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Original Articles

Gankyrin drives metabolic reprogramming to promote tumorigenesis, metastasis and drug resistance through activating β-catenin/c-Myc signaling in human hepatocellular carcinoma

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ABSTRACT

Gankyrin plays important roles in tumorigenicity and metastasis of hepatocellular carcinoma (HCC). We have for the first time investigated the effects of Gankyrin on glycolysis and glutaminolysis both *in vitro* and *in vivo*, including in patient-derived xenografts. We reported Gankyrin increases glucose consumption, lactate production, glutamine consumption and glutamate production in HCC through upregulating the expression of the transporters and enzymes involved in glycolysis and glutaminolysis, including HK2, GLUT1, LDHA, PKM2, ASCT2 and GLS1. We further demonstrated that Gankyrin drives glycolysis and glutaminolysis through upregulating c-Myc via activating β-catenin signaling. Importantly, we found c-Myc mediated metabolic reprogramming might contribute to the tumorigenicity, metastasis and drug resistance induced by Gankyrin. c-Myc inhibitor synergizes with Sorafenib or Regorafenib to suppress HCC PDX tumors with high Gankyrin levels. We detected a significant correlation between Gankyrin and β-catenin expression levels in a cohort of HCC biopsies, and combination of these two parameters is a more powerful predictor of poor prognosis. Collectively, our results uncovered that Gankyrin functions as an essential regulator in glycolysis and glutaminolysis via activation of β-catenin/c-Myc to promotes tumorigenesis, metastasis and drug resistance in human HCC.

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1. Introduction

Hepatocellular carcinoma (HCC) is the most common primary malignancy of liver, and is a major cause of cancer-related death worldwide [1]. Although early-stage disease is often amenable for transplantation or surgical resection, many patients diagnosed at advanced stages miss the optimal time for surgery [2]. It is therefore important to elucidate the underlying mechanisms of hepatocarcinogenesis and identify molecular targets to develop novel diagnostic, and therapeutic strategies.

Increasing evidence suggested that metabolism alterations play a critical role in cancer [3]. The “Warburg effect”, a phenotype of cancer cells prefer using glycolysis even under normoxic conditions, has been investigated for nearly a century. Quantitative researchers are attempting to demonstrate the reasons that cancer cells engage in “Warburg effect”. It is reported that enhanced aerobic glycolysis is believed to be due to the changes of catalytic enzymes and glucose transporters, including hexokinase 2 (HK2), the M2 isoform of pyruvate kinase (PKM2), lactate dehydrogenase A (LDHA) and glucose transporter 1 (GLUT-1) [4,5]. Warburg effect is usually accompanied by other metabolic alterations, particularly glutaminolysis, which is the process of glutamine catabolism. Glutaminase 1 (GLS1) is the first rate-limiting enzyme in glutaminolysis [6]. Alanine, serine, cysteine-preferring transporter 2 (ASCT2) is a major glutamine transporter in tumor [7], and activation of which captures large amounts of glutamine. Both of GLS1 and ASCT2 have been indicated to be overexpressed and correlated with improved glutamine utilization in cancer. Emerging studies has endeavored to investigate why cancer cells exhibit high rates of glycolysis and glutaminolysis, and evidence has suggested that both oncogenes and microenvironment are involved in the process [3]. However, the underlying molecular mechanism is still unclear.

Gankyrin (also known as PSMD10 or p28GANK), a small protein with seven ankyrin-repeat domains, has been shown to be overexpressed in different malignancies [8], including colorectal cancer [9,10], HCC [11–14], cholangiocarcinoma [15], oral cancer [16], esophageal cancer [17], pancreatic cancer [18], ovarian cancer [19] and breast cancer [20,21]. Gankyrin has been reported to engage in diverse biological processes to promote tumorigenesis and metastasis, including cell proliferation, migration, senescence, autophagy, cell cycle progression and differentiation [22]. Gankyrin could promote tumor progression through regulating multiple oncogenic pathways. In addition to the inhibition of two ubiquitous tumor suppressor proteins, Rb and p53 [22], multiple interacting partners of Gankyrin have also been identified, such as NF-κB [23], hepatocyte nuclear factor 4α (HNF4α) [24], IL-6/STAT3 [15], Farnesoid X receptor [25–27], Interleukin-1β/IRAK-1 [28], and RhoA/ROCK [29]. These interactions highlight the potential clinical relevance of targeting Gankyrin in cancer treatment. Increasing studies have suggested that Gankyrin is upregulated and plays important roles not only in the initiation but also in the progression of HCC [8]. However, the exact mechanisms of Gankyrin-mediated hepatocarcinogenesis and whether Gankyrin modulates metabolic reprogramming remain unknown. In this study, we intended to examine whether Gankyrin participates in glycolysis and glutaminolysis, and to clarify the molecular mechanism in HCC.

2. Materials and methods

2.1. Patients, cell lines and animals

After selection, 110 HCC patients were enrolled in this study as described in the Supplementary Materials. Six HCC cell lines, male BALB/c nude mice and NOD-*Prkdc*^{scid} *IL2rg*^{tm1}/Bcgen (NSG) mice were used in this study as described in the Supplementary Materials. The animal experimental protocol was reviewed and approved by the Committee on the Use of Live Animals in Teaching and Research of the Harbin Medical University, Harbin, China (SYSK 2010-012).

2.2. Quantitative real-time PCR and western blot

Quantitative real-time PCR was performed as described in the Supplementary Materials. Western blot analysis was performed as previously described [15].

2.3. Lentivirus-mediated delivery of small hairpin RNA against Gankyrin

The lentiviral shRNA (lenti-shRNA) vector system (pGCSIL-GFP) used in this experiment was constructed, packed, and purified by GeneChem Corporation (Shanghai, China), and manipulated according to the detailed protocol provided by the manufacturer as described in our previous study [15].

2.4. Anchorage-independent growth and cytotoxicity assays

Anchorage-independent growth assays and cytotoxicity assays were performed as described previously [15] in the Supplementary Materials.

2.5. Measurement of glucose uptake, the rate of glycolysis and lactate production

The detailed methodology has been described previously in the Supplementary Materials.

2.6. Migration and invasion assays

The transwell migration and invasion assays were performed in the BD Falcon 24-multiwell insert system (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instruction as described in previous studies [15].

2.7. Measurement of glutamine consumption and glutamate concentration

Concentrations of glutamine and glutamate were determined with the glutamine/glutamate determination kit (GLN-1; Sigma-Aldrich) as described in the Supplementary Materials.

2.8. Subcutaneous and orthotopic HCC experiments

To establish subcutaneous xenograft tumors, 3–6 × 10^6 cells suspended in 100 μl of PBS were injected into the flank of mice. Subcutaneous xenograft tumors can be visualized after 1 week. Tumor size was monitored by using Vernier calipers until all animal were killed. To establish the orthotopic HCC models, subcutaneous tumors were resected and diced into 1 mm^3 cubes, which were then implanted into the left lobe of the liver in mice. The mice bearing subcutaneous or orthotopic HCC tumors (n = 10/group) were observed for tumor formation and treatment response assays. The tumor appearance time, volume and the wet weight of each tumor were determined for each mouse as described in the Supplementary Materials.

2.9. Establishment and the treatments of HCC PDX models

The HCC PDX models were established using the B-NSG mice. In brief, each tumor fragment (diameter, 2–3 mm) was inoculated subcutaneously into the right flank of NSG mice. When the tumors reached approximately 130–150 mm^3 (Day 1), the mice were randomly allocated to treatment groups; mice then received vehicle or different treatments as described in the Supplementary Materials.

2.10. Histological analysis

Tumor tissue samples were fixed in 10% neutral formalin, embedded in paraffin, sectioned at 4 μm thickness, and stained with

hematoxylin and eosin (H&E staining) or antibodies (Gankyrin, AFP, GPC3 and β-catenin) as described in the Supplementary Materials.

2.11. Statistical analysis

Statistical analysis was performed with the GraphPad Prism software package (v. 4.02; GraphPad Prism Software Inc, San Diego, CA) or SPSS 16.0 software (SPSS, Chicago, IL, USA), and $P < 0.05$ was considered to be statistically significant.

