TIGAR Enhanced Free Ca^{2+} Concentration in Hepatocellular Carcinoma Cells to Accelerate the Sustained Proliferation and Drug Resistance

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Abstract

Background: To study the role of TP53-induced glycolysis and apoptosis regulator (TIGAR) in hepatocellular carcinoma (HCC) and drug resistance.

Methods: HCC cells (HepG2 and SMMC7721) were used in this study. Fura 2-AM was used to assess cytosolic free Ca^{2+} concentrations ($[Ca^{2+}]i$) within the two HCC cell lines. Nimodipine (NMDP), a Ca^{2+} antagonist, was used to reduce cytosolic $[Ca^{2+}]i$ level. Proliferation of HCC was measured using cell counting kit-8 (CCK-8). The roles of TIGAR and Ca^{2+} in drug resistance of HCC cells were assessed using epirubicin (Epi), 5-fluorouracil (5-FU), or cisplatinum (DDP).

Results: Knockdown of TIGAR significantly suppressed cell viability, reduced $[Ca^{2+}]i$, restrained protein expression of Ca^{2+}-activated cysteine proteinases (Calpain1 and 2), as well as blocked the activation of nuclear factor kappa B (NF-κB) through an increase of cytoplasmic NF-κB and reduction of nuclear NF-κB. However, overexpression of TIGAR (oeTIGAR) resulted in the opposite. Evidence also shows that oeTIGAR suppressed the sensitivity of HCC to Epi, which was retarded by NMDP as an additional treatment. TIGAR interference could enhance the sensitivity of HCCs with high TIGAR expression to drugs.

Conclusions: TIGAR promoted HCC progression and induced drug resistance, and the mechanism involved was $[Ca^{2+}]i$-mediated activation of Calpain 1 and 2 and NF-κB signaling.

Background

Hepatocellular carcinoma (HCC) is commonly caused by alcohol or viral infections (e.g., Hepatitis B and C virus), and accounts for 2.4% of all malignancies and 9% of cancer-related deaths(1). Patients with HCC usually receive a three-drug chemotherapy protocol consisting of cisplatinum (DDP) and 5-fluorouracil (5-FU), followed by partial surgical resection. However, 70% of cases recur due to drug resistance (2, 3). This necessitates a deeper analysis and better understanding of the molecular processes that favor HCC progression and the development of drug resistance to improve treatment of HCC.

TP53-induced glycolysis and apoptosis regulator (TIGAR) is an oncogene that is associated with progression and drug resistance of various cancers (4–6). In HCC progression, TIGAR promotes proliferation by inhibiting glycolysis, promoting anti-oxidative activities, reducing ROS as well as retarding ROS-associated DNA damage, apoptosis, and autophagy (7–9). Moreover, TIGAR also reduces the sensitivity of HCC cells to epirubicin (Epi) (8), suggesting that TIGAR serves as a promoter of drug resistance in HCC. These studies suggest that TIGAR can be a candidate for targeted cancer therapy.

An increase in free Ca^{2+} concentrations ($[Ca^{2+}]i$) acts as a second messenger to stimulate cytosolic events that participate in HCC proliferation, apoptosis, and migration(10, 11). Inhibition of Ca^{2+} signaling in HCC also suppresses multi-drug resistance by suppressing epithelial-mesenchymal transition, blunting

hypoxia-inducible factor1-α signaling as well as attenuating DNA damage repair (12). TIGAR functions similar to fructose-2,6 bisphosphatase, whose activity is enhanced by high [$Ca2^{+}$]i in human fibroblasts (13). Whether [Ca^{2+}]i plays a role in HCC proliferation and drug resistance remains to be proven.

To explore the role of Ca^{2+} in TIGAR's promotion of cancer cell proliferation in HCC *in vitro*, siRNA-mediated TIGAR knockdown (siTIGAR) and lentivirus-mediated TIGAR overexpression (oeTIGAR) were transfected into human HCC cells (HepG2 and SMMC7721). Nimodipine (NMDP) was used as Ca^{2+} inhibitor to decrease cytosolic ([Ca^{2+}]i). Calpains, also known as Ca^{2+}-activated cysteine proteinases, as well as nuclear factor κB (NF-κB) in the nucleus and cytoplasm were assessed. The sensitivity of HCC cells to Epi were also detected under oeTIGAR transduction or NMDP treatment.

Methods

Bioinformatics analysis

To study the expression and association of TIGAR, Calpain 1, and Calpain2 in HCC progression, tumor tissue and surrounding normal tissue were taken from patients with HCC (n = 13). Quantitative real-time polymerase chain reaction (qRT-PCR) was conducted to assess mRNA levels of TIGAR, Calpain 1, Calpain 2, and NF-κB in hepatic tissues. Pearson's correlation coefficients (r) and linear regression were carried out to analyze expression correlation between TIGAR and Calpain 1, as well as TIGAR and Calpain2.

Isolation of primary HCC cells

Primary HCC cells were separated from tumor tissue according to previously reported methods (14, 15). Resected HCC specimens were sliced into 1 cm^3sections, which were suspended in RPM11640 medium (Hyclone, Logan, UT, USA) containing 100 U/ml of penicillin and 100 μg/ml of streptomycin (Solarbio, Beijing, China). Tissue sections were digested into single cells by a collagenase/hyaluronidase solution (Thermo Fisher Scientific, Waltham, MA, USA) followed by 0.25% trypsin-EDTA at 37℃ for 2 h, then filtered using a 120 mesh sieve. The filtrate was centrifuged at 1000r/min for 10 min at 37℃. Primary HCC cells in the precipitate were further purified from stromal cells using a magnetic bead isolate system (Beijing Percans Oncology Research Co., Ltd.). In this study, 10 primary HCC cell lines were divided into low and High groups (n =5 per group), according to TIGAR mRNA levels assessed by RT-PCR.

Cell Culture and treatment

HepG2 (American Type Culture Collection (ATCC), Manassas, VA, USA) and SMMC7721 cells (ATCC) as well as primary HCC cells were suspended in DMEM (Hyclone) with 10% FBS (GIBCO) and 100 U/ml penicillin (Beijing Solarbio Science, China) until reaching 80% confluency at 37℃ under 5% CO_2.

To study the involvement of Ca^{2+} in TIGAR-induced cancer cell proliferation, HepG2 cells were transfected with TIGAR and then exposed to 10 μM of NMDP (Shanghai yuanye Bio-technology Co., Ltd, access No. 100270). To study the involvement of [Ca^{2+}]i in TIGAR-induced drug resistance, HepG2 or primary HCC

cells with oeTIGAR were treated with 10 μM of NMDP plus either Epi 2.5 μg/mL, 5-FU 5 μg/mL, or DDP 1mg/L. Epi, 5-FU, and DDP were all purchased from Selleck (https://www.selleck.cn/) with catalog numbersS1223, S1209, and S1166, respectively.

