Original Article

NDRG2 inhibition of glycolysis in liver tumor cells is regulated by SIRT1

Le Zhang[#], Jiangang Kang[#], Benge Xin, Wei Cao

Department of Interventional Radiology, The Second Affiliated Hospital of Air Force Medical University, Xi'an, China

Contributions: (I) Conception and design: W Cao; (II) Administrative support: W Cao; (III) Provision of study materials or patients: L Zhang, J Kang, W Cao; (IV) Collection and assembly of data: L Zhang, J Kang; (V) Data analysis and interpretation: B Xin; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

[#]These authors contributed equally to this work.

Correspondence to: Wei Cao. Department of Interventional Radiology, The Second Affiliated Hospital of Air Force Medical University, 569 Xinsi Road, Baqiao District, Xi'an 710038, China. Email: Cawe-001@163.com.

Background: N-Myc downstream-regulated gene 2 (NDRG2) is a tumor suppressor, highly expressed in normal tissues but downregulated in many cancers. However, it has been shown to be involved in regulating glycolytic enzymes in clear cell renal cell carcinoma and colorectal cancer, although the mechanism is still not clear, and the function of NDRG2 in liver tumor glycolysis is completely unknown.

Methods: Liver tumor tissues were obtained from resected tumors and confirmed by pathological review. Immunohistochemical staining was performed to assess the protein expression of NDRG2. NDRG2-overexpressed and knockdown HepG2/SMMC-7721 cell lines were infected by lentivirus and cultured, before measurement of glucose uptake, lactate production, lactase dehydrogenase activity, and oxygen consumption rate. NDRG2 and SIRT1 proteins were analyzed by western blot.

Results: Both the mRNA and protein levels of NDRG2 as a tumor suppressor were downregulated in the liver tumors and the survival rate of patients negatively correlated with the expression of NDRG2. In the NDRG2-overexpressed and knockdown cell lines, NDRG2 showed inhibition of glycolysis in liver tumor cells. Our experimental data suggested the expression of SIRT1 negatively correlated with that of NDRG2.

Conclusions: Our study findings enrich the understanding of the role of NDRG2 in tumor growth and of the mechanism by which NDRG2 regulates glycolysis. SIRT1, a deacetylase that plays an important role in glycolysis regulation, may be negatively regulated by NDRG2 in liver tumors.

Keywords: N-Myc downstream-regulated gene 2 (NDRG2); liver tumor; glycolysis; SIRT1

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Introduction

Liver tumor ranks among the top three malignant tumors due to their high heterogeneity, high mortality, strong invasiveness, difficult cure, and poor prognosis (1).

N-Myc downstream-regulated gene 2 (NDRG2) is a known tumor suppressor that is downregulated or even absent in various types of cancer (2,3). Overexpression of NDRG2 can inhibit the growth, and promote the apoptosis of cancer cells (4). Moreover, in liver tumors, researchers have demonstrated that overexpression of NDRG2 can inhibit cell migration and invasion by downregulating CD24 expression (5) and matrix metalloproteinases (MMPs) (6,7). All these reports prove the correlation between NDRG2 and the prognosis of liver tumors.

Glycolysis is a hallmark of cancer cells after metabolic reprogramming (8,9). Two studies reported the regulatory role of NDRG2 on glycolysis in colorectal cancer cells (10,11). Overexpression of NDRG2 in colorectal cancer cells reduced glucose consumption, lactate production, and

increased oxygen consumption; knockdown of NDRG2 increased glucose consumption, lactate production increased, and decreased oxygen consumption, suggesting that NDRG2 has an inhibitory effect on glycolysis in tumor cells. Studies show that NDRG2 reduces the proliferation of colorectal cancer cells, possibly by downregulating glucose transport and metabolism-related enzymes such as glycolysis-related hexokinase 2 (HK2), pyruvate kinase M2 isoform (PKM2), lactate dehydrogenase A (LDHA) and GLUT1, and upregulating the expression of TNXIP (10,11). Shi et al demonstrated that NDRG2 inhibits glycolysis in clear-cell renal cell carcinoma in the same way (12).

