Gankyrin and TIGAR cooperatively accelerate glucose metabolism toward the PPP and TCA cycle in hepatocellular carcinoma

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Abstract

Oncogene-derived metabolic reprogramming is important for anabolic growth of cancer cells, which is now considered to be not simply rely on glycolysis. Pentose phosphate pathway and tricarboxylic acid cycle also play pivotal roles in helping cancer cells to meet their anabolic and energy demands. The present work focused on gankyrin, a relatively specific oncogene in hepatocellular carcinoma (HCC), and its impact on glycolysis and mitochondrial homeostasis. Metabolomics, RNA-seq analysis, and subsequent conjoint analysis illustrated that gankyrin regulated the pentose phosphate pathway (PPP), tricarboxylic acid (TCA) cycle, and mitochondrial function and homeostasis, which play pivotal roles in tumor development. Mechanistically, gankyrin was found to modulate HCC metabolic reprogramming via TIGAR. Gankyrin positively regulated the transcription of TIGAR through Nrf2, which bound to the antioxidant response elements (AREs) in the promoter of TIGAR. Interestingly, TIGAR feedback regulated the transcription of Nrf2 and subsequently gankyrin by promoting nuclear importation of PGC1α. The loop between gankyrin, Nrf2, and TIGAR accelerated glucose metabolism toward the PPP and TCA cycle, which provided vital building blocks, such as NADPH, ATP, and ribose of tumor and further facilitated the progression of HCC.
1 | INTRODUCTION

Glucose is involved in glycolysis, the pentose phosphate pathway (PPP), and tricarboxylic acid (TCA) cycle and considered to be the main energy and nutrition source of cells. Metabolic reprogramming satisfies the energy and nutrition need of cancer cells for stress tolerance, proliferation, and microenvironment alteration. The activity or expression of metabolic enzyme is increased in cancer cells. However, the drivers of cancer metabolic changes remain unclear. Growing evidence shows that oncogene (such as myc, Ras, and BRAF)-directed metabolic reprogramming, rather than passive responses to damaged mitochondria, is required to support anabolic metabolism of cancer cells. Our previous work found gankyrin increased the quantity and stabilized the function of mitochondria in HCC. To understand the metabolism change caused by gankyrin, metabolomics, RNA-seq analysis, and subsequent integrative analysis were performed, and it was found that both the PPP and TCA cycle were depressed in gankyrin-knockdown cells.

Both the PPP and TCA cycle are important for cancer cells, as they supply building blocks for the rapid proliferation of cancer cells, such as nucleic acid synthesis, ATP, and NADPH. A proteomic study of breast cancer brain metastases detected increases in expression of enzymes involved in glycolysis, TCA cycle, oxidative phosphorylation, and PPP. The PPP, which branches from glycolysis at the first committed step of glucose metabolism, is required for the synthesis of ribonucleotides and is a major source of NADPH. Unconventionality activation of the PPP has been found in a wide variety of cancer and associated with invasion, metastasis, angiogenesis, and resistance of chemotherapy and radiotherapy. Distinct from the original concept of the Warburg effect, growing literature considers that mitochondria play a key role in oncogenesis. The TCA cycle is a central hub for energy metabolism, macromolecule synthesis, and redox balance, providing indeed materials for tumor anabolism, redox, and calcium homeostasis; participating in transcriptional regulation; and governing cell death. Increased α-ketoglutarate converted from glutamine feeds into TCA cycle for ATP synthesis, fatty acid and nucleotide synthesis is found and explored as a therapeutic target in tumors.

Mitochondrial function was intimately related to mitochondrial homeostasis. Mitophagy is a mitochondrial form of autophagy that is critical for mitochondrial quality control (QC) and homeostasis. Certain mitophagy receptors or mediators, such as MFN1, MFN2, DRP1, and PGAM5 were changed and mediated with therapeutic resistance in cancers. Gankyrin was reported to drive glycolysis and glutaminolysis and promote tumorigenesis, metastasis, and drug resistance through β-catenin/c-myc signaling in HCC. Therefore, we proposed that gankyrin accelerated glucose metabolism and generated ATP, NADPH, and ribose to meet the needs of rapid proliferation of cancer cells.

In this research, we determined the role of gankyrin in metabolism reprogramming, revealing that it regulated the PPP and mitochondrial function through TIGAR. A positive feedback loop between gankyrin and TIGAR, linked by Nrf2, was discovered to promote the PPP and maintain mitochondrial homeostasis in HCC cells. The loop between gankyrin, Nrf2, and TIGAR was particularly related to tumorigenesis and prognosis of HCC, which could be a potential therapeutic target of HCC.

