**Malignant progression of liver cancer progenitors requires KAT7-****acetylated and cytoplasm-translocated G-protein** **GαS**

Ye Zhou, Kaiwei Jia, Suyuan Wang, Zhenyang Li, Yunhui Li, Shan Lu, Yingyun Yang, Liyuan Zhang, Mu Wang, Yue Dong, Luxin Zhang, Wannian Zhang, Nan Li, Yizhi Yu, Xuetao Cao, and Jin Hou

**Materials and methods**

**Cell lines**

Hepatoma cell line HepG2, hepatocyte cell line HHL5, and mouse fetal liver cell line BNL CL.2 were obtained from cell bank of Chinese Academy of Sciences. HepG2 was cultured in RPMI 1640 with 10% FBS as routine(38). HHL5 was cultured in DMEM with 10% FBS. BNL CL.2 was cultured in DMEM with 10% FBS and NAA.

**Reagents**

Antibodies specific to phospho-STAT3 Y705 (4113), STAT3 (9139), SOCS3 (52113), JAK1 (3344), JAK2 (3230), SHP1 (3759), SHP2 (3397), SOCS1 (3950), PIAS1 (3550), PIAS3 (9042), KAT7 (58418), β-actin (4970), normal rabbit IgG (2729) and horseradish peroxidase-coupled secondary antibodies (7074 and 7076) were from Cell Signaling Technology (Danvers, MA). Antibody specific to GαS (sc-135914) and normal mouse IgG (sc-2025) for immunoprecipitation were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody specific to ATP1A1 (14418-1-AP) was from Proteintech Group (Wuhan, China). Antibody specific to Flag-tag (F1804) was from Sigma (St. Louis, MO). Antibodies specific to V5-tag (ab9116), GαS (ab83735) and GαS (ab283266) were from Abcam (Cambridge, MA). Antibody specific to acetyl-GαS (K28) was constructed by PTM Biolabs Inc (Hangzhou, China). Antibody specific to CD44-PE (130102606) and Anti-PE MicroBeads (130048801) were from Miltenyi Biotec. Protein G Agarose (P4691), ANTI-FLAG M2 Affinity Gel (A2220), Nethylmaleimide (NEM) (040260-5G), hydroxylamine (HAM) solution (467804), and DEN (N0258) were from Sigma.Recombinant human IL-6 (200-06) was from PeproTech (Rocky Hill, NJ). Recombinant mouse IL-6 (575706) was from BioLegend (San Diego, CA). NF449 (1391) was from Tocris Bioscience (Shanghai, China)(39). MEM non-essential animo acid solution (100×) (NAA, M7145) was from Sigma. Biotin-BMCC (C100222) was purchased from Sangon Biotech (Shanghai, China). Fetal Bovine Serum (FBS, 10099), RPMI 1640 Medium (11875-093) and DMEM (11965-092) were from Gibco (Shanghai, China).

**Transfection**

5×104 or 2×105 cells were seeded into each well of 24-well or 6-well plates respectively and incubated overnight, and then transfected with RNAs or plasmids using INTERFERin or jetPEI (Polyplus-transfection, France) respectively as we described previously(19, 38). The human GαS specific siRNA were 5’-GCA GCU ACA ACA UGG UCA UTT-3’ (sense) and 5’-AUG ACC AUG UUG UAG CUG CTT-3’ (antisense); the mouse GαS specific siRNA were 5’-GCA AAU CGA AGA UUG AGG ATT-3’ (sense) and 5’- GCA AAU CGA AGA UUG AGG ATT-3’ (antisense); the scrambled control RNA sequences were 5’-UUC UCC GAA CGU GUC ACG UTT-3’ (sense) and 5’-ACG UGA CAC GUU CGG AGA ATT-3’ (antisense). siRNA was synthesized from GenePharma (Shanghai, China). siRNA duplexes were transfected at a final concentration of 10 nM.

