N-myc downstream-regulated gene 2 expression is associated with glucose transport and correlated with prognosis in breast carcinoma

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Abstract

Introduction: N-myc downstream-regulated gene 2 (NDRG2), a novel tumour suppressor and cell stress-related gene, is involved in many cell metabolic processes, such as hormone, ion and fluid metabolism. We investigated whether NDRG2 is involved in any glucose-dependent energy metabolism, as well as the nature of its correlation with breast carcinoma.

Methods: The correlations between NDRG2 expression and glucose transporter 1 (GLUT1) expression in clinical breast carcinoma tissues were analysed. The effects of NDRG2 on glucose uptake were assessed in breast cancer cells and xenograft tumours. The consequences of NDRG2-induced regulation of GLUT1 at the transcription and translation levels and the interaction between NDRG2 and GLUT1 were examined.

Results: Data derived from clinical breast carcinoma specimens revealed that (1) patients with high NDRG2 expression had better disease-free survival and overall survival than those with low NDRG2 expression and (2) NDRG2 expression was negatively correlated with GLUT1 expression in these breast carcinoma tissues. NDRG2 inhibited glucose uptake by promoting GLUT1 protein degradation without affecting GLUT1 transcription in both breast cancer cells and xenograft tumours. In addition, NDRG2 protein interacted and partly colocalised with GLUT1 protein in cell cytoplasm areas.

Conclusions: The results of our study support the notion that NDRG2 plays an important role in tumour glucose metabolism, in which GLUT1 is a likely candidate contributor to glucose uptake suppression and tumour growth. Targeting the actions of NDRG2 in cell glucose-dependent energy delivery may provide an attractive strategy for therapeutic intervention in human breast carcinoma.
glucocorticoid-induced kinase 1 (SGK1) [10,16]. NDRG2 was also found to induce amiloride-sensitive Na\(^+\) transport in *Xenopus laevis* oocytes and Fischer rat thyroid cells [17]. In a previous study, we found that NDRG2 promoted Na\(^+\)/K\(^-\)-ATPase activity to promote cell Na\(^+\) transport and fluid balance [14]. We also identified that NDRG2 acted as a key molecule in pancreatic \(\beta\) cells and was involved in Akt-mediated protection of \(\beta\) cells against lipotoxicity [11]. The evidence described herein suggests that NDRG2 is a metabolism-related gene and plays important roles in cellular physiological metabolism. Furthermore, NDRG2 was recently shown to respond to cellular stress under a series of environmental stress conditions [1]. However, very little information is available regarding the function of NDRG2 in tumour metabolism. Mammalian cells depend on glucose as a major substrate for energy production [18]. Warburg showed that tumour cells could metabolise many orders of magnitude larger amounts of glucose than their differentiated normal counterparts [19,20]. The transport of glucose across the plasma membrane is the first rate-limiting step for glucose metabolism and is mediated via glucose transporter proteins (GLUTs) [18]. At present, 14 members of the GLUT family have been identified [21]. GLUT1 is broadly expressed in the body tissues and is involved in glucose uptake in the basic state. Elevated levels of GLUT1 have been shown to be present in many human cancers, including head and neck, breast, lung and ovarian [22,23]. Moreover, several reports have suggested that GLUT1 represents potential regulatory targets of oncogenes or tumour suppressors [24-26].

We posited the following questions: (1) whether NDRG2 expression is associated with any GLUT expression, as well as the nature of its correlation with breast carcinoma; (2) whether and why NDRG2 affects the glucose uptake; (3) what would be the significance of the interactions between NDRG2 and the GLUTs; and (4) whether this regulation of NDRG2 on the GLUTs exists in vivo. In our present study, we tested the hypothesis that a possible mechanism of NDRG2 induces its participation in cancer cell energy metabolism through the regulation of GLUTs in breast carcinoma.

**Methods**

**Tissue samples and study cohort**

This study was approved by the Ethics Committee of the Fourth Military Medical University. All patients from whom we obtained the 30 pairs of breast carcinoma and adjacent normal breast tissue specimens, as well as the 269 breast carcinoma sample study cohort, provided their full consent to participate in the study at the Xijing Hospital of the Fourth Military Medical University (Xi’an, China). NDRG2 and GLUT1 expression were detected in all specimens. Tissue specimens were examined separately by two pathologists under double-blinded conditions without prior knowledge of the clinical status of the specimens.

**Immunohistochemistry detection**

Immunohistochemistry (IHC) was performed using the avidin-biotin-peroxidase complex method on all breast carcinoma samples. All sections were deparaffinised in xylenes and dehydrated through a gradient concentration of alcohol before endogenous peroxidase activity was blocked using 0.5% H\(_2\)O\(_2\) in methanol for 10 minutes. After nonspecific binding was blocked, the slides were incubated with NDRG2 antibody (1:200; Abnova, Taipei, Taiwan) or GLUT1 antibody (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA) in phosphate-buffered saline (PBS) at 4°C overnight in a humidified container. Biotinylated goat anti-rabbit immunoglobulin G (IgG) (1:400; Sigma-Aldrich, St Louis, MO, USA) was incubated with the sections for 1 hour at room temperature and detected using a streptavidin-peroxidase complex. The brown colour indicative of peroxidase activity was developed by incubation with 0.1% 3,3′-diaminobenzidine (Sigma-Aldrich) in PBS with 0.05% H\(_2\)O\(_2\) for 5 minutes at room temperature. The appropriate positive and negative controls were included in each run of IHC.

**Staining evaluation**

An immunoreactivity score system based on the proportion and intensity of positively stained cancer cells was applied. The two extensional standards taken were as follows: (1) the number of positively stained cells ≤5%, scored 0; 6% to 25%, scored 1; 26% to 50%, scored 2; 51% to 75%, scored 3; and >75%, scored 4; and (2) the intensity of stain colourless, scored 0; pallideflavens, scored 1; yellow, scored 2; and brown, scored 3. Extensional standards (1) and (2) were multiplied, and the staining grade was stratified as absent (score 0), weak (score 1 to 4), moderate (score 5 to 8) or strong (score 9 to 12). Specimens were rescored if the difference of scores from the two pathologists was greater than 3. Tumours with moderate or strong immunostaining were classified as having high expression, and tumours with absent or weak immunostaining were classified as having low expression.