Silent mating type information regulator 1 (SIRT1) belongs to the Sirtuin family and is an NAD+-dependent histone deacetylase (13-15). SIRT1-mediated deacetylation inhibits the functions of multiple tumor suppressors, including P53 (16), P73 (17), and HIC1 (18), suggesting that SIRT1 promotes tumorigenesis and progression. Emerging studies have shown that tumor progression affected by SIRT1 may be an important way of regulating glycolysis. SIRT1 stimulates the expression of glycolysis-related genes, such as GLUT1 and GAPDH, thus promoting glycolysis in tumors (19-21). Furthermore, SIRT1 can also interact with GAPDH and retains it in the cytosol, thus protecting the enzyme from nuclear translocation, and promoting glycolysis (22). Based on these reports, a possible correlation between NDRG2 and SIRT1 attracted our attention.

In the present study, we focused on the role of NDRG2 in regulating glycolysis and in regulating SIRT1 expression in liver tumor cells. Our data indicated a novel pathway of NDRG2 regulation of glycolysis in liver tumors. We present the following article in accordance with the MDAR reporting checklist (available at https://jgo.amegroups.com/article/view/10.21037/jgo-23-149/rc).

Highlight box

Key findings

- We find that NDRG2 can inhibit glycolysis in liver tumor cells via SIRT1.

What is known and what is new?

- We know that NDRG2 is a tumor suppressor, but the function of NDRG2 in liver tumor glycolysis is completely unknown.
- This manuscript reported that SIRT1 is a key regulator involved in glycolysis in liver tumor cells.

What is the implication, and what should change now?

- This study findings enrich the understanding of the role of NDRG2 in tumor growth and of the mechanism by which NDRG2 regulates glycolysis.

Methods

Patient information and tissue specimens

The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). This study was approved by the Ethics Committee of the Second Affiliated Hospital of Air Force Medical University (No. GKJ-Y-140) and informed consent was taken from all the patients. Fresh liver cell carcinoma specimens were collected from tumors resected from 143 patients at the Second Affiliated Hospital of Air Force Medical University (Xi'an, China) from 2004 to 2008. Liver tumor tissues and corresponding non-carcinoma tissues were confirmed by pathological review and staged according to the American Joint Cancer Committee/Union for International Cancer Control (AJCC/UICC) classification guidelines. The grading and histopathology subtyping of liver tumor specimens was based on the WHO criteria.

Immunohistochemistry

Immunohistochemical staining was performed to assess the protein expression of NDRG2 as described previously (23). Formalin-fixed tumor tissues were embedded in paraffin, and serial 4-mm sections were obtained using a Leica microtome. For staining, tumor sections were dewaxed in toluene, rehydrated in an alcohol gradient, permeabilized in citrate buffer (pH 6.0), quenched with 3% H_2O_2 for 5 min to eliminate endogenous peroxidase activity, washed in phosphate-buffered saline (PBS), incubated overnight with different antibodies and then with biotinylated goat anti-rat or anti-rabbit IgG antibody for 15 min. After washing, sections were incubated with streptavidin peroxidase, lightly counterstained with hematoxylin, and observed under a photomicroscope.

Cell lines and cell culture

HepG2 and SMMC-7721 and HEK-293T cells were purchased from Merck Millipore (Billerica, MA, USA) and cultured in Dulbecco's modified Eagle's medium (Invitrogen Life Technologies, USA) supplemented with 10% fetal bovine serum (FBS, Invitrogen Life Technologies) at 37 ℃

in an incubator (5% CO_2, 21% O_2 and 74% N_2).

Lentivirus generation and infection

Recombinant lentiviral vectors were constructed with Invitrogen's ViraPower™ Lentiviral System in our laboratory. The cDNAs of human NDRG2 were cloned and subcloned into the vector pLenti6. Short hairpin RNAs (shRNA) against human NDRG2 were designed using a small interfering RNA design program and then subcloned into the EcoR I/Age I sites of the pLKO-TRC vector. The shRNA sequences specific for NDRG2 were: shNDRG2-1 forward: 5'-CCGGGAGGACATGCAGGAAATCATTCT CGAGAATGATTTCCTGCATGTCCTCTTTTTG-3'; shNDRG2-1 reverse: 5'-AATTCAAAAAGAGGACATGC AGGAAATCATTCTCGAGAATGATTTCCTGCATGT CCTC-3'; shNDRG2-2 forward: 5'-CGGGATCCAAAAA AGCCACCTCAAGCGTCCGTCCTAGCAACAGCAA GCTTCCTGTTGCCAGGACAGACGCCTGAGGCG GCGGTGTTTCGTCCTTTCCACAA-3'; shNDRG2-2 reverse: 5'-CCCTCGAGCCCCAGTGGAA-3'. The sequences for the control nonsense shRNA were: control forward: 5'-CCGGAAGGTCTTGTCCTCATCAACACT CGAGTGTTGATGAGGACAAGACCTTTTTTTG-3'; control reverse: 5'-AATTCAAAAAAAGGTCTTGTCC TCATCAACACTCGAGTGTTGATGAGGACAAGA CCTT-3'.