Details for other Materials and methods used in this study are given in the Supplementary Materials.

3. Results

3.1. Gankyrin promotes glycolysis and increases GLUT1, HK2, PKM2, and LDHA expression in HCC

To determine the influence of Gankyrin on glycolysis, pCMV-Tag2-Gankyrin plasmid was transfected into HCC cell lines with relatively low Gankyrin expression (SMMC-7721, HuH-7 and MHCC-97L) [19,28]. After being selected, stable clones were gained and western blot confirmed remarkable Gankyrin overexpression (Fig. 1a). Accordingly, Gankyrin overexpression greatly stimulated the Warburg effects;

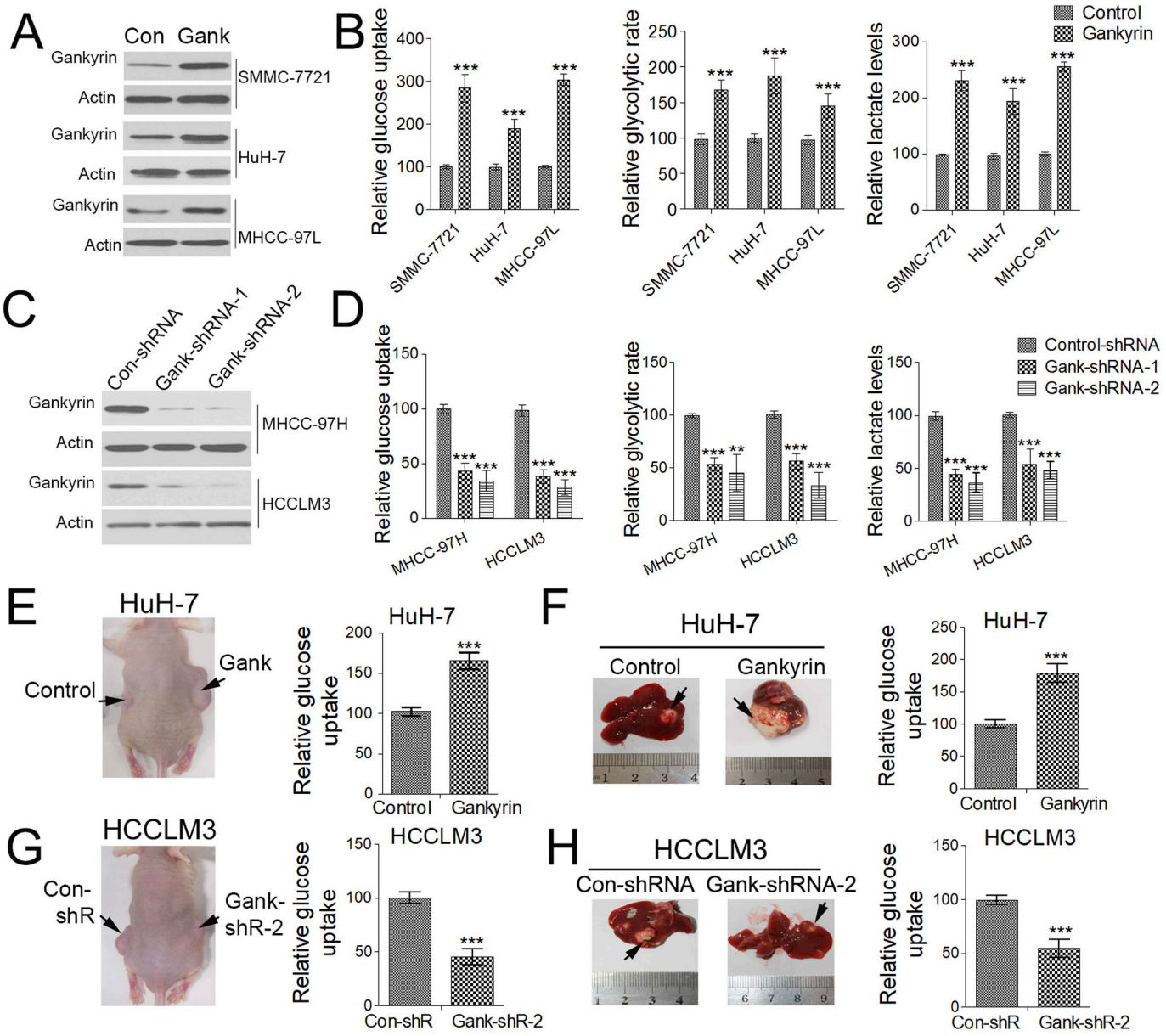

Fig. 1. *Gankyrin stimulates glycolysis both in vitro and in vivo.* (a) The overexpression of Gankyrin in indicated cell lines were confirmed using Western blotting. **(b)** Ectopic expression of Gankyrin enhanced glucose uptake, glycolytic rate and lactate production in indicated cells. **(c)** Knockdown of Gankyrin by two different shRNAs in MHCC-97H and HCCLM3 cells were confirmed using Western blotting. **(d)** Glucose uptake, glycolytic rate and lactate production were inhibited by Gankyrin-knockdown in MHCC-97H and HCCLM3 cells. **(e)** Left panels: Representative images of the tumors formed by HuH-7-Gankyrin or Control cells. Right panels: Glucose uptake was stimulated in HuH-7-Gankyrin xenograft tumors. **(f)** Left panels: Representative images corresponding to the orthotopic hepatic tumors formed by HuH-7-Gankyrin or Control cells in the liver of nude mice. Right panels: Glucose uptake was stimulated in HuH-7-Gankyrin orthotopic hepatic tumors. **(g)** Left panels: Representative images of the tumors formed by HCCLM3-Gank-shR-2 or Con-shR cells. Right panels: Glucose uptake was inhibited in HCCLM3-Gank-shR-2 xenograft tumors. **(h)** Left panels: Representative images corresponding to the orthotopic hepatic tumors formed by HCCLM3-Gank-shR-2 or HCCLM3-Con-shR cells in the liver of nude mice. Right panels: Glucose uptake was inhibited in HCCLM3-Gank-shR-2 orthotopic hepatic tumors. All data are the means ± SD of three separate experiments. ***, $P < 0.001$ compared with controls.

much higher levels of glucose uptake, glycolytic rate and lactate production were observed in Gankyrin-overexpressed cells compared with that in control cells (Fig. 1b). To further explore the role of Gankyrin in glucose metabolism, we also utilized Gankyrin-targeted shRNAs to knockdown Gankyrin expression in HCC cells with relatively high Gankyrin expression (HCCLM3 and MHCC-97H) [19,28]. To exclude the off-target effects of shRNA, we selected two individual shRNAs against Gankyrin (Fig. 1c) as described [15]. We observed that knockdown of Gankyrin inhibited glycolysis, as indicated by decreased glucose consumption and lactate production (Fig. 1d). To investigate the effects of Gankyrin on glycolysis *in vivo*, we developed the subcutaneous xenograft and orthotopic hepatic tumor models. Consistent with previous studies, we observed that Gankyrin overexpression could promote HCC tumor growth, whereas Gankyrin knockdown inhibited tumor growth both in subcutaneous xenograft and orthotopic hepatic tumor models (Additional file 1: Fig. S1). Importantly, the stimulating effect of Gankyrin on Warburg effect was observed in subcutaneous xenograft (Fig. 1e) and orthotopic hepatic tumors (Fig. 1f). Accordingly, decreased glucose uptake was observed in the subcutaneous xenograft (Fig. 1g) and orthotopic hepatic tumors (Fig. 1h) formed by Gankyrin-knockdown cells compared with the tumors formed by control cells. To further evaluate the influence of Gankyrin on glucose uptake *in vivo*, nude mice with Gankyrin-overexpressed HuH-7 tumors were prepared for ^{18}F-FDG PET scanning. MicroPET scanning demonstrated that ^{18}F-FDG accumulation was markedly increased by Gankyrin overexpression in orthotopic hepatic tumors (Additional file 2: Fig. S2). The results indicated that the mRNA levels of GLUT1, LDHA, HK2 and PKM2 were increased in Gankyrin-overexpressed cells (Fig. 2a), whereas were significantly decreased in Gankyrin-knockdown cells (Fig. 2b) compared with that in the control. We also examined the protein levels of them after Gankyrin overexpression or knockdown, and found that the changes were consistent with mRNA levels (Fig. 2c and d). In addition, we found that Gankyrin overexpression increases GLUT1, LDHA, HK2 and PKM2 in the HuH-7 subcutaneous xenograft and orthotopic hepatic tumors (Fig. 2e), whereas Gankyrin knockdown could decrease them in HCCLM3 subcutaneous and orthotopic hepatic tumor models (Fig. 2f).