**Cell cultures and reagents**

T-47D and SK-BR-3 cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in a humidified incubator under 5% CO\(_2\) in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen/Life Technologies, Carlsbad, CA, USA) supplemented with 10% foetal bovine serum (FBS) and 2 mM L-glutamine. NDRG2 antibody was purchased from Abnova. GLUT1, hemagglutinin (HA), actin, flag and tubulin antibodies were obtained from Santa Cruz Biotechnology.
Gene transfection
The cells (1 × 10⁶ cells/well) were seeded into six-well plates and transfected with the following constructs using Lipofectamine 2000 (Invitrogen/Life Sciences) according to the manufacturer's instructions as follows: NDRG2 expression plasmid (pCMV-flag-NDRG2), GLUT1 expression plasmid (pCMV-eGFP-GLUT1), HA-ubiquitin or small interfering RNA (siRNA) that targeted NDRG2. The target sequences of NDRG2 siRNA and control siRNA are given in Additional file 1: Table S1.

Gene infection
Cells were seeded into six-well plates at a density of 1 × 10⁶ cells/well and incubated to reach approximately 80% confluence. After the medium was removed, adenovirus expressing NDRG2 (Ad-NDRG2) or the negative control adenovirus expressing LacZ (Ad-LacZ) was added to serum-free DMEM, incubated for 2 hours, replaced with fresh DMEM supplemented with 10% FBS and incubated for another 48 hours. Recombinant adenoviruses carrying NDRG2 or LacZ were purchased from Benyuan Zhengyang Gene Technology Company (Beijing, China).

Immunoblotting
Both cells and breast tissues were lysed in radioimmunoprecipitation assay buffer (0.05 M Tris-HCl (pH 7.4), 0.15 M NaCl, 0.25% deoxycholic acid, 1% Nonidet P-40, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml aprotinin and 10 mg/ml leupeptin). Protein concentrations were measured using a bicinchoninic acid protein assay (Pierce Biotechnology) or the Odyssey Imaging System (LI-COR Biosciences, Lincoln, NE, USA). The RNA extracted from cells with TRIzol reagent (Invitrogen/Life Technologies, Carlsbad, CA, USA) was converted to combinational cDNA with the RevertAid First Strand cDNA Synthesis Kit (Fermentas/Thermo Scientific; Pittsburgh, PA, USA). Real-time PCR analysis was performed using the Prism 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) and the SYBR Premix Ex Taq II (Tli RNase H Plus) kit (TaKaRa Bio, Shiga, Japan) according to the manufacturer’s instructions. The relative gene expression levels were calculated using the 2^(-ΔΔCt) method, in which Ct represented the threshold cycle and β-actin was used as a reference gene. The primer sequence is given in Additional file 1: Table S1.

Cell proliferation assay
Cell growth following transfection was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were seeded into a 96-well plate (1 × 10⁴ cells per well) and incubated for 24 hours. The cells were then incubated with 0.5 mg/ml MTT (Sigma-Aldrich). Four hours later, the medium was replaced with 100 µl of dimethyl sulfoxide (Sigma-Aldrich) and vortexed for 10 minutes. Absorbance was then recorded at 490 nm using an Easy Reader 340 AT plate reader (SLT-Lab Instruments, Salzburg, Austria). Relative values of optical density were calculated as a percentage of the control. All experiments were performed three times independently.

Glucose uptake assay
Prior to being harvested, adherent cultures of control and NDRG2 adenovirus- or siRNA-treated cells in DMEM containing 25 mM glucose were washed twice with cold PBS and then lysed with ion-free H₂O for 5 minutes on ice. The glucose content was measured with a D-glucose measurement kit (GAHK-20; Sigma-Aldrich) according to the manufacturer’s protocol.

Immunofluorescence assay
Cells were fixed in a freshly prepared solution of 4% paraformaldehyde, rinsed and permeabilised with 0.1% Triton X-100 in PBS. Permeabilised cells were then incubated with horse serum in PBS to block nonspecific binding. After being washed with PBS, the cells were incubated overnight at 4°C with mouse anti-NDRG2 antibody (diluted 1:150), rabbit anti-GLUT1 antibody (diluted 1:150) and fluorescein isothiocyanate (FITC)-conjugated anti-mouse antibody (diluted 1:400; Sigma-Aldrich) or cyanine 3 (Cy3)-conjugated anti-rabbit antibody (diluted 1:400; Sigma-Aldrich). The isotype mouse and rabbit IgGs were used as negative controls. Dual-colour detection was performed using a laser confocal microscope after treatment with 4′,6-diamidino-2-phenylindole (DAPI) to label nuclear DNA.

Immunoprecipitation
Transfected or untransfected cells were incubated with 1 ml of lysis buffer containing 150 mM NaCl, 50 mM Tris-HCl (pH 7.4), 1% Lubrol (polyethylene glycolmonocetyl ether; MP Biomedicals, Solon, OH, USA) and 5 mM EDTA, as well as protease inhibitors, for 30 minutes at 4°C. The insoluble fraction was eliminated through centrifugation at 10,000 × g for 30 min at 4°C. After centrifugation, the lysates were incubated with the antibody of interest, and protein A or G was conjugated to sepharose (Pierce Biotechnology) for 8 hours at 4°C.
To quantify the total amount of protein loaded, 20 μl of the lysates was saved. Beads were washed four times with lysis buffer. Proteins were eluted in SDS-PAGE sample buffer and separated by SDS-PAGE for immunoblot analysis. The blots were then probed with peroxidase-conjugated goat anti-mouse or goat anti-rabbit antibodies and visualised by using an enhanced chemiluminescence reagent (Pierce Biotechnology).

**Xenograft study in nude mice**

For inoculation into nude mice, SK-BR-3 or MDA-MB-231 cells were washed with PBS, digested with trypsin and resuspended in serum-free DMEM. After centrifugation (800 rpm), the cell pellets were resuspended in DMEM. The cell suspension (1 × 10^6 cells in a 100-ml volume of PBS) was injected subcutaneously into the hind legs of 4-week-old female BALB/c athymic (nu/nu) mice (SLAC Laboratory Animal Company, Shanghai, China). When the tumours reached a volume of approximately 200 mm^3, the mice were arbitrarily assigned to different groups (n = 6 each) to receive intratumoural injections of 0.5, 1 or 2 × 10^9 plaque-forming units (PFU) of Ad-NDRG2, 2 × 10^9 Ad-LacZ or PBS. Intratumoural injections were repeated every 3 days for a total of 21 days. Tumours were measured (perpendicular diameters) every 3 days, and their volumes were calculated. On day 21, the mice were killed and their tumours were removed for analysis. Tumour volumes were calculated based on caliper measurements of the length and width of the lesions using the following formula: 0.5 × length × width^2. The tumour growth curve was then derived from these data.

All of the experimental procedures were conducted in accordance with the Detailed Rules for the Administration of Animal Experiments for Medical Research Purposes issued by the Ministry of Health of China and received ethical approval by the Animal Experiment Administration Committee of the Fourth Military Medical University (Xi’an, China). All efforts were made to minimise the animals’ suffering and reduce the number of animals used.

