N-Myc downstream-regulated gene 2 restrains glycolysis and glutaminolysis in clear cell renal cell carcinoma

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Abstract. Glycolysis and glutaminolysis are heavily involved in the metabolic reprogramming of cancer cells. The activation of oncogenes and inactivation of tumor suppressor genes has a marked effect on the cellular metabolic processes glycolysis and glutaminolysis. N-Myc downstream-regulated gene 2 (NDRG2) is a tumor suppressor gene that previous studies have demonstrated can inhibit the growth, proliferation and metastasis of clear cell renal cell carcinoma (ccRCC) cells. However, the function of NDRG2 in ccRCC metabolism remains unknown. In the present study, NDRG2 significantly inhibited the consumption of glucose and glutamine, as well as the production of lactate and glutamate in ccRCC. NDRG2 significantly suppressed the expression of glucose transporter 1, hexokinase 2, pyruvate kinase M2, lactate dehydrogenase A, glutamine transporter ASC amino acid transporter 2 and glutaminase 1 at the mRNA (by quantitative polymerase chain reaction) and protein level (by western blot analysis), all of which are key regulators and enzymes in glycolysis and glutaminolysis. Data from the present study also revealed that overexpression of NDRG2 suppressed cell proliferation in ccRCC *in vitro* and *in vivo*, demonstrated by colony formation assays, wound healing assay and nude mouse transplantation tumor experiment. The present findings demonstrate for the first time that NDRG2 acts as a key inhibitor of glycolysis and glutaminolysis in ccRCC and could be a promising target for the metabolic treatment of ccRCC.

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Abbreviations: NDRG2, N-Myc downstream regulated gene 2; ccRCC, clear cell renal cell carcinoma; GLUT1, glucose transporter 1; HK2, hexokinase 2; PKM2, M2 isoform of pyruvate kinase; LDHA, lactate dehydrogenase A; GLS1, glutaminase 1; ASCT2, alanine-serine-cysteine (ASC) amino acid transporter 2

Key words: NDRG2, metabolism, glycolysis, glutaminolysis, ccRCC

Introduction

Altered metabolism is considered to be a hallmark of cancer cells, aiding the maintenance of uncontrolled growth and proliferation by providing sufficient biomass and energy (1). Since the altered metabolism of transformed cells significantly contributes to cellular proliferation, targeting metabolic pathways of cancer cells is a promising area in cancer therapeutics (2). A key metabolic alteration exhibited by the majority of cancer cells is enhanced aerobic glycolysis, a phenomenon known as the Warburg effect, which provides several metabolic benefits to proliferating cancer cells (3). Additionally, cancer cells exhibit increased glutamine metabolism, which has important roles in bioenergetic and biosynthetic processes of cancer cells (4).

Clear cell renal cell carcinoma (ccRCC) is the most common malignancy of the kidney (5). Previous studies revealed that renal cancer tissues exhibited a different metabolic profile from normal tissues; RCC tissues exhibited the Warburg glycolytic phenotype and higher glucose levels than normal tissues (6). To meet the unique energetic requirements of cancer cells, changes in glycolysis and glutaminolysis alter the intracellular carbon flux (7,8). During the initiation and progression of cancer, the inactivation of tumor suppressor genes and the activation of oncogenes results in multiple intracellular signaling shifts, affecting glycolytic flux and glutaminolysis in cancer cells (9-12). An improved understanding of the molecular mechanisms involved in tumor metabolism may facilitate the identification of novel diagnostic approaches and treatment strategies for targeted cancer therapy.

The most critical amino acid in the metabolism of cancer cells is glutamine, the deprivation of which can cause apoptosis of neuroblastoma cells (13). The catabolism of glutamine is catalyzed by glutaminase 1 (GLS 1) and glutamate dehydrogenase. ASC amino-acid transporter 2 (ASCT2) is the primary glutamine transporter in cancer cells (14). The activation of ASCT2 can transport large amounts of glutamine into cancer cells to

support their proliferation (15); on the basis of this mechanism, inhibiting glutamine transportation by ASCT2 has the potential to be a cancer therapy (16). In glutaminolysis, glutaminase 1 (GLS1) is the first rate-limiting enzyme, and is regulated by v-myc avian myelocytomatosis viral oncogene (c-Myc) (17-19).