**Primers sequences for mice identification**

Genotyping was done by PCR analysis on genomic DNA extracted from mice tails as previously described(40). miR-143-/- forward: 5’-AGG GTG AGT AGG TGG TTT GG-3’, reverse: 5’-AGT GTC GTA TCG TTG GGT TG-3’; *Socs3F/F* forward: 5’-CGG GCA GGG GAA GAG ACT GT-3’, reverse: 5’-TCG ACT GTC CTC GGT CAC-3’; *Il6-/*- forward: 5’-TTC CAT CCA GTT GCC TTC TTG G-3’, reverse: 5’-TTC TCA TTT CCA CGA TTT CCC AG-3’ and 5’-CCG GAG AAC CTG CGT GCA ATC C-3’; *Il6raF/F* forward: 5’-GAA GGA GGA GCT TGA CCT TGG-3’, reverse: 5’-AAC CAT GCC TAT CAT CCT TTG G-3’; *GαSF/F(OVER)* forward: 5’-TGC TGA GCC AGA CCT CCA T-3’ and 5’-TGC ATC GCA TTG TCT GAG TA-3’, reverse: 5’-GAC AAA ACC GAA AAT CTG TGG-3’; Alb-cre forward: 5’-TGC AAA CAT CAC ATG CAC AC-3’ and 5’-GAA GCA GAA GCT TAG GAA GAT GG-3’, reverse: 5’-TTG GCC CCT TAC CAT AAC TG-3’; *GαSF/F(KO)* forward: 5’-GAG ATA CTT CAG GTA GAT GCT A-3’, reverse: 5’-CAG GCT TGT CAC ACT TCA-3’; *Kat7F/F* forward: 5’-CTG CGG AGG CTG GAG GTA TTT GG -3’, reverse: 5’-AAA TCA AAA TGG TCT GGC TAG TC-3’.

**DEN-induced HCC model**

For DEN-induced primary HCC, mice at postnatal day 15 were injected intraperitoneally with 25 mg/kg DEN, and then maintained on regular chow food as reported(38). Livers and tumors were photographed and harvested for analysis eight months after the initial injection. As with high-dose DEN-induced liver injury, 2-months old mice were injected intraperitoneally with 100 mg/kg DEN, and serum and liver tissues were obtained in the indicated time post injection(41).

**Isolation of primary hepatocytes and HcPCs**

Primary hepatocytes were isolated through hepatic perfusion methods with type IV collagenase digestion, and 1×107 cells were seeded into each well of 6-well plates and incubated overnight. Cell aggregates were isolated by filtration through 70 and 40 μm sieves. After dispersed the aggregates into single cells, HcPCs were enriched for CD44+ cells using magnetic beads as reported(4).

**Transplantation of HcPCs**

Eight-week-old C57BL/6J mice pretreated with Retrorsine (70 mg/kg) were injected intrasplenically with 103 viable CD44+ HcPCs. After two weeks, the transplanted mice were injected weekly with 3×0.5 ml/kg CCl4 intraperitoneally to induce liver inflammation and drive tumorigenesis as reported(4). Mice were sacrificed five months later, and tumor numbers and diameter were examined.

**RNA isolation and Real-time PCR analysis**

Total RNA was extracted from frozen HCC tissues, mouse liver tissues, and cell lines using TRIzol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Real-time quantitative RT-PCR (qRT-PCR) analysis was performed using LightCycler (Roche, Switzerland) and SYBR RT-PCR kit (Takara, Dalian, China) as previously described(19). For gene and miRNA expression analysis, q-PCR primers were human *GαS* forward: 5’-TGC CTC GGG AAC AGT AAG AC-3’, reverse:5’-GCC GCC CTC TCC ATT AAA C-3’; mouse *GαS* forward: 5’-CAG AGC CTC CAT TGG GGT C-3’, reverse: 5’-GCT TCT CGC TCA ACT GGG G-3’; mouse *Saa1* forward: 5’-AGG GTT TTT TTC ATT TGT TC-3’, reverse: 5’-TCT GAG TTT TTC CAG TTA GC-3’; mouse *Il-6* forward: 5’-TGA TGC ACT TGC AGA AAA CA-3’, reverse: 5’-ACC AGA GGA AAT TTT CAA TAG GC-3’; mouse *Tnf-α* forward: 5’-CAG GCG GTG CCT ATG TCT C-3’, reverse: 5’-CGA TCA CCC CGA AGT TCA GTA G-3’); mouse *Hgf* forward: 5’-ACT TCT GCC GGT CCT GTT G-3’, reverse: 5’-CCC CTG TTC CTG ATA CAC CT-3’; internal control human *β-actin* forward: 5’-GGC GGC ACC ACC ATG TAC CCT-3’, reverse: 5’-AGG GGC CGG ACT CGT CAT ACT-3’; mouse *β-actin* forward: 5’-AGT GTG ACG TTG ACA TCC GT-3’, reverse: 5’-GCA GCT CAG TAA CAG TCC GC-3’); miR-143 RT: 5’-GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACG AGC T-3’; miR-143 forward: 5’-GCT GAG ATG AAG CAC TGT-3’, reverse: 5’-GTG CAG GGT CCG AGG T-3’; internal control U6 RT: 5’-AAC GCT TCA CGA ATT TGC GT-3’; U6 forward: 5’-CTC GCT TCG GCA GCA CA-3’, reverse: 5’-AAC GCT TCA CGA ATT TGC GT-3’. The relative expression level of the individual genes was normalized to that of internal control by using 2-ΔΔCt cycle threshold method in each sample(42).