2 | MATERIALS AND METHODS

2.1 | Cell culture, transfection, and lentivirus infection

Human HCC cell lines Huh7 and MHCCLM3 were purchased from the Cell Bank of the Chinese Academy of Sciences. Cells were cultivated in DMEM (10% fetal bovine serum, 37°C and humidified air containing 5% CO₂). Plasmid pCDNA3.1A-gankyrin was constructed in our laboratory. Plasmid shTIGAR-1, shTIGAR-2, shgankyrin-1 and shgankyrin-2 were purchased from genechem Ltd. TIGAR/gankyrin overexpression/knockdown lentiviruses were purchased from genechem Ltd. Knockdown of gankyrin/TIGAR/Nrf2 was determined as shGank/shTIGAR/shNrf2, while overexpression of gankyrin/TIGAR/Nrf2 was determined as ovGank/ovTIGAR/ovNrf2. The sequences for shRNA were as follows: shgankyrin-1, 5′-TGGCTGAAGACACTGAGGGTAAACCTCTTTTACATGCTCATATTAGTCAAGAGACTAATATGAGCATGTCCTTGCTTTTTT-3′; shgankyrin-2, 5′-GAGATCGCTGTCATGTTACACATGCTCATATTAGTCAAGAGACTAATATGAGCATGTCCTTGCTTTTTT-3′; shTIGAR-1, 5′-GCAAGGACATGCTCATATTAGTTCAGAGACTAATATGAGCATGTCCTTGCTTTTTT-3′; shTIGAR-2, 5′-GGCCCTTCTTTTGAAAGATGTTCAGAGACTAATATGAGCATGTCCTTGCTTTTTT-3′.

2.2 | RNA collection, cDNA synthesis, and real-time PCR analysis

Total RNA was extracted from cell lines using Trizol (Invitrogen). cDNA synthesis was performed using random hexamers (Roche) and SuperScriptII reverse transcription (Invitrogen). qRT-PCR was performed using ABI 7900 Fast Real-Time PCR System (Applied Biosystems) and SYBR Green PCR kit (Takara Bio Inc.). The primer sequences were as follows: hG6PD: 5′-TGACCTGGCCAAGAAGAAGA-3′, 5′-GACATTCTTTACAAAGAAGGCCCTTTTTT-3′; hIDH2: 5′-CAAGAAGTCCTCCAGCTTG-3′; hME2: 5′-CTGCCTGTCATTTCTGGATGT-3′, 5′-ACCTCTTACTCTCTCTCT-3′; hTIGAR: 5′-CTACTTTGTTGGCCACTGACTGACGAGTGTTACACTGCTTCTTT-3′; hNrf2: 5′-CAAGAAGTCCTCCAGCTTG-3′; shTIGAR-1, 5′-GGCATTTCACTAAACACAA-3′; shTIGAR-2, 5′-GGATTTCCTACTAAAACACAA-3′; shgankyrin-1, 5′-TGCTGAAGACACTGAGGGTAAACCTCTTTTACATGCTCATATTAGTCAAGAGACTAATATGAGCATGTCCTTGCTTTTTT-3′; shgankyrin-2, 5′-GGCCCTTCTTTTGAAAGATGTTCAGAGACTAATATGAGCATGTCCTTGCTTTTTT-3′; ovTIGAR-1, 5′-GCAAGGACATGCTCATATTAGTTCAGAGACTAATATGAGCATGTCCTTGCTTTTTT-3′; ovTIGAR-2, 5′-GGCCCTTCTTTTGAAAGATGTTCAGAGACTAATATGAGCATGTCCTTGCTTTTTT-3′; ovNrf2, 5′-AGATCGCTGTCATGTTACACATGCTCATATTAGTCAAGAGACTAATATGAGCATGTCCTTGCTTTTTT-3′; ovNrf2-2, 5′-GGCCCTTCTTTTGAAAGATGTTCAGAGACTAATATGAGCATGTCCTTGCTTTTTT-3′.
2.3 | Chromatin immunoprecipitation (ChiP) assay