Groups

The cell experiments were divided into four parts. Part 1 examined the effects of siTIGAR on HepG2 and SMMC7721 cells. The two cell lines were transfected with siTIGAR, cultured for 72 h, then harvested at 0, 24, 48, and 72 h after treatment. The proliferation of cells at each time point was tested by CCK-8 assay. The $[Ca^{2+}]i$ content was evaluated using flow cytometry, and the protein levels of Calpain1, Calpain 2, cleaved-caspase 3 (c-caspase 3), Bax, Bcl2, nuclear and cytoplasmic NF-κB, total NF-κB, proliferating cell nuclear antigen (PCNA), H3, and GAPDH were detected by western blot at 48 h after treatment. Furthermore, the rescue assays of siTIGAR in the events involved in HCC were administrated by treatment with oeTIGAR in HepG2 cells, and the above indices were detected.

Part 2 examined the effects of oeTIGAR on drug resistance (NMDP) of HepG2. The first step tested the expression of TIGAR in HepG2 cells after oeTIGAR. The HepG2 cells were treated with an empty vector (Vector) and oeTIGAR, and the cells treated with the same amount of medium were used as control (Control). The cells were harvested at 48 h after treatment and the expression of TIGAR was measured. The second step divided the HepG2 cells into four groups: Vector group, oeTIGAR group, empty Vector + NMDP treatment group, and oeTIGAR + NMDP treatment group. The cells were transfected with empty Vector or oeTIGAR, and NMDP (10 μM) was added to the groups at the same time. The cell samples were harvested at 0, 24, 48, and 72 h after treatment. The proliferation of cells at each time point was tested by CCK-8 assay. The $[Ca^{2+}]i$ content was evaluated using flow cytometry, and the protein levels of Calpain1, Calpain 2, nuclear NF-κB, cytoplasmic NF-κB, total NF-κB, PCNA, H3, and GAPDH were detected by western blot at 48 h after treatment.

Part 3 assessed the effects of oeTIGAR on drug resistance of HepG2 cells against NMDP (10 μM) plus Epi 2.5 μg/mL, 5-FU 5 μg/mL, or DDP 1 mg/L. The cells were divided into six groups: Vector, oeTIGAR, Vector + Epi, oeTIGAR + 5-FU, Vector + NMDP + DDP, and oeTIGAR + NMDP + drug. The cell samples were harvested at 0, 24, 48, and 72 h after treatment. The proliferation of cells at each time point was tested by CCK-8 assay. Further, the protein levels of PCNA and H3 were detected using western blot at 48 h after treatment.

In part 4, 10 primary HCC cell lines were divided into low (A1–A5) and High (B1–B5) groups, according to TIGAR mRNA levels assessed by RT-PCR. All cell lines were treated with anti-HCC drugs (Epi, 5-Fu, and DDP) with or without NMDP, and CCK-8 was used to assess proliferation of primary HCC at 48 h. In addition, we assessed the effects of siTIGAR on drug resistance of two higher TIGAR expression cell lines (B4 and B5 cells) against NMDP (10 μM) plus 5-FU or DDP. Each cell line was divided into six groups: siNC, siTIGAR, siNC + drug, siTIGAR + drug, siNC + NMDP + drug, and siTIGAR + NMDP + drug. The cell samples were harvested at 48 after treatment. The proliferation of cells at each time point was tested by

CCK-8 assay, and the protein levels of PCNA and H3 were detected using western blot at 48 h after treatment.

Production and transfection of oeTIGAR vectors

The primers of the human TIGAR gene (NM_020375.3) are TIGAR-F: 5' -CGGAATTCATGGCTCGCTTCGCTCTG-3' and TIGAR-R: 5'-CGGGATCCTTAGCGAGTTTCAGTCAGTCCATTTAG-3', which were implanted into pLVX-Puro vector (Clontech, Palo Alto, USA). After the DNA sequence of TIGAR in the pGEM-T vector was confirmed, the gene fragment was ligated into the pLVX-Puro transfer plasmid (Lenti-X™; Clontech Laboratories, Mountain View, CA, USA) and packaged into lentiviruses according to the manufacturer's proposal, and transfected into HCC cells using 293fectin™ transfection reagent (Invitrogen, Carlsbad, USA). Stably expressing Flag-TIGAR cell line was identified by RT-PCR and western blotting. Meanwhile, cells transfected with pLVX-Puro vector without TIGAR expression were the control.

TIGAR transfection and RNA interference

Two siRNA target TIGAR (siTIGAR-1 and siTIGAR-2) genes were chosen and transfected into HepG2 and SMMC7721, according to a previous study’s methods (8). siTIGAR-1 was used in the following experiments as siTIGAR.

Isolation of nuclear and cytoplasmic fractions

Cytoplasmic and nuclear protein extracts of HCC cells were separated through NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (#78835, Thermo Fisher Scientific, Waltham, MA, USA) according to a previously reported study (16).

Western blot

A total of 30 mg of cytoplasmic and nuclear proteins from HCC cells were quantified by BCA protein assay kit (PICPI23223, Thermo, IL, USA), then separated by SDS-PAGE. Electrophoretic pure containing TIGAR, Calpain 1, Calpain 2, nuclear NF-κB, and cytoplasmic NF-κB were transferred to PVDF membranes. After blocking in 5% skim milk for 1 h, the membrane was incubated with anti-TIGAR (Ab37910, Abcam Cambridge, MA, USA), anti-NF-κB antibody (Ab16502, Abcam), anti-Calpain 1 antibody (Ab108400, Abcam), anti-Calpain 2 antibody (Ab236650, Abcam), anti-GAPDH antibody (60004-1-1G, Proteintech, Wuhan, China), and anti-H3 antibody (#4499, Cell Signaling Technology, Danvers, MA, USA) at 4℃ overnight, followed by secondary antibodies (A0208 and A0216, Beyotime Biotechnology, Shanghai, China) for another 1 h. Immunoreactive bands for TIGAR, Calpain 1, Calpain 2, and nuclear and cytoplasmic NF-κB were analyzed by ECL system (GE Healthcare/Amersham Biosciences, Pittsburgh, PA, USA) with Image J software (NIH, Bethesda, USA). TIGAR, Calpain 1, Calpain 2, and cytoplasmic NF-κB were normalized using GAPDH while nuclear NF-κB was normalized using H3.