**Immunoprecipitation and Western blot**

Cells or tissues were harvested and lysed with cell lysis buffer (Cell Signaling Technology) supplemented with protease inhibitor cocktail (Calbiochem). Protein concentrations of the lysates were measured with bicinchoninic acid (BCA) assay (Pierce) and equalized with the lysis buffer. Equal amount of the extracts was used for immunoprecipitation, or loaded and subjected to SDS-PAGE, transferred onto nitrocellulose membranes, and then blotted as described previously(41). Protein levels were quantified using Image J software and β-actin was used as a loading control.

**Tandem Mass Tag (TMT)-based quantitative proteomic analysis**

Aggregates and nonaggregates protein samples from four groups of male mice (five mice per group) five months post initial DEN injection were digested by trypsin and the peptide was labeled according to the manufacturer’s protocol for TMT kit. The tryptic peptides were fractionated into fractions by high pH reverse-phase HPLC and then processed LC-MS/MS analysis. The resulting MS/MS data were processed using Maxquant search engine (v.1.5.2.8). Tandem mass spectra were searched against human uniprot database concatenated with reverse decoy database. TMT-based quantitative proteomic analysis was conducted by PTM Biolabs Inc. The mass spectrometry proteomics data have been deposited in the ProteomeXchange Consortium via the iProX partner repository(43), with the dataset identifier PXD022611.

**Tissue microarray and Immunohistochemistry (IHC)**

Protein levels of GαS and STAT3 Y705 phosphorylation in tissues was determined using tissue microarray (TMA) as described previously(39, 44). The GαS antibody (ab283266, Abcam) and STAT3 pY705 antibody (4113, CST) were diluted 1:200 in PBS containing 1% bovine serum albumin and incubated at 4°C overnight. On the next day, immunostaining was performed using Dako ChemMate EnVision Detection Kit Peroxidase/Diaminobenzidine (DAB) Rabbit/Mouse (Dako, Glostrup, Denmark), which resulted in a brown-colored precipitate at the antigen site. Subsequently, sections were counterstained with hematoxylin (Zymed Laboratories, San Francisco, CA), mounted in non-aqueous mounting medium and cover slipped. Quantitative scanning approach to evaluate the staining was Aperio ImageScope from Leica Company, and the positivity value ×100 represented the quantitative levels of GαS and pSTAT3 Y705 as we described previously(41, 44).

**Dual-luciferase reporter assay**

pMIR-promoter-Firefly plasmids, RL-TK-Renilla vector, and indicated RNAs were co-transfected into HEK293T cells. After 24 hours, the activities of firefly and Renillaluciferases are measured sequentially from a single sample as the DLR™ Assay Kit (Promega) protocol described.

**Confocal microscopy**

HHL5 cell slides were labeled with rabbit specific antibody against V5 and mouse antibody against Flag, and then labeled with Alexa Fluor 546 goat anti-mouse IgG (Invitrogen A-11003) and Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen A-11034) following the standard protocol. Tissue slides of mouse livers in Fig. 6E and primary hepatocytes in Fig. 5E were labeled with rabbit specific antibody against GαS (Abcam ab283266), and then labeled with Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen A-11034). Tissue slides of mouse livers in Figure 5F were labeled with mouse specific antibody against Flag, and then labeled with Alexa Fluor 546 goat anti-mouse IgG (Invitrogen A-11003). Labeled slides were viewed with a Leica TCS confocal laser microscope.