Chromatin immunoprecipitation assays using anti-Nrf2 antibody were performed using a ChiP assay kit (Beyotime, P0013B). Primers used for ChiP assays were as follows: ARE1: 5'-AGTCCCGACTTCGGA-3', 5'-CCGGAACATTCTCAAGTTG-3'; ARE2: 5'-CTTCTATAGAAAGGTCGGCTCTATCC-3', 5'-CTGTGCACTCTGTCCTCC-3'; ARE3: 5'-ACCGACACACACACACACACAC-3', 5'-CACACAAAGACGTTGGCCATA-3'; ARE4: 5'-CCGGAACATTCTCAAGTTG-3'; ARE5: 5'-GGAGGGTGGTTACATTTACAGGAT-3', 5'-GGGCTCTTCTCTCTGACTTCC-3'; ARE6: 5'-CCGGAACATTCTCAAGTTG-3'; ARE7: 5'-CCGGAACATTCTCAAGTTG-3'; ARE8: 5'-GGGCTCTTCTCTCTGACTTCC-3'; ARE9: 5'-CCGGAACATTCTCAAGTTG-3'; ARE10: 5'-GGGCTCTTCTCTCTGACTTCC-3'.

2.4 | Immunoblotting, antibodies, and chemicals

Whole-cell lysis was prepared with RIPA buffer (Beyotime, P0013B) and centrifuged at 12,000 g for 15 minutes. Protein concentrations were measured using Pierce® BCA protein assay kit (Thermo Scientific, 23225). Immunoblot was performed using specific primary antibodies and fluorescein-conjugated secondary goat anti-rabbit/mouse antibody (Gene, 926-32211) and then detected using Odyssey fluorescence scanner.

Anti-Parkin (4211), anti-Ub (3936), anti-p-Ub (Ser65) (62802), and anti-PARP (9532) were purchased from Cell Signaling Technology. Anti-gankyrin (sc-10498), anti-BNIP3 (sc-56167), anti-β-actin (sc-8432), and anti-TIGAR (sc-166290) were purchased from Santa Cruz Biotechnology, Inc. Anti-G6PD (A-11234), anti-PGD (A-0563), anti-TKT (A-13553), anti-FUNDC1 (A-16318), anti-HSP60 (A-0564), and anti-TALDO (A-13551) were purchased from Abclonal. Anti-Nrf2 (sc-166290) and anti-TOMM20 (11802-1-AP) were purchased from Proteintech Inc.

2.5 | Immunofluorescence

Cells were fixed with 4% formaldehyde for 10 minutes, treated with 0.02% Triton X-100 for 5 minutes, incubated with goat serum for 45 minutes, hatched with anti-Parkin, anti-LC3, or anti-HSP60 overnight, and then incubated with specific goat anti-rabbit (Alexa Fluor 594)/goat anti mouse (Alexa Fluor488) for 45 minutes. DAPI was ultimately added for 10 minutes. Photos were gathered by a confocal laser scanning microscope (Olympus).

2.6 | Untargeted metabolomics by liquid chromatography coupled to high-resolution mass spectrometry (LC-HRMS)

Five million cells were harvested, washed once with 0.9% NaCl, transferred into 2 ml EP tube, 1 ml of solution (acetonitrile:methanol H2O = 2:2:1, v/v/v) was added and vortexed for 30 seconds. The samples were frozen in liquid nitrogen for 5 minutes and thawed at room temperature; then, they were put into a tissue grinder for 2 minutes at 55 Hz. The operation was repeated twice. The samples were then centrifuged at 4°C for 10 minutes at 13,500 g, and 850 μl of supernatant was transferred into another tube and concentrated to dry in vacuum. The samples were dissolved with 300 μl of 2-chlorobenzylalcohol solution (4 ppm) prepared with acetonitrile: 0.1% FA (1:9 v/v) (~20°C) and then filtered through a 0.22-μm membrane. From each sample, 20 μl was taken as the QC sample. The remaining samples were used for LC-MS detection.

Chromatographic separation was accomplished in an Thermo Ultimate 3000 system equipped with an ACQUITY UPLC® HSS T3 (150×2.1 mm, 1.8 μm, Waters) column maintained at 40°C. The temperature of the autosampler was 8°C. Gradient elution of analytes was carried out with 0.1% formic acid in water and 0.1% formic acid in acetonitrile or 5 mM ammonium formate in water and acetonitrile at a flow rate of 0.25 ml/min. Injection of 2 μl of each sample was done after equilibration. An increasing linear gradient of solvent B (v/v) was used as follows: 0-1 minutes, 2% B/D; 1-9 minutes, 2%-50% B/D; 9-12 minutes, 50%-98% B/D; 12-13.5 minutes, 98% B/D; 13.5-14 minutes, 98%-2% B/D; 14-20 minutes, 2% D-positive model (14-17 min, 2% B-negative model). The electrospray tandem mass spectrometry (ESI-MSn) experiments were executed on the Thermo Q Exactive Plus mass spectrometer with a spray voltage of 3.5 and −2.5 kV in positive and negative modes, respectively. Sheath gas and auxiliary gas were set at 30 and 10 arbitrary units, respectively. The capillary temperature was 325°C. The analyzer scanned over a mass range of m/z 81-1000 for full scan at a mass resolution of 70,000. Data-dependent acquisition (DDA) MS/MS experiments were performed with HCD scan. The normalized collision energy was 30 eV. Dynamic exclusion was implemented to remove some unnecessary information in MS/MS spectra. All the process was performed at BioNovoGene Ltd.