The N-Myc downstream-regulated gene (NDRG) family is comprised of four members, all of which exhibit high expression in normal brain, heart, skeletal muscle and kidney tissues (20). The expression of NDRG family member 2 (NDRG2) differs markedly between tumor and healthy tissue. The expression level of NDRG2 is positively correlated with the differentiation and development grade of an organ and negatively correlated with the proliferative capacity of cells (21). Higher expression of NDRG2 mRNA is clinically associated with less aggressive tumors in meningioma (22) and higher survival rates in high-grade gliomas (23). In previous studies, the expression level of NDRG2 mRNA and protein in ccRCC was found to be downregulated (24), indicating that NDRG2 may have a critical function in the development of ccRCC. However, to the best of our knowledge, the mechanism of NDRG2 inactivation in cancer has not been explained.

The objective of the present study was to investigate the underlying mechanism behind the inhibition of glycolysis and glutaminolysis by NDRG2 in ccRCC. Presented in the current study are novel results revealing that NDRG2 can suppress glycolysis and glutaminolysis in ccRCC by inhibiting glucose transporter 1 (GLUT1), hexokinase 2 (HK2), pyruvate kinase isoform M2 (PKM2), lactate dehydrogenase (LDHA), ASCT2 and GLS1 gene expression. In light of the present data, NDRG2 could be considered as a promising therapeutic target for cancer treatment.

Materials and methods

Cell cultures. The ccRCC 786-O and Caki-1 cell lines were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). The 786-O cells were cultured in RPMI-1640 medium (HyClone Laboratories; GE Healthcare Life Sciences, Logan, UT, USA); Caki-1 cells were cultured in McCoy's 5A Modified medium (HyClone Laboratories; GE Healthcare Life Sciences). The two media were supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The cell lines were incubated in a humidified atmosphere of 5% CO_2 at 37˚C.

Plasmid construction, virus packaging and infection. Recombinant lentiviral vectors were constructed in the present laboratory using the Invitrogen ViraPower™ Lentiviral System (Thermo Fisher Scientific, Inc.). Human full-length NDRG2 DNA was subcloned into a plenti6 vector in HEK-293T cells (ATCC). HEK-293 cells were transfected with pLenti6-mCherry/pLenti6-NDG2, PMD2G and PAX2 lentiviral vectors via Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The viral supernatants were collected, filtered (through a 0.45-μm filter; Millipore; Merck KGaA, Darmstadt, Germany), and introduced into 786-O and Caki-1 cells after 48 h.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was used to isolate total RNA from cells. cDNA was synthesized from the isolated RNA using AMV reverse transcriptase (Promega Corporation, Madison, WI, USA), according to the manufacturer's protocol. The cDNA was used as a template for qPCR using ABI Prism 7500 (Applied Biosystems; Thermo Fisher Scientific, Inc.). The specific primer pairs were as follows: NDRG2 forward, 5'-GAGATATGCTCTTAACCACCCG-3' and reverse, 5'-GCTGCCCAATCCATCCAA-3'; GLUT1 forward, 5'-ACCATTGGCTCCGGTATCG-3' and reverse 5'-GCTCGCTCCACCACAAACA-3'; hexokinase 1 forward, 5'-TGGAGTCCGAGGTTTATG-3' and reverse, 5'-TTTGGATTGTTGGCAAGG-3'; HK2 forward, 5'-CCAGTTCATTCACATCATCAG-3' and reverse, 5'-CTTACACGAGGTCACATAGC-3'; PKM1 forward, 5'-CGAGCCTCAAGTCACTCCAC-3' and reverse, 5'-GTGAGCAGACCTGCCAGACT-3'; PKM2 forward, 5'-CTGTGGACTTGCCTGCTGTG-3' and reverse, 5'-TGCCTTGCGGATGAATGACG-3'; LDHA forward, 5'-CTGGGAGTTCACCCATTAAGCT-3' and reverse, 5'-CAGGCACACTGGAATCTCCAT-3'; LDHB forward, 5'-AGGGAGTGTGTATATTTGAGTT-3' and reverse, 5'-TCAAACTTACCTATAAACCAAA-3'; ASCT2 forward, 5'-CCGCTTCTTCAACTCCTTCAA-3' and reverse 5'-ACCCACATCCTCCATCTCCA-3'; GLS1 forward, 5'-GCTGTGCTCCATTGAAGTGACT-3' and reverse, 5'-TTG GGCAGAAACCACCATTAG-3'; and β-actin forward, 5'-CGCGAGAAGATGACCCAGAT-3' and reverse, 5'-GTACGGCCAGAGGCGTACAG-3'. The following thermocycling conditions were maintained: 95˚C for 3 min; 95˚C for 10 sec and 60˚C for 30 sec for 39 cycles; and melting curve analysis using increase from 65.0 to 95.0˚C in 0.5˚C increments for 5 sec. Independent experiments were repeated three times. The relative expression levels of mRNA were analyzed using Bio-Rad CFX Manager v3.1 software (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with the $2^{-\Delta\Delta Cq}$ method (25).

