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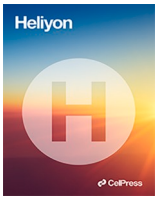

Research article

TFAIP6 facilitates hepatocellular carcinoma cell glycolysis through upregulating c-myc/PKM2 axis

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ABSTRACT

Background: Hepatocellular carcinoma (HCC) is the most prevalent liver cancer. Despite of the improvement of therapies, the durable response rate and survival benefit are still limited for HCC patients. It's urgent to clarify the molecular mechanisms and find therapeutic strategies to improve the clinical outcome. TNFα-stimulated gene-6 (TNFAIP6) plays a critical role in the prognosis of various tumors, but its roles in HCC are still unclear.

Methods: Quantitative real-time PCR (qRT-PCR) and immunohistochemistry (IHC) analysis were employed to evaluate the clinical relevance of TNFAIP6 expressions in HCC patients. Cell counting kit-8 (CCK-8), Edu assay, and transwell assay were performed to evaluate the malignancy of HCC cells. Glucose uptake, lactate production, ATP production, extracellular acidification rate (ECAR) by Seahorse XF analyzer were employed to evaluate the role of TNFAIP6 in the regulation of aerobic glycolysis. The expressions of key proteins involved in glycolysis were examined by Western blot. Co-immunoprecipitation (Co-IP) and chromatin immunoprecipitation (ChIP) were used for protein-protein interactions or protein-RNA interactions respectively. Knockdown and overexpression of TNFAIP6 in HCC cells were employed for analyzing the functions of TNFAIP6 in HCC.

Results: TNFAIP6 was significantly upregulated in HCC and predicted a poor clinical prognosis. Knockdown of TNFAIP6 inhibited *in vitro* cell proliferation, invasion, migration, as well as glycolysis in HCC cells. Mechanistically, we clarified that TNFAIP6 interacted with heterogeneous nuclear ribonucleoprotein C (HNRNPC), stabilized *c-Myc* mRNA and upregulated pyruvate kinase M2 (PKM2) to promote glycolysis.

Conclusions: Our study reveals a molecular mechanism by which TNFAIP6 promotes aerobic glycolysis, which is beneficial for malignance of HCC and provides a potential clinical therapy for disease management.

1. Introduction

Liver cancer remains a health challenge worldwide, and its occurrence is rapidly rising [1]. Many risk factors lead to liver cancer, including chronic virus infection (hepatitis B virus (HBV) and hepatitis C virus (HCV)), smoking, chronic alcohol consumption, obesity, and others [2]. Due to the asymptomatic property of the early HCC, patients are commonly diagnosed at intermediate or advanced

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stages, and unfortunately miss the opportunity for curative treatment (hepatic resection or liver transplantation) [3]. HCC patients diagnosed with advanced stages could be treated by combining radiotherapy, chemotherapy, local ablation, and molecular targeted therapies [4], but the durable survival benefit and response rate are still limited for HCC patients. It's urgent to explore a new therapeutic strategy to improve the clinical outcomes in HCC.

As a hallmark of cancer cells, energy metabolism reprogramming also contributes to the malignant nature of cancer. Despite abundant oxygen, cancer cells use a specific glycolytic strategy to survive which is also known as "Warburg effect" and characterized by increased glucose uptake and lactate production [5,6]. Although it is less efficient to produce adenosine triphosphate (ATP) during aerobic glycolysis, it remains the primary route of ATP supply in many types of tumors [7]. A further understanding of "Warburg effect" in HCC will help to reveal the molecular mechanisms and provide potential therapeutic strategies for HCC. However, detailed molecular mechanism underlying "Warburg effect" in HCC remains poorly understood.

Recently, a critical enzyme regulating glycolysis named PKM2 has gained attentions [8–10]. *PKM2* is an alternative splicing of *PKM* gene variant, highly expressed in different cancers [11,12]. PKM2 plays crucial roles in cell cycle progression and metabolic reprogramming of cancer cells. PKM2 regulates the expression of glycolytic enzymes in renal cell carcinoma [8]. In HCC, mRNA splicing of PKM2 is facilitated by protein phosphatase 1 regulatory subunit 26 (PPP1R26) to drive glycolysis [10]. It also interacts with transcription factor p65 (NF-κB/p65) and hypoxia-inducible factor 1α (HIF-1α), ultimately promotes VEGF-A secretion in human pancreatic adenocarcinoma [13]. Overexpression of PKM2 in cancer cells results in increasing glucose uptake and lactic acid production, thereby promotes cell survival and proliferation [9]. PKM2 depletion significantly impairs cell proliferation, migration and invasion of cancer cells [8,13–15]. *c-Myc* transcription factor is a well-known oncogene for its critical roles in stem cell maintenance and tumorigenesis [16,17]. Dysregulation of *c-Myc* is found in many commonly occurring human cancers including breast cancer [18], colon cancer [19,20], gastric cancer [21] and pancreatic cancer [22]. *c-Myc* contributes to the development of various human cancers by regulating genes related with the biogenesis of ribosomes and mitochondria, as well as glucose and glutamine metabolism [23]. TNFAIP6, also known as TSG-6, is an inflammation-associated protein secreted by mesenchymal stem cells or monocytes/macrophages and can be detected in a variety of tissue injury models and inflammatory diseases [24]. High expression of TNFAIP6 predicts poor overall survival (OS) in patients with various cancer types, including urothelial carcinoma [25] and gastric carcinoma [26]. It has been reported that in mice with acute liver injury, TNFAIP6 suppresses hepatic stellate cells activation and promotes liver regeneration [27]. Furthermore, TNFAIP6 inhibits oxidative stress and induces M2 polarization through suppressing STAT3 activation in hepatic macrophages, suggesting it to be a potential target for the treatment of alcoholic hepatitis [28]. However, the effects of TNFAIP6 in HCC patients and the molecular mechanisms have not been comprehensively investigated. In this study, we found expression of TNFAIP6 in HCC tissues was higher than in normal tissues. We also assessed the co-relation between TNFAIP6 expressions and the clinical parameters and prognosis of HCC patients. High expression of TNFAIP6 contributed to the malignant properties and glycolysis in HCC cells. Our data also clearly clarified a molecular mechanism, which was that TNFAIP6 stabilized c-Myc through interaction with HNRNPC, and thus upregulated the activation of PKM2 to facilitate glycolysis and promoted malignant behavior of HCC cells. Our investigation suggests that TNFAIP6 serves as a novel prognosis indicator and potential therapeutic target for HCC.

2. Materials and methods

2.1. Patients and specimens

A total of 60 HCC tumorous and paracancerous tissues were collected from HCC patients who underwent curative resection from March 2019 to March 2022. All tissues were collected immediately after resections and then transported into liquid nitrogen, and all samples were stored at −80 °C. Tumor stage was determined according to the 6th edition of the Tumor-Node-Metastasis (TNM) classification of the International Union Against Cancer. The research was approved by the Ethics Committee of Third Affiliated Hospital of Naval Medical University (EHBHKY2022-H009-P001), and written informed consent was obtained from each patient.

2.2. Cell culture

The standard liver cell line THLE-2 and HCC cell lines HepG2，SMMC-7721，MHCC97, Huh7，and Hep3B were purchased from China Center for Type Culture Collection (CCTCC). Cell lines were maintained in DMEM or RPMI 1640 (Giboco, Waltham, MA, USA) supplemented with 10 % FBS (Gibco, Waltham, MA, USA) and 1X Penicillin-Streptomycin solution at 37 °C in a humidified chamber containing 5 % CO_2.