**Generation of antibody specific to K28-acetylated GαS**

The synthesized acetyl-modified peptides and a non-modified control peptide were cross-linked with the carrier protein keyhole limpet hemocyanin (KLH) to enhance immunogenicity. New Zealand white rabbits were immunized four times respectively on day 1, 21, 35, and 54. Rabbit serum was obtained for multi-step affinity purification. After the serum was filtered with a filter, the sample was loaded on the balanced protein A chromatography column, and washed with PBS buffer solution and eluted with 150 mM glycine buffer, and then the eluate was collected and added with neutralization buffer solution to adjust the pH to 7. The crude IgG obtained after protein A purification was loaded onto the balanced antigen peptide affinity chromatography column to specifically enrich the target antibody. The target antibody was loaded onto a non-modified affinity chromatography column, and the effluent was collected directly to remove non-specific antibody components. The purified antibodies were tested for quality using Dot Blot and Western blot. The antibodies were generated by PTM Biolabs Inc(45).

**Acyl-Biotin Exchange (ABE) assay**

The ABE assay was performed as previously reported(46). Briefly, HHL5 cells transfected with Flag-tagged GαS, GαS C3S, GαS C3Y, GαS K28A, or GαS K28Q were lysed with the cell lysis buffer (CST) supplemented with protease inhibitor cocktail (Calbiochem) and NEM (50 mM, Sigma) for 1.5 hours at 4℃, respectively. Protein concentrations of the lysates were measured with bicinchoninic acid (BCA) assay (Pierce) and equalized with the lysis buffer. The supernatants were incubated with anti-Flag M2 Affinity Gel (Sigma) at 4℃ for 3 hours. After incubation, the gel was washed five times with cell lysis buffer (pH 7.5) and then three times with cell lysis buffer (pH 7.2). Then, beads were incubated with cell lysis buffer supplemented with HAM (1 M) at room temperature for 1 hour and washed four times with cell lysis buffer (pH 7.2) and three times with cell lysis buffer (pH 6.2). Finally, the gel was treated with Biotin-BMCC (5 μM) in cell lysis buffer (pH 6.2) at 4℃ for 1 hour. Equal amount of the extracts was used for immunoprecipitation, or loaded and subjected to SDS-PAGE, transferred onto nitrocellulose membranes, and then blotted as described previously using anti-Flag M2-antibody (Sigma) and streptavidin-HRP (Beyotime)(46).

**Accession number and Data availability**

The accession number of mass spectrometry proteomics data reported in this paper is PXD022611 (ProteomeXchange, http://proteomecentral.proteomexchange.org). All the original unprocessed gels and images, and all the original source data of figures have been deposited and available at the public research database Mendeley Data Reserved https://data.mendeley.com/datasets/hdkhhsjv8n/2

**References**

[38] **Hou J**, **Zhou Y**, **Zheng Y**, Fan J, Zhou W, Ng IO, et al. Hepatic RIG-I predicts survival and interferon-α therapeutic response in hepatocellular carcinoma. Cancer Cell 2014;25:49-63.

[39] Hohenegger M, Waldhoer M, Beindl W, Böing B, Kreimeyer A, Nickel P, et al. Gsalpha-selective G protein antagonists. Proc Natl Acad Sci U S A 1998;95:346-351.

[40] **Zheng Q**, **Hou J**, Zhou Y, Li Z, Cao X. The RNA helicase DDX46 inhibits innate immunity by entrapping mA-demethylated antiviral transcripts in the nucleus. Nat Immunol 2017;18:1094-1103.

[41] **Li Z**, **Zhou Y,** **Zhang L**, Jia K, Wang S, Wang M, et al. microRNA-199a-3p inhibits hepatic apoptosis and hepatocarcinogenesis by targeting PDCD4. Oncogenesis 2020;9:95.

[42] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCt method. Methods 2001;25:402-408.