The HEK-293T cells were transfected with the pLenti6-Cherry/NDRG2, pLKO-Scramble/NDRG2-shRNA, PAX2, and PMD2G lentiviral vectors using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen). After 48 h, the lentiviral supernatants were collected, filtered (0.45-μm size filter; Millipore, Billerica, MA, USA), and added to the HepG2/SMMC-7721 cells in the presence of 2 μg/mL Blasticidin (Sigma-Aldrich, USA) or 1 μg/mL Polybrene (Sigma-Aldrich, USA) for 6–8 h. Two rounds of infection were performed. After infection, the cells that survived this treatment were selected and collected after1 week had passed, and then analyzed for NDRG2 expression by Western blot.

Western blot analysis

The cells were harvested from 60-mm culture dishes and prepared by lysis in 200 mL RIPA buffer [0.05 M Tris-HCl (pH 7.4), 0.15 M NaCl, 0.25% deoxycholic acid, 1% Nonidet P-40, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 mg/mL aprotinin and 10 mg/mL leupeptin], supplemented with 100:1 (v/v) ratio of a protease inhibitor cocktail and phosphatase inhibitor cocktail. The protein concentrations in the lysates were measured using the bicinchoninic acid (BCA) protein assay. The proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes, which were saturated with Tris-buffered saline with 0.1% Tween 20 and 3% bovine serum albumin (TBST-BSA) and were then probed with the appropriate antibodies: NDRG2 (1:2,000, Cell Signaling Technology, USA), β-actin (1:2,000, Cell Signaling Technology, USA), SIRT1 (1:1,000, Cell Signaling Technology, USA), followed by incubation with species-matched secondary antibodies. The bands were detected using enhanced chemiluminescence (Pierce Rockford, USA) or the Odyssey Imaging System (LiCor Biosciences). The band intensities were quantified with Kodak Digital Science 1D 3.0 (Eastman Kodak, USAT).

Measurement of glucose uptake, lactate production, lactate dehydrogenase (LDH) activity and oxygen consumption rate

Cells were seeded on 6-well plates at a density of 2×10^5 cells per well and incubated at 37 ℃ for 24 h. The concentrations of glucose and lactate in the culture medium were measured by Glucose TestKit (Invitrogen) and Lactate Assay Kit (Jiancheng Bioengineering, China) respectively. Harvested cells were digested with 0.25% trypsin and washed with PBS. The cell suspension was homogenized on ice. LDH activity was measured by colorimetric assay using a specific test kit (Solarbio, China) according to the manufacturer's instructions. The absorbance of LDH was measured at 450 nm and its activity in the control group was normalized to 1.0.

The cellular activity and oxygen consumption rate (OCR) were determined using the Seahorse XFe 96 Extracellular Flux Analyzer (Seahorse Bioscience, USA). Experiments were performed according to the manufacturer's instructions. OCR was measured with the Seahorse XF Cell Mito Stress Test Kit (Seahorse Bioscience). Briefly, 2×10^5 cells were plated onto Seahorse plates, maintained overnight, and then washed with Seahorse buffer. Next, Seahorse buffers including oligomycin (Oligo), p-trifluoromethoxy carbonyl cyanide phenylhydrazone (FCCP), and rotenone + antimycin A (Rot + AA) were sequentially injected. The results were analyzed using software XF-96 wave (Seahorse Bioscience). All experiments