2.3. Cell transfection

To establish Hep3B or Huh7 cells overexpressing or knocking down of TNFAIP6 respectively, pcDNA3.1 plasmid expressing TNFAIP6 or short hairpin RNA (shRNA) with the sequence of GCAGCAGGCGTATACCATAGA targeting *TNFAIP6* was generated by RiboBio (Guangzhou, China). The empty plasmid (vector) or that expressing control shRNA (sh-NC) was used as the corresponding negative control. Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) was employed for transfection of plasmids or shRNAs respectively following the manufacture's manuals. Briefly, 8×10^5 cells were plated into a 6-cm plate overnight, and then shRNAs or plasmids were transfected using the lipofectamine reagents in culture media without antibiotics. Cells were incubated for another 48 h before harvest.

2.4. CCK-8 cell viability assay

CCK-8 (Sigma-Aldrich, St Louis, MO, USA) was used for cell viability assay. 2000 cells were seeded into 96-well plates and transfected for indicated days, followed by adding 10 μl CCK-8 reagents to each well and incubated at 37 °C for 3 h. The absorbance at 450 nm was recorded and analyzed on a microplate reader.

2.5. Click-iT Edu assay

Click-iT® Plus EdU Alexa Fluor® 555 Imaging Kit (Invitrogen, Carlsbad, CA, USA) was used for cell proliferation assay following the manufacture's manuals. Briefly, Huh7 or Hep3B cells were incubated with 10 μM Edu for 2 h, and then cells were fixed with 4 % paraformaldehyde (PFA) and permeabilized with 0.5 % Triton-X 100 respectively, followed with the detection of Edu using Click-iT® Plus reaction cocktails according to the user guide. For nuclear staining, Hoechst 33342 was incubated at room temperature (RT) for 30 min.

2.6. Quantitative real-time PCR

TRIzol (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA from cells or tissues, following the manufacture's user guide. M-MLV-RTase from Promega was used for RNA reverse transcription. SYBR Master Mixture (TAKARA, Tokyo, Japan) was used to perform qRT-PCR on the Agilent MX3000p Real time PCR system. The sequence of qRT-PCR primers could be found in Supplementary Table 1.

2.7. Co-immunoprecipitation

Co-IP was performed using Pierce™ Classic Magnetic IP/Co-IP Kit (Thermofisher Scientific, Rockford, IL, USA) following the manufacturer's instructions. Briefly, cell lysates were incubated with HNRNPC antibody for 1 h at 4 °C. Then, protein A/G magnetic beads were added to bind antigen-antibody complex at RT for another 1 h, followed by washing the beads with IP lysis/wash buffer twice, and finally the complex was eluted.

2.8. Glucose uptake, ATP levels, and lactate production

Huh7 or Hep3B cells were plated at a density of 5×10^5 cells/well and incubated for 24 h. Cell lysates were used to analyze glucose uptake (AAT Bioquest, Sunnyvale, CA, USA) and ATP levels (Beyotime, Shanghai, China). Lactate production in the culture medium was determined using Lactic acid Assay Kit (Elabscience, Wuhan, China). All experiments were following the manufacturer's instructions and all procedures were performed on ice.

2.9. Seahorse assay

Cell glycolysis was monitored using the Seahorse Bioscience Analyzer (XF24, Seahorse Bioscience Inc., North Billerica, MA, USA). Briefly, Huh7 or Hep3B cells were plated in a Seahorse XF96 Cell Culture Microplate (Agilent, Santa Clara, CA, USA) at a density of 4×10^4 per well and incubated at 37 °C overnight. Next day, cells were washed and incubated with unbuffered media for 1 h at 37 °C without CO_2. The machine ran a 3 min mixture and 30 s waiting time as a cycle. First three cycles were recorded for basal respiration measurement. Then ECAR were measured. During the assay, various compounds were injected into the chamber. Three ECAR values were recorded after injection with glucose (10 mM), oligomycin (2 μM), and 2DG (100 mM).

2.10. Trans-well assay

Trans-well assays were used to determine cell migration and invasion. For cell migration assay, 3×10^4 cells were seeded into the upper chamber of 24-well inserts (Corning, Lowell, MA, USA) and Ham′s F–12K or DMEM/F-12 complete medium supplemented with 10 % FBS were added into the lower chambers to serve as a chemoattractant. Cells were incubated for 48 h, then cells migrating to the other side of the filter were stained and counted. For Cell invasion experiment, we seeded 1×10^4 cells per 100 μl serum-free medium into the upper surface of the chambers pre-coated with Matrigel (BD Biosciences, Bedford, MA, USA). After incubation for 48 h, the culture medium and the cells on the upper surface were removed, and cells attached on the lower surface were fixed by methanol for 30 min, followed by staining with crystal violet for another 30 min. The images of migrant and invasive cells were collected under an inverted microscope.

2.10.1. ChIP assay

Pierce Magnetic ChIP Kit (ThermoFisher Scientific, Rockford, IL, USA) was employed to perform ChIP assay, the experiment followed the manufacture's user guide. Briefly, 4×10^6 cells were crosslinked using 1 % formaldehyde for 10 min at RT, and then the crosslinking reaction was stopped by glycine. Cell lysates were incubated with Dynabeads conjugated with PKM2 antibody (15822-1-AP, Proteintech, Rosemont, IL, USA) for 2 h at 4 °C. Then, the beads were washed with IP wash buffer, and eluted with elution buffer to reverse cross-linking of DNA-protein complexes at 65 °C for 1.5 h. Purified DNA was subjected to qPCR analysis.

2.11. Western blot analysis

Proteins were lysed with RIPA buffer containing proteinase inhibitors. Protein concentrations of lysates were determined by Bradford (Sigma-Aldrich, St. Louis, MO, USA). Subsequently, a total of 20 μg protein was added to each well of 10 % SDS-PAGE gel. Then proteins were transferred to NC or PVDF membranes, and blocked with 5 % non-fat milk at RT for 1 h. Membranes were incubated with TNFAIP6 (ab267469, Abcam, Oxford, UK), E-Cadherin (ab40772, Abcam, Oxford, UK), N-Cadherin (ab76011, Abcam, Oxford, UK), Twist 1 (ab50887, Abcam, Oxford, UK), PKM2(ab85555, Abcam, Oxford, UK), HK2 (ab228819, Abcam, Oxford, UK), LDHA (ab52488, Abcam, Oxford, UK), PFK1 (ab119796, Abcam, Oxford, UK) and GAPDH (ab8245, Abcam, Oxford, UK) primary antibodies at 4 °C overnight respectively, followed by incubation with secondary antibody (Invitrogen, Carlsbad, CA, USA) at RT for 60 min.

2.12. Immunohistochemistry

IHC was performed on paraformaldehyde-fixed, paraffin-embedded normal liver or HCC tumor tissues. Sections were treated with 3 % H_2O_2 for 15 min at RT to block endogenous peroxidase activity. Then sections were blocked with goat serum for an hour, incubated with primary antibodies overnight at 4 °C. After incubation, the sections were washed with PBS for 3 times, and subsequently incubated with HRP-conjugated secondary antibodies for an hour at RT. After washing with PBS three times for 5 min each, diaminobenzidine tetrahydrochloride (DAB) was used as a substrate for signals detection. Nuclear was counterstained with hematoxylin, and sections were sealed. Images were captured with an Olympus X71 inverted microscope (Olympus Corperation, Tokyo, Japan).

