells

AR overexpression in SUN449 cells. AR is overexpressed in SUN449 cells by infection of lentiviral AR. Shown is immunoblot of SUN449 infected with a control lentivirus and AR lentivirus. GAPDH serves as a loading control.

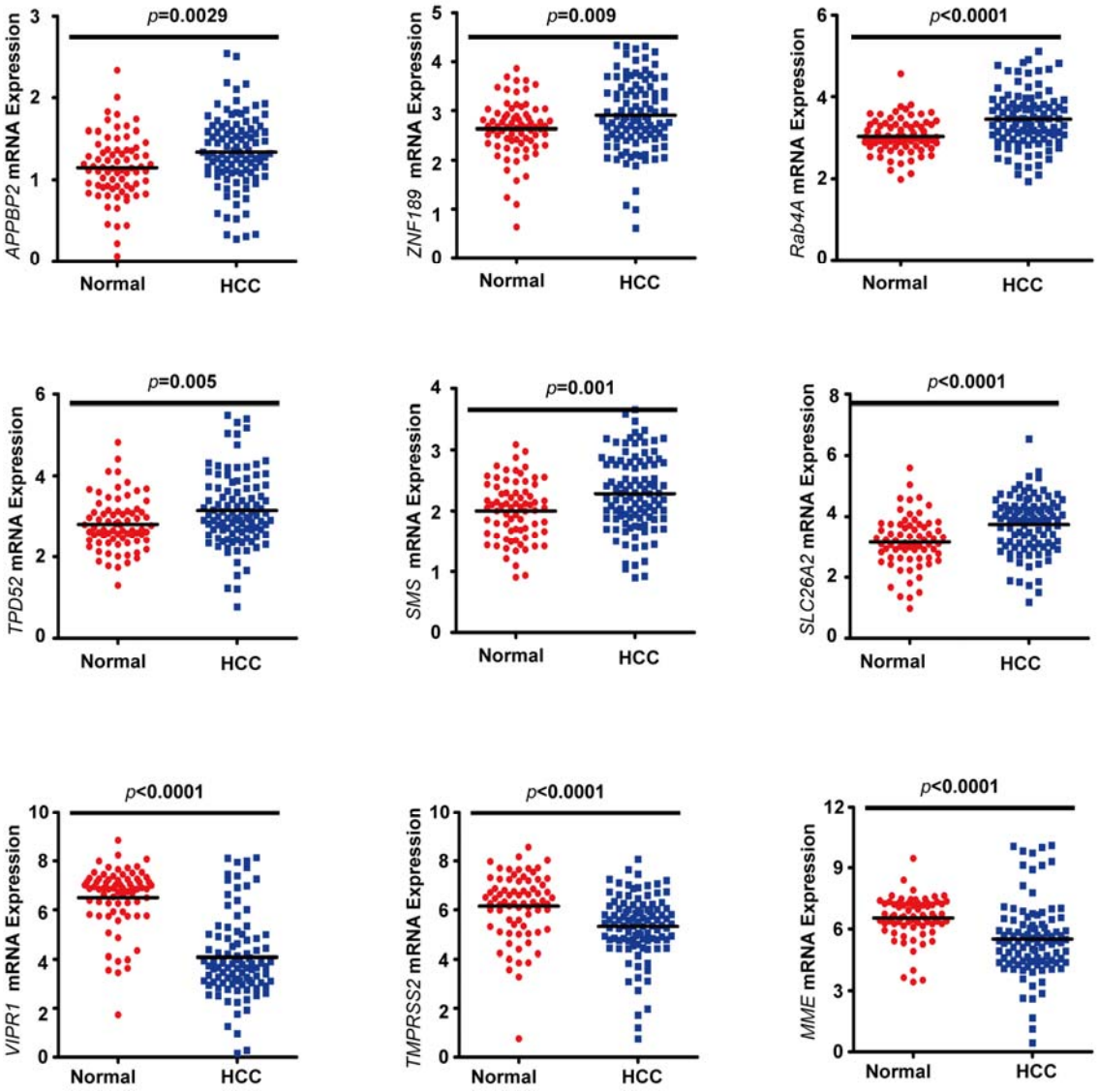

Fig. S2. The expression of AR target genes are significantly altered in HCC

mRNA expression data for AR target genes APPBP2, Rab4A, SMS, ZNF189, SLC26A2, TPD52, VIPR1, MME and TMPRSS2 were downloaded from an Oncomine transcriptome dataset that includes 104 HCC and 76 normal liver tissues. The results were analyzed by Student's T-test (bar represents mean value).