RT-PCR

Total RNA from HCC tissues or cell samples was extracted via Trizol regent (1596-026, Invitrogen, Carlsbad, CA, USA), and subjected to reverse transcription using Revert Aid First Stand cDNA Synthesis kit (Fermentas, USA). Quantified analysis of mRNA levels of TIGAR, Calpain1, Calpain2, NF-κB, and GAPDH were conducted using SYBR Green PCR Mix (Thermo, Shanghai, China) on ABI Prism 7300 SDS system (Applied Biosystem, Foster City, CA, USA). Primers used in RT-PCR are listed in Table 1.

Table 1
Primers used in RT-PCR analysis.

Gene	Primer (5'-3')
TIGAR (NM_020375.3)	Forward: TCCCAAGGATCTCCAAGC
460-600, 141 bps	Reverse: AGCACCGTGACTCACAAC
Calpain1 (NM_001198869.1)	Forward: TCGTGCTCGCCCTTATGC
1256-1525, 270 bps	Reverse: AGTCGCCCTCCTTGTTGG
Calpain2 (NM_001748.5)	Forward: ATTGCCTCCCTCACCTTG
331-515, 185 bps	Reverse: TCGGCTGAATGCACAAAG
NF-κB (NM_021975.4)	Forward: GGGGACTACGACCTGAATG
448-556, 109 bps	Reverse: TGTCAAAGATGGGATGAGAAAG
GAPDH (NM_002046.7)	Forward: GGATTGTCTGGCAGTAGCC
25-241, 217 bps	Reverse: ATTGTGAAAGGCAGGGAG

Cell counting kit-8 (CCK-8)

After culture of HCC cells (HepG2 and SMMC7721) or primary HCC cells (3×10^3 cells/well) within a stipulated time, CCK-8 reagent (10% v/v, obtained from CP002, SAB, https://www.sabbiotech.com/) was placed in each well for 1 h at 37°C, and absorbance (OD) at 450 nm was documented using a plate reader (Bio-Tek, Winooski, VT, USA).

Intracellular $[Ca^{2+}]i$ measurements

HCC cells were incubated with 10 μM of Fura 2-AM (F1241, Life Technologies, Carlsbad, CA, USA) for 1h at 37°C under 5% CO_2. $[Ca^{2+}]i$ content were evaluated using flow cytometry (Accuri C6, BD, Biosciences, USA) with Flowjo (Tree Star, Version 9.2, Ashland, OR, USA) software.

Statistical analysis

Data were calculated using Graphpad Prism 6 (GraphPad Software Inc., USA) and presented as mean ± SEM. Difference effect between groups was determined using one-way ANOVA with Tukey–Kramer method, and P value < 0.05 being significant. Correlations between TIGAR and Calpain1, as well as TIGAR and Calpain2 in HCC tumor samples were assessed by Pearson r analysis.

Results

Expression of TIGAR, Calpain 1, and Calpain 2 in HCC

Expression and association of TIGAR, Calpain 1, and Calpain 2 in hepatic tissues of HCC patients were assessed. Figure 1A–D indicated that TIGAR, Calpain 1, Calpain 2, and NF-κB were significantly enhanced in HCC ($P < 0.01$). Furthermore, Figure 1E **& F** show that the correlation between TIGAR and both Calpains were significantly positive.

siTIGAR inhibited HCC cell proliferation, down-regulated Ca^{2+}signaling, and prevented NF-κB activation

As shown in Figure 2, siTIGAR remarkably reduced HCC cell proliferation (Figure 2A**&B**), suppressed $[Ca^{2+}]i$ (Figure 2C**&D**), and down-regulated Calpain1, Calpain 2, Bcl2, PCNA, and nuclear NF-κB, and up-regulated c-caspase 3, Bax, and cytoplasmic NF-κB (Figure 2E**&F**) in the two cells. In addition, rescue assays of TIGAR demonstrated that siTIGAR-induced changes were significantly reversed by additional oeTIGAR treatment in HepG2 cells (Figure 3A–C).

oeTIGAR promoted HCC cell proliferation, increased Calpains, and accelerated NF-κB activation via enhancing Ca^{2+} concentration

Significant increases in TIGAR mRNA and protein were observed in HepG2 cells after TIGAR overexpression (Figure 4A), suggesting a successful establishment of oeTIGAR. oeTIGAR-transfected HepG2 cells were treated with 10 μM of NMDP. Cell proliferation, Ca^{2+} concentration, as well as expression of Calpain1, Calpain 2, PCNA, nuclear NF-κB, total NF-κB, and cytoplasmic NF-κB were then detected. Our data indicated that oeTIGAR evoked cell proliferation (Figure 4B), enhanced $[Ca^{2+}]i$ and $[Ca^{2+}]i$-dependent protein expression of Calpains, and PCNA (Figure 4C**&D**), as well as exacerbated nuclear NF-κB and reduced cytoplasmic NF-κB (Figure 4D). However, oeTIGAR effects were dramatically reversed by NMDP, suggesting that oeTIGAR enhanced $[Ca^{2+}]i$ to promote HCC progression and activation of Calpains and the NF-κB pathway.

oeTIGAR enhanced drug resistance of HCC cells via the Ca^{2+} pathway

To study whether TIGAR acted on $[Ca^{2+}]i$ to induce drug resistance in HCC cells, oeTIGAR-transfected HepG2 cells were exposed to NMDP + anti-HCC drugs (Epi, 5-FU, or DDP). The carcinogenesis of HepG2 cells were assessed by proliferation assay. Figure 5A–C indicated that anti-HCC drugs reduced the proliferation of HepG2 cells with or without oeTIGAR (All $P < 0.01$). HepG2 proliferation was significantly

enhanced in oeTIGAR + drug group in comparison to Vector + drug group, but was remarkably reduced in oeTIGAR + drug + NMDP group, in comparison to oeTIGAR + drug group. Moreover, the protein level of PCNA at 48 h after treatment showed the same trends with cell proliferation, as shown in Figure 5D–F. Given the role of oeTIGAR in regulating Ca^{2+} (Figure 4C), our data suggested that oeTIGAR evoked the Ca^{2+} pathway to promote drug resistance against HCC medications.