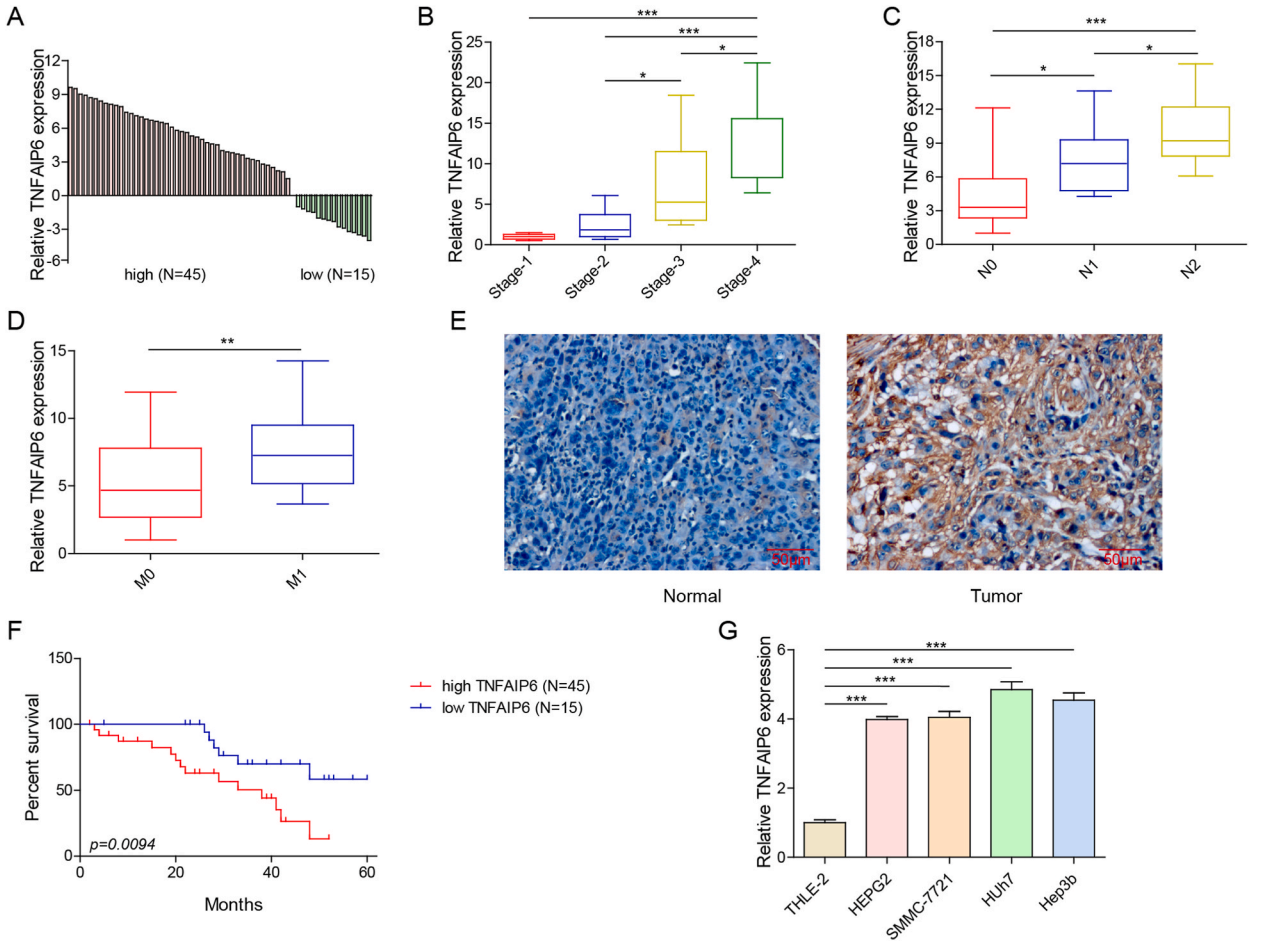

Fig. 1. Ectopically overexpressed TNFAIP6 indicates the poor prognosis of HCC patients. (A) The expression of TNFAIP6 was examined by qRT-PCR, compared between 60 pairs of HCC and matched noncancerous liver tissues. (B,C,D) TNFAIP6 expression was evaluated in patients with different stages of HCC (B), lymph node metastasis (C), and distant metastasis (D) respectively. (E) The positive expression of TNFAIP6 was examined by immunohistochemistry in HCC tissues and normal tissues. (F) The overall survival of patients was analyzed by Kaplan-Meier analyses. (G) The expression of TNFAIP6 was examined by qRT-PCR in normal liver cell line and HCC cell lines. n = 60, *, P < 0.05; **, P < 0.01; ***, p < 0.001.

2.13. Luciferase assay

The JASPAR software was used to predict potential binding site of *c-Myc* or *PKM2* promoter. The wild type *PKM2* promoter or *PKM2* promoter mutant was amplified and cloned into a pGL3-report vector (Promega Corporation, Madison, WI, USA). Equal amount of the pGL3 and pRL-TK plasmids (Promega Corporation, Madison, WI, USA) were co-transfected into 293T cells for 48 h, then luciferase activity was measured using the Dual-Glo Luciferase Assay system (Promega Corporation, Madison, WI, USA).

2.14. Statistical analysis

GraphPad prism 6.0 software was employed for data analysis. To compare quantitative data, post hoc Tukey test was used for One-Way ANOVA assay. The data was shown as the mean ± SD of three independent experiments. $p < 0.05$ was considered as statistical significance.