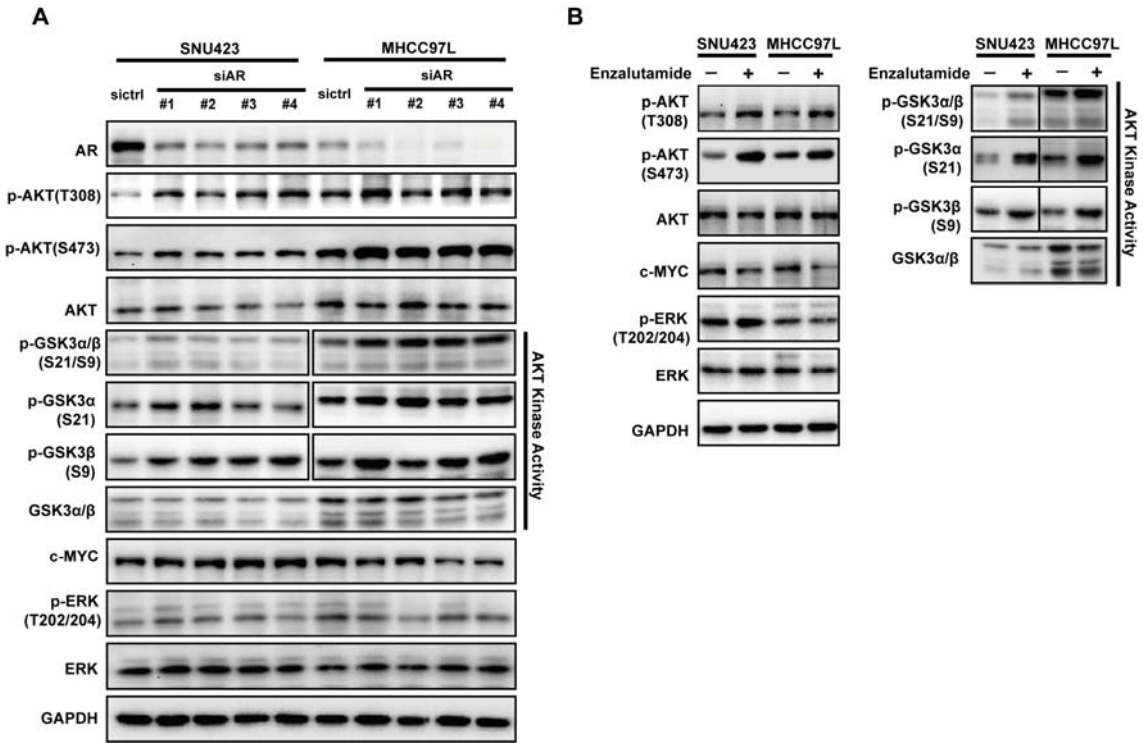

Fig. S3. AR inhibition leads to activation of AKT pathway but not c-MYC and ERK pathways

(A) siRNA-mediated knockdown of AR activates AKT pathway, but not c-MYC and ERK pathway. SUN423 cells was transfected with four independent AR-specific siRNAs, and analyzed for AR knockdown efficiency and effect on AKT, c-MYC and ERK pathways by immunoblot. GAPDH is used as a loading control.

(B) Enzalutamide activates AKT pathway, but not c-MYC and ERK pathway. SUN423 cells was treated with enzalutamide, and analyzed for AR knockdown efficiency and effect on AKT, c-MYC and ERK pathways by immunoblot. GAPDH is used as a loading control.