**Statistical analysis**

*In vitro* experiments were performed three times, and each experiment was performed in triplicate. Data from all quantitative assays are expressed as the means ± SD and were analysed statistically using one-way analysis of variance, independent samples t-test or Student’s t-test. In the clinical specimens study, the associations between NDRG2 expression and categorical variables were analysed using the χ² test or Fisher’s exact test as appropriate. Correlations between NDRG2 expression and the expression of other molecules were analysed by using the Spearman correlation test. Kaplan–Meier analysis was used to evaluate disease-free survival and overall survival. P < 0.05 was considered statistically significant.

**Results**

**Relationship between NDRG2 expression and clinical histopathological characteristics in breast carcinoma**

To assess the significance of NDRG2 protein expression in the development and progression of breast cancer, we compared the histopathological characteristics of 269 breast cancer samples with available NDRG2 protein status. The correlations between NDRG2 expression and different clinical histopathological factors are presented in Table 1. Significant correlations were found between low NDRG2 expression and advanced TNM staging (P < 0.0001), high proliferation index (Ki67 status; P = 0.006), positive human epidermal growth factor receptor 2 (HER2) status (P = 0.010) and poor histological differentiation (P < 0.0001). However, high NDRG2 expression was correlated with positive oestrogen receptor (ER) status (P = 0.010). Correlation coefficients are presented in Table 2. Furthermore, Kaplan–Meier analysis was used to evaluate the disease-free survival and overall survival of patients with breast cancer and NDRG2 protein expression. The results show that patients with high NDRG2 expression in breast tumour tissues had better disease-free survival than those with low NDRG2 expression (P = 0.0066 by logrank test) (Figure 1A). Breast cancer patients with low NDRG2 expression had a higher risk of relapse than those with high NDRG2 expression. A statistically significant association between long overall survival and high NDRG2 protein levels was found in breast cancer patients. Breast cancer patients with high NDRG2 expression had longer overall survival than patients with low NDRG2 expression P = 0.0007 by logrank test) (Figure 1B).

**Correlation of NDRG2 expression with GLUT1 expression in breast carcinoma**

Tumour cells depend on glucose as a major substrate for energy production, and many tumour suppressor genes play important roles in the regulation of glucose metabolism [27]. GLUT1 is broadly expressed in the body tissues and is also involved in glucose uptake in the basic state, especially so for tumour glucose metabolism [18]. In our present study, we first investigated this relationship between NDRG2 and GLUT1 expression by IHC and immunoblotting in 30 pairs of breast carcinoma and adjacent normal breast tissue specimens. Positive staining of anti-NDRG2 was found predominantly in the cytoplasm of both normal breast cells and breast cancer cells, but weaker staining for NDRG2 was observed in the cancer specimens compared with normal tissues. Conversely, strong positive staining of GLUT1 was observed in the cytoplasm and cytosolic membrane of tumour cells, and
weaker staining was found in noncancerous breast tissue (Figure 1C and Additional file 2: Figures S1 and S2). Furthermore, immunoblotting data revealed that NDRG2 protein expression in cancer samples was much lower than that in the paired adjacent normal tissue. However, GLUT1 expression was higher in cancer tissue than in the paired adjacent normal tissue (Figure 1D and Additional file 2: Figure S3). Indeed, in normal tissue and cancer tissue, the means of immunoreactivity score of NDRG2 IHC staining were 6.03 and 1.97 (Figure 1E), respectively, and the means of immunoreactivity score of GLUT1, IHC Staining were 3.13 and 6.53 (Figure 1F), respectively. Similarly, we identified the degree of correlation between NDRG2 expression and GLUT1 expression in 269 breast cancer specimens. The Spearman correlation analysis indicated that NDRG2 protein expression was inversely correlated with GLUT1 expression (Pearson correlation coefficient $r_s = -0.179; P = 0.003$) (Table 3). The data imply that NDRG2 expression might be involved in the development and progression of breast cancer and that NDRG2 expression is inversely correlated with GLUT1 expression in breast carcinoma.

NDRG2 inhibits the growth of breast cancer cells in both high- and low-glucose medium

Next, we sought to explore the regulatory mechanism of NDRG2 in tumour glucose metabolism. To investigate the effect of NDRG2 on breast cancer cell proliferation in high-glucose (25 mM) or low-glucose (5.5 mM) medium, we designed the following assays. First, the

| Variables                  | NDRG2 expression |   |   |   |
|----------------------------|-----------------|---|---|---|
|                           | Low expression, n (%) | High expression, n (%) |   |   |
| Age (years)               |                 |   |   |   |
| ≤50                       | 112             | 64.3 | 35.7 | 0.104<sup>b</sup> |
| >50                       | 157             | 54.1 | 45.9 |   |
| Tumour size               |                 |   |   |   |
| ≥2 cm                     | 121             | 56.2 | 43.8 | 0.536<sup>c</sup> |
| >2 cm                     | 148             | 60.1 | 39.9 |   |
| TNM stage                 |                 |   |   |   |
| I to II                   | 114             | 42.1 | 57.9 | <0.0001<sup>b</sup> |
| III to IV                 | 155             | 70.3 | 29.7 |   |
| Lymph node metastasis     |                 |   |   |   |
| Negative                  | 114             | 49.1 | 50.9 | 0.009<sup>b</sup> |
| Positive                  | 155             | 65.2 | 34.8 |   |
| Histology                 |                 |   |   |   |
| Poorly differentiated      | 103             | 72.8 | 27.2 | <0.0001<sup>c</sup> |
| Moderately differentiated  | 83              | 62.7 | 37.3 |   |
| Well differentiated        | 83              | 36.1 | 63.9 |   |
| Tumour invasion           |                 |   |   |   |
| No                        | 134             | 61.2 | 38.8 | 0.387<sup>c</sup> |
| Yes                       | 135             | 55.6 | 44.4 |   |
| ER status                 |                 |   |   |   |
| Negative                  | 79              | 70.9 | 29.1 | 0.010<sup>b</sup> |
| Positive                  | 190             | 53.2 | 46.8 |   |
| Her-2 status              |                 |   |   |   |
| Negative                  | 201             | 53.7 | 46.3 | 0.010<sup>b</sup> |
| Positive                  | 68              | 72.1 | 27.9 |   |
| Ki67 status               |                 |   |   |   |
| Negative                  | 119             | 48.7 | 51.3 | 0.006<sup>b</sup> |
| Positive                  | 150             | 66.0 | 34.0 |   |