Western blot analysis. Total protein was isolated from cell lines and clinical ccRCC tissue samples for western blot analysis using RIPA buffer (Beyotime Institute of Biotechnology, Haimen, China). Immunoblotting was the performed as described below. Total protein content was measured using a BCA protein assay then 50 μg/lane was separated using 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The membranes were incubated with 5% non-fat milk for 1 h at room temperature, followed by washing with TBS with Tween-20. The blots were then incubated with primary antibodies for 12 h at 4˚C followed by incubation with the secondary antibody for 2 h at room temperature. The following primary antibodies were used: Polyclonal rabbit anti-human HK2 (dilution, 1:2,000; cat. no. 2106S; Cell Signaling Technology, Inc., Danvers, MA, USA); polyclonal rabbit anti-human PKM2 (dilution, 1:2,000; cat. no. 3198S; Cell Signaling Technology, Inc.); polyclonal rabbit anti-human LDHA (dilution, 1:2,000; cat. no. 2012S; Cell Signaling Technology, Inc.); polyclonal rabbit anti-human ASCT2 (dilution, 1:2,000; cat. no. 5345S; Cell Signaling, Inc.); polyclonal rabbit anti-human GLS1 (dilution, 1:2,000, cat. no. ab93434; Abcam, Cambridge, UK); monoclonal rabbit anti-human GLUT1 (dilution; 1:1,000; cat. no. ab115730; Abcam); monoclonal mouse anti-human NDRG2 (dilution,

1:5,000, cat. no. H57447-M03; Abnova, Taipei, Taiwan); and polyclonal rabbit anti-human β-actin (dilution, 1:1,000; cat. no. bs-0061R; Beijing Biosynthesis Biotechnology Co., Ltd., Beijing, China). Horesradish peroxidase-conjugated secondary antibodies polyclonal goat anti-rabbit IgG (dilution, 1:3,000; cat. no. 7074S; Cell Signaling Technology, Inc.) and polyclonal horse anti-mouse IgG (dilution, 1:3,000; cat. no. 7076S; Cell Signaling Technology, Inc.) were used. An ECL kit (Beyotime Institute of Biotechnology) was used to perform chemiluminescence detection according to the manufacturer's protocol.

Glucose consumption and lactate production test. NDRG2-overexpressing- and cherry-control- 786-O, and Caki-1 cells were seeded on 6-well plates at a density of $1x10^5$ cells/well, and the culture Dulbecco's modified Eagle's medium (DMEM; HyClone Laboratories; GE Healthcare Life Sciences) was changed to fresh DMEM following incubation at 37˚C for 12 h. The concentrations of glucose and lactate in culture medium were measured after 24 h using the Glucose Test kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) and the Lactate Assay kit (Nanjing Jiancheng Bioengineering Institute), respectively, according to the manufacturer's protocol.

Glutamine/glutamate concentration test. NDRG2-overexpressing and cherry-control-786-O, and Caki-1 cells were cultured for 24 h in 6-well plates in phenol red-free medium. The culture medium was collected and cells were lysed with RIPA buffer. Concentrations of glutamine in the medium and in the cell lysate were determined using the Glutamine/Glutamate Determination kit (cat. no. GLN-1; Sigma-Aldrich; Merck KGaA). All protein levels were determined spectrophotometrically using a standard curve, using absorbance measurements at 340 nm. The absorbance is proportional to the ratio of NAD^+:NADH, which accompanies the oxidation of glutamate to α-ketoglutarate, as catalyzed by glutamic dehydrogenase. Glutamine levels were determined from the amount of glutamine converted to glutamate via GLS1. The glutamine consumption was calculated as the difference between the initial and final glutamine levels of the cells in culture. Glutamate production was calculated as the difference between the final and initial levels of glutamate.

Colony formation assay. NDRG2-overexpressing and cherry-control- 786-O cells were seeded into a 6-well plate at a density of 100 cells/well. The cells were grown for 14 days in DMEM under the same incubation conditions as described in the aforementioned culture method. The colonies were dried and stained for 10 min at 37˚C with 0.5% crystal violet. The colonies formation efficiency was calculated by a standard formula: Colony formation efficiency (%)=(colonies/seeded cells)x100%.