3.2. Gankyrin promotes glutaminolysis and increases ASCT2 and GLS1 expression

In addition to Gankyrin-mediated promotion on glycolysis, we found that Gankyrin overexpression could stimulate glutaminolysis, as indicated by increased glutamine consumption, glutamate concentration in the culture medium and intracellular glutamate concentration (Fig. 3a). Accordingly, Gankyrin knockdown inhibited glutaminolysis in HepG2, HCCLM3 and MHCC-97H cells (Fig. 3b). To demonstrate the underlying mechanisms through which Gankyrin regulate glutaminolysis, we analyzed the expression of glutamine transporters and glutaminolytic pathway-related enzyme. We observed that the mRNA levels of ASCT2 and GLS1 were increased in Gankyrin-overexpressed cells (Fig. 3c), whereas were decreased in Gankyrin-knockdown cells (Fig. 3d). Consistent with the changes of mRNA levels, the protein levels of ASCT2 and GLS1 were increased in Gankyrin-overexpressed cells (Fig. 3e), whereas were decreased in Gankyrin-knockdown cells. These results suggested that ASCT2 and GLS1 contributed to Gankyrin-mediated glutaminolysis in HCC.

3.3. c-Myc mediates the promotion of Gankyrin in glycolysis and glutaminolysis

As reported, oncogene c-Myc controls many aspects of cellular metabolism, including glycolysis and glutaminolysis, through transcriptionally activating GLUT1, ASCT2, HK2, PKM2, LDHA and GLS1 [30]. A previous study reported that that Gankyrin decrease could result in the downregulation of β-catenin and c-Myc expression [31]. We hypothesized that Gankyrin might promote glycolysis and glutaminolysis through upregulating c-Myc. We found that Gankyrin overexpression consistently increased c-Myc expression in SMMC7721 and HuH-7 cells while Gankyrin knockdown decreased c-Myc in HCCLM3 and MHCC-97H cells (Fig. 4a and b). We next investigated the role of c-Myc in Gankyrin-dependent glycolysis and glutaminolysis. The results indicated that c-Myc knockdown by siRNA largely abolished the promoted effects of Gankyrin on glycolysis and glutaminolysis as indicated by decreased glucose consumption, glycolytic rates and lactate production, glutamine consumption and glutamate concentration (Fig. 4c and d). Importantly, we observed that c-Myc inhibitor (10058-F4) also largely abolished the promoting effects of Gankyrin on glycolysis and glutaminolysis (Additional file 3: Fig. S3). Furthermore, c-Myc knockdown decreased GLUT1, HK2, PKM2, LDHA, ASCT2 and GLS1 expression in Gankyrin-overexpressed HuH-7 cells (Fig. 4e). Taken together, we have identified that Gankyrin promoted glycolysis and glutaminolysis through regulating c-Myc.

3.4. Gankyrin increases c-Myc expression through activating β-catenin signaling

As reported, aberrant activation of the Wnt signaling gives rise to β-catenin accumulation and promotes the transcription of c-Myc to drive tumorigenesis and metastasis [30]. We investigated whether Gankyrin activate c-Myc through regulating â-catenin. Results indicated the mRNA and protein levels of β-catenin were increased in Gankyrin-overexpressed HCC cells, whereas were decreased in Gankyrin-knockdown cells (Fig. 4f and Additional file 4: Fig. S4). Accordingly, the cytoplasmic and nuclear β-catenin were increased in Gankyrin-overexpressed cells, whereas was decreased in Gankyrin-knockdown cells (Fig. 4g). Gankyrin is a subunit of proteasome without transcription activity. It is interesting to investigate through which mechanisms Gankyrin increase β-catenin mRNA levels. It has been reported RNA-binding proteins, such as HuR (ELAVL1), hnRNPAB, CUGBP2, TTP, and TIA1, could stabilize β-catenin mRNA to increase its expression [32]. Therefore, we hypothesize that Gankyrin may stabilize β-catenin mRNA level indirectly through regulating RNA-binding proteins. Interestingly, our results suggested that Gankyrin could upregulate HuR expression in SMMC-7721 and MHCC-97L cells. Further results indicated HuR knockdown by shRNA could attenuate the increase of â-catenin mRNA levels induced by Gankyrin overexpression in HCC cells (Additional file 4: Fig. S4). Accordingly, the expression level of c-Myc was increased in β-catenin-overexpressed cells, whereas was decreased in β-catenin-knockdown cells (Fig. 4h), which is similar as in previous studies [31]. We observed that β-catenin knockdown by siRNA abolished the increase of c-Myc by Gankyrin in HuH-7 and MHCC-97L cells (Additional file 5: Fig. S5).

3.5. c-Myc inhibition largely abolishes the promoting effect of Gankyrin on tumorigenesis, metastasis and drug resistance

To further determine the causal role of Gankyrin-dependent metabolic reprogramming in HCC, we treated Gankyrin overexpressed HuH-7 cells with or without 2-DG (glycolytic inhibitor) or BPTES (GLS1 inhibitor). We got the results that inhibition of glycolysis or glutaminolysis by 2-DG or BPTES alone could partially attenuate the promoting effect of Gankyrin on anchorage-independent cell growth and migration (Additional file 6: Fig. S6). Importantly, we observed that the combination of the two agents could robustly diminish the effects of Gankyrin on proliferation and invasion. Altogether, these results suggested that Gankyrin-dependent glycolysis and glutaminolysis might play important roles in HCC progression. Further results indicated that c-Myc pathway inhibition by shRNA or 10058-F4 greatly reduced the promoting effect of Gankyrin on anchorage-independent cell growth of HuH-7 cells in soft agar (Fig. 5a). c-Myc shRNA or 10058-F4 could also largely abolish the promoting effect of Gankyrin on the growth of subcutaneous and orthotopic hepatic tumors formed by HuH-7 cells