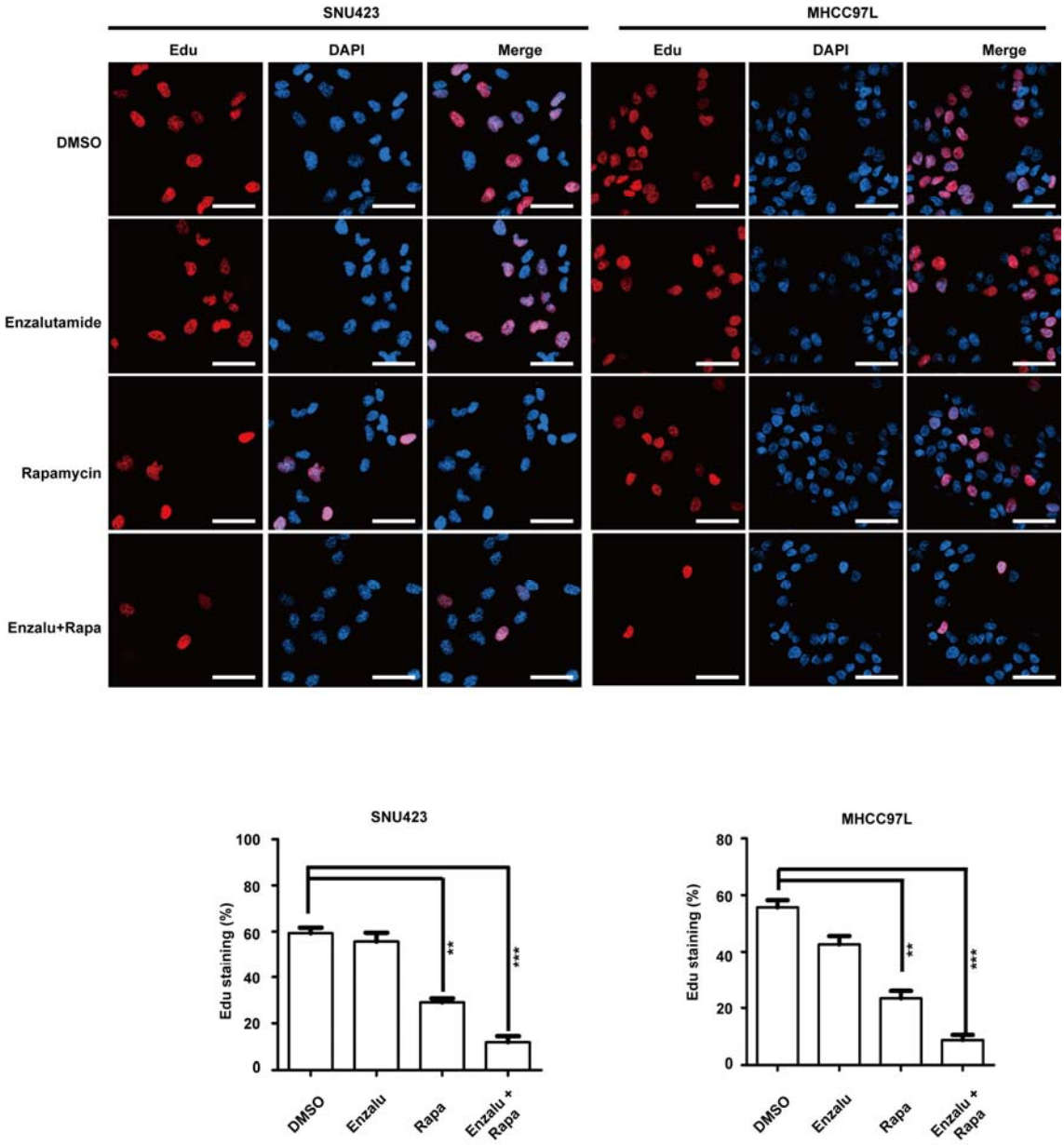

Fig. S4. Enzalutamide and rapamycin inhibit HCC cell proliferation in vitro

SNU423 and MHCC-97L cells were treated with drug carrier (DMSO), enzalutamide (MDV3100), rapamycin, or the combination of two drugs for 3 days, and measured for cell proliferation by the EdU proliferation assay. Data (mean ± SD, n = 5) were analyzed by One-way ANOVA test; ** $p < 0.01$. Scar bar, 50 μm.