Wound healing assay. NDRG2-overexpressing and control-cherry 786-O cells were cultured at a density of $1.0x10^6$ cells/well in 6-well plates. Once the cells had grown to a fully confluent monolayer, the cell monolayer was carefully scraped using a sterile tip to create a wound (scratch) and washed twice with fresh DMEM to remove any debris. Cells were then incubated for 24 h. Images of the wound and the surrounding area were captured immediately (0 h) and 24 h after scraping.

In vivo tumorigenicity assay. A subcutaneous injection of $1x10^7$ cells was administered to six 6-week-old athymic nude female mice (weighing 18-22 g) in the right hind limb (all the animals were kept in the animal center of The Fourth Military Medical University, Xi'an, China). All animals were raised in a sterile environment in laminar flow cabinets with disinfectant-treated baskets and bedding, adequate feed and drinking water, aseptic operation, constant temperature (18-20˚C) and constant humidity (50-60%). Tumor growth was quantified by measuring tumor size with vernier calipers weekly for 1 month. Tumor volume was calculated using a standard formula: Tumor volume (mm^3) = width (mm^2) x length (mm) x 0.5. At the end of the experiment, tumors and tumor tissues were harvested and analyzed once a tumor size of 2x2 cm was achieved. The assessment was performed by weighing the tumors and measuring protein expression via western blot analysis.

Statistical analysis. Statistical analyses were performed using SPSS v.19.0 software (IBM Corp., Armonk, NY, USA) for Windows. All data shown are the mean ± standard error of triplicate values from three separate experiments. $P<0.05$ was considered to indicate a statistically significant difference. Independent Student's t-tests were used to compare the variables between two groups.

Results

NDRG2 inhibits glycolysis and glutaminolysis in ccRCC. To study the function of NDRG2 in the metabolic reprogramming of ccRCC, NDRG2 was successfully overexpressed in two ccRCC cell lines (786-O and Caki-1) through lentivirus transfection (Fig. 1A). The results of the present study reveal that NDRG2 inhibits aerobic glycolysis, as indicated by the decrease in glucose consumption and lactate production in 786-O and Caki-1 cells (Fig. 1B). Additionally, overexpression of NDRG2 also inhibits glutaminolysis, as indicated by decreased glutamine consumption and glutamate concentration in the culture medium of NDRG2-overexpressing 786-O and Caki-1 cells, compared to controls (Fig. 1C).

NDRG2 inhibited expression of glycolysis and glutaminolysis genes in ccRCC cells. To identify the molecular targets involved in NDRG2-regulated aerobic glycolysis, the expression of glucose transporters and enzymes in glycolysis, and glutamine transporters and glutaminolysis pathway enzymes was assessed in NDRG2-overexpressing 786-O and Caki-1 cells. Compared with the control group, overexpression of NDRG2 significantly reduced the expression of GLUT1, HK2, PKM2 and LDHA genes in 786-O and Caki-1 cell lines (Fig. 2A and B). Overexpression of NDRG2 also significantly decreased the expression of ASCT2 and GLS1 genes in 786-O and Caki-1 cells (Fig. 2C and D). Tissue analysis from the tumor-formation experiment, in which mice were injected with 786-O cells overexpressing NDRG2, agreed with the *in vivo* result (Fig. 3A).