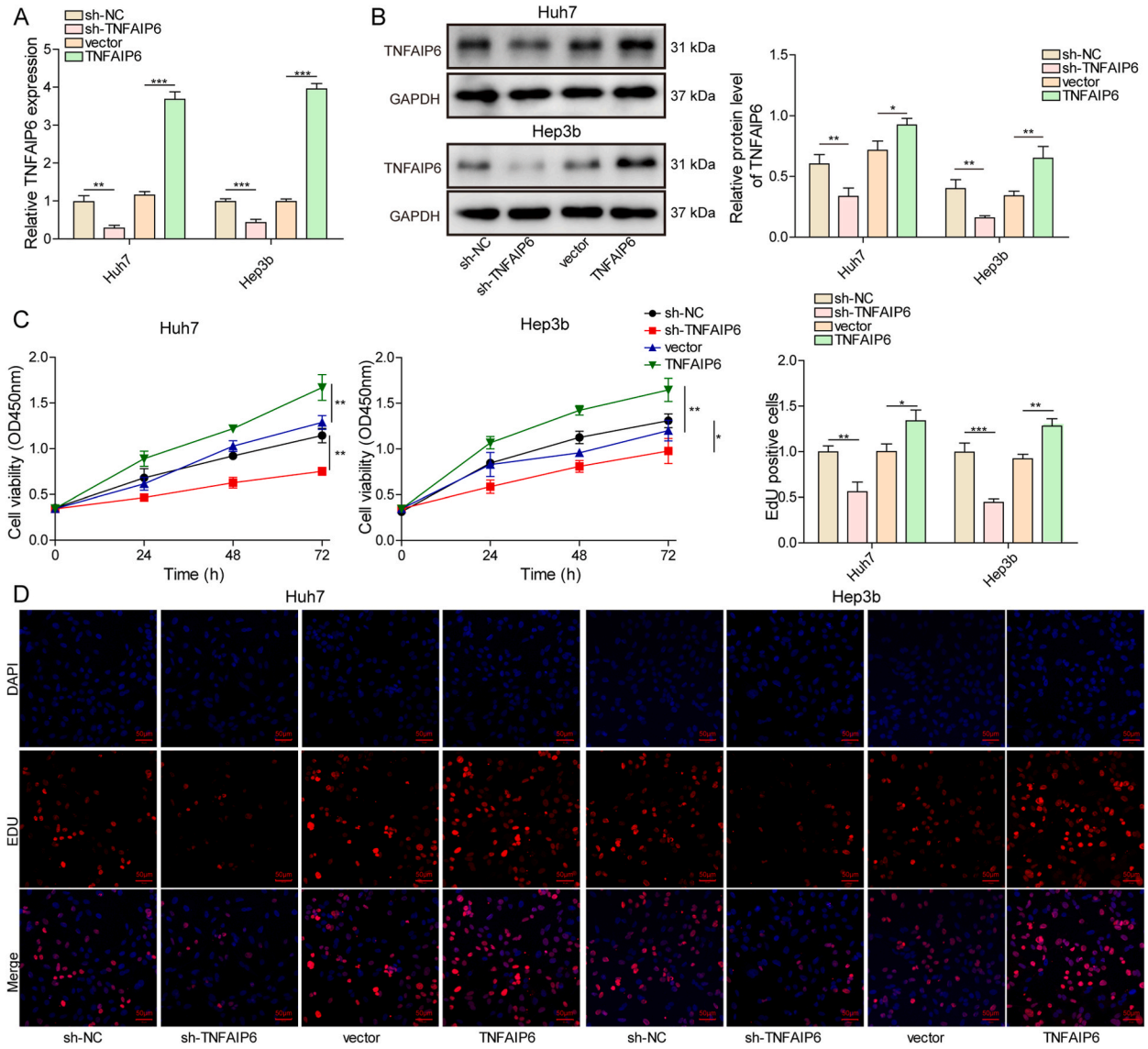

Fig. 2. TNFAIP6 confers to the proliferation, migration and invasion of HCC cells. Huh7 and Hep3B cells were transfected with vector, TNFAIP6, sh-NC or sh-TNFAIF6 respectively. (A) Analysis of mRNA level of TNFAIP6 using qRT-PCR. (B) Analysis of protein level of TNFAIP6 upon over-expression or knockdown using Western blot. Cell proliferation was detected by CCK-8 analysis (C) and Edu assays (D). (E) *Trans*-well analysis of migration and invasion. (F) The expression of EMT related proteins was analyzed by Western blot. n = 3, mean ± SEM *, $P < 0.05$; **, $P < 0.01$; ***, $p < 0.001$.

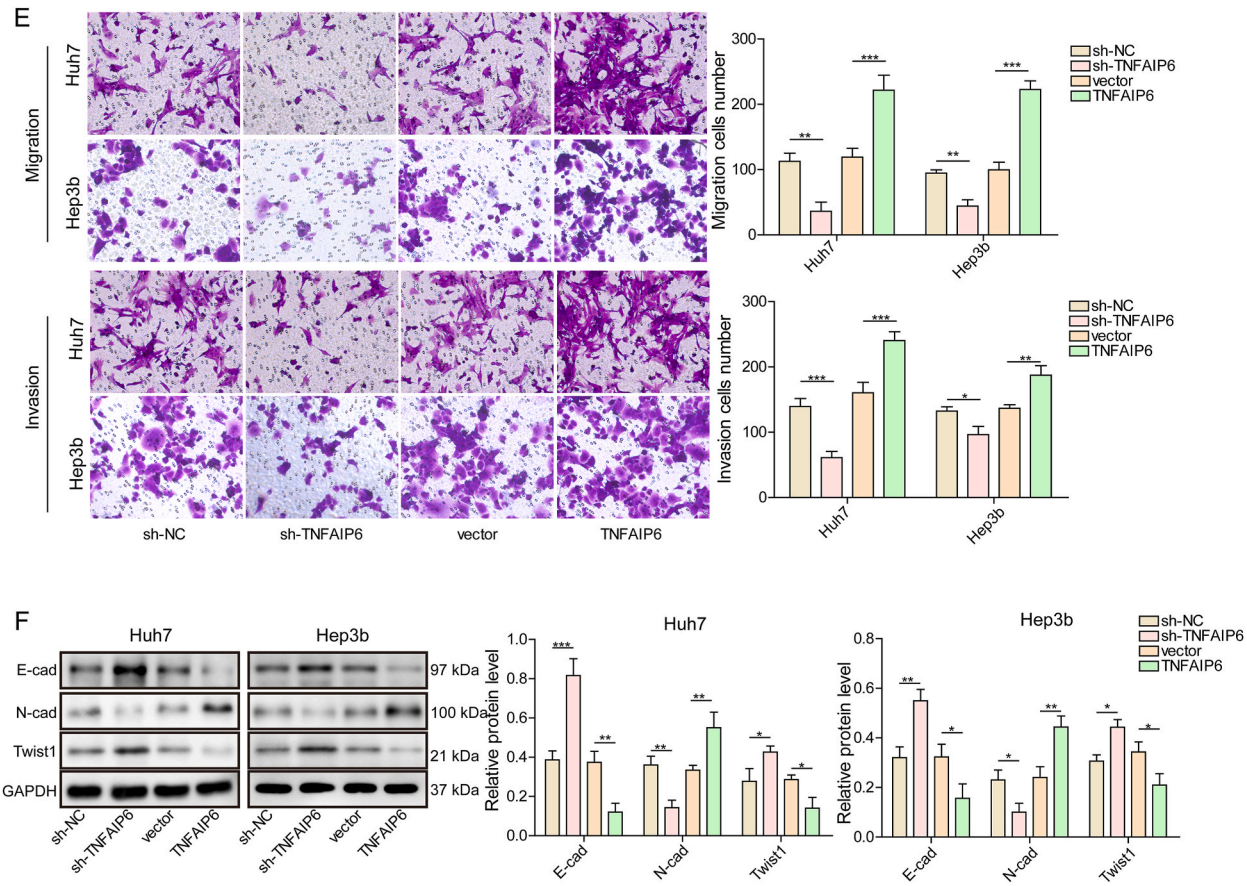

Fig. 2. (*continued*).

3. Results

3.1. Ectopically overexpressed TNFAIP6 indicates the poor prognosis of HCC patients

To explore the relationship between TNFAIP6 expression level and HCC, we collected 60 patients' HCC and paracancerous tissues. qRT-PCR results showed that 45 of 60 HCC tissues showed a high expression of TNFAIP6, and 15 of 60 HCC tissues showed a relative low expression of TNFAIP6 (Fig. 1A). Interestingly, TNFAIP6 expression was significantly elevated in patients with advanced stages (Fig. 1B), lymph node metastasis (Fig. 1C) and distant metastasis (Fig. 1D). The immunochemistry data also indicated a significant increase of TNFAIP6 expression in HCC tissues (Fig. 1E). Then, we further explored the relationship between TNFAIP6 expression and prognosis of HCC patients. Overall survival (OS) was obviously shorter in patients with high expression of TNFAIP6 than in patients with low expression of TNFAIP6 (Fig. 1F). We next analyzed the TNFAIP6 expression level in HCC cell lines including HepG2, SMMC-7721, Huh7 and Hep3B. Compared to normal cells (THLE-2), HCC cell lines exhibited a higher TNFAIP6 expression. These data indicated that a higher TNFAIP6 expression level was significantly co-related with HCC patients.