2.7 | Assessment of cell death

Cells were treated with PBS or 0.5 mM H2O2 for 5 hours to detect the cleaved PARP. Cells were also incubated with PI (Sigma-Aldrich,
P4170) for 30 minutes and then analyzed by the flow cytometry performed with MoFlo XDP Cell Sorter (Beckman Coulter).

2.8 | Tumor xenograft experiment

Approximately $1 \times 10^7$ LM3-NC, ovGank, and ovGankshTIGAR cells in 0.1 ml PBS were injected into the right flank of mice. The volume of tumors was measured every 3 days. Tumor was harvested when the volume reached 1-1.5 cm$^3$. All experiments were performed with at least five mice in each group, and all experiments were repeated three times.

2.9 | Nuclear and mitochondria isolation

Nuclei were isolated using NE-PER® nuclear and cytoplasmic extraction reagents (Thermo Scientific, 78833), and mitochondria were isolated using Cell Mitochondria Isolation Kit (Beyotime, C3601), which were then analyzed by Western blot.

2.10 | Patients, specimens, and tissue microarrays (TMAs)

A total of 269 HCC specimens were collected from the Eastern Hepatobiliary Surgery Hospital (Shanghai, China) from 2003 to 2005. The patients were selected according to the following inclusion criteria: WHO performance status 0-1; Child-Pugh class A; and no chemotherapy or radiotherapy before hepatectomy and diagnosis of HCC by pathological examination. Immunohistochemistry was performed as previously described.\(^{17}\)

2.11 | Measurement of reactive oxygen species (ROS) levels

Cells were incubated with 5 mM CM-H2DCF DA (Beyotime, S0033S) for 30 minutes and analyzed by the flow cytometry performed with MoFlo XDP Cell Sorter (Beckman Coulter).

2.12 | Statistical analysis

All the statistical analyses were performed with SPSS version 20.0 (IBM Corp.) The associations between gankyrin and TIGAR expression and clinical data were determined using the $\chi^2$ test. Kaplan-Meier analysis with log-rank tests was used to determine disease-free survival (DFS) and overall survival (OS). The data represent mean values of at least three independent experiments. Error bars represent mean ± SEM. Statistical significance was set at $p<0.05$.

3 | RESULTS

3.1 | Gankyrin maintains the PPP and mitochondrial function and homeostasis in HCC

Conjoint analysis of metabolomics and RNA-seq data showed that mitochondrial function and PPP were both changed in gankyrin-knockdown HCC cells (Figure S1A,B). Metabolites involved in glucose metabolism were significantly decreased in gankyrin-knockdown cells (Figures 1A and S1C). Gankyrin increased NADPH, ATP, and GSH/GSSG ratio in HCC cells (Figure 1B-D). The expression of enzymes that execute the PPP were decreased in gankyrin-knockdown cells while elevated in gankyrin-overexpression cells (Figures 1E,F and S2A). Genes involved in TCA, such as ME2, IDH2, and MTHFD2, were reduced in gankyrin-knockdown cells (Figure 1G,H).

RNA-seq analysis showed regulation of mitophagy was also influenced (Figure S1A). Electron microscope assay showed that the quantity of mitochondria was reduced in gankyrin-knockdown cells (Figure 1I). The mRNA level of 16S, a mitochondrial-specific gene, was also decreased in gankyrin-knockdown cells (Figure S2B). Accordingly, gankyrin increased the expression of HSP60 and TOMM20, which are intrinsic genes of mitochondria (Figure 1J). Further, the critical role of gankyrin in maintaining mitochondrial number was confirmed with MT-green/MT-red staining of mitochondria (Figures 1K and S2C,D). To further assess the effects of gankyrin on the regulation of mitochondrial oxidative phosphorylation, we measured oxygen consumption rate (OCR) in MHCCLM3 cells. Gankyrin significantly enhanced the rate of oxidative phosphorylation (Figure 1L).