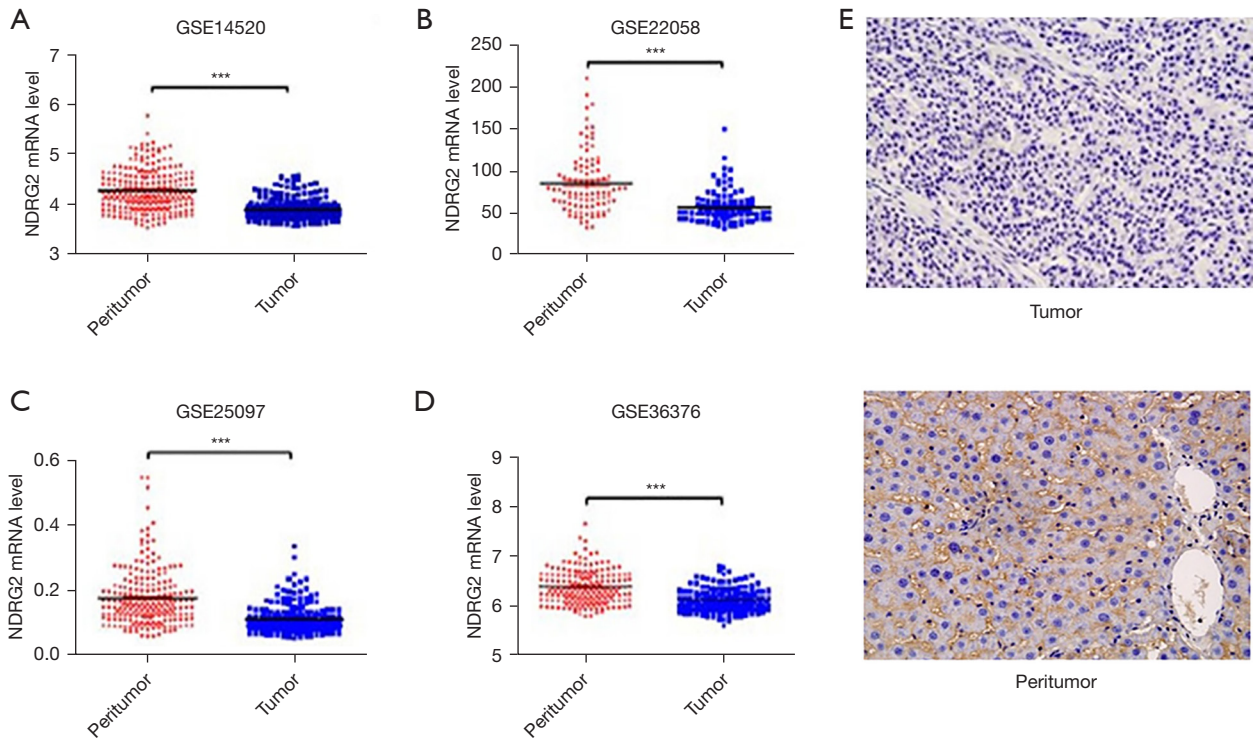

Figure 1 NDRG2 mRNA expression levels in liver tumor and peritumor analyzed in four sub-databases of the Gene Expression Omnibus (A-D). Immunohistochemical assays was performed to detected the protein expression levels of NDRG2 in human liver tumor tissues (E, ×200). ***, P<0.05. NDRG2, N-Myc downstream-regulated gene 2.

were repeated at least 3 times.

Statistical analysis

The chi-square test or Fisher's exact test and Student's *t*-test were utilized to determine the significance of the differences between groups. The survival rates were analyzed using Kaplan-Meier analysis and log-rank test. The *t*-test method was used to compare the differences between groups. Statistical analysis was performed using SPSS software, Version 16.0 (Chicago, USA). Statistical significance was based on P<0.05.

Results

NDRG2 expression in liver tumor cells

We first analyzed NDRG2 mRNA levels in patients using Gene Expression Omnibus (GEO), a database repository of gene expression profiles. As shown in *Figure 1A-1D*, the mRNA level of NDRG2 (P<0.05) was significantly decreased in liver tumors compared with peritumor from four sub-databases (GSE14520, GSE22058, GSE25097, GSE36376). The protein level of NDRG2 was assessed in 143 liver carcinoma tissues and corresponding non-carcinoma tissues. The results of immunohistochemical staining showed distinct staining of the cytoplasm of non-carcinoma tissues, but only faint or no staining in the liver tumor tissues (*Figure 1E*). Thus, we concluded that NDRG2 as a tumor suppressor was downregulated at both the mRNA and protein levels in liver tumors.