Effect of TIGAR on primary HCC cells with drugs and NMDP

From the RT-PCR assay of TIGAR mRNA levels in 10 primary HCC cell lines, we confirmed A1–A5 as low TIGAR expression group (n = 5) and B1–B5 as high TIGAR expression group (n = 5) (Figure 6A). Figure 6B-D indicated the inhibited effect of anti-HCC drugs on proliferation of B1–B5 cell lines was significantly reduced in comparison to A1–A5 cell lines; however, they were obviously enhanced with additional NMDP treatment. Moreover, the proliferation of primary HCC cell lines B4 and B5 were reduced by NMDP and anti-HCC drugs, and the inhibited effect of anti-HCC drugs was further aggravated by TIGAR interference **(**Figure 6E**)**. The protein levels of PCNA in B4 and B5 cells showed same trend with the proliferation **(**Figure 6F**&G)**.

Discussion

TIGAR is a proliferation inducer and anti-apoptosis regulator in HCC (7), and can function as fructose-2,6 bisphosphatase to contribute to energy metabolism in tumors. Evidence suggest that the blockade of the intracellular Ca^{2+} release pathway favored apoptosis of HCC cells, based on a study on human fibroblasts that indicated that enhanced $[Ca^{2+}]i$ is associated with the increased levels of fructose 2,6-bisphosphate(13).Thus, in this study, we investigated whether $[Ca^{2+}]i$ also played a role in HCC progression, in addition to TIGAR.

Calpains are a class of Ca^{2+}-dependent cysteine proteases and exist primarily in two characterized forms (Calpain 1 and 2, also known as μ- and m-calpain, respectively). Calpain is activated and associated with the retarding of extrinsic apoptotic signaling in hepatitis C virus-related HCC (17). In this study, we confirmed the increased expression of TIGAR, Calpain 1, and Calpain 2 in HCC progression (7, 18), and reported the positive correlations between TIGAR and Calpains1 and 2 for the first time (Figure 1).

We also investigated whether Ca^{2+}-mediated Calpain was involved in TIGAR-induced HCC progression. Our data showed that siTIGAR suppressed HCC proliferation, down-regulated anti-apoptotic protein Bcl2, and up-regulated pro-apoptotic proteins c-caspase 3 and Bax. However, oeTIGAR led to the opposite, confirming the role of TIGAR inhibition in regulating HCC cell death (7, 8). These indicate that TIGAR plays an important role in HCC, and suppressing TIGAR expression could be a probable target to treat HCC. More importantly, TIGAR enhanced $[Ca^{2+}]i$ and elevated levels of Calpain 1 and Calpain 2, while NMDP exposure remarkably reduced TIGAR's effects (Figure 4). These data showed that $[Ca^{2+}]i$-mediated Calpain signaling was the primary mechanism by which TIGAR exerts its effects on HCC cells.

Activation of NF-κB frequently occurs in tumors, especially in those with drug or radiotherapy resistance (19–21). The dimeric NF-κB complexes are localized to the cytosol through interaction with Inhibitor of NF-κB (IκBα). Decreased IκBα expression and activity favors the release of NF-κB dimers, which are subjected to nuclear translocation and result in subsequent activation of NF-κB target genes. Evidence suggests that TIGAR inhibits NF-κB activation in murine adipocyte cells by suppressing the activity of IKKβ (adaptor protein for IκBα degradation) (22). In bladder cancer cells, elevated $Ca^{2+}$is essential for NF-κB activation (23). In this study, we showed that TIGAR-activated NF-κB depended on the elevated $[Ca^{2+}]i$ levels in HCC cells (Figure 4).

Inhibition of Ca^{2+} entry SOCE (a key pathway for extracellular Ca^{2+} influx) enhanced sensitivity of HCC cells to chemotherapy (24). Thus, we studied whether Ca^{2+} was involved in TIGAR-induced drug resistance. TIGAR confers resistance to Epi, as evidenced by enhanced proliferation of HCC in the oeTIGAR + Epi group when compared to Vector + Epi, which is consistent with a previous study (8). However, the anti-cancer effect of Epi in oeTIGAR + Epi was strongly augmented with additional NMDP treatment, substantiating that TIGAR enhanced Ca^{2+} levels to induce drug resistance in HCC cells. Moreover, Figure 5 shows that primary HCC with high TIGAR expression was less sensitive to Epi, and abrogation of the Ca^{2+} pathway using NMDP potentiated the death-inducing ability of Epi, which further verified that the resistance of primary HCC to Epi-induced killing was attributed to TIGAR-mediated Ca^{2+} overload. Furthermore, we obtained similar results using 5-FU and DDP (Figure 5 & 6), suggesting that TIGAR-mediated enhancement of $[Ca^{2+}]i$ was involved in drug resistance in HCC.

Conclusions

In summary, TIGAR evoked Ca^{2+}-dependent Calpain and activated NF-κB in HCC cells. Elevated Ca^{2+} appears to be the mechanism in which TIGAR promotes proliferation and drug resistance in HCC. Our study suggests that targeting TIGAR is a therapeutic strategy for HCC chemotherapy.

Abbreviations

Abbreviations	Definition
TIGAR	TP53-induced glycolysis and apoptosis regulator
HCC	Hepatocellular carcinoma
[Ca2+]i	Cytosolic free Ca2+ concentrations
NMDP	Nimodipine
CCK-8	Cell counting kit-8
Epi	Epirubicin
5-FU	5-fluorouracil
DDP	Cisplatinum
NF-κB	Nuclear factor kappa B
oeTIGAR	Overexpression of TIGAR
siTIGAR	SiRNA-mediated TIGAR knockdown
c-caspase 3	Cleaved-caspase 3
PCNA	Proliferating cell nuclear antigen
IκBα	Inhibitor of NF-κB

Declarations

Ethical Approval and Consent to participate

Our experimental protocol was approved by the Second Affiliated Hospital of Soochow University in accordance with the Declaration of Helsinki.

Consent for publication

Written informed consent for publication was obtained from all participants.

Availability of supporting data

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

Competing interests

The authors declare no competing interests.

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Authors' contributions

H.Z., Z.Y.Y. and W.C. conceived and designed the research. J.M.X., Y.Q.S. and X.Y.F. performed experiments, analyzed, and interpreted data. Z.Y.F., W.L. and X.M.M. analyzed data. D.K.G. and P.D. interpreted data. W.C. wrote the paper. All authors edited the paper for submission.