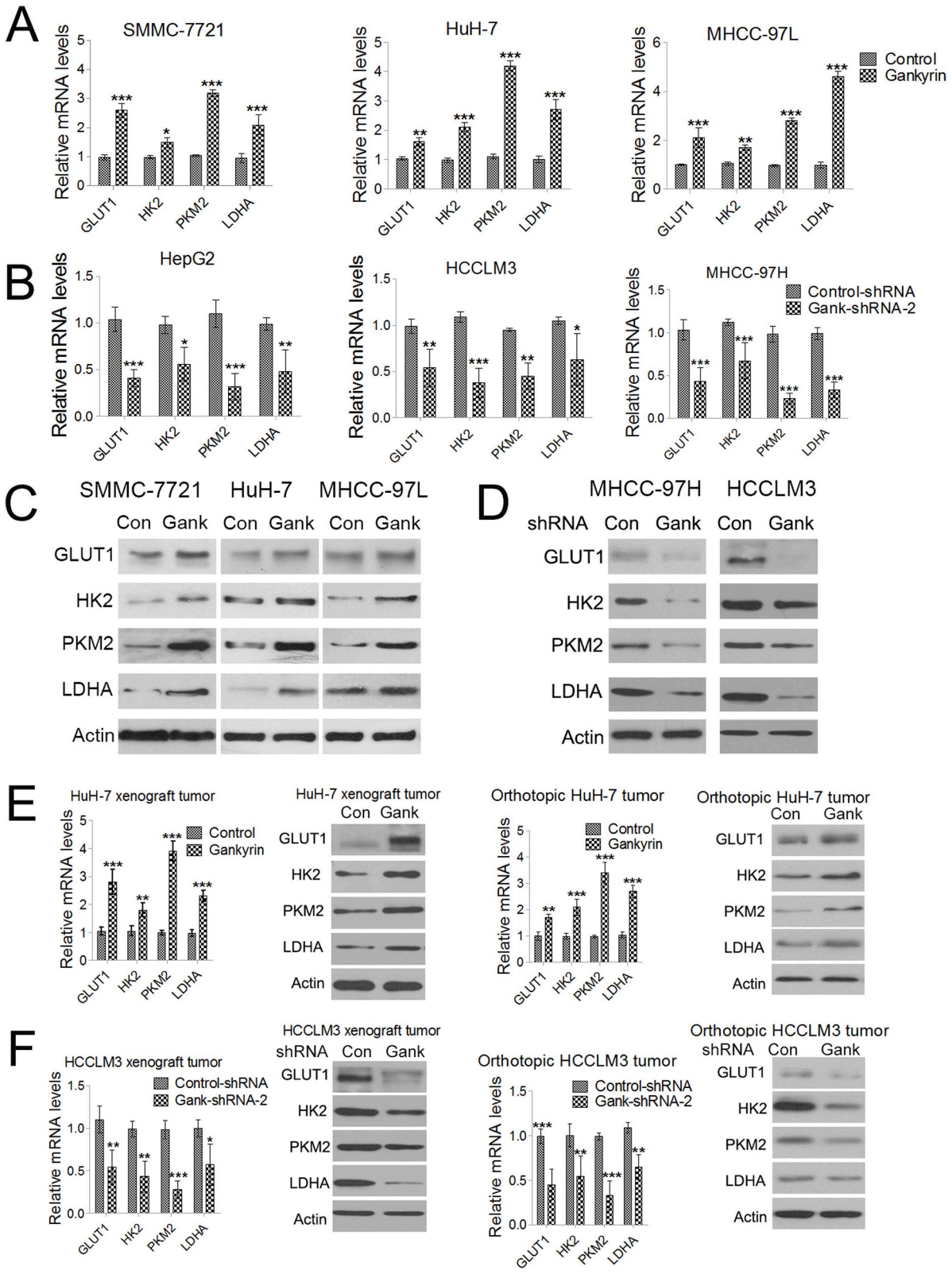

Fig. 2. *Gankyrin increases the expression of glucose transporters, and glycolytic enzymes both in vitro and in vivo.* The mRNA levels of GLUT1, HK2, PKM2, and LDHA were determined after Gankyrin overexpression in SMMC-7721, HuH-7 and MHCC-97L cells **(a)**, or after Gankyrin knockdown in HepG2, HCCLM3 and MHC-97H cells **(b)**. The protein levels of GLUT1, HK2, PKM2, and LDHA were determined after Gankyrin overexpression **(c)**, or after Gankyrin knockdown in indicated cells **(d)**. **(e)** The mRNA and protein levels of GLUT1, HK2, PKM2, and LDHA were assayed in HuH-7-Gankyrin xenograft tumors (left panels) or orthotopic hepatic tumors (right panels). **(f)** The mRNA and protein levels of GLUT1, HK2, PKM2, and LDHA were assayed in HCCLM3-Gank-shR-2 xenograft tumors (left panels) or orthotopic hepatic tumors (right panels). All data are the means ± SD of three separate experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ compared with controls.

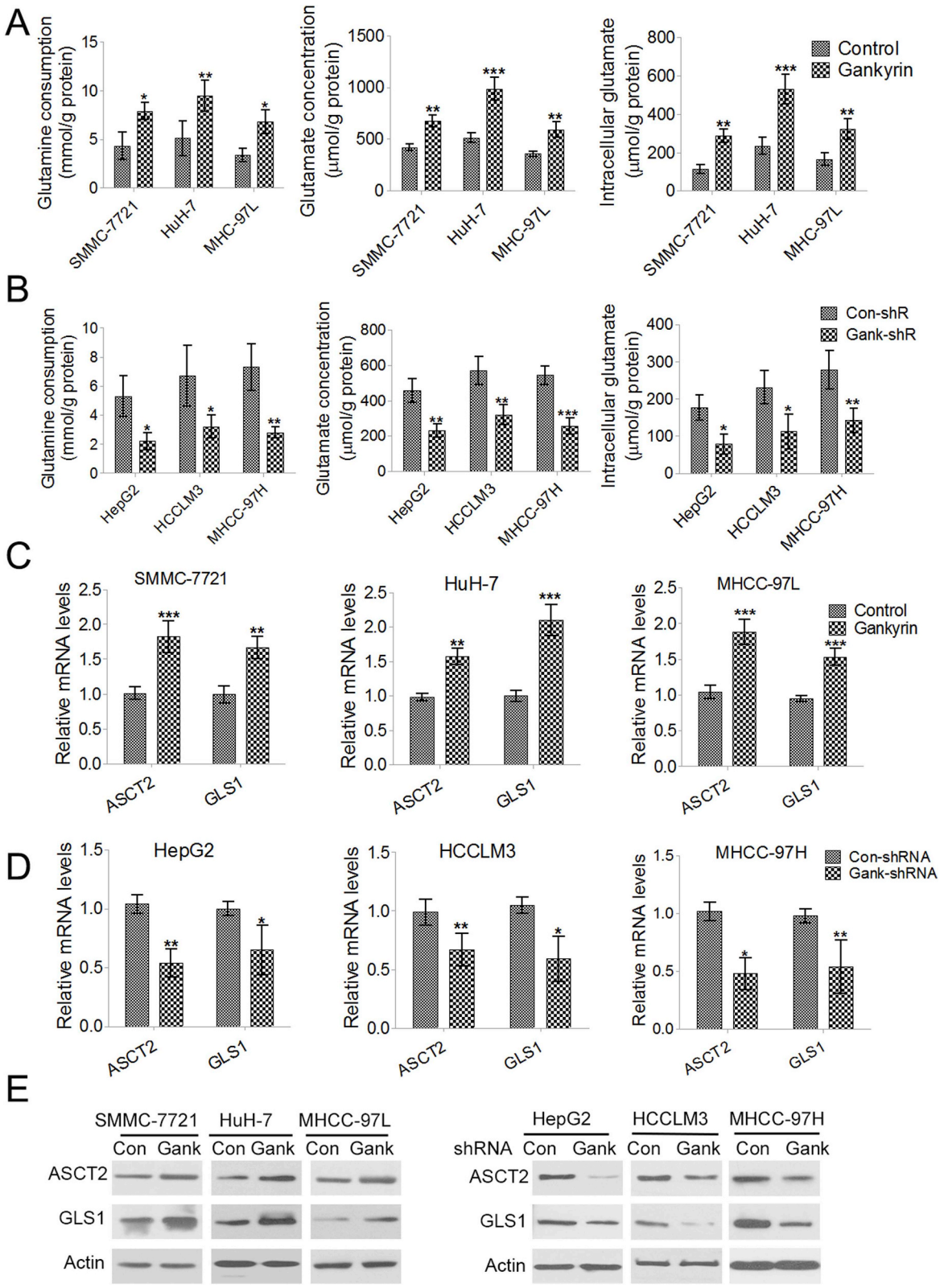

Fig. 3. *Gankyrin stimulates glutaminolysis in HCC cells.* (a) Ectopic expression of Gankyrin in indicated cells enhanced glutamine consumption, glutamate concentration and intracellular glutamate. **(b)** Glutamine consumption, glutamate concentration and intracellular glutamate were inhibited by Gankyrin-knockdown. The mRNA levels of ASCT2 and GLS1 were determined after Gankyrin overexpression in SMMC-7721, HuH-7 and MHCC-97L cells **(c)**, or **(d)** after Gankyrin knockdown in HepG2, HCCLM3 and MHCC-97H cells. The protein levels of ASCT2 and GLS1 were determined in indicated cells **(e)**. All data are the means ± SD of three separate experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ compared with controls.