**FIGURE 1** Metabolomics and RNA-seq analysis synthetically showed gankyrin impressed the pentose phosphate pathway (PPP) and mitochondrial function. (A) Heat map visualization for metabolites involved in glucose metabolism in MHCCLM3 control and shGank cells. (B–D) Relative NADPH/NADP$^+$ ratio (B), GSH/GSSG ratio (C), and amount of ATP (D) in MHCCLM3 control, shGank, and ovGank cells. E, qRT-PCR analysis of G6PD, PGD, TKT, TALDO, and gankyrin in MHCCLM3 control and shGank cells. (F) Western blot (WB) analysis of key genes in PPP, G6PD, TKT, and TALDO between MHCCLM3 and Huh7 control and shGank cells. (G) qRT-PCR analysis of ME1, IDH1, MTHFD2, and gankyrin in MHCCLM3 control and shGank cells. (H) WB analysis of ME2 and IDH2 in MHCCLM3 control and shGank cells. (I) Mitochondria ultrastructure was taken by electron microscopy in MHCCLM3 control, shGank, and ovGank cells. Bars: 2 μm. (J) WB analysis of HSP60 and TOMM20 in control, shGank, and ovGank cells. (K) Fluorescence microscopy revealed the Mt-Green levels in MHCCLM3 and Huh7 control and shGank cells. Bar, 100 μm. (L) Oxygen consumption rate (OCR) was measured using Seahorse Bioscience Extracellular Flux Analyzer in NC, shGank, and ovGank cells. OCR curves were obtained after treatment with oligomycin, FCCP, and rotenone/antimycin A. Black arrows indicate the time of treatment ($n = 3$). (M) The quantity and location of Parkin and HSP60 were detected by confocal microscopy in control, shGank, and ovGank cells stimulated with CCCP (10 nM, 12 h). Bar, 10 μm.
Genes involved in mitophagy were detected as RNA-seq analysis showed that mitophagy regulatory pathways were affected in gankyrin-knockdown cells. Parkin, PINK1, BNIP3, and FUNDC1 expressions were not influenced as gankyrin was depleted (Figure S2E). However, p-Ub (Ser65), which participates in PINK1-Parkin–induced mitophagy, was significantly accumulated in gankyrin-knockdown cells (Figure S2F). The results above suggested that gankyrin may affect mitophagy through the PINK1-Parkin pathway, while the regulation did not rely on the expression alteration of PINK1 and Parkin. Subsequently, we found Parkin and LC3 gathered on mitochondria and colocalized with HSP60 by immunofluorescence assay under stimulation with CCCP (Figures 1M and S2G). At the same time, when challenged with CCCP, p62 was increased, while LC3II level was decreased as gankyrin was depleted (Figure S2H,I).

3.2 | Gankyrin regulated the PPP and mitochondrial function and homeostasis through TIGAR

To inquire how gankyrin influenced metabolism, we analyzed RNA-seq data and found TIGAR was decreased in gankyrin-knockdown cells, which was associated with the PPP and mitochondrial function (Figure 2A). TIGAR was reported to play an important role in NADPH production as it regulates the PPP. Moreover, literature also reported that TIGAR influenced mitochondrial function and mitophagy. By RT-PCR and Western blot assays, we revealed that gankyrin increased the expression of TIGAR (Figure 2B–D). To verify that gankyrin participated in metabolism reprogramming through TIGAR, we analyzed the effect of TIGAR on mitophagy and the PPP in gankyrin-modified cells. Metabolomic assay showed that the amount of PPP- and TCA cycle-related metabolites that reduced in gankyrin-knockdown cells increased again with the introduction of TIGAR (Figure 2E). TIGAR supplement markedly increased GSH, NADPH, and ATP levels in gankyrin-knockdown cells (Figure 2F–H). As TIGAR plays a critical role in eliminating ROS, we detected the ROS levels by flow cytometry in HCC cells. TIGAR supplementation notably reduced the ROS elevation caused by knockdown of gankyrin (Figure 2I). Expressions of key genes in the PPP in gankyrin-knockdown cells were partially restored by TIGAR (Figure 2J). Correspondingly, knockdown of TIGAR resulted in decreased expression of PPP key genes that were upregulated by gankyrin overexpression (Figure 2K).