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Figures

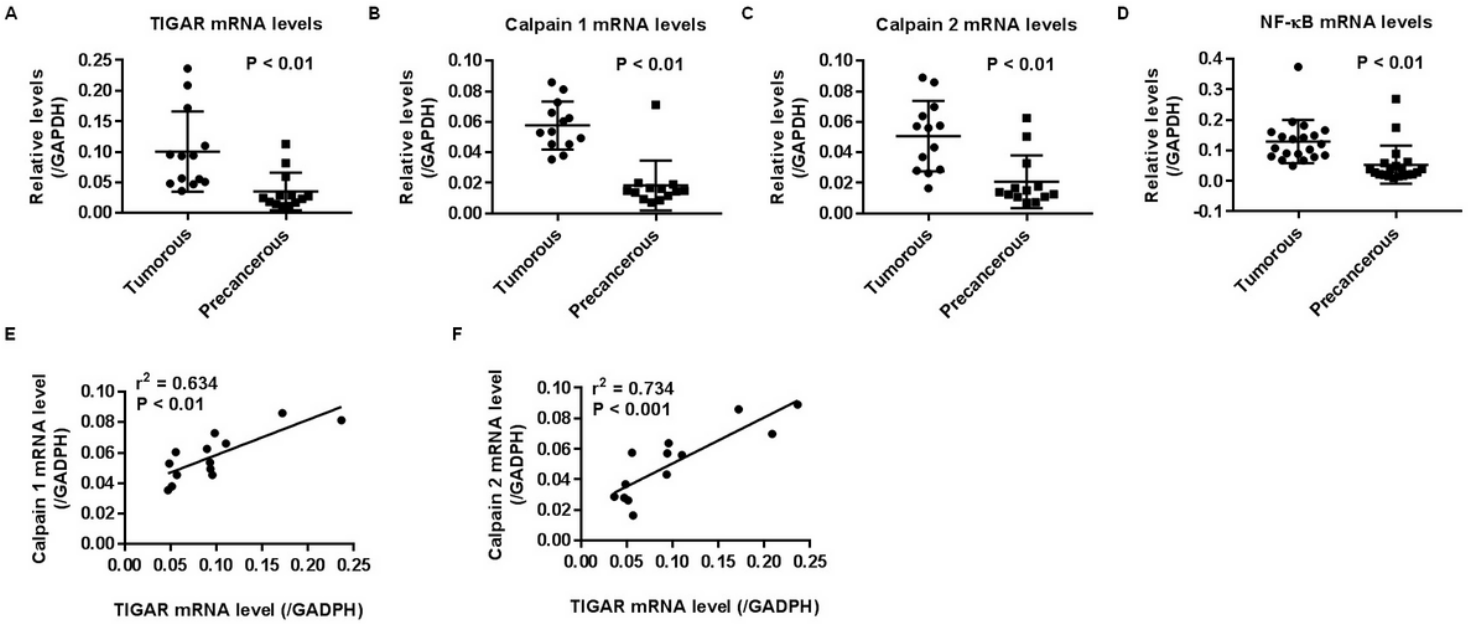

Figure 1

Expression of TIGAR and its correlation to Calpain 1 and Calpain 2 in HCC.

A-D. mRNA levels of TIGAR, Calpain1, Calpain2, and NF-κB in 13 pairs of malignant and premalignant tissues from patients with HCC recruited from the Second Affiliated Hospital of Soochow University were assessed by qRT-PCR. **E&F**. Pearson r analysis showing the correlation between TIGAR and Calpain1, as well as the correlation between TIGAR and Calpain 2 in HCC tumor tissue. Difference between groups was determined using one-way ANOVA with Tukey–Kramer method. Correlations between TIGAR and Calpain1, as well as TIGAR and Calpain2 in HCC tumor samples were assessed by Pearson r analysis. $P < 0.01$ vs. Tumorous.

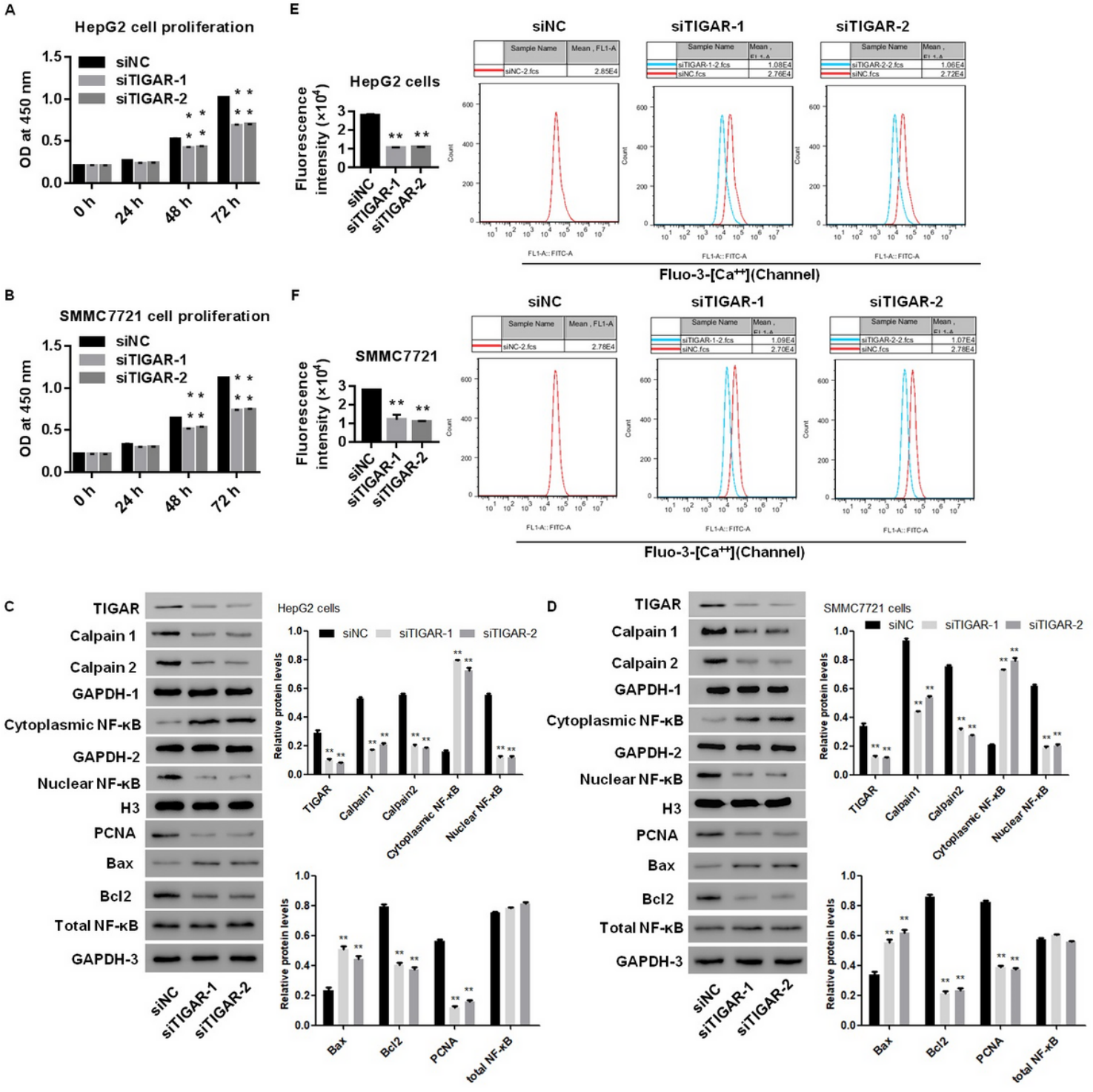

Figure 2

siTIGAR inhibited proliferation of HCC cells, blocked Ca^{2+} signaling, and suppressed NF-κB activation.