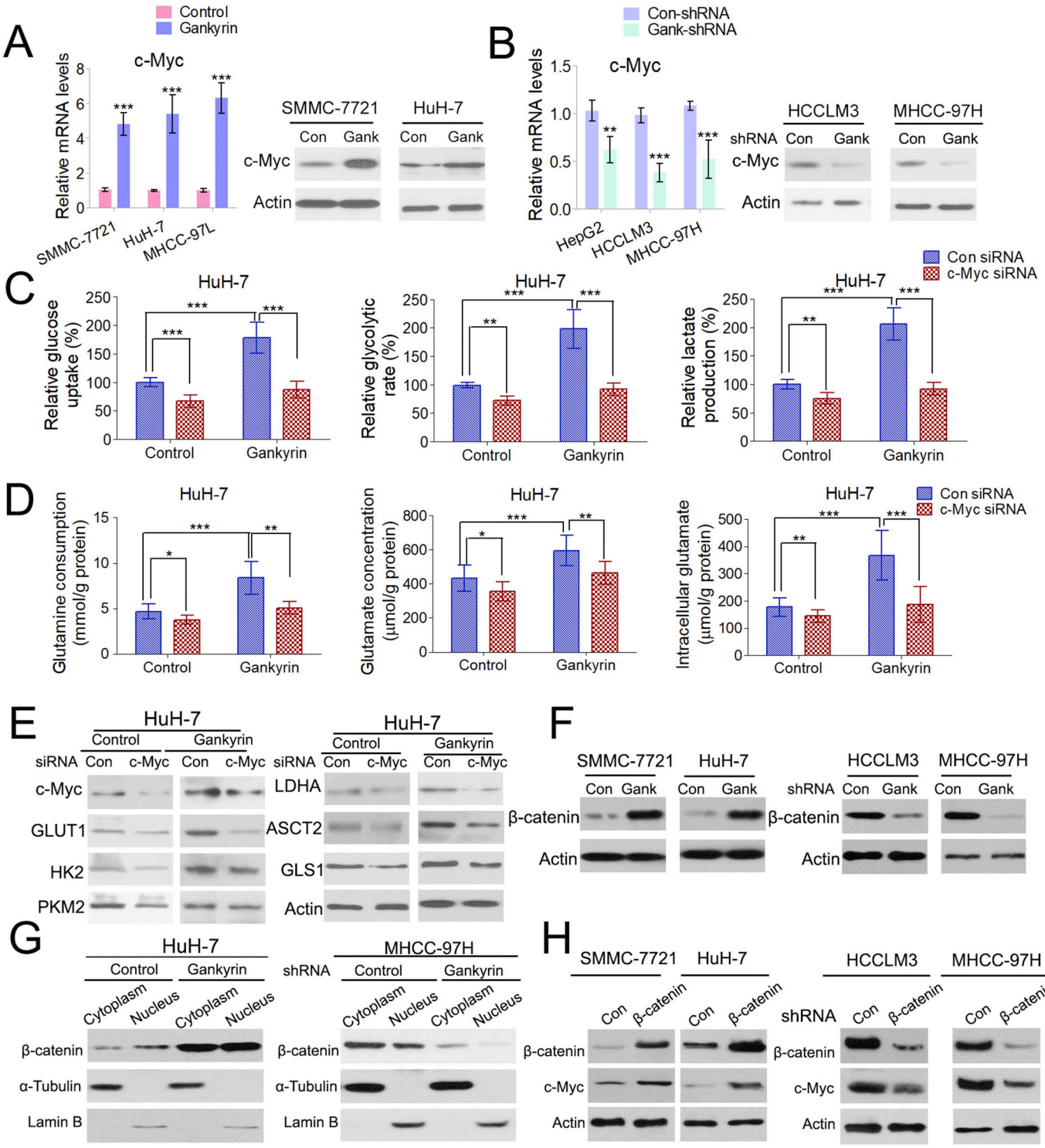

Fig. 4. *Gankyrin stimulates glycolysis and glutaminolysis through activating β-catenin/c-Myc signaling.* The mRNA and protein levels of c-Myc was determined after Gankyrin overexpression **(a)**, or after Gankyrin knockdown **(b)**. **(c)** Glucose uptake, glycolytic rate and lactate production were determined in indicated cells. **(d)** Glutamine consumption, glutamate concentration and intracellular glutamate were determined. **(e)** Protein levels of GLUT1, HK2, PKM2, LDHA, ASCT2 and GLS1 in HuH-7-Gankyrin or HuH-7-Control cells after transfection of c-Myc siRNA or control siRNA. **(f)** The protein level of β-catenin was determined after Gankyrin overexpression or Gankyrin knockdown in indicated cells. **(g)** The subcellular distribution of β-catenin in cytoplasm and nucleus of HuH-7 cells after Gankyrin overexpression, or MHCC-97H cells after Gankyrin knockdown, α-tubulin and Lamin B served as internal control of cell cytoplasm and nucleus accordingly. **(h)** The protein levels of β-catenin and c-Myc were assayed in SMMC-7721 and HuH-7 cells after β-catenin over expression, or in HCCLM3 and MHCC-97H cells after β-catenin siRNA. All data are the means ± SD of three separate experiments. **, $P < 0.01$; ***, $P < 0.001$.

(Fig. 5b and c, and Additional file 7: Fig. S7). Furthermore, c-Myc inhibition largely blocked the promoting effect of Gankyrin on invasion and migration in HuH-7 cells (Fig. 5d and e). Sorafenib and Regorafenib are currently acknowledged worldwide as the standard first-line therapy and second-line setting for advanced HCC [33]. Several studies suggested that Gankyrin could promote sorafenib resistance in HCC