<sup>a</sup>ER, Oestrogen receptor; Her-2, Human epidermal growth factor receptor 2; NDRG2, N-myc downstream-regulated gene 2; TNM, tumour, node, metastasis. <sup>b</sup>Fisher’s exact test was used for statistical analyses. <sup>c</sup>P < 0.05 were considered statistically significant. <sup>d</sup>Pearson’s χ² test was used for statistical analyses.
Table 2 Correlation of NDRG2 expression with clinical histopathologic characteristics in 269 breast cancer specimens

| Variables              | Correlation coefficient ($r_s$) | P-value |
|------------------------|---------------------------------|---------|
| Age (years)            | 0.101                           | 0.097   |
| Tumour size            | −0.040                          | 0.516   |
| TNM stage              | −0.283                          | 0.0001  |
| Lymph node metastasis  | −0.161                          | 0.008   |
| Differentiation status | 0.301                           | 0.0001  |
| Tumour invasion        | 0.057                           | 0.350   |
| ER status              | 0.164                           | 0.007   |
| Her-2 status           | −0.162                          | 0.008   |
| Ki67 status            | −0.174                          | 0.004   |

*ER, Oestrogen receptor; Her-2, Human epidermal growth factor receptor 2; NDRG2, N-myc downstream-regulated gene 2; TNM, Tumour, node, metastasis. Spearman's correlation test was used for statistical analyses. $P < 0.05$ was considered statistically significant.

basic NDRG2 expression in five breast cancer cell lines was examined by immunoblotting. The results showed that the expression of NDRG2 was relatively high in T-47D cells and relatively low in SK-BR-3 cells (Figure 2A). In the following studies, we chose T-47D and SK-BR-3 cells as experimental models. Next, the adenovirus carrying NDRG2 (Ad-NDRG2) or the siRNA targeting NDRG2 (NDRG2 siRNA) was applied to upregulate or knock down, respectively, the level of NDRG2. After infection by Ad-NDRG2 in SK-BR-3 or transfection by NDRG2 siRNA in T-47D cells, the expression of NDRG2 was successfully increased or decreased (Figure 2B). Moreover, these effects were more prominent with increasing concentrations of Ad-NDRG2 or NDRG2 siRNA (Figures 3A to 3D). The results of the MTT assays showed that in both high- and low-glucose medium, the overexpression of NDRG2 inhibited SK-BR-3 cells proliferation (Figure 2C) and silencing NDRG2 promoted T-47D cell proliferation (Figure 2D). In high-glucose medium, however, the effects of NDRG2 on cell proliferation were more prominent.

NDRG2 could decrease the intracellular glucose level of breast cancer cells

Given that the antiproliferative effects of NDRG2 on breast cancer cells were more prominent in high-glucose medium, we investigated whether NDRG2 would affect the capacity of these cells to take in glucose. First, the effect of the glucose culture environment on NDRG2 expression was investigated. Immunoblot analysis revealed that, after exposure to different concentrations of glucose medium, the transcription and translation levels of NDRG2 increased with increasing glucose levels (Figures 2E and 2F). In addition, we checked the intracellular glucose content to assess the extent to which NDRG2 contributed to cell glucose uptake. Glucose uptake was decreased with increasing Ad-NDRG2 treatment in SK-BR-3 cells (Figure 2G). T-47D cells were transfected with NDRG2 siRNA at different concentrations, and glucose uptake was significantly augmented with increasing NDRG2 siRNA administration (Figure 2H). These data suggest that a high-glucose microenvironment could promote NDRG2 expression and that NDRG2 could inhibit glucose intake in breast cancer cells.

NDRG2 negatively regulates GLUT1 levels in breast cancer cells

The transport of glucose across the plasma membrane is the first rate-limiting step for glucose metabolism and is mediated via GLUTs. GLUTs play critical roles in glucose uptake, especially in tumour cells. To investigate whether GLUTs are regulated by NDRG2, GLUT1/2/3/4 protein levels were measured in SK-BR-3 cells infected with Ad-NDRG2 or T-47D cells transfected with NDRG2 siRNA in a high-glucose medium. Immunoblot analysis revealed that GLUT1 protein levels were decreased by increases in Ad-NDRG2-mediated NDRG2 overexpression, but that GLUT1 protein levels were increased with NDRG2 siRNA treatment (Figures 3A and 3B). There were no changes in the intensity of anti-GLUT2 and anti-GLUT4 bands from treated cells compared with the untreated controls. GLUT3 was nearly undetectable in both the SK-BR-3 and T-47D cells (Figures 3A and 3B). However, the data obtained from real-time PCR showed that GLUT1 transcriptional levels were not significantly changed with NDRG2 up- or downregulation (Figures 3E and 3F). Because the amount of GLUT1 protein can be modified by changes in the rate of synthesis or degradation, we hypothesised that NDRG2 may exert an effect on the degradation of GLUT1. To test this hypothesis, we added the ubiquitin-proteasome inhibitor MG-132 with the Ad-NDRG2-treated SK-BR-3 cells. As shown in Figure 3G, the addition of MG-132 rescued GLUT1 protein levels, indicating that the decrease in GLUT1 in response to NDRG2 overexpression was due to proteasome-dependent degradation. GLUT1 protein is synthesised and degraded in the cytosol, whereas GLUT1 protein enacts its biological function primarily when it is transported to the cell membrane.

To determine GLUT1 protein distribution, we separated the membrane and cytosolic fractions of SK-BR-3 and T-47D cells infected with Ad-NDRG2 (Figure 3H). Immunoblot analysis showed that GLUT1 protein was significantly decreased in the cytosolic fraction of NDRG2-overexpressed SK-BR-3 cells compared with the controls. The amount of GLUT1 protein in the membrane fraction of the NDRG2-overexpressed SK-BR-3 cells was not significantly different from that in the control cells. These results suggest that NDRG2 is able to promote GLUT1 ubiquitination and degradation and can lead to decreased GLUT1 protein level.
Figure 1 NDRG2 is correlated with increased survival and negatively correlated with GLUT1 in breast carcinoma. Kaplan–Meier analysis was carried out according to N-myc downstream-regulated gene 2 (NDRG2) expression levels of disease-free survival (A) and overall survival (B). (C) Serial immunostained sections for NDRG2 and glucose transporter 1 (GLUT1) in breast cancer and normal tissues were analysed. Original magnification, 40x (top) and 400x (bottom); scale bars = 50 μm. (D) Protein was extracted from matched breast tumour tissue (T) and adjacent normal tissue (N) and subjected to immunoblot analysis to examine NDRG2 and GLUT1 expression. β-actin served as a loading control. P: patient. Relative expression levels of NDRG2 (E) and GLUT1 (F) in human breast cancer and adjacent normal tissue are shown. Immunoreactivity score distribution of cancer and adjacent normal tissue were represented with black and brown closed circles, respectively. The horizontal lines presented are means; error bars represented SD from 30 samples. P < 0.01 was considered a statistically significant difference.
NDRG2 interacts with GLUT1