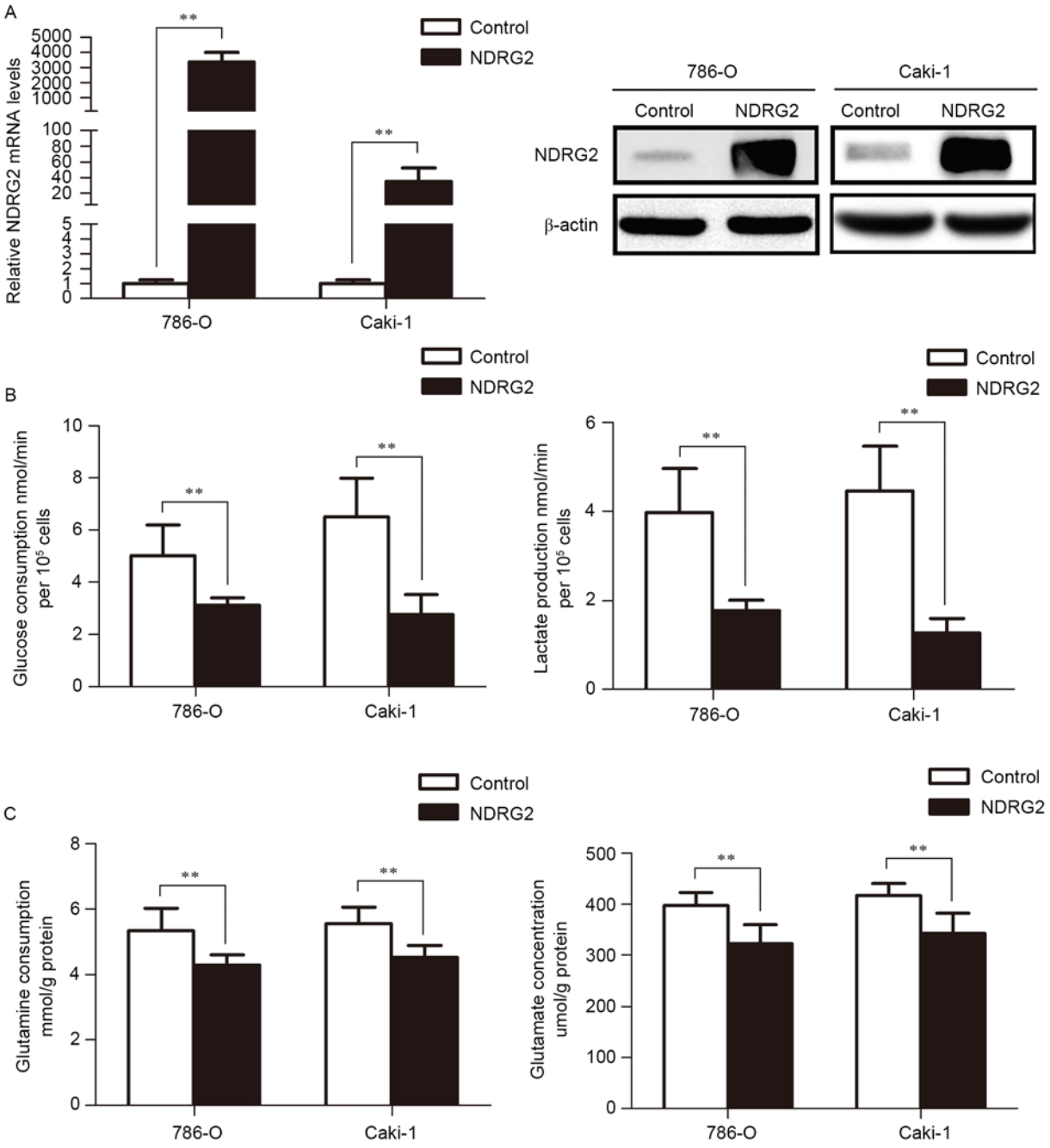

Figure 1. Glucose and glutamine consumption, lactate production rate, and glutamate concentration status of NDRG2-overexpressing clear cell renal cell carcinoma cell lines. (A) NDRG2 mRNA levels and protein levels in 786-O and Caki-1 cells infected with lentivirus containing NDRG2 or mCherry (control), and β-actin. β-actin acted as an internal control to ensure equal loading. (B) Quantification of glucose consumption and lactate production in NDRG2-overexpressing 786-O and Caki-1 cells. (C) Quantification of glutamine consumption and glutamate concentration in NDRG2-overexpressing 786-O and Caki-1 cells. **P<0.01. NDRG2, N-myc downstream-regulated gene 2 protein.

NDRG2 inhibited the growth and proliferation of ccRCC. Colony formation assays, wound-scratch assays and tumor-formation experiments in nude mice *in vivo* were used to investigate whether NDRG2 regulates the growth and proliferation of ccRCC cells. The results of the present study indicate that overexpression of NDRG2 significantly inhibits the efficiency of colony formation in the 786-O cell line (Fig. 3B). Data also revealed that overexpression of NDRG2 clearly reduced the migratory ability of the 786-O cell line (Fig. 3C). Additionally, overexpression of NDRG2 significantly suppressed tumor growth in nude mice: Tumor formation in mice injected with NDRG2-overexpressing 786-O cells progressed much more slowly than it did in the control groups (Fig. 3D). At the end of fourth week after injection of tumor cells, mice injected with 786-O cells over-expressing NDRG2 exhibited a statistically significant decrease in mean tumor volume compared with the control groups (Fig. 3D). In addition, the mean tumor weight in mice injected with NDRG2-overexpressing 786-O cells was significantly lower than that of mice injected with control 786-O cells (Fig. 3D). These data indicated that NDRG2 effectively suppresses the growth and proliferation of ccRCC cells *in vitro* and *in vivo*.