Correlation between NDRG2 expression and prognosis of liver tumor patients

We then investigated the correlation between NDRG2 expression and survival status of liver tumor patients using Kaplan-Meier curves. *Figure 2* shows that patients with higher NDRG2 expression had good overall survival (OS) (P<0.001), disease-specific survival (DSS) (P<0.001), and relapse-free survival (RFS) (P<0.01) compared with those with lower NDRG2 expression. In detail, the mean OS of patients with high and low NDRG2 protein expression

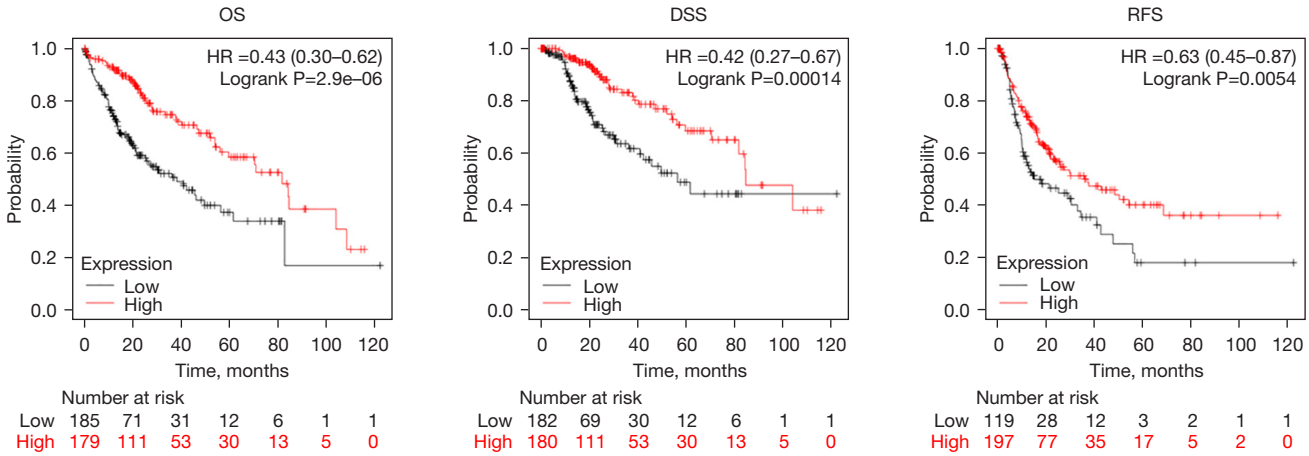

Figure 2 Kaplan-Meier curves of survival rates of liver tumor patients with NDRG2 expression patterns. Results were acquired from online database Kaplan-Meier Plotter (kmplot.com). NDRG2, N-Myc downstream-regulated gene 2; OS, overall survival; DSS, disease-specific survival; RFS, relapse free survival.

levels were 81.9 and 38.3 months, respectively. The mean DSS of patients with high and low NDRG2 protein expression levels were 84.7 and 56.5 months, respectively. The mean RFS of patients with high and low NDRG2 protein expression levels were 37.2 and 15.17 months, respectively.

NDRG2 inhibits glycolysis in liver tumor cells

Glycolysis is a major feature of tumor cell metastasis (8,9). To prove whether NDRG2 can also inhibit glycolysis in liver tumor cells as in colorectal cancer cells (10), we constructed two liver tumor cell lines with stable knockdown and overexpression of NDRG2 by using an NDRG2-shRNA plasmid and NDRG2 eukaryotic expression plasmid, respectively (*Figure 3A,3B*). According to our data, overexpression of NDRG2 in the liver tumor cell line (SMMC-7721) inhibited glycolysis as suggested by decreased glucose uptake rate (*Figure 3C*), decreased lactate production (*Figure 3D*), decreased LDH activity (*Figure 3E*), and increased OCR (*Figure 3F*). Furthermore, NDRG2 knockdown by shRNA in liver tumor cells (HepG2) promoted glycolysis as indicated by decreased OCR (*Figure 3G*). Taken together, these data demonstrated that NDRG2 inhibited glycolysis in liver tumor cells.