Our observation that NDRG2 regulated GLUT1 protein stability prompted us to examine the interaction between NDRG2 and GLUT1 and their subcellular distribution. Confocal microscopy was applied to observe the subcellular localisation of NDRG2 and GLUT1 in SK-BR-3 cells. We found that a portion of endogenous NDRG2 and endogenous GLUT1 was colocalised in the cytoplasmic region of SK-BR-3 cells (Figure 4A). The colocalisation suggests that NDRG2 and GLUT1 may physically interact with each other. To establish whether NDRG2 physically associates with GLUT1, coimmunoprecipitation was performed using lysates prepared from SK-BR-3 cells. A 41-kDa protein that corresponds to NDRG2 was precipitated by anti-GLUT1 antibody and probed by anti-NDRG2 antibody, and a 55-kDa protein corresponding to GLUT1 was precipitated by the anti-NDRG2 antibody and probed by anti-GLUT1 antibody (Figure 4B). We also demonstrated that exogenous GLUT1 coimmunoprecipitated with exogenous NDRG2 in HEK293 cells that were cotransfected with pCMV.Flag-NDRG2 and pCMV-eGFP-GLUT1 plasmids and vice versa (Additional file 2: Figure S5). Collectively, the results of these experiments indicated that NDRG2 bound to GLUT1.

NDRG2 decreases glucose uptake and GLUT1 protein level in subcutaneous xenograft tumours

We found that NDRG2 could induce GLUT1 protein degradation to decrease glucose uptake in breast cancer cell lines, but whether this regulatory mechanism also functions in the tumour microenvironment must be studied in vivo. We injected Ad-NDRG2 at the concentrations of 0.5, 1 and 2 × 10⁹ PFU or 2 × 10⁹ PFU Ad-LacZ every 3 days into preestablished human SK-BR-3 breast tumours (approximately 200 mm³) grown in nude mice. As shown in Figure 5A, the Ad-NDRG2 group that received injections at 2 × 10⁹ PFU achieved a sustained and significant arrest of tumour growth (68% decrease in mean tumour volume on day 21 compared with Ad-LacZ group). The mice were killed at 21 days after the first intratumoral injection, and the tumours were removed for analysis of the glucose uptake and protein levels of NDRG2 and GLUT1. We found that the glucose uptake of tumour cells was inhibited significantly with increased Ad-NDRG2 compared with the Ad-LacZ group (Figure 5B). Consistent with the results of in vitro experiments (Figure 3A), increased NDRG2 was correlated with decreased GLUT1 in the xenograft tumours (Figure 5D and Additional file 2: Figure S6). In addition, obvious positive NDRG2 staining was detected by IHC in tumours excised from mice in the 2 × 10⁹ PFU Ad-NDRG2 group. However, GLUT1 staining was weaker in the Ad-NDRG2 group compared with the Ad-LacZ group (Figure 5C). We repeated the xenograft tumour experiments with MDA-MB-231 cells, a highly metastatic cell line, and the results (Additional file 2: Figure S7) were consistent with those of SK-BR-3 cells.

Discussion

Understanding the mechanisms involved in cancer cell energy metabolism may provide a reasonable interpretation for the function of NDRG2 as a tumour suppressor. To the best of our knowledge, we report for the first time that NDRG2 participated in cellular glucose uptake by regulating the protein stability of GLUT1. We also found that NDRG2 adenovirus can be used to treat breast cancer by inhibiting cellular glucose uptake in a nude mouse xenograft model. Consistent with the cell- and animal-based results, a significantly inverse correlation between NDRG2 and GLUT1 expression was observed in clinical breast cancer tissue specimens.

Much of the data obtained from tissue of breast cancer patients presented herein are supportive of previously published work by Oh et al. [6]. Although researchers in several studies have reported that NDRG2 inhibited breast cancer cell survival and other malignant activities [5,28-30], the available clinical data before the publication by Oh et al. were very limited [6]. Liu et al. previously reported that there was a reduction in NDRG2 mRNA levels in 5 of 21 breast cancer tissue samples tested compared with normal tissues [31]. Anders et al. found that NDRG2 protein was reduced in breast cancer tissue specimens. A 41-kDa protein that corresponds to NDRG2 was precipitated by anti-GLUT1 antibody and probed by anti-NDRG2 antibody, and a 55-kDa protein corresponding to GLUT1 was precipitated by the anti-NDRG2 antibody and probed by anti-GLUT1 antibody (Figure 4B). We also demonstrated that exogenous GLUT1 coimmunoprecipitated with exogenous NDRG2 in HEK293 cells that were cotransfected with pCMV.Flag-NDRG2 and pCMV-eGFP-GLUT1 plasmids and vice versa (Additional file 2: Figure S5). Collectively, the results of these experiments indicated that NDRG2 bound to GLUT1.

| Variable | N | Low expression, n (%) | High expression, n (%) | p<sup>b</sup> |
|----------|---|----------------------|-----------------------|-------------|
| GLUT1    |   |                      |                       |             |
| Low expression | 130 | 64 (49.2)               | 66 (50.8)              | 0.003<sup>c</sup> |
| High expression | 139 | 93 (66.9)               | 46 (33.1)              |             |