A&B. Proliferation of HepG2 and SMMC7721**,** assessed by CCK-8. **C&D**. Western blot showed that siTIGAR significantly reduced TIGAR, Ca^{2+}-dependent proteases (Calpain 1 and Calpain 2), Bcl2, PCNA, and nuclear NF-κB. However, it increased cytoplasmic NF-κB, suggesting a successful establishment of TIGAR silencing similar to blockade of Ca^{2}+ signaling and the inhibition NF-κB activation. TIGAR, Bcl2,

Bax, Calpain 1, and Calpain 2 were normalized by GAPDH-1; cytoplasmic NF-κB was normalized by GAPDH-2, PCNA and nuclear NF-κB was standardized by H3. **E&F**. Intracellular [Ca^{2+}]i assessed by flow cytometry using Fura 2-AM regent. Difference between groups was determined using one-way ANOVA with Tukey–Kramer method. **P < 0.01 vs. siNC.

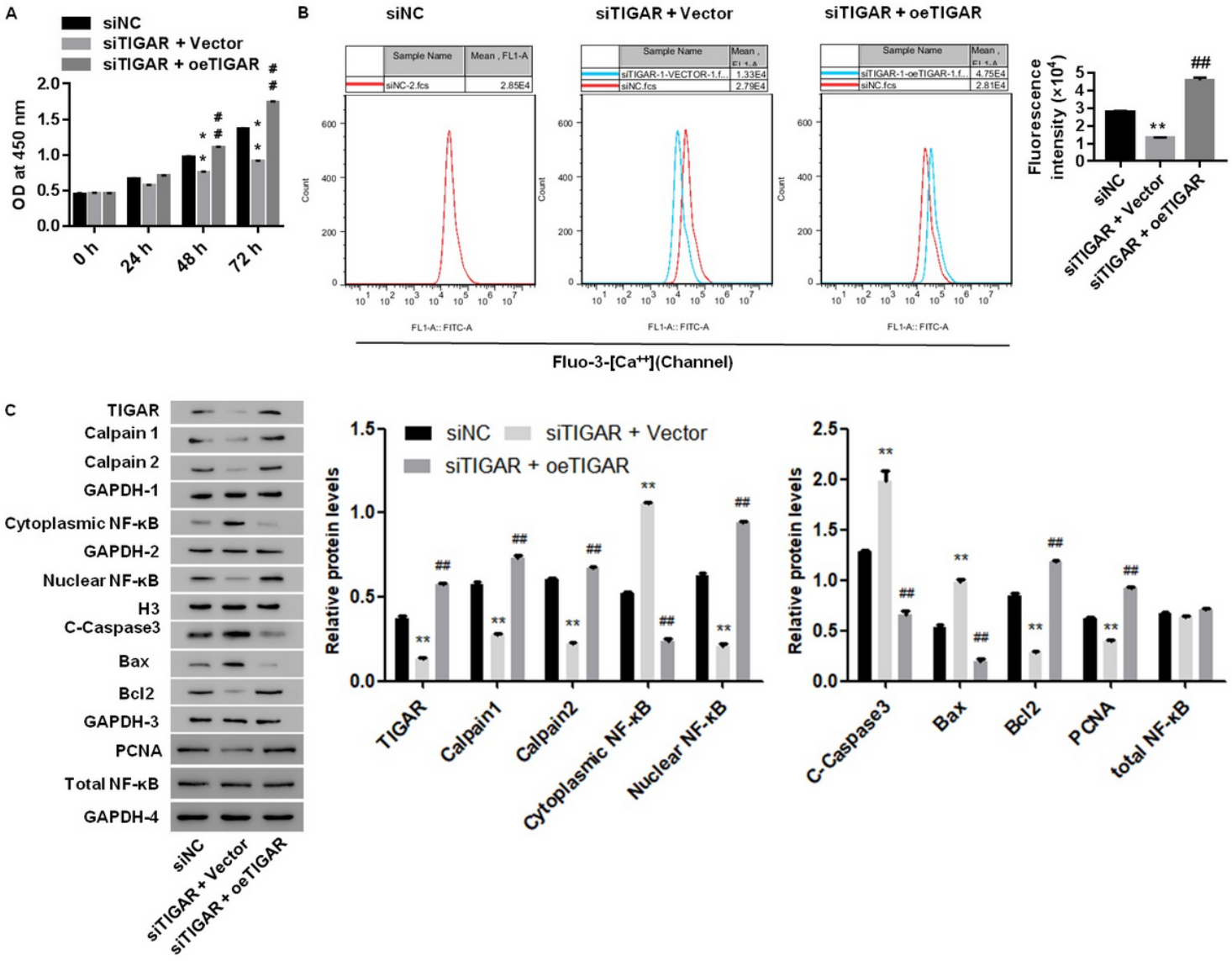

Figure 3

Rescue assays of TIGAR in the events involved in HCC.

A. Proliferation of HepG2, assessed by CCK-8. **B**. Intracellular [Ca2 +]i assessed by flow cytometry using Fura 2-AM regent. **C**. Relative protein levels. Difference between groups was determined using one-way ANOVA with Tukey–Kramer method. **P <0.01 vs. siNC; ##P < 0.01 vs. siTIGAR+ Vector.