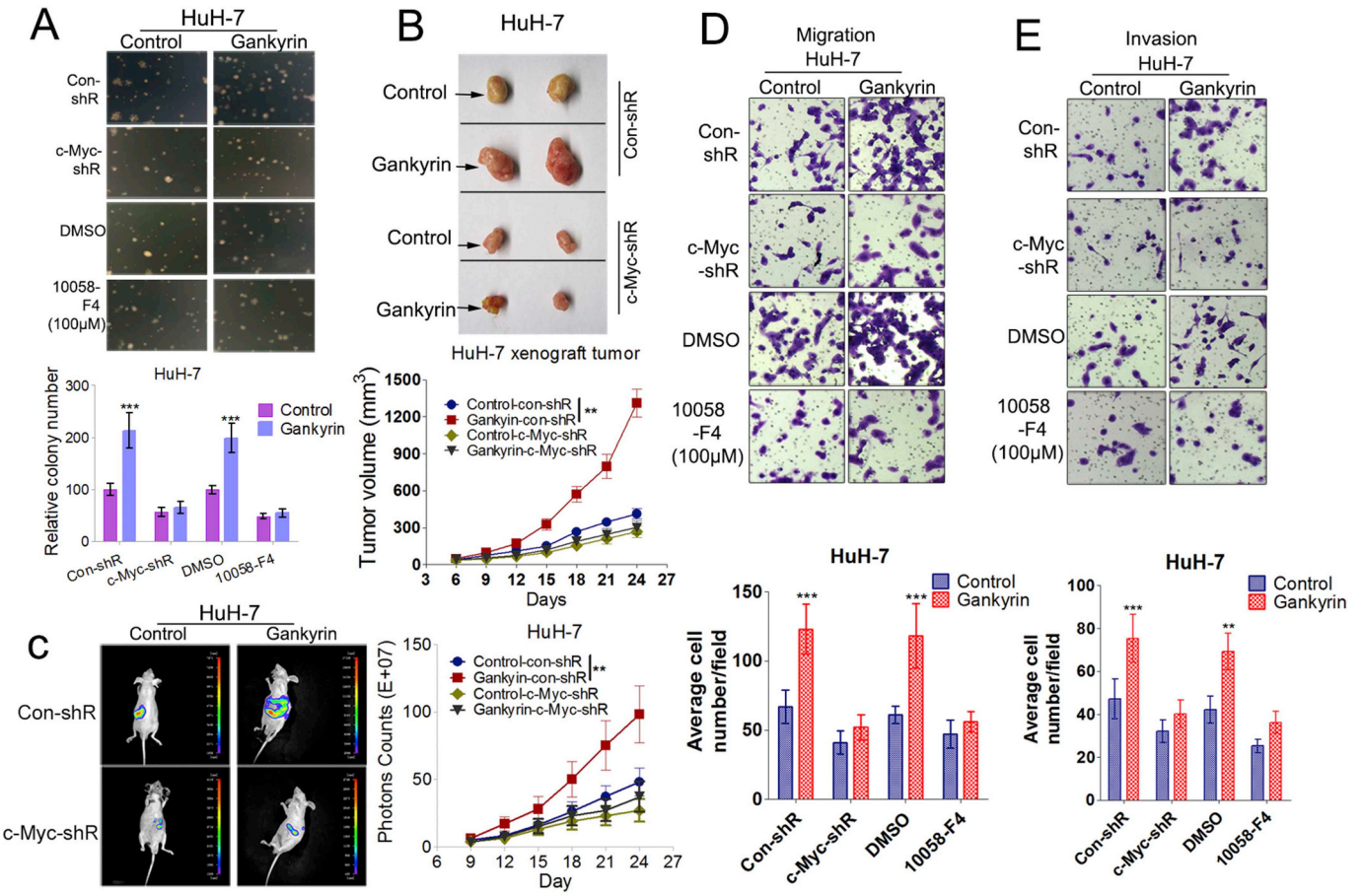

Fig. 5. *Blocking c-Myc inhibits the promoting effect of Gankyrin on tumorigenesis and metastasis in HCC.* (a) c-Myc inhibition by shRNA or 10058-F4 largely blocked the promoting effect of Gankyrin on anchorage-independent growth of HuH-7 cells in soft agar. c-Myc inhibition by shRNA largely abolished the promoting effect of Gankyrin on the growth rate of xenograft HuH-7-Gankyrin tumors **(b)** and HuH-7-Gankyrin orthotopic hepatic tumors **(c)**. c-Myc inhibition by shRNA or 10058-F4 largely largely abolished the effect of Gankyrin on migration **(d)** and invasion **(e)** abilities of HuH-7 cells. All data are the means ± SD of three separate experiments. **, $P < 0.01$; ***, $P < 0.001$ compared with controls.

[34,35], we here also found that Gankyrin overexpression could decrease the sensitivity of HuH-7 cells to sorafenib or regorafenib (Fig. 6a and b). The *in vivo* results indicated that regorafenib-treated mice displayed significant tumor inhibition compared with the mice treated with vehicle (Fig. 6c and d). Gankyrin overexpression reduced the inhibitory effects of regorafenib on subcutaneous and orthotopic hepatic tumor. Importantly, c-Myc shRNA largely abolished the resistance to regorafenib mediated by Gankyrin *in vivo*. Regorafenib treatment induced slight increase of glucose uptake. Importantly, Gankyrin overexpression increase glucose uptake in mice treated with Regorafenib, whereas c-Myc shRNA reduced the glucose uptake mediated by Gankyrin both in the subcutaneous and orthotopic hepatic tumors treated with Regorafenib (Fig. 6e and f). Taken together, these results demonstrate that Gankyrin activates c-Myc pathway, which in turn promotes tumorigenesis, metastasis and resistance to Sorafenib or Regorafenib in HCC.

3.6. c-Myc inhibitor synergizes with sorafenib or regorafenib to repress HCC patient-derived xenograft (PDX) tumors with high Gankyrin levels

After establishing the HCC PDX models successfully as described in Supporting Information (Additional file 8: Fig. S8), we performed experiments in two selected PDX models with different Gankyrin levels (Fig. 7a). Glucose uptake assays and ^{18}F-FDG PET scanning results indicated that the glycolysis levels were much higher in the PDX#6 than that in PDX#9 tumors (Fig. 7b). Accordingly, the expression levels of β-catenin, c-Myc, GLUT1, HK2, LDHA, ASCT2 and GLS1 in PDX#6 were higher than that in PDX#9 (Fig. 7c). Ten mice in each group were implanted for PDX#6 or PDX#9 model and were randomly distributed for the treatment with Sorafenib, Regorafenib alone or in combination with 2-DG, BPTES or 10058-F4 for 21 days. The results indicated that Sorafenib, Regorafenib, 2-DG, BPTES or 10058-F4 could inhibit the tumor growth of PDX models (Fig. 7d and Additional file 9: Fig. S9). Interestingly, we observed that 2-DG, BPTES or 10058-F4 could only synergize with Sorafenib or Regorafenib to repress PDX#6 tumor growth, but not in the PDX#9 group. Importantly, the synergistic effects were more significant in the group treated with combination of 10058-F4 and Sorafenib or Regorafenib, suggesting that c-Myc inhibition might be an excellent therapeutic strategy for HCC patients with high Gankyrin expression, especially combined with Sorafenib or Regorafenib.

3.7. Combination of Gankyrin and β-catenin levels has better prognostic value for HCC

We further analyzed the expression levels of Gankyrin and β-catenin in clinical HCC samples as described in the Supporting Information. Tissue microarray analysis of 110 patient specimens revealed a strong correlation of Gankyrin expression with β-catenin levels ($r = 0.4238$, $P < 0.0001$) (Fig. 8a and b). Interestingly, Kaplan-Meier curve results indicated that patients whose tumors expressed above-average levels of Gankyrin or β-catenin exhibited decreased trend in overall survival