<sup>a</sup>GLUT1, Glucose transporter protein 1; NDRG2, N-myc downstream-regulated gene 2. <sup>b</sup>Spearman correlation test was used for statistical analyses. <sup>c</sup>P < 0.05 was considered statistically significant. <sup>d</sup>Spearman correlation coefficient (r_s) = −0.179.
from 189 breast carcinoma patients, we show in our present study that, in specimens obtained from 269 breast cancer patients, low NDRG2 expression was associated with advanced TNM stage, high Ki67 and HER2 expression and poor histological differentiation. We also found that breast cancer patients with high...
Figure 3 NDRG2 downregulates GLUT1 by promoting its ubiquitination. (A), (C) and (E) SK-BR-3 cells were infected with an adenovirus carrying N-myc downstream-regulated gene 2 (Ad-NDRG2) at 1, 5 and 10 multiplicity of infection (MOI) or Ad-LacZ for 48 hours. (B), (D) and (F) T-47D cells were transfected with NDRG2 small interfering RNA (siRNA) 10, 25 and 100 pmol or control siRNA for 48 hours. Next, cell proteins or mRNA were extracted and analysed by immunoblotting (A) and (B) or by real-time PCR (C) to (F). β-actin was used as a loading control. (C) – (F) The data presented are the means ± SD of three independent experiments; error bars represent SD from 3 replicative wells. *P < 0.05 and **P < 0.01 versus control group. (G) SK-BR-3 cells were infected with 10 MOI Ad-NDRG2 or Ad-LacZ for 48 hours and then treated with 2 μM, 6 μM or 8 μM MG-132 for 4 hours. Next, the protein was extracted and analysed by immunoblotting. (H) Cell fractions were prepared from the SK-BR-3 cells infected with 10 MOI Ad-NDRG2 or Ad-LacZ for 48 hours, and the membrane and cytosolic fractions of endogenous glucose transporter 1 (GLUT1) protein were detected. Tubulin and β-actin served as loading controls. (I) SK-BR-3 cells were transfected with hemagglutinin (HA)-ubiquitin plasmid for 6 hours and infected with Ad-NDRG2 or Ad-LacZ for another 48 hours. Subsequently, the cell lysates were collected and analysed by immunoprecipitation (IP) and immunoblotting with GLUT1 and HA antibodies. WB, Western blot.
NDRG2 expression had longer disease-free survival and better overall survival compared with patients with low NDRG2 expression. However, by Oh et al. showed that high NDRG2 expression correlated only with favourable recurrence-free survival, not with overall survival [6]. Further observations are needed to confirm the correlation of NDRG2 and breast carcinoma prognosis. Additionally, in our present study, NDRG2 expression was inversely correlated with GLUT1 expression in patient specimens, which is in agreement with our cell- and animal-based results.

Many tumour suppressor genes play important roles in the regulation of glucose metabolism, in addition to their established roles in cell survival and apoptosis. p53, one of the most highly studied tumour suppressors, which can upregulate NDRG2 expression [4], has been reported to reduce intracellular glucose levels by inhibiting the expression of GLUTs [27]. For example, p53 directly represses the transcriptional activity of GLUT1 and GLUT4 gene promoters [33]. In addition, p53 represses GLUT3 gene expression indirectly by preventing the activation of the inhibitor of the nuclear factor κB pathway [34]. In our present study, we show that NDRG2 could regulate GLUT1 posttranslational modification without affecting other glucose transporters, including GLUT2, GLUT3 and GLUT4. We also found that NDRG2 decreased GLUT1 protein stability by promoting the ubiquitin-mediated protein degradation pathway, whereas the transcription levels of both GLUT1 and other GLUT genes were not affected.

Investigators in previous studies have shown that the expression of NDRG2 is regulated by some transcription factors, including p53 [4], Myc [35] and Hif-1 [36]. NDRG2 is a novel p53-inducible target involved in the p53-mediated apoptosis pathway in lung cancer cells [4], and the expression of NDRG2 was upregulated by Hif-1 in tumour cells under hypoxic conditions [36]. However, the expression of human NDRG2 is downregulated by
Myc via transcriptional repression [35]. c-Myc directly transactivates genes encoding GLUT1 protein and increases glucose uptake in Rat1 fibroblasts [37]. Under hypoxic conditions, a transcription factor complex including Hif-1α was shown to bind the GLUT1 promoter to upregulate GLUT1 mRNA expression [38]. Interestingly, among the above-mentioned transcription factors, p53 might promote NDRG2 expression [4] and inhibit GLUT1 transcriptional activity [33], and Myc might suppress NDRG2 expression [35] and transactivate GLUT1 [37]. NDRG2 and GLUT1 were inversely regulated by p53 [4,33] and Myc [35,37], which suggests that NDRG2 may function as a tumour suppressor by decreasing glucose uptake. Surprisingly, Hif-1 increases the expression of both NDRG2 [36] and GLUT1 [38]. We cannot explain why Hif-1 positively regulates both NDRG2 and GLUT1 in a manner different from p53 or Myc. We hypothesise that this difference is due to the fact that Hif-1-related experiments were performed under different hypoxic conditions and cell physiological contexts. Whether hypoxia-inducible factors are involved in NDRG2-mediated GLUT1 content and glucose intake regulation in breast cancer needs to be directly determined in future studies.

NDRG2 appears to be broadly involved in stress responses, cell proliferation and cell differentiation [1]. The proteins with which NDRG2 interacts may provide important information contributing to understanding its precise molecular and cellular functions. Our previous study characterised a cell-cycle-dependent transcription factor, MSP58, as a binding partner of NDRG2. NDRG2 may colocalise with MSP58 in the nuclear region of the HeLa cell during cell stress [39]. In another of our previous studies, we found that the β1 subunit of Na⁺/K⁺-ATPase interacted and colocalised with NDRG2 in the perinuclear cytoplasmic region in human salivary cells and that NDRG2 could protect the β1 subunit protein and inhibit its degradation [14]. In that previous study, we detected that NDRG2 bound to and partly colocalised with GLUT1 in the cytoplasmic region of breast cancer cells. We showed that NDRG2 can decrease GLUT1 protein stability and promote the ubiquitination and degradation of GLUT1. Collectively, these experiments imply that NDRG2 might act as some kind of chaperone molecule that is involved in regulating protein stability in different cell physiological contexts.

Figure 5 NDRG2 decreases the glucose uptake and GLUT1 protein levels in SK-BR-3-based subcutaneously xenograft tumours. The experiments illustrated are described in the Methods section. (A) Tumour growth was assessed every 3 days until day 21 treatment by measuring two perpendicular diameters and calculating the volume in cubic centimetres. Ad-LacZ, adenovirus expressing LacZ; Ad-NDRG2, adenovirus expressing NDRG2; PFU, Plaque-forming units. The data presented are means ± SD; error bars represent SD from 6 mice. *P < 0.05 and **P < 0.01 versus phosphate-buffered saline (PBS) or Ad-LacZ. (B) Tumour cells were dissociated from xenograft tumours and suspended in PBS after the number of cells was counted. Next, the glucose uptake of cells in each group was detected. The data presented are means ± SD of three independent experiments; error bars represent SD from 6 mice. *P < 0.05 and **P < 0.01 versus PBS or Ad-LacZ. (C) Intratumoural protein expression was assessed by N-myc downstream-regulated gene 2 (NDRG2) and glucose transporter 1 (GLUT1) IHC staining. Representative images are shown. Original magnification: 400 x; Scale bars = 50 μm. (D) Proteins of the xenograft tumours from each group were extracted and analysed by immunoblotting to quantify NDRG2 and GLUT1 protein changes.
does not indicate any known motif or domain in NDRG2 [40], and, to the best of our knowledge, there is no published literature indicating that NDRG2 is an E3 ubiquitin ligase. Mass spectrometric analysis could be used to screen for the ubiquitin-related proteins that interact with both NDRG2 and GLUT1. However, this hypothesis must be determined directly in future studies.