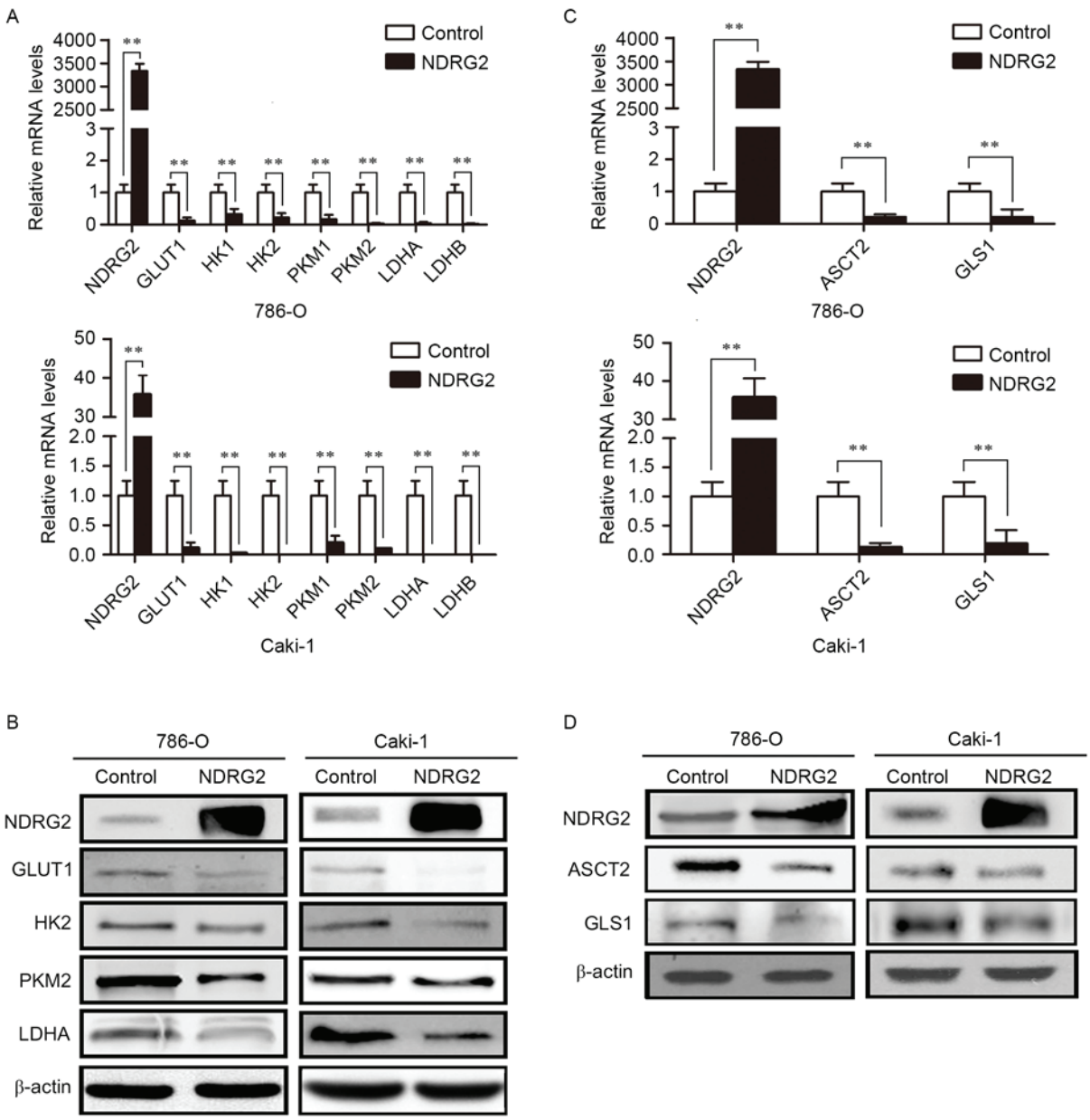

Figure 2. Expression levels of various metabolic enzymes in NDRG2-overexpressing clear cell renal cell carcinoma cell lines. (A) NDRG2, GLUT1, HK1, HK2, PKM1, PKM2, LDHA and LDHB mRNA expression in 786-O and Caki-1 cells transfected with lentivirus containing NDRG2 or mCherry (control). (B) Western blot analysis showing NDRG2, GLUT1, HK2, PKM2 and LDHA protein levels in 786-O and Caki-1 cells. β-actin acted as an internal control to ensure equal loading. (C) NDRG2, ASCT2 and GLS1 mRNA expression of 786-O and Caki-1 cells transfected with lentivirus containing NDRG2 or mCherry, and β-actin acted as an internal control to ensure equal loading. (D) Western blot analysis showing protein expression of NDRG2, ASCT2 and GLS1 in 786-O and Caki-1 cells. β-actin acted as an internal control to ensure equal loading. **P<0.01. NDRG2, N-myc downstream-regulated gene 2 protein; GLUT1, glucose transporter 1; HK1, hexokinase-1; PKM1, pyruvate kinase isoform 1; LDHA, lactate dehydrogenase A chain; GLS1, glutaminase 1; ASCT2, alanine-serine-cysteine transporter 2.

Discussion

One of the hallmarks of cancer tissues is the metabolic reprogramming phenotype, in which glucose consumption and lactate production are significantly increased. Glucose and glutamine are key metabolites in the metabolic processes that generate energy for the cell. In cancer cells, metabolic reprogramming assists in maintaining uncontrolled growth and proliferation by providing sufficient energy (1).

A previous study revealed that the activation of oncogenes and inactivation of tumor suppressor genes are closely associated with tumor metabolic reprogramming (12). NDRG2 is a tumor suppressor gene that can inhibit tumor cell proliferation and invasion (26). In 2015, Xu *et al* (27) found that NDRG2 could inhibit glycolysis and glutaminolysis in colorectal cancer cells through the inhibition of c-Myc. However, the inhibitory function of NDRG2 in the metabolism of renal cancer remains unclear.

The present study demonstrates that NDRG2 can down-regulate glycolysis and glutaminolysis in ccRCC by inhibiting the expression of GLUT1, HK2, PKM2, LDHA, ASCT2 and GLS1. The data produced by the current study demonstrate that NDRG2 acts as a key inhibitor of glycolysis and glutaminolysis in ccRCC.