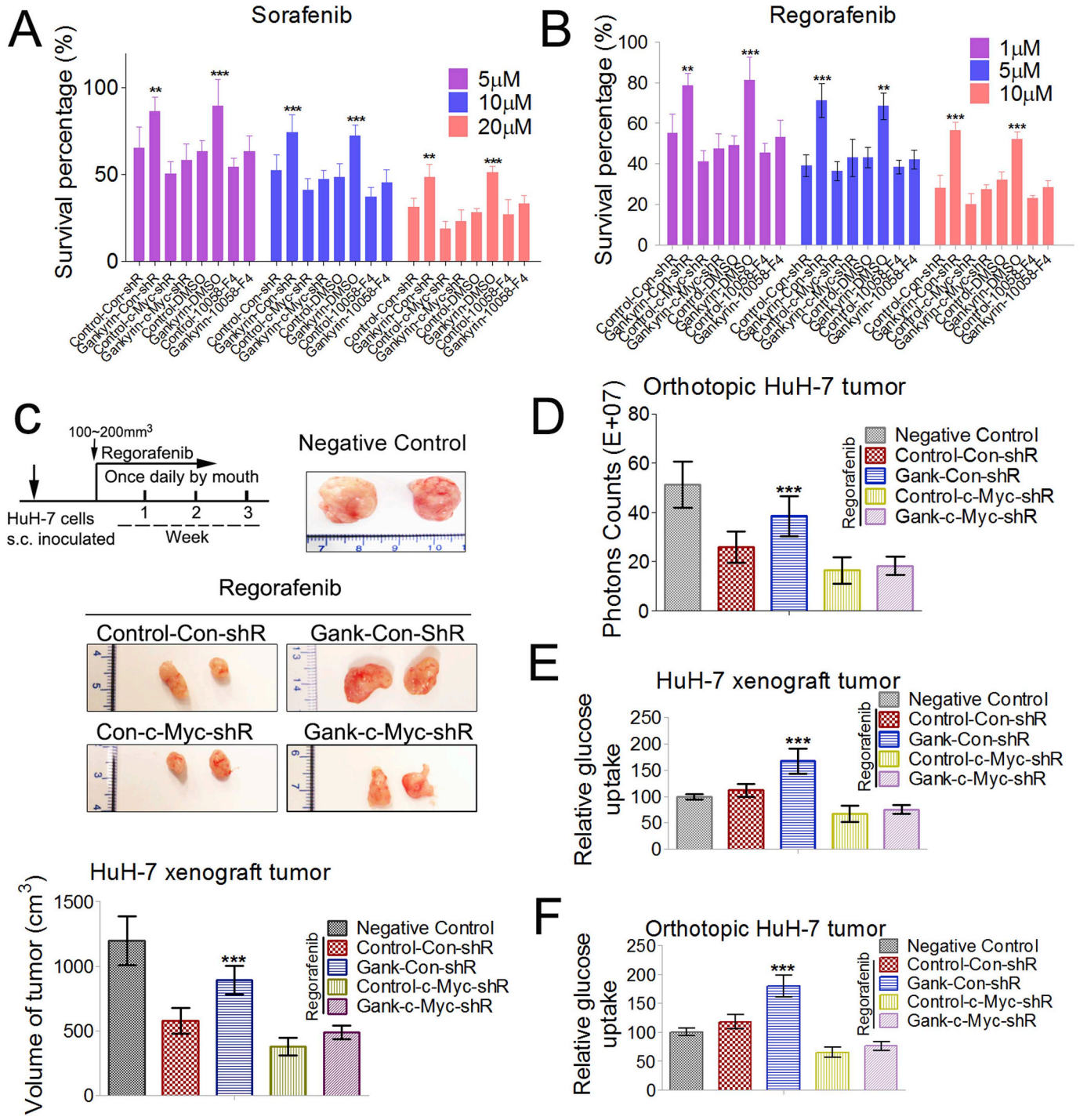

Fig. 6. *Blocking c-Myc abolished the resistance to Sorafenib or Regorafenib mediated by Gankyrin in HCC.* (a–b) The sensitivity of indicated cells to Sorafenib or Regorafenib was significantly enhanced after c-Myc knockdown or 10058-F4 treatment. **(c)** Representative images of HuH-7-Con-Con-shR, HuH-7-Gank-Con-shR, HuH-7-Control-c-Myc-shR, and HuH-7-Gank-c-Myc-shR xenograft tumors treated with Regorafenib, and the volume of tumors was determined. **(d)** Volume of the indicated orthotopic HCC tumors treated with vehicle or Regorafenib was determined. The glucose uptake was determined in indicated xenograft **(e)** or orthotopic tumors **(f)**. All data are the means ± SD of three separate experiments. **, $P < 0.01$; ***, $P < 0.001$ compared with controls.

(OS) attributed to HCC-related death (Additional file 10: Fig. S10). For patients whose tumors had above-average levels of both Gankyrin and β-catenin, adverse outcomes were exacerbated. Using the combination of these two parameters increased the prognostic value, as compared to Gankyrin or β-catenin overexpression alone (Fig. 8c). In conclusion, evaluation of both Gankyrin and β-catenin expression is a powerful predictor of poor prognosis for HCC.

4. Discussion

Gankyrin has been reported to play a pivotal role in HCC progression through regulating multiple oncogenic signaling pathways [8]. Metabolic deregulation is considered as a hallmark of cancer [3]. However, the exact molecular mechanisms leading to metabolism reprogramming are not well defined. In this study, we have for the first

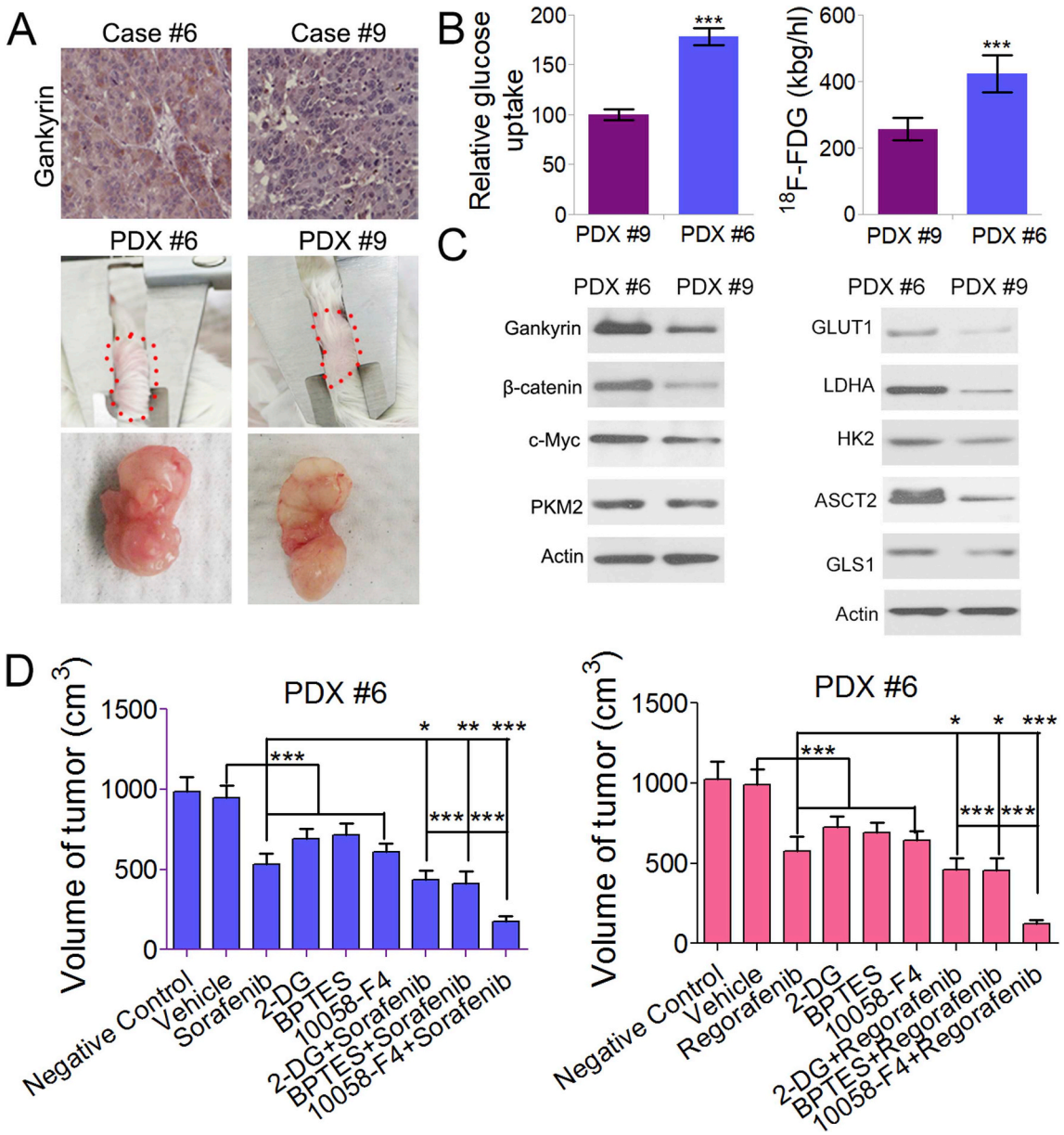

Fig. 7. *HCC PDXs with high Gankyrin levels exhibited enhanced glucose uptake and are sensitive to c-Myc inhibitor combined with Sorafenib or Regorafenib.* (a) HCC PDX models (#6 and #9) with different Gankyrin levels used in this study. Gankyrin protein levels in samples from the indicated patients were determined by IHC (upper panels). Representative images of PDX tumor masses were shown (lower panels). **(b)** MicroPET and glucose uptake assays were performed in PDX #6 and #9 models. **(c)** The protein levels of indicated proteins were determined in PDX #6 and #9 models. **(d)** Volume of the indicated HCC PDX#6 tumors (10 mice/group) treated with vehicle, Sorafenib (20 mg/kg), Regorafenib (15 mg/kg), 2-DG (1000 mg/kg), BPTES (12.5 mg/kg), 10058-F4 (20 mg/kg), Sorafenib + 2-DG, Sorafenib + BPTES, Sorafenib + 10058-F4, Regorafenib + 2-DG, Regorafenib + BPTES, Regorafenib + 10058-F4 was determined. All data are the means ± SD of three separate experiments. **, $P < 0.01$; ***, $P < 0.001$.

time discovered that Gankyrin participates in the promoting effects on glycolysis and glutaminolysis. Gankyrin increases the expression of glucose transporter GLUT1 and glycolytic key enzymes HK2, PKM2 and LDHA, the expression of ASCT2 and GLS1 at the transcriptional level both *in vivo* and *in vitro*.