3.2. TNFAIP6 confers to the proliferation, migration and invasion of HCC cells

To demonstrate the specific role of TNFAIP6 in HCC cells, Huh7 and Hep3B cells were used for further investigation because of their relative high endogenous expression of TNFAIP6. Overexpression with plasmid transfection or knockdown with specific shRNA of TNFAIP6 was performed in Huh7 and Hep3B cells, then mRNA or protein expression levels were validated by qRT-PCR (Fig. 2A) or Western blot (Fig. 2B or Fig. 2B in Supplementary Fig. 2) respectively. Overexpression of TNFAIP6 resulted in an increased viability, while knockdown of TNFAIP6 led to a decreased cell viability and proliferation in CCK-8 analysis (Fig. 2C). To further explore the role of TNFAIP6 in cell proliferation, Edu assay was also performed. Edu positive rates in cells overexpressing with TNFAIP6 were 3 times higher than normal cells (Fig. 2D). The degree of malignance and aggressiveness of cancers could be reflected by the extent of metastasis and invasion. Next, we analyzed the ability of migration and invasion by *trans*-well assays. Compared to normal cells, cells overexpressing with TNFAIP6 exhibited about 3 times higher capability of migration and invasion. On the other hand, the capability of migration and invasion was notably decreased in cells with sh-TNFAIP6 (Fig. 2E). Next, we tested the expression level of some

epithelial-mesenchymal transformation (EMT) related proteins in HCC cells by Western blot. Interestingly, overexpression of TNFAIP6 increased the expression level of N-Cad, but decreased the expression of E-Cad and Twist 1, both in Huh7 and Hep3B cells, and vice versa (Fig. 2F or Fig. 2F in Supplementary Fig. 2).

3.3. TNFAIP6 promotes HCC cells glycolysis

As a new hallmark of cancer cells, glycolysis plays important roles in tumorigenic functions. TNFAIPs are involved in tumor development and progression [29], and as a member of TNFAIP family, TNFAIP8 has been previously reported to increase glycolysis in prostate cancer [30]. Thus, we speculated TNFAIP6 might function similarly with TNFAIP8 and then we investigated if TNFAIP6 played important roles in glycolysis. Compared to cells from negative control groups, knockdown of TNFAIP6 in Huh7 and Hep3B cells attenuated glucose uptake (Fig. 3A), lactate production (Fig. 3B) and ATP productions (Fig. 3C). In the other hand, when we over-expressed TNFAIP6 in Huh7 and Hep3B cells, compared to control cells, the glucose uptake (Fig. 3A), lactate production (Fig. 3B) and ATP productions (Fig. 3C) were significantly increased. We also perform the seahorse assay to measure the ECAR to determine glycolysis. As seen in Fig. 3D, TNFAIP6 gene knockdown can effectively reduce the rate of ECAR compared with sh-NC group. After overexpression of TNFAIP6, ECAR was significantly increased. To further explore the role of TNFAIP6 in glycolysis, we analyzed the expression level of some key proteins in glycolysis. As reported, depletion of HK2 inhibits glycolysis in HCC [31]. LDHA regulates aerobic glycolysis in pancreatic cancer [32]. Both PKM2 and PFK1 are key regulators contributing to glycolysis [13,33]. As seen in Fig. 3E or Fig. 3E in Supplementary Fig. 2, knockdown of TNFAIP6 decreased the expression level of HK2, LDHA, PKM2 and PFK1. Meanwhile, overexpression of TNFAIP6 resulted in increased expression of these proteins. Taken together, the results strongly proved that TNFAIP6 functions as an oncogenic driver in HCC.

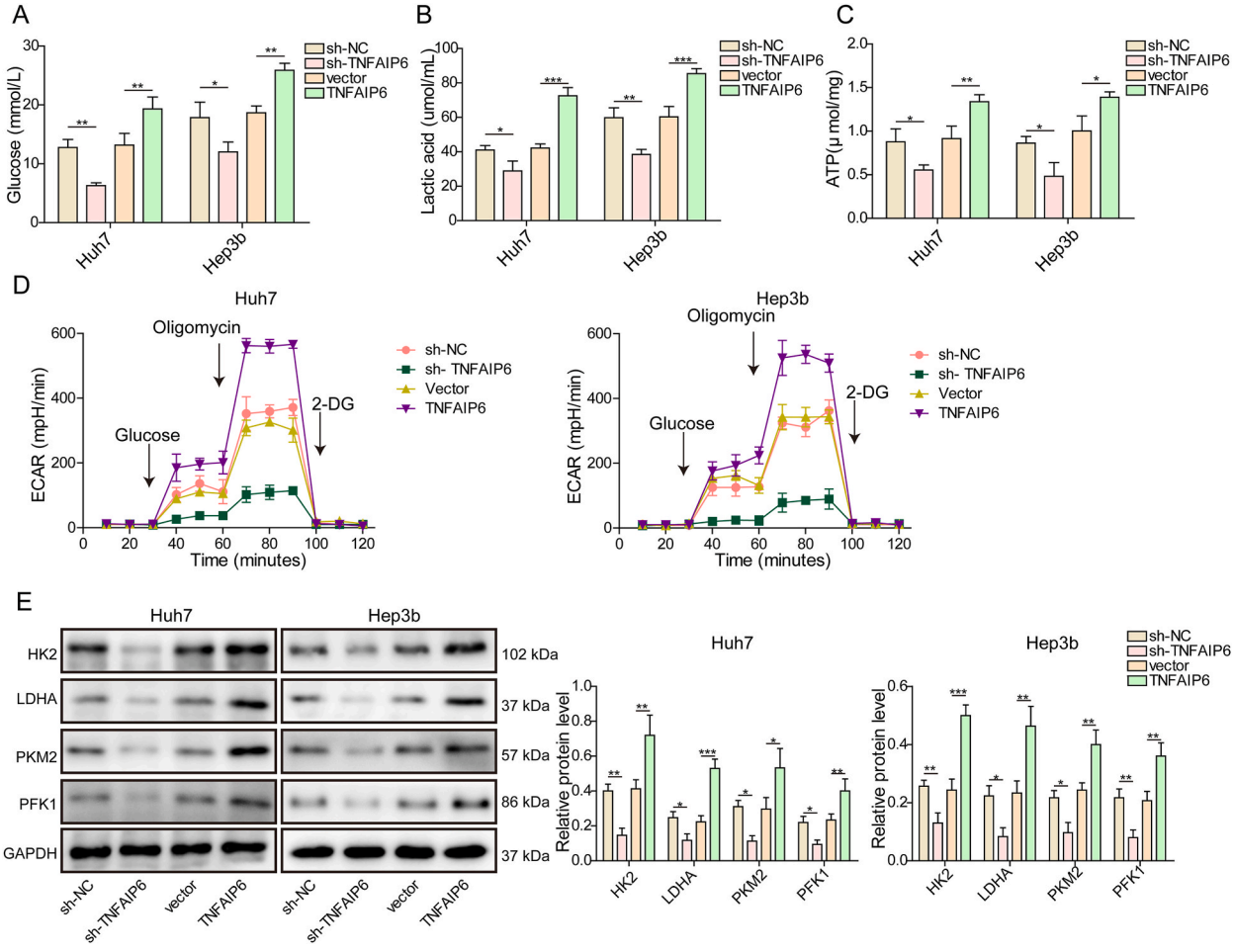

Fig. 3. TNFAIP6 promotes HCC cells glycolysis. Huh7 and Hep3B cells were transfected with vector, TNFAIP6, sh-NC or sh-TNFAIF6 respectively. (A,B,C) Glucose uptake levels (A), Lactic acid (B) and ATP production (C) was measured by commercial kits. (D) The ECAR was measured by seahorse assay. (E) The expression of glycolysis related proteins was analyzed by Western blot n = 3, mean ± SEM, *, P < 0.05; **, P < 0.01; ***, p < 0.001.