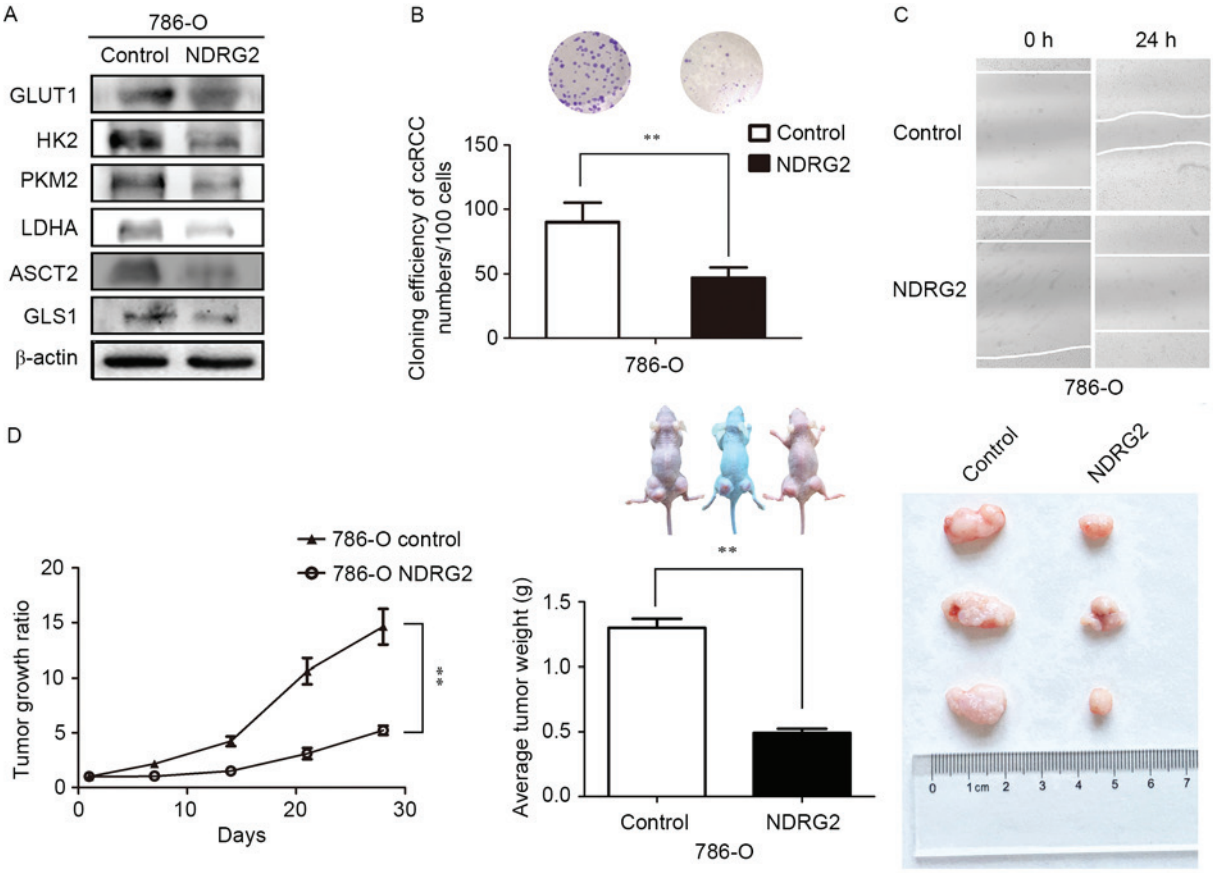

Figure 3. Expression levels of various metabolic enzymes and tumor growth ratio of NDRG2-overexpressing 786-O cells in a tumor bearing experiment; in addition to the effects of NDRG2 overexpression on the proliferative, and migratory abilities of 786-O cells. (A) Levels of GLUT1, HK2, PKM2, LDHA, ASCT2 and GLS1 protein in tumor tissues were assessed *ex vivo* via western blot analysis; β-actin acted as an internal control to ensure equal loading. (B) Equal numbers of NDRG2-overexpressing and control 786-O cells were seeded onto a 60-mm dish and after 14 days, the cells were fixed and stained with crystal violet. (C) Equal numbers of NDRG2-overexpressing and control 786-O cells were seeded onto 6-well plates, then the cell motility of NDRG2-overexpressing and control 786-O cells were determined by wound migration assay. $^{**}P<0.01$. (D) Tumor volume was calculated by the formula (width^2 x length x 0.5). The tumor weight of mice was measured 28 days after injection, at which point each tumor was photographed. NDRG2, N-myc downstream-regulated gene 2 protein; GLUT1, glucose transporter 1; HK2, hexokinase-2; PKM2, pyruvate kinase isoform 2; LDHA, lactate dehydrogenase A chain; GLS1, glutaminase 1; ASCT2, alanine-serine-cysteine transporter 2.

Xu *et al* (27) demonstrated that the NDRG2-dependent inhibition of glycolysis and glutaminolysis in colorectal cancer cells occurs through inhibition of c-Myc. Although associations between NDRG2 and c-Myc in ccRCC were not examined in the present study, previous studies have revealed that the majority of the enzymes and transporters involved in glycolysis and glutaminolysis can be regulated by c-Myc (9,28-31). For example, the expression and