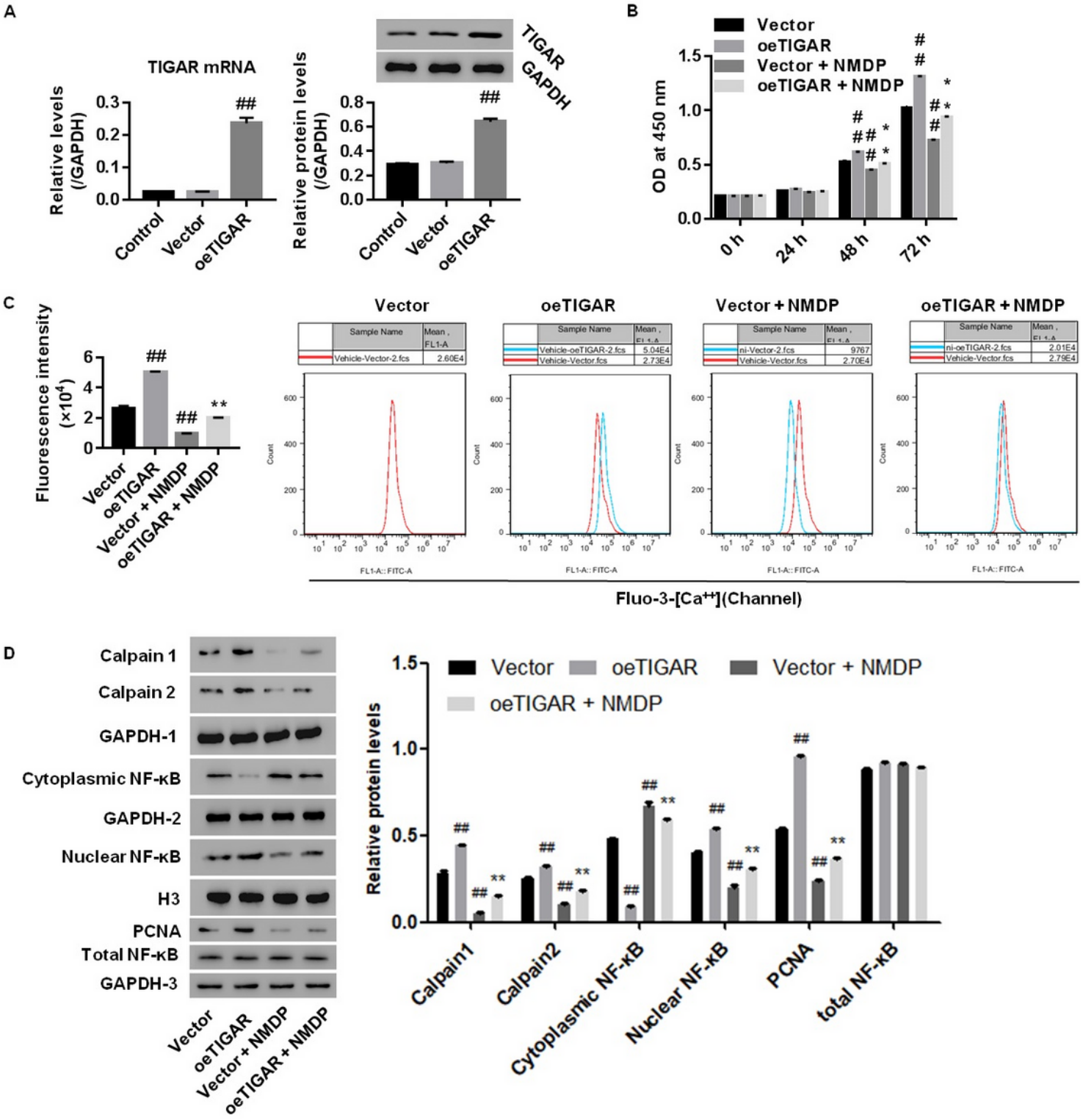

Figure 4

oeTIGAR stimulated Ca^{2+} signaling to exacerbate HCC.

HepG2cells were transfected with oeTIGAR for 24h. **A**. oeTIGAR dramatically enhanced TIGAR mRNA and protein, suggesting a successful construction of oeTIGAR. **B**. Proliferation of cells. **C**. Intracellular $[Ca^{2+}]i$, **D**. Protein expression of PCNA, Calpain 1, Calpain 2, as well as total NF-κB, nuclear NF-κB, and

cytoplasmic NF-κB. Difference between groups was determined using one-way ANOVA with Tukey–Kramer method. ##P < 0.01 vs. Vector; **P < 0.01 vs. Vector + NMDP.