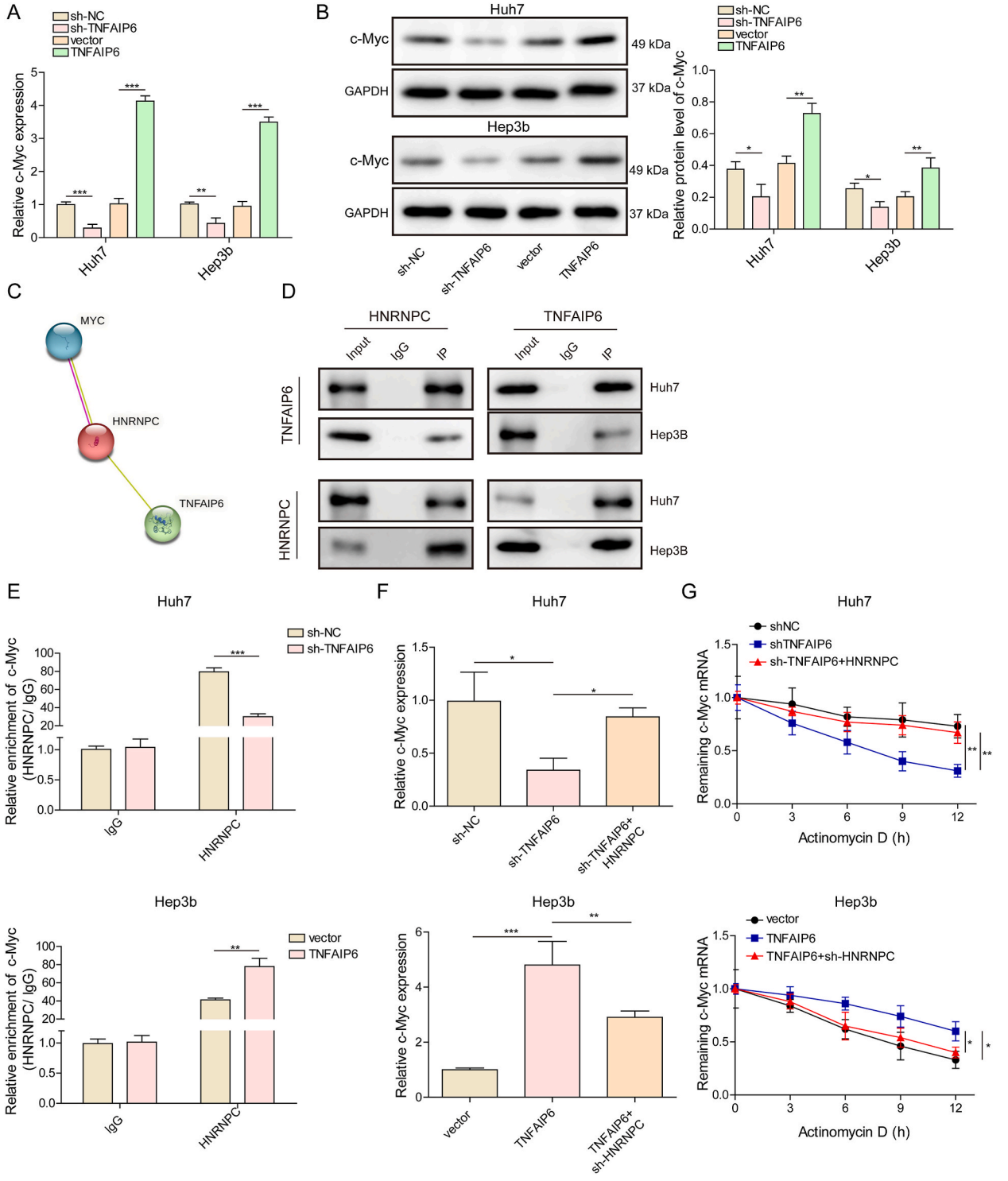

Fig. 4. TNFAIP6 stabilizes c-Myc mRNA by interacting with HNRNPC. (A) c-Myc mRNA expression level was detected by qRT-PCR. (B) c-Myc protein expression level was detected by Western blot. (C) The STRING online software predicted the interactions among TNFAIP6, HNRNPC and c-Myc. (D) The potential interaction between HNRNPC, TNFAIP6 and c-Myc was assessed by Co-IP assay. (E) The interaction between HNRNPC and c-Myc after TNFAIP6 overexpression or knockdown was assessed by Co-IP assay. (F) c-Myc mRNA expression level was examined by qRT-PCR. (G) The mRNA stability of c-Myc was examined by qRT-PCR after treating with actinomycin D for indicated time periods. n = 3, mean ± SEM, *, $P < 0.05$; **, $P < 0.01$; ***, $p < 0.001$.