[43] **Ma J**, **Chen T**, Wu S, Yang C, Bai M, Shu K, et al. iProX: an integrated proteome resource. Nucleic Acids Res 2019;47:D1211-D1217.

[44] **Han D**, **Li J**, Wang H, Su X, Hou J, Gu Y, et al. Circular RNA circMTO1 acts as the sponge of microRNA-9 to suppress hepatocellular carcinoma progression. Hepatology 2017;66:1151-1164.

[45] **Wang L**, **Wen M**, Cao X. Nuclear hnRNPA2B1 initiates and amplifies the innate immune response to DNA viruses. Science 2019;365: eaav0758.

[46] **Lu Y**, **Zheng Y**, **Coyaud É**, **Zhang C**, Selvabaskaran A, Yu Y, et al. Palmitoylation of NOD1 and NOD2 is required for bacterial sensing. Science 2019;366:460-467.

**Supporting Figures and Figure Legends**

**Supporting Figure 1. Increased GαS in HcPCs is mediated by impaired targeting of miR-143.**

(A) The consistent eight upregulated proteins and eight downregulated proteins in the aggregates compared to nonaggregates were shown.

(B) *Cd44*, *Ly6d, Epcam, and Afp* mRNA expression was examined by qRT-PCR in normal hepatocytes, nonaggregated hepatocytes and HcPCs (n=4, one-way ANOVA and Tukey’s multiple comparisons test).

(C) STAT3 phosphorylation was examined by Western blot in the nonaggregated hepatocytes and HcPCs from DEN-treated mice for five months.

(D) *GαS* mRNA expression was examined by qRT-PCR in normal hepatocytes, nonaggregated hepatocytes and HcPCs (n=4, one-way ANOVA and Tukey’s multiple comparisons test).

(E, F) HEK293T cells were co-transfected with negative control/miRNA mimics as indicated, together with pRL-TK and pMIR-Report firefly luciferase reporter construct containing the targeting region of *GαS*. Relative firefly luciferase activity was measured and normalized by Renilla luciferase activity 24 hours post transfection (n=3, unpaired *t*-test).

(G) HHL5 and BNL CL.2 cells were transfected with negative controls, miR-143 mimics or inhibitors for 72 hours. GαS protein level was examined by Western blot.

(H) *GαS* mRNA expression was examined by qRT-PCR in HHL5 and BNL CL.2 cells transfected with negative control or miR-143 mimics (n=3, unpaired *t*-test).

(I) miR-143 mRNA expression was examined by qRT-PCR in normal hepatocytes, nonaggregated hepatocytes, and HcPCs (n=4, one-way ANOVA and Tukey’s multiple comparisons test).

(J, K) Constructions of *GαS* hepatocyte-specific knockin (J) and knockout (K) mice.

Data are shown as mean ± SD or photographs from one representative of three independent experiments. \*\*, p < 0.01.

**Supporting Figure 2. GαS-promoted hepatocarcinogenesis is dependent on IL-6.**

(A) Serum ALT and AST were analyzed in two-month-aged male *GαSF/F(OVER)* and *GαShep+/+*mice intraperitoneally injected with DEN (n=3, unpaired t-test).

(B) Construction of miR-143 knockout mice.

(C) miR-143 expression was examined by qRT-PCR in mouse primary hepatocytes and Kupffer cells of male wild-type and miR-143-/- mice (n=3, unpaired t-test).

(D) *GαS* mRNA expression was examined by qRT-PCR in the livers of male wild-type and miR-143-/- mice (n=3, unpaired t-test).

(E) GαS protein level was examined by Western blot in liver tissues and primary hepatocytes of male wild-type and miR-143-/- mice.

(F) Tumor incidence (chi-square test), number and maximum diameter (unpaired t-test) of DEN-induced HCC in male wild-type, miR-143-/-, *Il6*-/-,andmiR-143-/-*Il6*-/- mice were analyzed (n=10).

(G) Productions of IL-6, TNF-α, and HGF in liver tissues were examined by qRT-PCR in two-month-aged male *GαSF/F(OVER)* and *GαShep+/+*mice treated with DEN (n=3, unpaired t-test).