Downregulation of SIRT1 by NDRG2

To further explore the role of NDRG2 in glucose metabolism, the protein interaction network of NDRG2 was achieved using the STRING database (https://string-db.org/) (*Figure 4A*). Impressively, SIRT1 was found in this network despite unknown or predicted interactions with NDRG2. Hence, we conceived a correlation between NDRG2 and SIRT1(24) that may contribute to glucose metabolism in liver tumor cells. Subsequent western blot analysis showed increased SIRT1 expression in NDRG2 knockdown cells but a decrease in NDRG2-overexpressed cells (*Figure 4B,4C*). Our experimental data suggested the expression of SIRT1 negatively correlated with that of NDRG2.

Discussion

NDRG2 is a known tumor suppressor involved in energy metabolism, especially glucose metabolism (25,26). In this study, the mRNA and protein expression levels of NDRG2 were analyzed in liver carcinoma tissues and non-carcinoma tissues, with the results reconfirming its downregulated expression in liver tumors (*Figure 1*).

Recently, it was reported that NDRG2 is involved in cellular glucose metabolism through insulin signal transduction based on NDRG2 being a substrate of kinase Akt and serum- and glucocorticoid-induced kinase 1 (SGK1) (27,28). According to the Warburg effect, cancer cells are more prone to glycolysis than oxidative phosphorylation for glucose metabolism (29,30). Glycolysis contributes to cancer progression (29), and glycolysis inhibition is