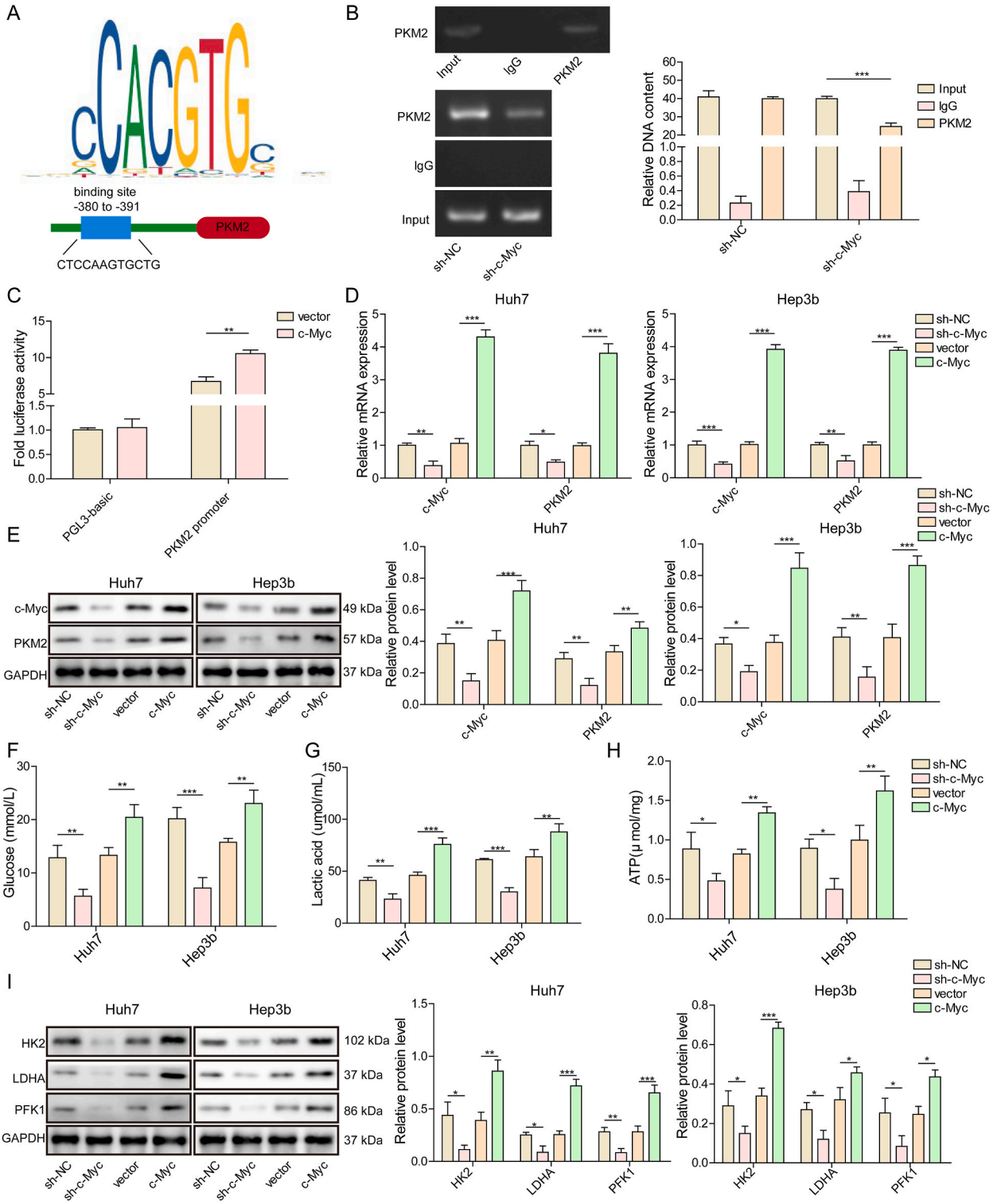

Fig. 5. c-Myc transcription activates PKM2 to induce HCC cells glycolysis. (A) JASPAR software predicted the binding site of c-Myc to the PKM2 promoter region. (B) The binding between c-Myc and PKM2 promoter region was validated by ChIP assay. (C) Luciferase reporter assay to measure activity of 293T cells expressing wild type PKM2 or PKM2 mutant. (D and E) mRNA and protein expression levels of c-Myc and PKM2 in c-Myc overexpressing or silencing cells. (F,G,H) Glucose uptake levels (F), Lactic acid (G) and ATP production (H) were measured in Huh7 and Hep3B cells overexpressing or knocked-down c-Myc by commercial kits. (I) The expression of Glycolysis related proteins was analyzed by Western blot. n = 3, mean ± SEM, *, P < 0.05; **, P < 0.01; ***, p < 0.001.

3.4. TNFAIP6 stabilizes c-Myc mRNA by interacting with HNRNPC

Next, to clarify the mechanism of TNFAIP6's oncogenic role, we assessed the impacts of TNFAIP6 on serval HCC oncogenes including *TP53*, *c-MYC*, *ZEB1*, *HIF1α* and *STAT3* using qPCR. We found that knockdown of TNFAIP6 in HCC cells did not affect expressions of *TP53*, *ZEB1* and *STAT3*, but it suppressed the expression of *c-Myc* and *HIF1α*. The most significant effect was observed on *c-MYC* expression (Supplementary Fig. 1). *c-Myc* gene dysregulation is frequently found in human hepatocellular carcinoma [34]. To explore whether TNFAIP6 is associated with dysregulation of *c-Myc*, we analyzed expression levels of *c-Myc* upon TNFAIP6 overexpression or knockdown. qRT-PCR and Western blot results showed that silence of TNFAIP6 down-regulated the expression level of c-Myc, both in mRNA and protein levels. Meanwhile, overexpression of TNFAIP6 led to an increase of c-Myc expression (Fig. 4A and B or Fig. 4B in Supplementary Fig. 2). To reveal the relationship between TNFAIP6 and c-Myc in depth, we used STRING software to predict the interactions between them (Fig. 4C). We found that they both interacted with HNRNPC. As an RNA binding protein, HNRNPC belongs to the hnRNP family, and functions in mRNA splicing, stabilization and translation [35–37]. Emerging evidence suggests important roles of HNRNPC in different cancers, including prostate cancer [38], hepatocellular carcinoma [39] and breast cancer [40]. Next, the interactions between HNRNPC and c-Myc or TNFAIP6 were validated with Co-IP experiments in both Huh7 and Hep3B cells (Fig. 4D or Fig. 4D in Supplementary Fig. 2). To further determine the relationship between TNFAIP6 and c-Myc, we analyzed the enrichment of c-Myc RNA in TNFAIP6 overexpressed or knockdown cells. Compared to control cells, overexpression of TNFAIP6 increased the amount of c-Myc RNA, and vice versa (Fig. 4E). These data suggested TNFAIP6 stabilized c-Myc RNA. To clarify the mechanism, we overexpressed HNRNPC in TNFAIP6 knocked-down cells and found overexpression of HNRNPC could rescue the downregulation of c-Myc caused by TNFAIP6 deletion. Similarly, the silence of HNRNPC in TNFAIP6 overexpressed cells down-regulated the expression of c-Myc (Fig. 4F). Furthermore, deletion of TNFAIP6 promoted c-Myc RNA degradation, while overexpression of HNRNPC could attenuate the degradation. Correspondingly, overexpression of TNFAIP6 slowed down the degradation of c-Myc RNA, and knockdown of HNRNPC reversed the effects (Fig. 4G). Altogether, we came to a conclusion that TNFAIP6 stabilized c-Myc mRNA through interacting with HNRNPC.

3.5. c-Myc transcription activates PKM2 to induce HCC cells glycolysis

To further identify the reason why TNFAIP6 promoted glycolysis in HCC cells, we next investigated the association between c-Myc and glycolysis. As a last rate-limiting enzyme, PKM2 expressed highly in cancer cells and was always associated with a poor prognosis [41].

JASPAR database predicted a potential binding site between c-Myc and PKM2 promoter (Fig. 5A). To prove the interactions between c-Myc and PKM2 promoter, both ChIP assay and dual-luciferase reporter assay were performed. Anti-c-Myc ChIP assay detected a strong binding between c-Myc and the promoter region of PKM2 in HCC cells. Weak binding was detected with IgG, and the binding was markedly diminished after c-Myc knockdown (Fig. 5B). The luciferase activity also decreased in cells expressing the PKM2 mutant that doesn't contain c-Myc binding sites (Fig. 5C). To further explore the mechanism, we tested the relative expression levels of PKM2 in response to c-Myc overexpression or knockdown. Both Huh7 and Hep3B were used for the experiments. qRT-PCR and Western blot results showed that the relative expression of PKM2 was in accordance with c-Myc. When we overexpressed c-Myc, the expression level of PKM2 also increased. In contrast, when we deleted c-Myc in HCC cells, the expression level of PKM2 decreased (Fig. 5D and E or Fig. 5E in Supplementary Fig. 2). As previously reported, PKM2 promoted the progression of HCC through regulating glucose metabolism [14]. We next investigated if c-Myc could regulate glucose metabolism through PKM2. Knockdown of c-Myc efficiently inhibited glucose uptake, lactate production and ATP production. In contrast, overexpression of c-Myc enhanced glucose uptake, lactate and ATP production in both Huh7 and Hep3B cells (Fig. 5F, G and 5H). We also analyzed the key proteins in glycolysis.