(H) IL-6 mRNA level in HcPCs isolated from male *GαSF/F(OVER)* and *GαShep+/+*mice was examined by qRT-PCR analysis (n=3, unpaired t-test).

(I) Representative livers of male mice transplanted by intrasplenic injection with isolated HcPCs from male *GαSF/F(OVER)* and *GαShep+/+* mice or *GαSF/F(KO)* and *GαShep-/-* mice.

Data are shown as mean ± SD or photographs from one representative of three independent experiments. △, p>0.05; \*, p<0.05; \*\*, p < 0.01.

**Supporting Figure 3. GαS promotes IL-6 induced-STAT3 phosphorylation independent on its catalytic function.**

(A, B) Male wild-type and miR-143-/- mice were injected intraperitoneally with DEN or injected with recombinant IL-6 through hepatic portal vein for the indicated time periods. STAT3 phosphorylation in the liver tissues was examined by Western blot.

(C) *GαS* mRNA level was examined by qRT-PCR in HHL5 and BNL CL.2 cells transfected with human or mouse GαS siRNAs respectively (n=3, unpaired t-test).

(D) cAMP amount was examined by ELISA in HHL5 and BNL CL.2 cells treated with NF449 (50 μM) for 24 hours (n=3, unpaired t-test).

(E) IL-6-induced STAT3 phosphorylation was analyzed in HHL5 and BNL CL.2 cells treated with NF449.

(F) cAMP amount was examined by ELISA in HHL5 cells transfected with control, GαS, GαS LOF (loss-of-function), and GαS over-activation plasmids (n=3, one-way ANOVA and Tukey’s multiple comparisons test).

(G) IL-6-induced STAT3 phosphorylation was analyzed in HHL5 cells transfected as F.

Data are shown as mean ± SD or photographs from one representative of three independent experiments. \*, p<0.05; \*\*, p < 0.01.

**Supporting Figure 4. Cytoplasm-translocated GαS associates with STAT3 upon IL-6 stimulation to impede SOCS3-STAT3 interaction.**

(A) HHL5 and HepG2 cells were treated with recombinant IL-6 for the indicated time periods. The association between STAT3 with JAK1, JAK2, SHP1, SHP2, and SOCS3 were examined using co-IP analysis.

(B) HHL5 cells were transfected with control or GαS loss of AC function (LOF) construct, and the association between STAT3 with SOCS3 and GαS LOF were examined using co-IP analysis.

(C) HHL5 cells were treated with NF449, and the association between STAT3 with SOCS3 and GαS were examined using co-IP analysis.

(D) The expressions of SOCS3 in the primary hepatocytes from male *Socs3F/F* and *Socs3hep-/-* mice were confirmed by Western blot.

Data are shown as photographs from one representative of three independent experiments.

**Supporting Figure 5. IL-6-induced acetylation of GαS at K28 mediates its cytoplasm-translocation and association with STAT3.**

(A) Tandem mass spectrometry spectrum of GαS acetylated K28 fragment. Detected productions are indicated in red (b ions) and blue (y ions).

(B) Sequence alignment of GαS K28 from the indicated species.

(C) Synthesized peptides used to generate antibodies specific to acetylated GαS at K28, and for specificity examination. Dot blot analysis of antibodies specific to acetylated GαS at K28 or K28 unmodified (free) peptides as indicated.

(D) IL-6-induced cytoplasmic translocation of GαS was examined using Western blot in HHL5 cells transfected with Flag-tagged GαS mutants as indicated.

(E) Construction of GαS K28A or K28Q mutant mice.

(F) IL-6-induced STAT3 phosphorylation was evaluated in liver tissues from male GαS K28A or K28Q mutant mice upon IL-6 injection through hepatic portal vein.

(G) IL-6-induced GαS-STAT3 association and SOCS3-STAT3 interaction were examined using co-IP in the liver tissues from male wild-type, GαS K28A or K28Q mutant mice upon IL-6 injection through hepatic portal vein.

Data are shown as photographs from one representative of three independent experiments.

**Supporting Figure 6. IL-6-induced acetylation of GαS at K28 is mediated by acetyltransferase KAT7.**

(A) PAGE gel resolution of immunoprecipitated GαS and its associated proteins from HHL5 hepatocyte cell line treated with IL-6 for 30 minutes. Different bands were analyzed by MS. Arrow indicates the band of the protein detected by MS.