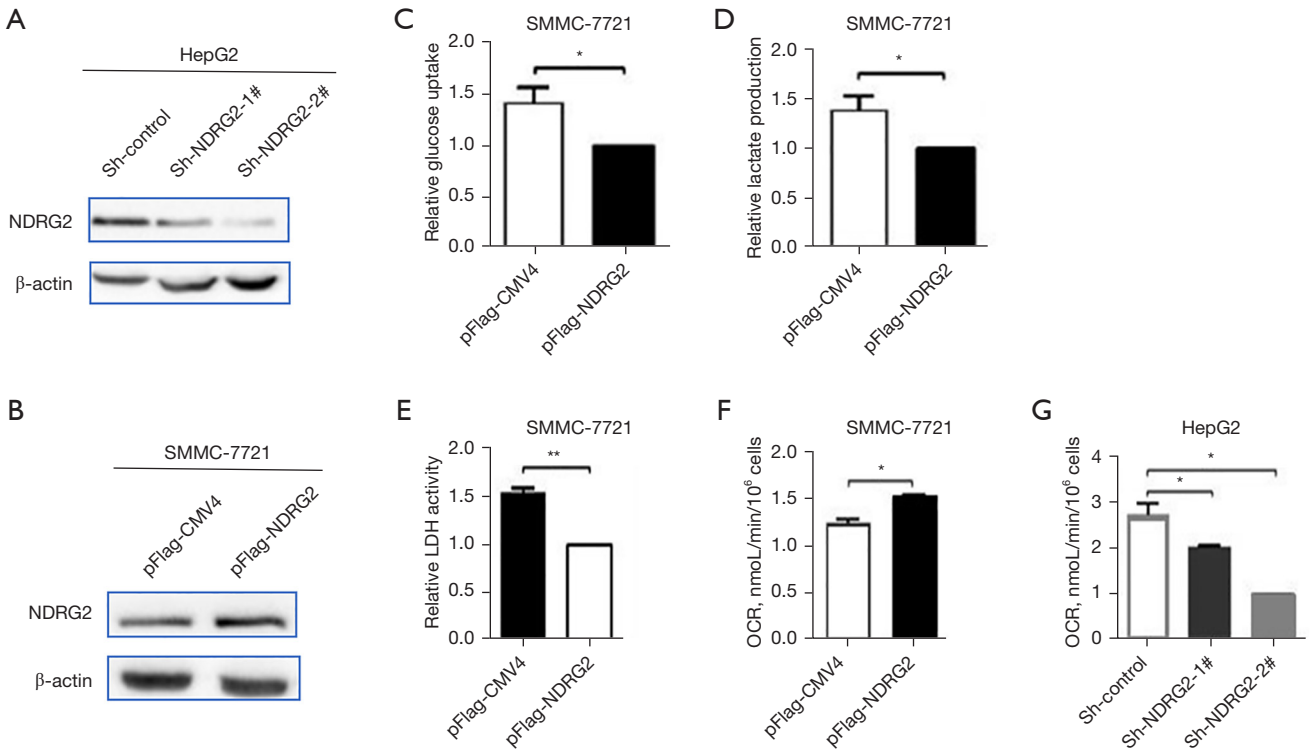

Figure 3 Mechanism of NDRG2 inhibition of glycolysis in liver tumor cells. NDRG2 downregulation via viral delivery of shRNA in HepG2 cells (A). NDRG2 upregulation via viral delivery of pFlag-NDRG2 in SMMC-7721 cells (B). NDRG2 overexpression inhibits glycolysis in SMMC-7721 cells as indicated by glucose uptake (C), lactate production (D), LDH activity (E), and OCR (F). NDRG2 downregulation decreases the oxygen consumption rate in HepG2 cells (G). *, P<0.05; **, P<0.01. NDRG2, N-Myc downstream-regulated gene 2; LDH, lactate dehydrogenase; OCR, oxygen consumption rate.

emerging as a promising area of cancer therapy (31). To date, including in our present study, NDRG2 has been shown to significantly inhibit glycolysis of tumor cells in colorectal cancer (10,11), clear cell renal cell carcinoma (12), and liver tumor (*Figure 3C-3F*). In light of these reports, the suppression of glycolysis by NDRG2 is through the regulation of glycolysis-related genes. NDRG2 was first identified to decrease glucose uptake in breast cancer by promoting GLUT1 protein degradation without affecting GLUT1 transcription (32). Subsequently, the expressions of glycolysis-related hexokinase 2 (HK2), pyruvate kinase M2 isoform (PKM2), and lactate dehydrogenase A (LDHA) were proved to be significantly suppressed by NDRG2 in colorectal cancer cells and clear cell renal cell carcinoma cells (10,12). Moreover, NDRG2 could stimulate TXINP expression to reduce glucose uptake (11).

Interestingly, although *NDRG2* is an N-My downstream-regulated gene, it is not repressed by transcription factor N-Myc but by C-Myc (10,33). C-Myc is a known viral oncogene in cancer energy metabolism (34,35) and mainly promotes the glycolysis of cancer cells through upregulating glycolysis gene expressions, such as LDH, HK2, GLUT1, and PKM2 (3). In addition, HIF-1 and P53, two other transcription factors, also play crucial roles in tumorigenesis by regulating the expression of glycolytic genes (36). HIF-1 promotes but P53 hinders these genes expression (37,38). Moreover, HIF-1 and P53 show negative and positive regulatory effects on NDRG2, respectively (39-42). Hence, we believe that the regulation by NDRG2 of glycolysis flux is accomplished by cooperating with C-Myc, HIF-1, and P53 to regulate the expression of glycolytic genes.

Sirtuins (e.g., SIRT1-7) play important roles in the Warburg effect and can regulate glycolytic genes through various effects (21). Sirtuins directly regulate the expression of glycolytic enzymes, alter the enzymatic activity of glycolytic genes via multiple post-translational modifications and affect the sub-location of the enzymes (19,21,43,44). For example, SIRT1promotes the expression of GLUT1,