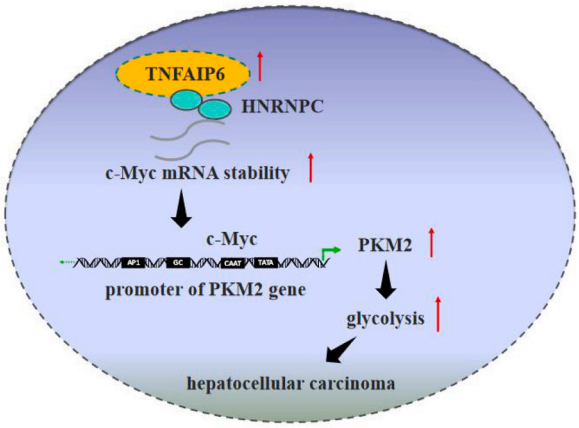

Fig. 6. The graphical abstract of TNFAIP6 in HCC. TNFAIP6 binds to HNRNPC, stabilizes c-Myc mRNA and upregulates c-Myc/PKM2 axis to promote glycolysis in hepatocellular carcinoma.

Compared to negative control, knockdown of c-Myc led to downregulation of HK2, LDHA and PFK1, while overexpression of c-Myc upregulated the expression of these proteins (Fig. 5I or Fig. 5I in Supplementary Fig. 2). Taken together, these data indicated that c-Myc promoted the glycolysis via modulating PKM2.

In summary, our study clearly demonstrated the molecular mechanisms of TNFAIP6 in hepatocellular carcinoma. TNFAIP6 binds to HNRNPC, stabilizes the interaction of c-Myc/PKM2, and promotes cell glycolysis in HCC (Fig. 6).

4. Discussion

Our data firstly certified the role of TNFAIP6 in HCC. It has been reported that TNFAIP6 plays an important role in many types of cancers [25,26], and always predicts a poor prognosis. Upregulated TNFAIP6 promotes cancer cell proliferation and cell invasion, which are the natures of cancer cells. However, whether TNFAIP6 functions in HCC is unknown. Here we investigated the roles of TNFAIP6 in HCC, and found that the expression of TNFAIP6 was higher in HCC patients and tissues. Kaplan-Meier analysis of survival indicated a poor prognosis with TNFAIP6 high-expression in HCC patients (Fig. 1). These indicated a critical role of TNFAIP6 in HCC. Additionally, we also found TNFAIP6 was required for cell proliferation, migration and invasion in HCC cells, and knockdown of TNFAIP6 decreased malignancy in HCC cells (Fig. 2). These data suggested TNFAIP6 played a crucial role in the proliferation and invasion of HCC. Additionally, our work also provided evidence that *TNFAIP6* might serve as an oncogene in a wide range of cancers, which needs to be further clarified.

Most cancer cells reprogram cellular glucose metabolism to support rapid cancer cell growth, invasion and survival [42]. We analyzed glucose uptake levels, lactic acid and ATP productions in TNFAIP6 overexpressing or silencing cells (Fig. 3), and the results suggested that upregulation of TNFAIP6 enhanced HCC cells aerobic glycolysis. Taken together, above findings demonstrated the oncogenic role of TNFAIP6 in hepatocellular carcinoma was via positive regulation of aerobic glycolysis, which will be a new support for “Warburg effect” in HCC. However, the ways of TNFAIP6 affecting HCC may be various, and more studies should be performed.

To clarify the molecular mechanisms of TNFAIP6 promoting HCC, we next found TNFAIP6 stabilized c-Myc mRNA by interacting with HNRNPC (Fig. 4). HNRNPC is the first member of a family discovered to regulate RNA splicing and has two isoforms (C1 and C2) in humans [43, 44]. It has been reported that HNRNPC is up-regulated in various cancers [40, 45, 46], suggesting its important role in the occurrence and progress of cancers. HNRNPC functions in cancer by many ways, including regulating the RNA stability [47], splicing [48] or RNA modification [49]. In this study, we found, as the downstream of TNFAIP6, HNRNPC regulated the c-Myc RNA stability. Silencing of TNFAIF6 led to the downregulation and instability of c-Myc RNA, which could be rescued by overexpression of HNRNPC (Fig. 4).

As a transcription factor, c-Myc is a well-known oncogene [16,17,19]. Intriguingly, although multiple studies have demonstrated that Myc directly binds as high as 30 % of all known gene promoters, only a portion of the bound genes actually respond (up-regulate or down-regulate) to Myc [50]. In our study, we mainly focused on the regulation of c-Myc in glucose metabolism. c-Myc interacted with PKM2 (Fig. 5A–C). As previously reported, PKM2 drives glycolysis in HCC [10] and regulates the expression of glycolytic enzymes in renal cell carcinoma [8]. Fig. 5 showed that knockdown of c-Myc affected metabolism reprogramming, decreased the glucose uptake levels, ATP and lactic acid production, and regulated the expressions of key proteins involved in glycolysis, which were consistent with the role of PKM2. Collectively, these data showed that upregulation of c-Myc facilitated cell glycolysis in HCC cells.

In summary, our data demonstrated the roles of TNFAIP6 in hepatocellular carcinoma. By identifying the upregulation of c-Myc/PKM2 downstream of TNFAIP6 to promote aerobic glycolysis in HCC and defining its regulation and relevance to clinical outcomes, we identified the TNFAIP6- HNRNPC-c-Myc-PKM2 axis, and provided a potential novel therapeutic approach for the treatment of HCC by targeting TNFAIP6. However, one limitation of this work was the lack of *in vivo* studies, which will provide more evidence for clinical approach. Thus, more efforts are still needed.

4.1. Ethics approval statement

This study was approved by the Ethics Review Committee of Third Affiliated Hospital of Naval Medical University (EHBHKY2022-H009-P001). All patients provided written informed consent, and the study was conducted in accordance with the Declaration of Helsinki. All authors confirm that all methods are carried out in accordance with relevant guidelines and regulations.

Consent for publication

Not applicable.

Conflict of interest disclosure

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data availability statement

Data will be made available on request.

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CRediT authorship contribution statement

Kecai Duan: Writing – review & editing, Writing – original draft, Resources, Methodology, Conceptualization. **Kunpeng Fang:** Writing – review & editing, Writing – original draft, Supervision, Investigation, Formal analysis. **Chengjun Sui:** Writing – review & editing, Supervision, Investigation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e30959.

Abbreviation

HCC	hepatocellular carcinoma
TNFAIP6	TNFα-stimulated gene-6
qRT-PCR	Quantitative real-time PCR
CCK-8	Cell Counting Kit-8
OCR	oxygen consumption rate
ECAR	extracellular acidification rate
Co-IP	Co-immunoprecipitation
ChIP	Chromatin immunoprecipitation
HNRNPC	heterogeneous nuclear ribonucleoprotein C
PKM2	pyruvate kinase M2
HBV	hepatitis B virus
HCV	hepatitis C virus
ATP	adenosine triphosphate
TNM	Tumor-Node-Metastasis
CCTCC	China Center for Type Culture Collection
shRNA	short hairpin RNA
PFA	paraformaldehyde
RT	room temperature
OXPHOS	cellular oxidative phosphorylation
DAB	diaminobenzidine tetrahydrochloride
OS	overall survival
EMT	epithelial-mesenchymal transformation
HIF-1α	hypoxia-inducible factor 1α

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