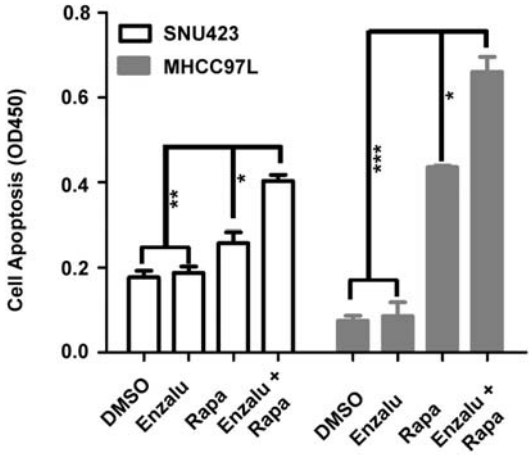

Fig. S5. Enzalutamide and rapamycin induce cell apoptosis

SNU423 and MHCC-97L cells were treated with enazlutamide and rapamycin individually or in combination for 6 days. DMSO was used as a drug carrier control. Apoptotic cell death was analyzed using the Cell Death Detection ELISA assay. Data (mean ± SD, n = 3) were analyzed by One-way ANOVA test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

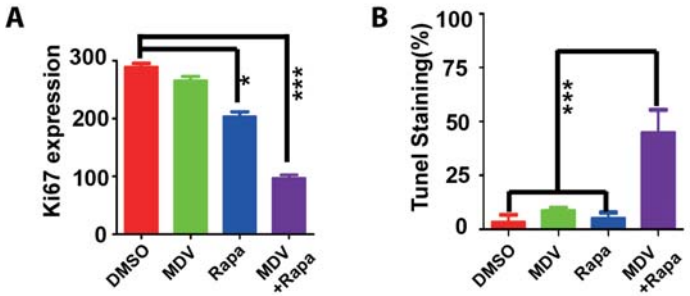

Fig. S6. Co-targeting AR and mTOR suppresses proliferation and induces apoptosis of xenograft HCC tumors

Mice bearing MHCC-97L xenograft tumors were treated with a drug carrier, enzalutamide (MDV3100), rapamycin or their combination. Shown are quantification results for Ki67 (A) and Tunel staining (B). Data (mean ± SD, n = 6) were analyzed by One-way ANOVA test; * $p < 0.05$, *** < 0.001.

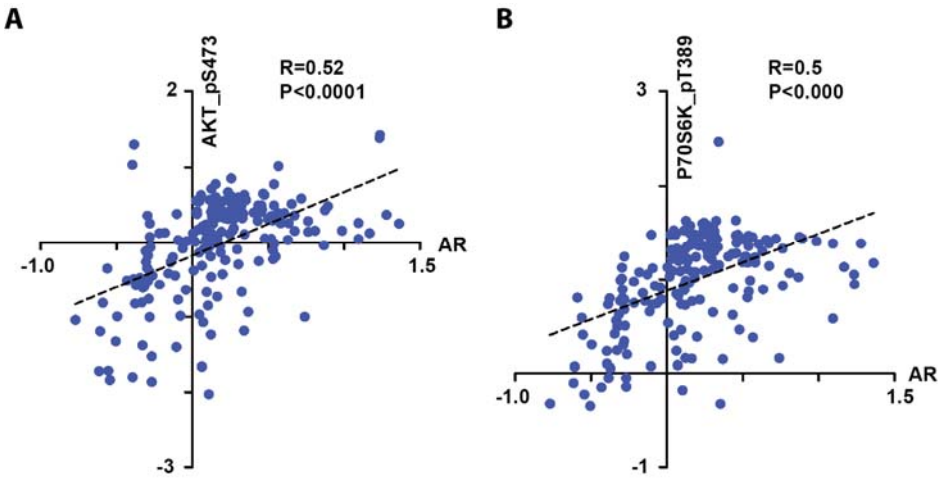

Fig. S7. AR expression is positively correlated with AKT-mTOR signaling in HCC tissues

Correlation analysis of AR protein expression versus p-AKT(S473) (left panel), and AR protein expression versus p-P70S6K(T389) (right panel) was performed with 184 primary human HCC tumor samples in the TCGA proteomic database. The correlation score was analyzed using Pearson correlation test.