Conclusions
To the best of our knowledge, the data produced in our present study provide the first evidence that NDRG2, a tumour suppressor, is negatively correlated with GLUT1 expression in breast carcinoma. This correlation is associated with a better prognosis in breast carcinoma patients. We have further demonstrated that NDRG2 plays an important role in tumour cell glucose transport, during which NDRG2 promotes the degradation GLUT1 protein to suppress glucose uptake in breast cancer cells in vivo and in vitro. Although the contribution of NDRG2 to tumour homeostasis is complicated and not yet fully understood, our findings provide a better understanding of the energy metabolism of tumours. NDRG2 may be viewed as an attractive therapeutic target for breast carcinoma.

Additional files

Additional file 1: Table S1. The sequences of small interfering RNA or primers.

Additional file 2: Figure S1. Negative control with human breast cancer tissues probed with isotype control IgG. Figure S2. NDRG2 is negatively correlated with GLUT1 in breast carcinoma. Figure S3. NDRG2 expression inversely correlated with GLUT1 expression in paired tumour and adjacent normal tissues of breast cancer patients. Figure S4. NDRG2 downregulates GLUT1 by promoting its ubiquitination. Figure S5. The interaction of exogenous NDRG2 and exogenous GLUT1. Figure S6. NDRG2 decreases the GLUT1 protein levels in SK-BR-3-based xenograft tumours. Figure S7. NDRG2 decreases the glucose uptake and GLUT1 protein levels in MDA-MB-231-based subcutaneous xenograft tumours.

Abbreviations
Akt: Protein kinase B; Cy3: Cyanine 3; DAPI: 4,6-diamidino-2-phenylindole; DMEM: Dulbecco’s modified Eagle’s medium; ER: Estrogen receptor; FBS: Foetal bovine serum; FITC: Fluorescein isothiocyanate; GLUT1: Glucose transporter 1; HA: Hemagglutinin; HER2: Human epidermal growth factor receptor 2; Hif-1: Hypoxia-inducible factor 1; IHC: Immunohistochemistry; IP: Immunoprecipitation; MT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NDRG2: N-myc downstream-regulated gene 2; SGK1: Serum- and glucocorticoid-induced kinase 1; siRNA: Small interfering RNA; TMV: Tumour, node, metastasis.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
All authors conceived of the study and participated in its design. JM, WCL and HG performed most of the experiments. SLI performed xenograft experiments. WC participated in all statistical analyses. XD and SLi performed formalin-fixed, paraffin-embedded, archived patient materials and conducted pathologic reviews and clinical data evaluations. WH performed immunostaining and quantitative analyses. NL and YL interpreted the data and drafted the manuscript. JM, WCL, HG, SLI, WC, XD, SLi, WH, LX and LY revised the manuscript critically. All authors read and approved the final manuscript.

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References
1. Yao L, Zhang J, Liu X: NDRG2: a Myc-repressed gene involved in cancer and cell stress. Acta Biochim Biophys Sin (Shanghai) 2008, 40:625–635.
2. Deng Y, Yao L, Chau L, Ng SS, Peng Y, Liu X, Au WS, Wang J, Li F, Ji S, Han H, Nie X, Li Q, Kung HF, Leung SY, Lin MC. N-Myc downstream-regulated gene 2 (NDRG2) inhibits glioblastoma cell proliferation. Int J Cancer 2003, 106:342–347.
3. Liu J, Yang L, Zhang J, Zhang J, Chen Y, Li K, Li Y, Li Y, Yao L, Guo G. Knock-down of NDRG2 sensitizes cervical cancer HeLa cells to cisplatin through suppressing Bc-2 expression. BMC Cancer 2012, 12:370.
4. Liu N, Wang L, Li X, Yang Q, Liu X, Zhang J, Zhang J, Wu Y, Ji S, Zhang Y, Yan A, Han H, Yao L. N-Myc downstream-regulated gene 2 is involved in p53-mediated apoptosis. Nucleic Acids Res 2008, 36:3535–3549.
5. Ma J, Liu W, Yan X, Wang Q, Zhao Q, Xue Y, Ren H, Wu L, Cheng Y, Li Y, S, Miao L, Yao L, Zhang J. Inhibition of endothelial cell proliferation and tumor angiogenesis by up-regulating NDRG2 expression in breast cancer cells. PLoS One 2012, 7:e32568.
6. Oh S, Kim D, Kim DH, Chang HH, Sohn KC, Kim KH, Jung SH, Lee BK, Kim JH, Kim XD. NDRG2 correlated with favorable recurrence-free survival inhibits metastasis of mouse breast cancer cells via attenuation of active TGF-β production. Cancerogenesis 2012, 33:1882–1888.
7. Nichols NR, Ndrg2, a novel gene regulated by adrenal steroids and antidepressants, is highly expressed in astrocytes. Ann N Y Acad Sci 2003, 1007:349–356.
8. Foletta VC, Prior MJ, Stupka N, Carey K, Segal DH, Jones S, Swinton C, Martin S, Cameron-Smith D, Walder KR. NDRG2, a novel regulator of myoblast proliferation, is regulated by anabolic and catabolic factors. J Physiol 2009, 587:1619–1634.
9. Takahashi K, Saitho A, Yamada M, Iwai T, Inagaki M, Yamada M. Desamethasone indirectly induces NDRG2 expression in rat astrocytes. J Neurosci Res 2012, 90:160–166.
10. Burchfield JG, Leonard AJ, Narasimhan S, Hughes WE, Wasinger VC, Corthals GL, Okuda T, Kondoh H, Biden TJ, Schmitz-Pfeiffer C. Akt mediates insulin-stimulated phosphorylation of NDRG2, evidence for cross-talk with protein kinase CB1. J Biol Chem 2004, 279:18623–18632.
11. Shen L, Liu X, Hou W, Yang G, Wu Y, Zhang R, Li X, Chen H, Lu Z, Zhang Y, Liu X, Yao L. NDRG2 is highly expressed in pancreatic β cells and involved in protection against lipotoxicity. Cell Mol Life Sci 2010, 67:1371–1381.
12. Sun Z, Tong G, Ma N, Li J, Li X, Li S, Zhou J, Xiong L, Cao F, Yao L, Wang H, Shen L: NDRG2: a newly identified mediator of insulin cardioprotection against myocardial ischemia-reperfusion injury. Basic Res Cardiol 2013, 108:341.
13. Boulkroun S, Le Moellie C, Blot-Chabaud M, Farman N, Courtois-Couthy N: Expression of androgen receptor and androgen regulation of NDRG2 in the rat renal collecting duct. PloS One 2012, 7(5):e38894.