Oxygen consumption rate measurement assay showed that knockdown of TIGAR partly suppressed gankyrin-enhanced mitochondrial oxidative phosphorylation capacity (Figure 3A,B). Interestingly, as gankyrin increased glycolytic rate, no significant differences were observed in the parameters of ECAR as modulation of TIGAR (Figure S3A,B). These results suggested that TIGAR participated in the gankyrin-promoted PPP and mitochondrial oxidative phosphorylation, but not glycolysis. With respect to mitochondria, TIGAR replenishment recovered the quantity of mitochondria in gankyrin-knockdown cells (Figure 3C). TIGAR also increased HSP60 expression in gankyrin-knockdown cells (Figure 3D). Given that previous studies reported TIGAR inhibited mitophagy through BNIP3 inactivation rather than PINK1-Parkin, we tested the location of the BNIP3 complex in mitochondria. Accumulation of BNIP3 complex on mitochondria induced by gankyrin-knockdown was blocked by overexpression of TIGAR, while mitochondria-located BNIP3 complex diminution caused by gankyrin overexpression was reversed by knockdown of TIGAR (Figure 3E). Consistently, knockdown of TIGAR reversed the reduction of parkin and LC3 on mitochondria caused by overexpression of gankyrin, while TIGAR also blocked the aggregation of parkin and LC3 on mitochondria caused by knockdown of gankyrin (Figures 3F,G and S3C,D). These results above indicated that TIGAR affected mitophagy through not only BNIP3 but also the PINK1-Parkin pathway in HCC.

3.3 | Gankyrin, TIGAR, and Nrf2 constitute a positive feedback loop in promoting metabolism

It seems a paradox, as TIGAR was the main target gene of p53, while gankyrin negatively regulated the expression of p53, it increased the expression of TIGAR (Figure S4A, Figure 2C, D). Therefore, we speculated that gankyrin regulated TIGAR through a novel p53-independent pathway. Using JASPAR (jaspar.genereg.net), AREs were found in TIGAR promoter region that Nrf2 can recognize and bind, and thereby promote transcription of downstream target genes (Figure 4A,B). ChIP analysis revealed that Nrf2 bound to ARE elements in promoter of TIGAR (Figure 4C). Besides, Nrf2 increased TIGAR expression (Figure 4D,E). TBHQ and sulforaphane, which activated Nrf2, could increase the expression of TIGAR (Figure 5B). Our previous work has reported that gankyrin could stabilize Nrf2 through competitive binding with Keap1. From the above data, we can infer that gankyrin regulated the expression of TIGAR through Nrf2.

Intriguingly, we found that overexpression of TIGAR could increase the expression of gankyrin and Nrf2 in gankyrin-knockdown cells (Figure 2J). Meanwhile, knockdown of TIGAR attenuated
Nrf2 and gankyrin expression in gankyrin-overexpression cells (Figure 2K). TIGAR depletion largely reduced the mRNA and protein level of Nrf2 and gankyrin, and overexpression obviously increased expression of gankyrin and Nrf2 (Figures 4F,G and S4C). It is reported that TIGAR depletion inhibited the nuclear transportation and activation of PGCl1a, the main transcription factor of Nrf2, through SIRT1.19 Indeed, TIGAR increased the expression of SIRT1, as well as subsequent activation and nuclear importation of PGCl1a (Figures 4H and S4D). A potent activator of PGCl1a, ZLN005, inhibited the decrease of Nrf2 and gankyrin caused by the knockdown of TIGAR (Figure S4E). In addition, TIGAR also impacted the ubiquitination of Nrf2 (Figure 4I). We previously reported that gankyrin inhibited Nrf2 degradation by competitive combination with Keap1.8 Gankyrin replenishment in TIGAR-knockdown cells inhibited the ubiquitination and degradation of Nrf2 (Figure 4I). In contrast, gankyrin depletion obviously promoted ubiquitination and degradation of Nrf2 even in a state of high TIGAR expression (Figure S4F). In conclusion, gankyrin, Nrf2, and TIGAR cooperatively promoted the PPP and maintained mitochondrial function in HCC.

3.4  |  Gankyrin and TIGAR cooperatively promoted tumorigenesis of HCC

To investigate the clinical relevance of gankyrin and TIGAR, the sensitivity of HCC cells to H2O2 was evaluated. TIGAR knockdown increased the sensitivity of gankyrin-overexpressed cells to H2O2, and TIGAR overexpression in gankyrin-knockdown cells helped restore resistance to H2O2 (Figure 5A). PARP cleavage was also decreased in gankyrin-knockdown cells by overexpression of TIGAR (Figure 5B). ovGank, ovGankshTIGAR, and control cells were subcutaneously inoculated into nude mice and tumor growth was periodically monitored. Gankyrin notably accelerated tumor progression, but combination with the silencing of TIGAR significantly attenuated gankyrin-driven tumor growth in vivo (Figure 5C).