Supplementary Materials and Methods

Cell culture, plasmids and transfection

Three human HCC cell lines, SNU423, SNU449, Bel7402, as well as the LO2 human immortal liver cell line, were cultured in RPMI 1640 Medium (Invitrogen, USA) plus 10% fetal bovine serum (FBS) (Biological Industries, Israel). MHCC-97L, Huh7 and PLC/PRF/5 human HCC cell lines were maintained in high-glucose DMEM (Invitrogen, USA) plus 10% FBS. Cells were incubated at 37°C in a humidified chamber containing 5% CO2. For the hormone-stimulation or AR inhibitor treatment experiment, cells were incubated in phenol-red free DMEM supplemented with 5% charcoal/dextran-treated FBS. AR-overexpressing lentiviral pCDH-AR was kindly provided by Dr. Bin He (Baylor College of Medicine, USA)(1).

Small interference RNA (siRNA) targeting AR (siAR-1: 5'-GACAGUGUCACACAUUGAATT-3'; siAR-2: 5'GCUGAAGAAACUUGGUAAUTT-3'; siAR-3: 5'- GCAGAAAUGAUUGCACUAUTT'; siAR-4: 5'CUGCUACUCUUCAGCAUUATT-3') or control sequence (5'-GTTCTCCGAACGTGTCAC-3') was purchased from Shanghai GenePharma. They weretransfected into HCC cells using Lipofectamine 3000 (Invitrogen, USA) according to the manufacturer's protocol. Human pCDH-AR lentivirus was generated in HEK293FT cells following the manufacturer's protocol. 2 days after transfection, viral supernatants were harvested from HEK293FT cell cultures. The stableAR overexpression in SNU449 and PLC/PRF/5 cells was established by infection with pCDH-AR lentivirus, followed by selection in 2μg/ml puromycin. AR luciferase reporter plasmid werepurchased from Shanghai YASEN.

Cell proliferation and colony formation assays

HCC cells were seeded at 1,000 cells each well of 96-well plate and cell proliferation was measured by the CCK-8 assay (Dojindo, Japan). For the colony formation assay, 1,000 HCC cells were cultured in 6-well plates for three weeks. Colonies were then fixed and counted

after staining with crystal violet. Three or more independent experiments were performed for each of the above assays.

Chemicals, antibodies and other related reagents

Rapamycin, enzalutamide and MG-132 were purchased from Selleck Chemicals (USA);cycloheximide and testosterone were purchased from Sigma (USA) and MedChem Express (USA), respectively. SNU423, MHCC-97L and SNU449 cells were treated with 100 nM Rapamycin, 10 μM enzalutamide, 20 μM MG132 and 50 nM testosterone, respectively. Antibodies against androgen receptor (#5153), GAPDH (#5174), p-S6 (S235/236), S6 (#2217), p-S6K (T398), S6K, p-AKT(T308) (#13038), AKT (#4691), p-mTOR(S2448) (#5536), mTOR (#2983) were purchased from Cell Signaling Technology (USA). Ki-67 antibodies were purchased from Abcam Inc. (USA). Antibodies against Flag (F1804) was purchased from Sigma (USA).

Immunoblot

Cell-free protein extracts were prepared with Cell Lysis Buffer (20 mMTris-HCl pH 7.5, 150 mMNaCl, 2.5mM sodium pyrophaosphate, 1% Triton, and 1mM Na_2EDTA) containing protease and phosphatase inhibitor cocktails. Protein concentration was determined by the BCA assay (Pierce, USA). 30 μg of protein extracts were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and blotted onto PVDF membrane. The membrane was blocked with 5% milk in Tris buffer (10 mMTris-HCl, pH7.4, containing 0.05% Tween-20) and then incubated with a primary antibody at 4°C overnight. After washing with the Tris buffer for 3 times, the membrane was incubated with a peroxidase-conjugated secondary antibody (Bentyl, USA), developed in Super-Signal West Pico Chemiluminescent Substrate, and analyzed and quantified by densitometric analyses using a Versadoc Imaging System (Model 3000).