(B) Tagged KAT7, GαS and their truncates were constructed and co-transfected into HEK293T cells as indicated, and their association was examined using co-IP analysis.

(C) Construction of *Kat7* hepatocyte-specific knockout mice. d, KAT7 protein level was examined by Western blot in the hepatocytes from *Kat7F/F* and *Kat7hep-/-*mice.

Data are shown as photographs from one representative of three independent experiments.

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**Supporting Figure 7. GαS expression is correlated to human HCC progression and prognosis.**

(A) Representative H&E staining of human normal liver tissues and hepatic dysplastic nodules. N, normal; ND, dysplastic nodule. Scale bar, 100 μm.

(B) The protein levels of GαS were densitometrically quantified by IHC in HCC and paired non-tumor tissues from HCC Cohort 1 and 2 (paired t-test). The horizontal lines in the box plots represent the median, the boxes represent the interquartile range, and the whiskers represent the 2.5th and 97.5th percentiles.

(C) The correlation between GαS and STAT3 phosphorylation in HCC tissues from Cohort 1 and 2 were analyzed by Pearson’s correlation coefficient assay.

(D, E) The correlation between GαS levels in HCC tissues and tumor TNM stage (D) or histological grade (E) were analyzed by Spearman’s correlation coefficient assay.

(F) Shown are Kaplan-Meier survival curves of overall and disease-free survival in HCC Cohort 1 and 2. The median values of GαS protein levels in each cohort were chosen as the cutoff points, with log-rank test for significance.

Data are shown as box plots, dot plots, or survival curves as indicated.

Supporting Table 1.Univariate and multivariate analysis of factors associated with overall survival

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Clinical variables | Cohort 1 n=131 | | | Cohort 2 n=129 | |
| Hazard ratio (95% CI) | p value | Hazard ratio (95% CI) | | p value |
| Univariate analysis |  |  | |  |  |
| GαS (higher vs. lower) | 4.6 (2.2-9.8) | <0.001 | | 3.6 (1.7-7.4) | 0.001 |
| Gender (male vs. female) | 1.2 (0.4-3.8) | 0.794 | | 0.9 (0.4-2.1) | 0.766 |
| Age (>50 vs. ≤50 years) | 0.7 (0.4-1.4) | 0.299 | | 0.4 (0.2-0.8) | 0.006 |
| HBV (positive vs. negative) | 1.6 (0.5-5.2) | 0.434 | | 1.0 (0.4-2.5) | 0.958 |
| Histological grade (>II vs. I/II) | 1.4 (0.7-2.8) | 0.331 | | 1.7 (0.7-3.8) | 0.208 |
| AFP (≥40 vs. <40 ng/ml) | 1.2 (0.7-2.3) | 0.527 | | 2.1 (1.1-4.2) | 0.033 |
| Capsule (incomplete vs. complete) | 1.9 (1.0-3.8) | 0.063 | | 1.1 (0.6-2.0) | 0.834 |
| Tumor size (>5 cm vs. ≤5 cm) | 3.1 (1.6-5.8) | 0.001 | | 3.1 (1.6-6.0) | 0.001 |
| Tumor number (>1 vs. 1) | 2.0 (1.1-3.8) | 0.030 | | 3.2 (1.7-6.0) | <0.001 |
| VI (positive vs. negative) | 4.1 (2.2-7.9) | <0.001 | | 3.1 (1.6-6.0) | 0.001 |
| TNM stage (>I vs. I) | 3.8 (1.9-7.7) | <0.001 | | 3.7 (2.0-7.1) | <0.001 |
| Multivariate analysis |  |  | |  |  |
| GαS (higher vs. lower) | 2.8 (1.2-6.7) | 0.022 | | 2.5 (1.1-5.6) | 0.031 |
| Age (>50 vs. ≤50 years) | 1.1 (0.6-2.2) | 0.778 | | 0.5 (0.2-1.0) | 0.057 |
| AFP (≥40 vs. <40 ng/ml) | 0.8 (0.4-1.7) | 0.583 | | 1.3 (0.6-2.7) | 0.480 |
| Tumor size (>5 cm vs. ≤5 cm) | 2.0 (1.0-3.9) | 0.046 | | 1.4 (0.6-3.1) | 0.466 |
| Tumor number (>1 vs. 1) | 1.5 (0.6-3.5) | 0.377 | | 1.7 (0.9-3.5) | 0.113 |
| VI (positive vs. negative) | 2.4 (0.7-8.4) | 0.163 | | 1.3 (0.5-3.3) | 0.648 |
| TNM stage (>I vs. I) | 0.8 (0.2-3.7) | 0.826 | | 1.5 (0.6-3.9) | 0.395 |