In clinical HCC TMA samples, the expressions of gankyrin and TIGAR were detected (Figure 5D,E). The patients were divided into four groups based on the expression of gankyrin and TIGAR: group I (n = 51), low gankyrin and low TIGAR; group II (n = 54), low gankyrin and high TIGAR; group III (n = 47), high gankyrin and low TIGAR; and group IV (n = 127), high gankyrin and high TIGAR. Patients in group IV had shorter DFS (the median DFS of group I, II, III, and IV were 12, 6, 3, and 2 months, respectively) and OS (the median OS of group I, II, III, and IV were 39, 24, 10, and 7 months, respectively) compared with the other groups (Figure 5F,G). To confirm the value of the loop between gankyrin and TIGAR on prognosis, we analyzed gankyrin, TIGAR, and survival of HCC patients in The Cancer Genome Atlas (TCGA) database and obtained consistent results (Figure S5). Consequently, we showed that TIGAR knockdown significantly inhibited the tumorigenicity of gankyrin-high cells, and the combination of gankyrin, Nrf2, and TIGAR could be a valuable prediction factor for the prognosis of HCC patients.

4  |  DISCUSSION

Metabolism reprogramming is an important hallmark of cancer, as metabolic pathways involved in carbohydrates, lipids, amino acids, and nucleotides biosynthesis, as well as energy production, are changed to meet the tumor’s specific nutritional and proliferation requirements.5 Cancer metabolism has been considered to rely on aerobic glycolysis. However, oncogene-directed metabolic reprogramming, rather than passive responses to damaged mitochondria, is required to support anabolic growth of cancer cells.20 Gankyrin is an oncogene related to apoptosis, proliferation, and prognosis of HCC. We reported that gankyrin participated in redox homeostasis through feedback regulation of Nrf2.8 This work focused on the role of gankyrin in metabolism reprogramming of HCC. Metabolomic analysis is a promising approach to identify specific changes and underlying mechanisms in metabolic pathways. Analysis of metabolites of glycolysis, TCA, and PPP was performed in MHCC-LM3 cells with or without gankyrin knockdown. Both RNA-seq analysis and metabolomics revealed that both PPP and TCA cycles were inhibited in gankyrin-knockdown cells.

The pentose phosphate pathway, which generates NADPH and RSP and provides materials for DNA synthesis, is often aberrantly activated in the process of tumorigenesis. The key enzymes transketolase-like 1 (TKTL1) and transaldolase (TALDO) are frequently overexpressed in cancer.21,22 Both oncogenes and tumor suppressors regulate PPP activity and further modulate cancer metabolism. For instance, Ras-driven transformation induces transcriptional upregulation of enzymes that mediate R-5-P biosynthesis.7 Wild-type, but not the mutant, p53 inhibits the PPP by directly binding and inactivating the rate-limiting PPP enzyme glucose-6-phosphate dehydrogenase (G6PD).23
Figure 4  Gankyrin, TIGAR, and Nrf2 constitute a positive feedback loop in promoting metabolism. (A) Analysis by JASPAR (jaspar.genereg.net) showing ARE sites in TIGAR promoter. (B) Schematic representation of TIGAR promoters in humans (up) and adjacent sequences (down) in TIGAR promoter. (C) ChIP were performed with anti-Nrf2 and analyzed with primers for ARE1-9. (D, E) qRT-PCR analysis (D) and Western blot (WB) analysis (E) of TIGAR and Nrf2 in MHCCLM3 control, shNrf2, and ovNrf2 cells. F, G, qRT-PCR (F) and WB analysis (G) of Nrf2, gankyrin, and TIGAR in control, shTIGAR-1, and shTIGAR-2 cells. (H) WB analysis of PGC1α in nucleus, cytoplasm, and whole-cell lysate in control and shTIGAR cells of MHCCLM3 and Huh7. (I, J) Whole-cell lysates of shTIGAR, control, and ovTIGAR cells (I), as well as shTIGAR and shTIGARovGank cells (J) were immunoprecipitated with anti-Nrf2 or IgG and analyzed by WB with anti-ubiquitin antibody.
FIGURE 5  Legend on next page
Tricarboxylic acid cycle is a central route for oxidative phosphorylation in cells, and fulfills their bioenergetic, biosynthetic, and redox balance requirements. Despite earlier dogma that cancer cells bypass the TCA cycle and primarily utilize aerobic glycolysis, emerging evidence demonstrates that certain cancer cells, especially those with deregulated oncogene and tumor suppressor expression, rely heavily on the TCA cycle for energy production and macromolecule synthesis. Tumor cells can exploit the TCA cycle to produce additional fuel sources, such as glutamine, to meet their metabolic demands.