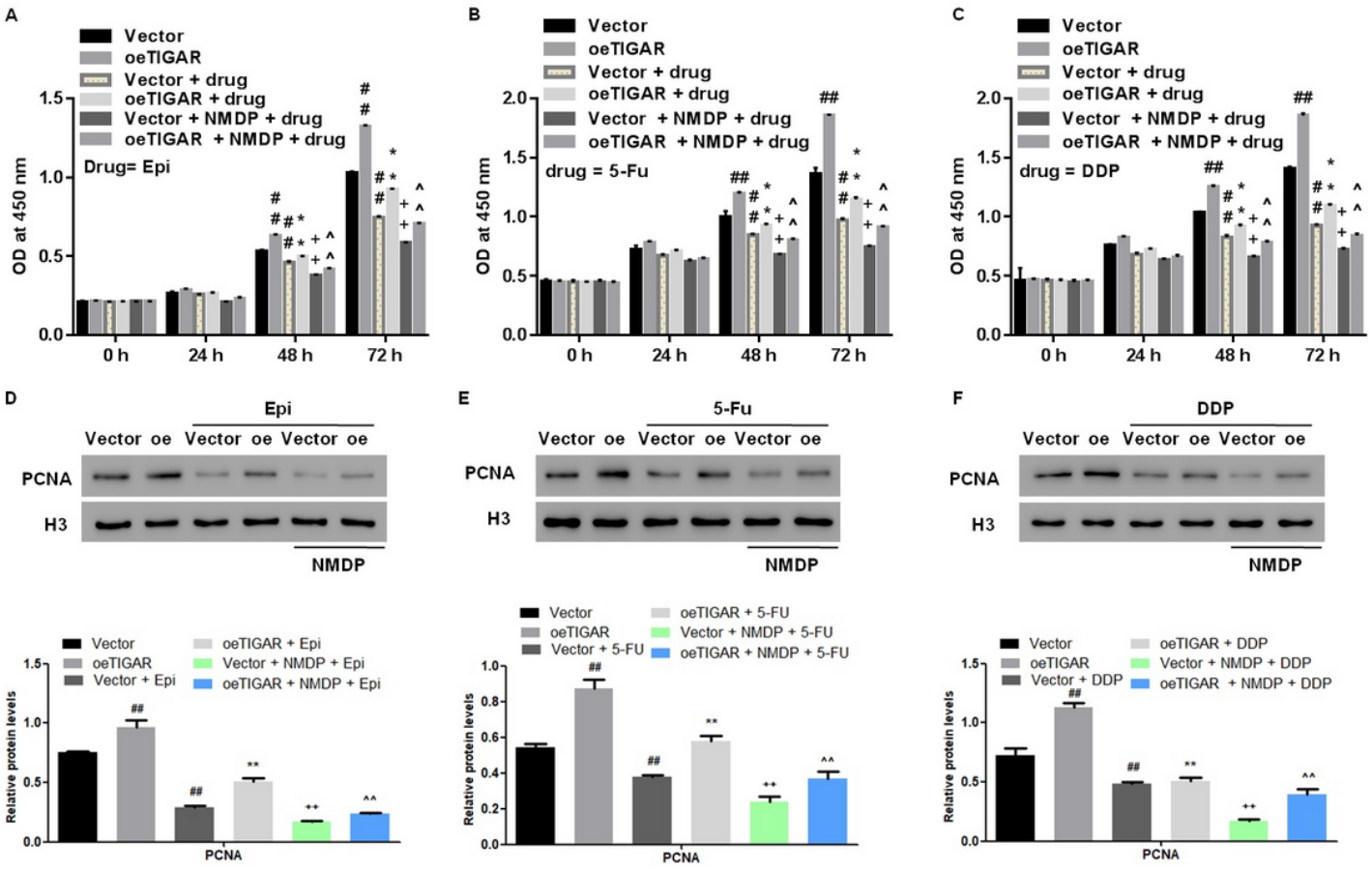

Figure 5

TIGAR triggered Ca^{2+} signaling to induce drug resistance in HCC cells.

oeTIGAR-transfected HepG2 cells were treated with Epi, 5-FU, and DDP, followed by exposure to10 μM of NMDP. **A-C**. Proliferation of HepG2 was assessed by CCK-8. **D-F**. Protein expression of PCNA at 48 h after treatment. Difference between groups was determined using one-way ANOVA with Tukey–Kramer method. ##P < 0.01 vs. Vector; **P < 0.01 vs. Vector + drug; ^^P < 0.01 vs. Vector + drug + NMDP.

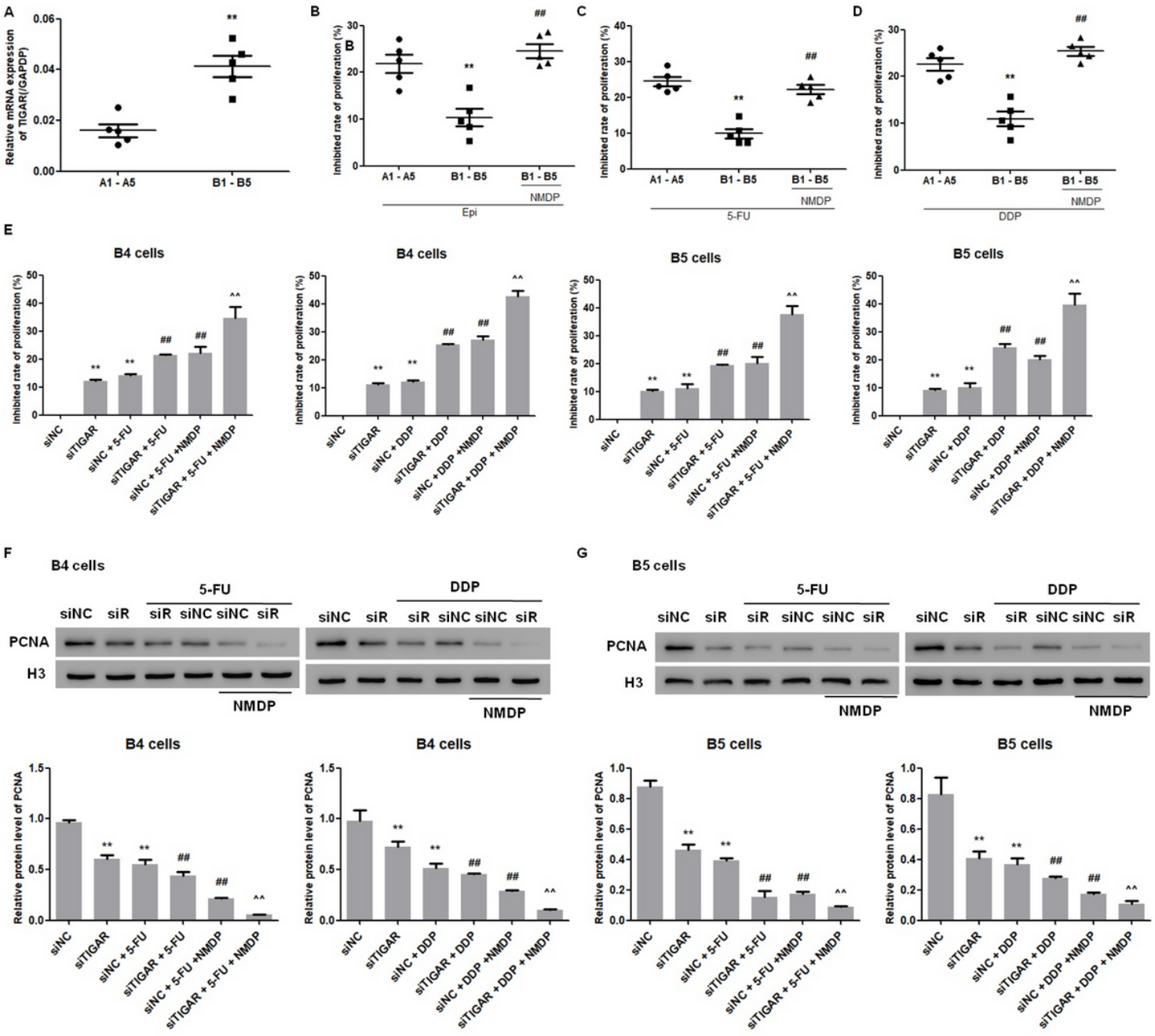

Figure 6

siTIGAR prevented drug resistance in HCC cells.

HCC cells with high TIGAR expression were transfected with siTIGAR and treated with 5-FU and DDP, followed by exposure to10 μM of NMDP. **A**. RT-PCR showing the mRNA levels of TIGAR in primary HCC cell lines (n = 10), isolated from tumor tissues of patients with HCC. **B-D**. The inhibited proliferation rate of primary HCC cell lines after exposure drugs plus NMDP (10 μM). **E.** The proliferation of cell lines B4 and B5 were reduced by NMDP and anti-HCC drugs. **F&G.** Protein levels of PCNA at 48 h after treatment. Difference between groups was determined using one-way ANOVA with Tukey–Kramer method. **P < 0.01vs. siNC; ##P < 0.01 vs. siNC + drug; ^^P < 0.01 vs. siNC + drug + NMDP.