Immunofluorescence (IF)

Cells were cultured in u-Slide 8 well (IBIDI, Germany) and fixed in 4% formaldehyde at room temperature for 40 min. After blocking with PBS containing 4 % bovine serum albumin (BSA) (Sigma, USA) for 90 min, cells were incubated with AR polyclonal antibody, followed

bya secondary antibody conjugated to Alexa Fluor 488 (Life Technologies, USA). Nuclei were stained using 4', 6-Diamidino-2-phenylindole dihydrochloride (DAPI) (Life technologies, USA). Images were acquired in multi-tracking mode using anOlympus scanning confocal microscope (FV1000, Olympus, Japan).

Immunohistochemistry (IHC)

IHC was performed as previously described (2, 3). Tumor tissues were dried at 60°C for 2 h and were then deparaffinized in xylenes and rehydrated in graded ethanol. The endogenous peroxidase activity was then blocked by incubation in 3% hydrogen peroxide in methanol for 10 min. The sections were then boiled in 10 mM citrate buffer (pH 6.0) for 20 min in a microwave oven. After cooling down to the room temperature (RT), tumor sections were incubated with anti-AR (1:100), anti-p-S6 (1:400) and anti-Ki-67 (1:600) antibody overnight at 4^{o}C,followed by peroxidase-linked second antibodies (DakoCytomation, CA) for 30 min at RT. Tumor sections were developed with 3, 5-diaminobenzidine (DAB) substrate and counterstained with Mayer's hematoxylin. IHC staining was quantified with semi-quantitative immunostaining score (ISS) method. The tumor staining results were analyzed independently by two experienced pathologists. The intensity of immunostaining was defined as 0–3 (0, negative; 1, weak; 2, moderate; 3, strong), and the percentage of positive immunostaining was scored as 0 to 100%. An overall expression score, ranging from 0 to 12, was calculated by multiplying the intensity score and extent score. ISS is calculated for each sample by the multiplication of the intensity score by the percentage score, resulting in an ISS between 0 (no staining) and 300 (maximum staining). Based on the final staining score, patients were divided into high- and low-expression groups by the median score.

TUNEL staining of tumor sections were carried out using the Roche TUNEL staining kit (Roche, Lewes, UK) following manufacturer's instruction. Briefly, after dewaxing and washing twice with PBS, tumor sections were incubated in poteinase K solution (Invitrogen) for 15 minutes at room temperature. Following washing twice in PBS, the TUNEL solution (Roche TUNEL kit; fluorescein labelled nucleotides and terminal deoxynucleotidyl transferase) was added and incubated for 1 hour at 37°C, which was followed by incubation with peroxide

blocker for 15 minutes at room temperature and susequently converter solution (Roche TUNEL kit) at 37°C for 30 minutes. Finally, 3, 5-diaminobenzidine (DAB) solution (Menarini, Wokingham, UK) was then applied for five minutes and the tumor sections were counterstained with Mayer's hematoxylin.

ARE luciferase reporter assay

$1x10^5$ SNU423 and MHCC-97L cells were seeded in 24-well plates and transfected with 1 μg pLUC-4XARE and 10 ngpRL-TK using Lipofectamine 3000 (Life Technologies, USA). Cells were collected 48 h post-transfection and assayed for luciferase activity using the Glomax 96 microplateluminometer (Promega, USA).

Profiling of key AR target genes in AR overexpressing HCC cells

Androgen receptor target gene profiling was performed using Human Androgen Receptor Signaling Targets PCR Array on 96-well plate (Qiagen, Germany). The array profiles the expression of 84 key AR target genes. A set of controls wasincluded on each plate, which enables data analysis using ΔΔCT method of relative quantification, assessment of reverse transcription performance, and assessment of PCR performance. The Androgen Receptor Signaling Targets PCR Array enables SYBR Green-based real-time PCR analysis using Roche Lightcycler 96 RT-PCR system (Roche Diagnostics, USA) as follows: 95°C for 10 min; 40 cycles at 95°C for 15 sec; 60°C for 1 min; 55°C for 30 sec and 95°C 30 sec; The relative expression was calculated using the ΔΔCT method (relative gene expression = 2-(ΔCT sample-ΔCT control)] and presented in fold increase relative to control.