Our previous work has reported that feedback loop between gankyrin and Nrf2 maintained mitochondrial function. Consistently, we found metabolites involved in the TCA cycle are decreased in gankyrin-knockdown cells, as well as mitochondrial protein, HSP60, and TOMM20, which indicated the diminishment of mitochondria. Mitophagy is a specialized form of autophagy, in which damaged, dysfunctional, or obsolete mitochondria are recognized by the autophagy machinery and eventually degraded by the lysosome. Most of the proteins involved in the mitophagic processes have been shown to be dysregulated in HCC patients, such as MFN1, MFN2, DRP1, and Opa1. Several genetic studies support the current view that both glucose-dependent metabolic pathways and mitochondria metabolism are pivotal in tumorigenesis.

As mentioned above, while some tumors rely mostly on aerobic glycolysis to meet their bioenergetic demands, they also...
strive to maintain mitochondria function to tune their metabolic and biosynthetic requirements. 33

By regulating the PPP and mitochondrial function, gankyrin also maintains high levels of NADPH in tumor cells. NADPH donates high energy electrons for antioxidant defense and reductive biosynthesis. Regeneration and maintenance of the cellular NADP(H) content is strongly implicated in a variety of diseases, such as diabe-
tes, cardiovascular disease, neurodegenerative diseases, and aging, especially in tumorigenesis and cancer progression. 34 Gankyrin was reported to drive glycolysis and glutaminolysis through β-catenin/c-Myc signaling in HCC. 35 Our work focused on the downstream and bypass of glycolysis and proposed that gankyrin accelerates the glu-
cose metabolism process and produces ATP, NADPH, and ribose to satisfy the demand of rapid proliferation of cancer cells.

Intriguingly, we identified TIGAR linking the PPP and mitochondrial function, as well as mitophagy, in HCC. TIGAR is a main tar-
get gene of p53, which plays an important and indispensable role in metabolism. However, TIGAR promotes tumorigenesis in various subtypes of cancer, and the underlying mechanism has mainly been reported to be the production of RSP and NADPH. 36 TIGAR inhibits glycolysis and promotes the PPP to exert oxidative resistance and anti-apoptosis. High levels of TIGAR have been observed in hemato-
pathy and solid tumors, including leukemia, breast cancer, colon cancer, and lung cancer. 36,37 TIGAR promotes chemoresistance in breast cancer and chronic lymphocytic leukemia. 38,39 Chemotherapy is accompanied with increased TIGAR, as well as TIGAR-derived NADPH and GSH production. 40,41 In addition, nuclear localization of TIGAR exerted antioxidation and provides R5P for DNA repair, allowing HCC to benefit from epirubicin treatment. 42 In the present work, we found a new mechanism that TIGAR increased transcription and expression of gankyrin through Nrf2, thereby promoting HCC tumorigenesis. We found a new circulation between gankyrin, Nrf2, and TIGAR participated in metabolism reprogramming of HCC. The synergistic reaction between gankyrin, TIGAR and Nrf2 increased our understanding the metabolism alternation in HCC as PPP and mitochondrial function were boosted and then promoted tumorigenesis. The synergy between gankyrin, Nrf2, and TIGAR ac-
celerated glucose metabolism toward the PPP and TCA cycle, which provided vital materials such as NADPH, ATP, and ribose for the pro-
gression of HCC, as shown in Figure 6. This work provides a novel mechanism for direct metabolic regulation caused by gankyrin, deepens the understanding of targeted metabolic reprogramming of oncogenes, and supplies a potential therapeutic target for HCC.

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DISCLOSURE
The authors have no conflict of interest.

ETHICAL APPROVAL
Approval of the research protocol by an Institutional Review Board: The study was conducted according to guidelines of the Declaration of Helsinki and approved by the Institutional Reviewer Board of Eastern Hepatobiliary Surgery Hospital, Naval Military Medical University (Protocol code: EHBHKY2017-K-006).

INFORMED CONSENT
Written informed consent was obtained from all donors who provided samples.

ANIMAL STUDIES
The animal protocol was approved by the Institutional Animal Care and Use Committee of Eastern Hepatobiliary Surgery Hospital, Naval Military Medical University.

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SUPPORTING INFORMATION
Additional supporting information can be found online in the Supporting Information section at the end of this article.

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