activation of glucose transporters, HK2, PKM2 and LDHA are all regulated by c-Myc (9,28-30). Additionally, lactate-induced activation of c-Myc can trigger the expression of the glutamine transporter ASCT2 and GLS1, resulting in increased glutamine uptake and catabolism (31). This association, in addition to the regulatory roles of NDRG2 in glycolysis and glutaminolysis, suggests that c-Myc could also regulate the same metabolic pathways in ccRCC. The present study therefore indicates that the association between NDRG2 and c-Myc on the inhibition of glutaminolysis and glycolysis in ccRCC should be a topic of future studies.

A previous study reported that NDRG2 could inhibit growth and proliferation of ccRCC cells (32). A similar effect was observed in 786-O and Caki-1 cells that overexpressed NDRG2, which were confirmed to exhibit reduced glycolysis and glutaminolysis metabolism *in vivo* and *in vitro*. Since the growth and proliferation of cancer cells rely on glycolysis and glutaminolysis (8), the inhibitory effect on the growth and proliferation of ccRCC may be due to NDRG2. However, the association between the inhibition of the growth and proliferation of ccRCC cells and the inhibition of glycolysis and glutaminolysis caused by overexpression of NDRG2 has not been confirmed; this association should therefore be explored further in future studies.

In summary, the present study illustrates the regulatory role of the tumor suppressor gene NDRG2 in the metabolic reprogramming of ccRCC. With these findings, the mechanism of tumor metabolic reprogramming can be further understood, with the pathway representing a novel metabolic target for cancer treatment.

Acknowledgements

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