Supporting Table 2. Univariate and multivariate analysis of factors associated with disease-free survival

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Clinical variables | Cohort 1 n=131 | | | Cohort 2 n=129 | |
| Hazard ratio (95% CI) | p value | Hazard ratio (95% CI) | | p value |
| Univariate analysis |  |  | |  |  |
| GαS (higher vs. lower) | 2.3 (1.4-3.9) | 0.001 | | 3.5 (1.9-6.3) | <0.001 |
| Gender (male vs. female) | 0.6 (0.3-1.3) | 0.215 | | 1.0 (0.5-2.2) | 0.991 |
| Age (>50 vs. ≤50 years) | 1.0 (0.6-1.7) | 0.882 | | 0.6 (0.3-1.0) | 0.033 |
| HBV (positive vs. negative) | 1.1 (0.5-2.3) | 0.886 | | 0.8 (0.4-1.7) | 0.565 |
| Histological grade (>II vs. I/II) | 1.4 (0.8-2.4) | 0.183 | | 2.3 (1.1-4.8) | 0.032 |
| AFP (≥40 vs. <40 ng/ml) | 1.5 (0.9-2.4) | 0.125 | | 2.2 (1.2-3.8) | 0.009 |
| Capsule (incomplete vs. complete) | 1.1 (0.7-1.9) | 0.601 | | 1.0 (0.6-1.7) | 0.975 |
| Tumor size (>5 cm vs. ≤5 cm) | 2.1 (1.3-3.4) | 0.004 | | 3.2 (1.8-5.7) | <0.001 |
| Tumor number (>1 vs. 1) | 2.0 (1.2-3.3) | 0.006 | | 2.7 (1.6-4.7) | <0.001 |
| VI (positive vs. negative) | 2.1 (1.3-3.5) | 0.003 | | 3.8 (2.2-6.8) | <0.001 |
| TNM stage (>I vs. I) | 2.2 (1.3-3.7) | 0.002 | | 3.4 (2.0-5.9) | <0.001 |
| Multivariate analysis |  |  | |  |  |
| GαS (higher vs. lower) | 1.9 (1.1-3.4) | 0.034 | | 2.1 (1.0-4.1) | 0.040 |
| Age (>50 vs. ≤50 years) | 1.3 (0.7-2.1) | 0.417 | | 0.7 (0.4-1.2) | 0.203 |
| AFP (≥40 vs. <40 ng/ml) | 1.2 (0.7-2.1) | 0.430 | | 1.3 (0.7-2.5) | 0.373 |
| Tumor size (>5 cm vs. ≤5 cm) | 1.7 (1.0-2.8) | 0.043 | | 1.6 (0.8-3.1) | 0.173 |
| Tumor number (>1 vs. 1) | 1.7 (0.8-3.7) | 0.142 | | 1.5 (0.8-2.6) | 0.205 |
| VI (positive vs. negative) | 1.4 (0.6-3.4) | 0.446 | | 1.8 (0.8-3.9) | 0.137 |
| TNM stage (>I vs. I) | 0.8 (0.3-2.5) | 0.722 | | 1.3 (0.6-2.7) | 0.552 |

Analysis was conducted on HCC patients of Cohorts 1 and 2. Hazard ratios (95% confidence interval) and p values were calculated using univariate or multivariate Cox proportional hazards regression in SPSS 17.0. The median values of GαS expression in HCC tissues in each cohort were chosen as the cutoff points. Abbreviations: HBV, hepatitis B virus; AFP, alpha-fetoprotein; VI, vascular invasion; TNM, tumor-node-metastasis.