Reverse transcription-PCR (RT-PCR) and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from HCC cells using TRIZOL (Invitrogen, USA). RNA samples were measured by a NanoDropTM 2000 spectrophotometer (Thermo Fisher Scientific, MA, USA). cDNA was synthesized by reverse transcription reaction according to the protocol of GoScriptTM Reverse Transcription System kit (Promega, MI, USA). RT-PCR was performed using GoTaq RT-PCR Master Mix (Promega, MI, USA). All samples were run in triplicate and underwent denature at 95°C for 10 min and then 40 amplification cycles at 95°C for 15

sec, 60°C for 1 min, 55°C for 30 sec and 95°C 30 sec on a Roche Lightcycler 96 RT-PCR system (Roche Diagnostics, IN, USA). For relative quantification, ΔΔCt was calculated using GAPDH as an internal standard control for each sample. For graphical representation, ΔΔCt values were normalized to controls and expressed as the difference of fold change.

The following primers were used to verify the expression of key AR target genes: AR, 5'-GGCAGGAGACGACAAACA-3' and 5'- GCGTGGCAATCAGGTAGA-3; GAPDH, 5'-AAGGTCGGAGTCAACGGATTT-3' and 5'-ACCAGAGTTAAAAGCAGCCCTG3'; FKBP5, 5'-CTCCCTAAAATTCCCTCGAATGC-3' and 5'-CCCTCTCCTTTCCGTTTGGTT-3', and NKX3-1, 5'-CCCACACTCAGGTGATCGAG-3' and 5'-GAGCTGCTTTCGCTTAGTCTT-3'; ORM1, 5'-ACACCACCTACCTGAATGTCC-3' and 5'-GTGAGCGAAATGCTCTTGGC-3'; ELL2, 5'- CATCACCGTACTGCATGTGAA-3' and 5'-ACTGGATTGAAGGTCGAAAAGG-3'. ELL2, 5'-CATCACCGTACTGCATGTGAA-3' and 5'-ACTGGATTGAAGGTCGAAAAGG-3'; TIPARP, 5'-AGAACGAGTGGTTCCAATCCA-3' and 5'-TGGGTGCAAAAGATCAGTCTG-3'; IGFBP5, 5'-ACCTGAGATGAGACAGGAGTC-3' and 5'-GTAGAATCCTTTGCGGTCACAA-3'; VAPA, 5'-TAGCAAAGCTGTTCCACT-3' and 5'- GAATGTGCTACCTTTCTGA-3'.

Cycloheximide (CHX) pulse-chase assay

Cycloheximide pulse-chase experiment was performed as described before(4). Cells were seeded on 6-well plate at a density of 2×10^5 cells per well. Cells were treated without or with 100 nM rapamycin in the presence of 50 μg/ml cycloheximide and analyzed for AR protein stability by immunoblot.

EdU proliferation and apoptosis assays

Cells were seeded in u-Slide 8 well (IBIDI, Martinsried, Germany) and cultured in complete medium for one day. Before adding Edu, cells were incubated at 37°C for 48 hrs in the presence or absence of Enzalutamide (10 μM), or rapamycin (100nM). EdU (10 μM in completed medium) was added into each well and incubated at 37°C for 4 hrs. Following EdU incubation,

cells were washed with PBS twice and fixed with 3.7% formaldehyde in PBS for 15 min. After permeabilizating with 0.5% Triton® X-100 for 10 min, cells were incubated with 50 μl Click-iT® reaction cocktail for 30 minutes at room temperature and followed by washing twice with PBS. Nuclei were stained using Hoechst33342 (10 μg/ml) for 30 min at the room temperature. Images were acquired in multi-tracking mode using a scanning confocal microscope (OLYMPUS FV1000, OLYMPUS, Japan) and number of positive cells was counted in 5 fields.

For the apoptosis assay, 1 x 10^3 cells were seeded in 96-well plates, incubated at 37°C overnight and then subjected to 24-hour serum deprivation. HCC cells continue to growth for 4 days (MHCC97L) or 6 days (SNU423) in the presence or absence of enzalutamide (10 μM), or rapamycin (100 nM). Cell apoptosis was analyzed with the Cell Death Detection Elisa Plus Kit (Roche, Lewes, UK) by measuring the absorbance at 405nm according to the manufacturer's instructions.

Reference

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