As an important transcription factor, through the direct upregulation of related genes, such as GLUT1, HK2, LDHA, ASCT2 and GLS1, c-Myc contributes to the aerobic glycolysis and glutaminolysis. A previous study demonstrated that Gankyrin establishes a positive feedback loop in â-catenin signaling, and thereby regulate c-Myc [31]. Interestingly, we found that Gankyrin could indeed increase the expression of c-Myc at transcriptional level through upregulating β-catenin both *in vitro* and in *vivo*. Our results further suggested that Gankyrin might increase the mRNA level of β-catenin through upregulating the RNA-binding protein HuR. However, the exact molecular mechanism through which Gankyrin upregulates HuR still requires further investigation. Importantly, c-Myc inhibition greatly abolished the promoting effects of Gankyrin on metabolic reprogramming and tumor progression. Further investigation showed that c-Myc-dependent energy reprogramming also contributes to the resistance of HCC to sorafenib and regorafenib mediated by Gankyrin both *in vitro* and *in vivo*. Interestingly, the results that inhibition of glycolysis and glutaminolysis could largely attenuate the promotion of cell proliferation and migration by Gankyrin suggested a causal role of Gankyrin-dependent metabolic changes in HCC progression.

Given Gankyrin exhibited the documented critical roles in tumor

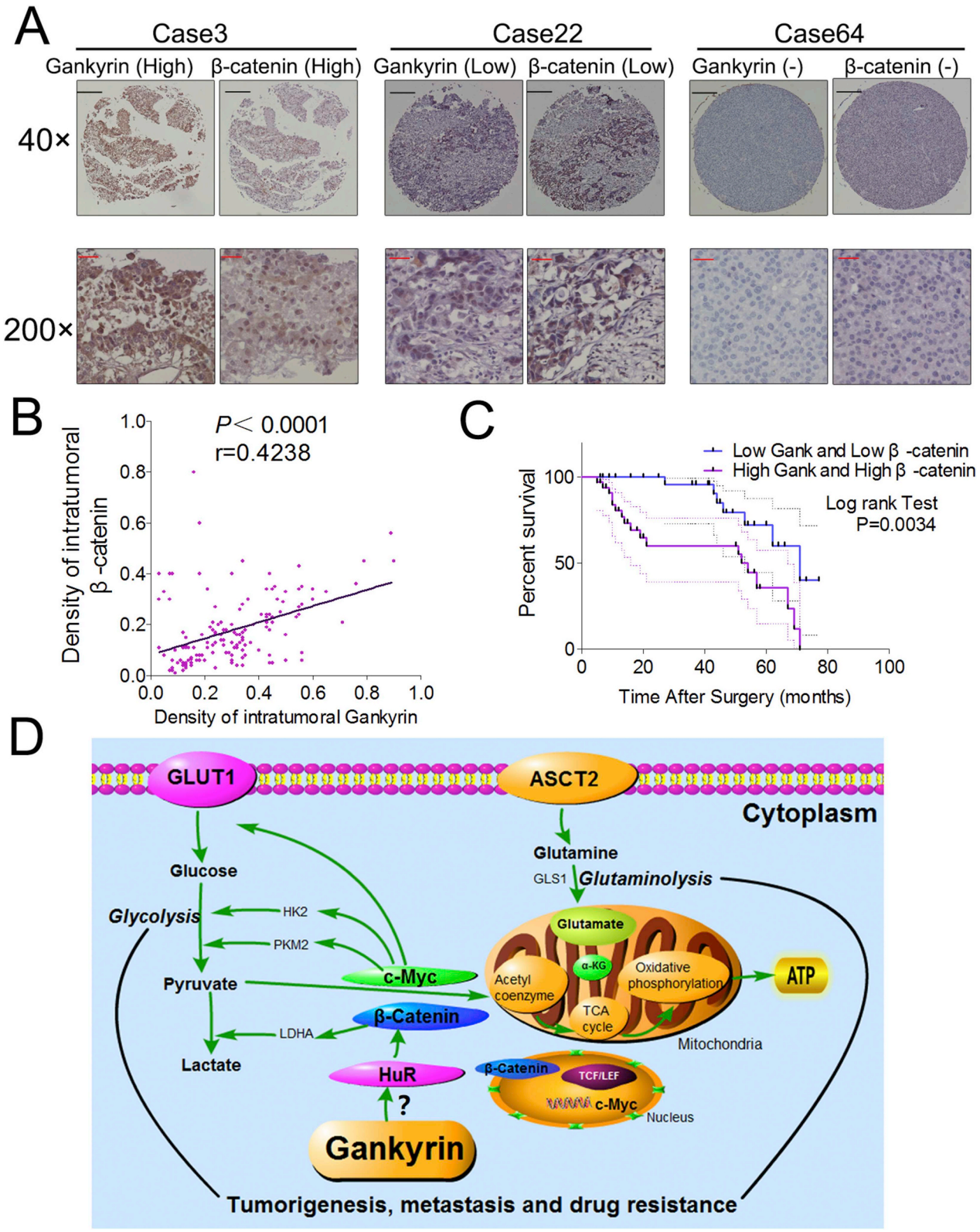

Fig. 8. *Combination of Gankyrin and β-catenin improves prognostic accuracy for HCC patients.* (**a**) Representative view of IHC analysis of Gankyrin and β-catenin expression in 110 HCC tissues. Black and red scale bar stands for 100 μm and 25 μm respectively. **(b)** Correlation between Gankyrin expression and β-catenin levels was analyzed by Pearson correlation analysis. **(c)** The Kaplan-Meier analysis of concurrent Gankyrin and β-catenin expression with overall survival. The median value for Gankyrin and c-Myc expression in HCCs was used to divide the patients into high (above median) and low (below median) Gankyrin or β-catenin groups. **(d)** Schematic representation for Gankyrin mediated glycolysis and glutaminolysis promotion in HCC is shown. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

initiation, and progression [8], Gankyrin inhibition via different methods such as use of siRNA or synthetic small inhibitor molecules might be a promising therapeutic strategy for HCC, especially in combination with current chemotherapy. Recently, Thakur et al. designed a potential ligand, which is exactly fitting in the cavity of Gankyrin and forming many close interactions to protein atoms including its active site residues [36]. However, further *in vivo* studies testing this inhibitor has not been reported yet. Having observed that c-Myc-mediated energy reprogramming contributes to the promotion of Gankyrin on tumorigenesis and metastasis, it will be interesting to investigate the effects of c-Myc inhibition on Gankyrin-overexpressed HCC. In this study, we established two PDX models from primary HCC patients with different Gankyrin levels. The xenografts were successfully serially transplanted while preserving the characteristics of the original patient tumors. We observed that PDX#6 tumors with high Gankyrin grow faster than PDX#9 tumors with relatively low Gankyrin levels. We also observed that the glucose uptake and the expression levels of β-catenin, c-Myc, GLUT1, HK2, LDHA, ASCT2 and GLS1 were much higher in PDX#6 tumors than that in PDX#9 tumors. The most interesting part of the results shown here is the remarkable inhibition of the PDX#6 tumors by the combination of Sorafenib or Regorafenib and c-Myc inhibitor, compared with the combination with 2-DG or BPTES. Importantly, the predictive range of Gankyrin expression combined with β-catenin was more sensitive than that of Gankyrin alone for OS strongly suggesting that the concerted activities of Gankyrin and β-catenin detected in our experiments.

In summary, our current study has for the first time illustrated the regulatory role of Gankyrin in metabolic reprogramming of HCC. The ability of Gankyrin to activate β-catenin/c-Myc signaling contributes to enhanced glycolysis and glutaminolysis, leading to the promotion of hepatocarcinogenesis, metastasis and drug resistance. We propose a scheme for Gankyrin-mediated glycolysis and glutaminolysis promotion in HCC (Fig. 8d). With these discoveries, we could further understand the mechanism of energy metabolic reprogramming and might provide novel metabolic targets for HCC treatment in the future.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2018.11.030.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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