14. Li Y, Yang J, Li S, Zhang J, Hou W, Zhai H, Guo Y, Liu X, Dou K, Shi U, Yao L: N-Myc downstream-regulated gene 2, a novel estrogen-targeted gene, is involved in the regulation of Na+/K-ATPase. J Biol Chem 2011, 286(52):5289–5300.

15. Boulkroun S, Fay M, Zennaro MC, Escoubet B, Jaisser F, Blot-Chabaud M, Farman N, Courtois-Couthy N: Characterization of rat NDRG2 (N-Myc downstream regulated gene 2), a novel early mineralocorticoid-specific induced gene. J Biol Chem 2002, 277(32):29136–29143.

16. Murray JT, Campbell DG, Morrice N, Auld GC, Shpiro N, Marquez R, Peggie M, Bannister AJ, Canfield AE, Behrens C: Characterization of rat GLUT1 as a therapeutic target in hepatocellular carcinoma. J Biol Chem 2006, 281(28):28289–28299.

17. Carvalho KC, Cunha IW, Rocha RM, Ayala FR, Cajaíba MM, Begnami MD, Carvalho A, Zaniboni AC, de Alencar FP, de Alencar LR, de Alencar AC, de Alencar LA: Expression of androgen receptor and androgen regulation of NDRG2 in the rat renal collecting duct. J Biol Chem 2002, 277(32):29136–29143.

18. Li Y, Yang J, Li S, Zhang J, Hou W, Zhai H, Guo Y, Liu X, Dou K, Shi U, Yao L: N-Myc downstream-regulated gene 2, a novel estrogen-targeted gene, is involved in the regulation of Na+/K-ATPase. J Biol Chem 2011, 286(52):5289–5300.

19. Warburg O, Wind F, Negelein E: The protein family of glucose transport facilitators: it's not only about glucose after all. J Physiol 2012, 618(1):1–33.

20. Augustin R: The protein family of glucose transport facilitators: it's not only about glucose after all. J Physiol 2012, 618(1):1–33.

21. Adelkova K, Rosen ST, Shanmugam M: GLUT1 as a therapeutic target in hepatocellular carcinoma. Expert Opin Ther Targets 2009, 13(11):1411–1427.

22. Basturol O, Singh R, Kayguusuz E, Balci S, Dursun N, Culhaci N, Adsay NV: GLUT1 expression in pancreatic neoplasic lesions: implications in pathogenesis, diagnosis, and prognosis. Pancreas 2011, 40(1):93–99.

23. Chen DA, Sutphin PD, Nguyen P, Turcotte S, Lai EW, Bann A, Reynolds G, Chi JT, Wu J, Solow-Cordero DB, Bonnet M, Flanagan J, Bouley DM, Graves EE, Denny WA, Hay MP, Giaccia AJ: Targeting GLUT1 and the Warburg effect in renal cell carcinoma by chemical synthetic lethality. Sci Transl Med 2011, 3(11):35.

24. Mann J, Hellerbrand C: GLUT1 as a therapeutic target in hepatocellular carcinoma. Expert Opin Ther Targets 2009, 13(11):1411–1427.

25. Shen L, Sun X, Fu Z, Yang G, Li J, Yao L: The fundamental role of the p53 pathway in tumor metabolism and its implication in tumor therapy. Clin Cancer Res 2012, 18(15):3915–3922.

26. Zheng J, Liu Q, Li Y, Yang J, Ma J, Yu F, Shi H, Ren Q, Zhang R, Zhang J, Yao J, Xue Y, Yao L, Jia J, Han Z, Guo Y, Liu X, Dou K, Shi U, Yao L: N-Myc downstream-regulated gene 2 (NDRG2) is a new HIF-1 target gene necessary for hypoxia-induced apoptosis in A549 cells. Cell Physiol Biochem 2008, 21:239–250.

27. Osthus RC, Shim H, Kim S, Li Q, Reddy R, Mukherjee M, Xu Y, Wonesy D, Lee LA, Dang CV: Deregulation of glucose transporter 1 and glycolytic gene expression by c-Myc. J Biol Chem 2000, 275(21):17977–17980.

28. Chuong FY, Huang MY, Yeh CS, Chang HJ, Cheng TL, Yen LC, Wang JY, Lin SR: GLUT1 gene is a potential hypoxic marker in colorectal cancer patients. BMC Cancer 2009, 9:241.

29. Zhang J, Liu J, Li X, Li F, Wang L, Zhang J, Li J, Li S, Yang A, Han H, Zhang Y, Zhang J, Han W, Liu X: N-Myc downstream-regulated gene 2 (NDRG2) is a new HIF-1 target gene necessary for hypoxia-induced apoptosis in A549 cells. Cell Physiol Biochem 2008, 21:239–250.

30. Qu X, Zhai Y, Wei H, Zhang C, Xing G, Yu Y, He F: Characterization and expression of three novel differentiation-related genes belong to the human NDRG family, Mol Cell Biochem 2002, 229:35–44.

31. Shon SK, Kim A, Kim KJ, Kim KJ, Yang Y, Lim JS: Bone morphogenetic protein-4 induced by NDRG2 expression inhibits MMP-9 activity in breast cancer cells. Biochem Biophys Res Commun 2009, 378:198–203.

32. Park Y, Shon SK, Kim A, Kim KJ, Yang Y, Cho DH, Lee MS, Lim JS: SOCS1 induced by NDRG2 expression negatively regulates STAT3 activation in breast cancer cells. Biochem Biophys Res Commun 2007, 363:361–367.

33. Liu N, Wang L, Liu X, Yang Q, Zhang J, Zhang W, Wu Y, Shen L, Zhang Y, Yang A, Han H, Zhang J, Yao L: Promoter methylation, mutation, and genomic deletion are involved in the decreased NDRG2 expression levels in several cancer cell lines. Biochem Biophys Res Commun 2007, 358:164–169.

34. Lorentzen A, Lewinsky RH, Bohnholt J, Vogel LK, Mitchellmore C: Expression profile of the N-myc downstream regulated gene 2 (NDRG2) in human cancers with focus on breast cancer. BMC Cancer 2011, 11:24.

35. Schwartzenberg-Bar-Yoseph F, Amromin M, Karniel E: The tumor suppressor p53 down-regulates glucose transporters GLUT1 and GLUT4 gene expression. Cancer Res 2004, 64:2627–2633.

36. Kawachi K, Araki K, Tobiume K, Tanaka N: p53 regulates glucose metabolism through an IRK-NEF-kB pathway and inhibits cell transformation. Nat Cell Biol 2008, 10:611–618.