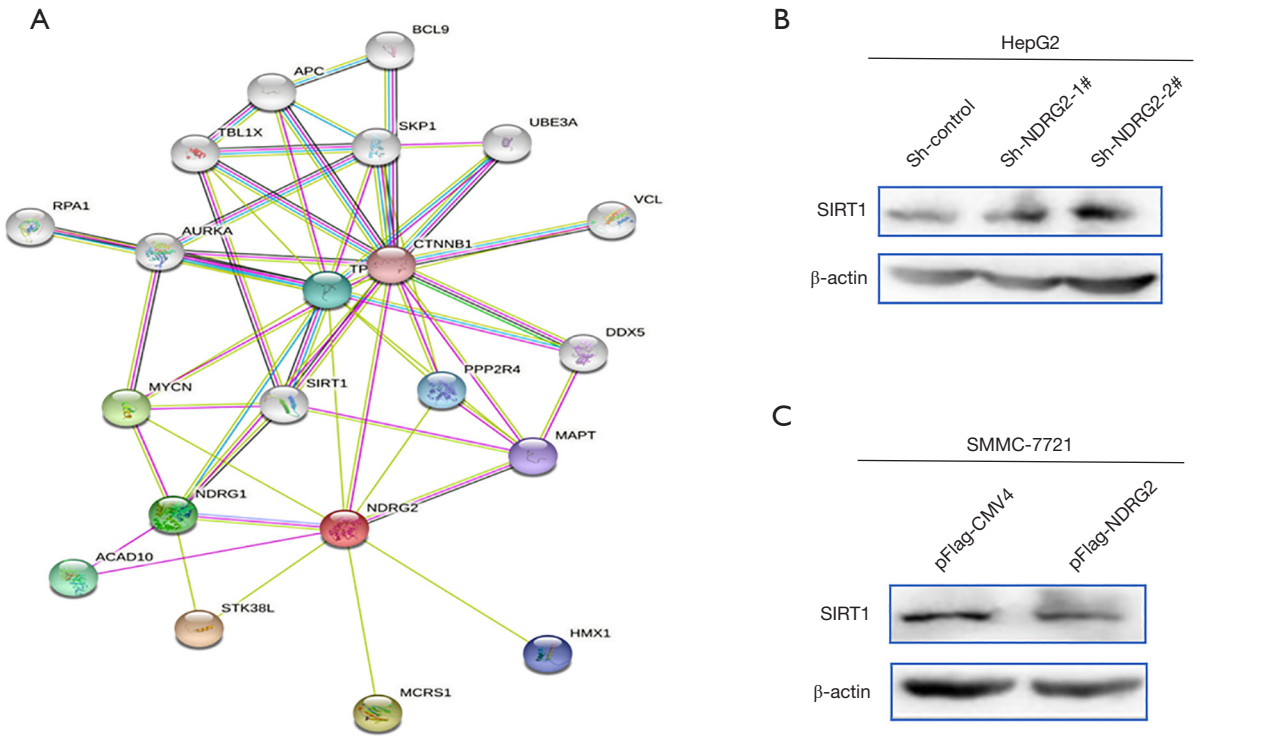

Figure 4 Negative regulation of SIRT1 expression by NDRG2. Analysis of NDRG2 protein interaction network using the online STRING database (A). Western blot analysis of SIRT1 expression in NDRG2 down-regulated (B) and up-regulated (C) cells. NDRG2, N-Myc downstream-regulated gene 2.

GAPDH, and LDHA to benefit glycolysis (19,43), interacts with GAPDH, and retains it in the cytosol, thus promoting glycolysis (22). NDRG2 and SIRT1 show opposing regulatory effects on glycolytic enzymes (*Figure 4B*,*4C*). The regulatory relationship between SIRT1 and glycolytic regulators is opposite to that between NDRG2 and glycolytic regulators. The expression of SIRT1 increases through C-Myc binding to the SIRT1 promoter, and then deacetylating C-Myc, and stimulating the transcriptional activity of C-Myc (21); SIRT1-mediated deacetylation suppresses the functions of P53 (45).

In this study, we found that SIRT1 regulates the inhibitory effects of NDRG2 on glycolysis in hepatocellular carcinoma cells, but further research on the relationship between SIRT1 and glycolysis is needed.

Conclusions

Overall, NDRG2 and SIRTI, as a pair of negative regulators, have opposing effects on tumor glycolysis, SIRT1 plays an important role in glycolysis regulation, is negatively regulated by NDRG2 in liver tumors.

Acknowledgments

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Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at https://jgo.amegroups.com/article/view/10.21037/jgo-23-149/rc

Data Sharing Statement: Available at https://jgo.amegroups.com/article/view/10.21037/jgo-23-149/dss

Peer Review File: Available at https://jgo.amegroups.com/article/view/10.21037/jgo-23-149/prf

Conflicts of Interest: All authors have completed the ICMJE

uniform disclosure form (available at https://jgo.amegroups.com/article/view/10.21037/jgo-23-149/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). This study was approved by the Ethics Committee of the Second Affiliated Hospital of the Air Force Medical University (No. GKJ-Y-140) and informed consent was taken from all the patients.

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