NDRG2 is a new HIF-1 Target Gene Necessary for Hypoxia-Induced Apoptosis in A549 Cells

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Key Words

NDRG2 gene • Hypoxia • HIF-1 • Apoptosis

Abstract

The NDRG2 gene belongs to a family of N-Myc downstream-regulated genes (NDRGs) and is expressed in many normal tissues. NDRG2 gene expression has been shown to be regulated in the stress response of certain cells. However, its function is not yet fully understood. Many studies have demonstrated that hypoxia, one of the stress responses, induced apoptosis in several cell types. In the current study, we investigated NDRG2 involvement in hypoxia response and found that NDRG2 expression was markedly up-regulated in several tumor cell lines exposed to hypoxic conditions or similar stresses at the mRNA and protein level. We also observed that the expression of NDRG2 was regulated by Hypoxia-inducible factor 1 (HIF-1) in tumor cells under hypoxia. Three hypoxia-responsive elements (HREs) in the NDRG2 promoter were

identified. HRE1 could directly bind Hif-1 in vivo. Importantly, we found that silencing or enforcing the expression of NDRG2 could strongly inhibit or increase apoptosis. In addition, our data also showed that NdrG2 was able to be translocated from the cytoplasm to the nucleus, and the segment from 101 to 178 amino acids of NdrG2 is responsible for its translocation. Taken together, this study suggests that NDRG2 is a Hif-1 target gene and closely related with hypoxia-induced apoptosis in A549 cells.

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Introduction

Hypoxia is an important physiological stress that influences responses of a wide range of pathologies, including stroke, infarction, and tumorigenesis. Tumor hypoxia is also closely associated with tumor propagation,

malignant progression, and resistance to therapy [1]. Given these factors, it is important to clarify the mechanism of hypoxia in tumor physiology and cancer treatment.

The *NDRG* gene family consists of 4 members: *NDRG1*, *NDRG2*, *NDRG3*, and *NDRG4*, which share 57–65% amino acid identity. A database search and a phylogenetic analysis revealed that *NDRGs* are highly conserved in plants, invertebrates, and mammals, suggesting that this gene family may have important cellular functions [2].

NDRG 2 (also named SYLD/KIAA1248) was first cloned in our laboratory in 1999 (GenBank Accession No. AF159092). This gene is strongly related to the N-Myc downstream-regulated gene 1 (*NDRG1*), which has been linked to cell stress responses, such as hypoxia [3], cell proliferation, and differentiation. *NDRG1* was proposed to be a candidate tumor suppressor gene due to its reduced expression in some cancer tissues and its induction of apoptosis in certain cancer cells [4–7]. A previous study indicated that *NDRG2* is a differentiation-related gene and could be repressed by c-myc [8]. Some evidence suggests that *NdrG2* may also play a role in embryo differentiation and development as well as Alzheimer's disease [9]. However, the function of *NdrG2* is not fully understood.

Apoptosis can be induced in response to hypoxia. In solid tumors, hypoxia is a common phenomenon. Cells in rapidly growing tumors are intermittently, or sometimes constantly, exposed to hypoxic conditions. In severe or prolonged hypoxia, cells will initiate the process of programmed cell death.

Hif-1 plays a very important role in hypoxia-induced apoptosis. It is a member of the basic helix-loop-helix transcriptional factor family [10] and specifically binds to HRE in the promoter or enhancer region of various hypoxia-inducible genes, including erythropoietin, vascular endothelial growth factor, and glucose transporters [11–15]. Furthermore, certain transition metals (Co^{2+} , Ni^{2+} , and Mn^{2+}) can also imitate the hypoxia effect and thus induce *HIF-1* gene expression.

In this study, we first analyzed the regulation of *NDRG2* expression in cells exposed to hypoxia and found that Hif-1 plays a role in *NDRG2* gene induction. Additionally, we demonstrated that the binding site of Hif-1 in the promoter of the *NDRG2* gene is crucial for *NDRG2*'s hypoxia response. We then investigated the function of *NDRG2* in apoptosis induced by hypoxia. Moreover, we found that hypoxia can induce the translocation of *NdrG2*. Our data suggest that the *NDRG2* gene is a target gene of Hif-1.

Materials and Methods

Materials

Aprotinin, leupeptin, phenylmethylsulfonylfluoride (PMSF), nickel chloride (NiCl_2), and cobalt chloride (CoCl_2) was purchased from Sigma/Aldrich. The luciferase assay substrate was purchased from Promega (WI). Lipofectamine™ 2000 was obtained from Invitrogen (Invitrogen Life Technologies, Carlsbad, CA). *NdrG2*, β -Actin, and α -Tubulin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Hif-1 α antibody was purchased from BD Biosciences (San Diego, CA). The adenovirus carrying wt*NDRG2* or negative control Ad-lac Z was produced by Benyuan Zhengyang (Beijing, China).

Cell Culture and hypoxia induction

A human lung cancer cell line (A549), human hepatocellular carcinoma cell line (HepG2), human breast carcinoma cell line (SKBR-3), and human embryonic kidney cell line (HEK293) were obtained from the American Type Culture Collection (Manassas, VA). Cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Sijiqing Biological Engineering Materials Co, Hangzhou, China). All cells were incubated at 37 °C in the presence of 5 % CO_2 -balanced air. To induce hypoxia, cells were rendered in a chamber with a gas mixture of 1%, 2%, 5%, or 10% O_2 and 5% CO_2 -balanced N_2 at 37°C. The level of oxygen in the chamber was verified using a gas monitor (SKC, Inc., Eighty Four, PA). To mimic hypoxia using chemicals, cells were cultured under 20% oxygen in the presence or absence of either 100 μM CoCl_2 or 1 mM NiCl_2 for a specified time period.

RT-PCR

The cells were collected at the indicated time point, and the total RNA was isolated from each sample using TRIzol (Invitrogen, Carlsbad, CA) and then quantified. We reverse-transcribed total RNA using reverse transcriptase (Promega, WI) according to the manufacturer's instructions. All PCR experiments were performed with *Taq* polymerase (Promega, WI). The primers used were as follows: *NDRG2* 5'- ATG GCG GAG CTG CAG GAG GTG C-3' (sense) and 5'- TGA GGA ACG AGG TCT GGG TGG G -3' (antisense); *NDRG1*, 5'-TCT CGG GAG ATG CAG GAT GTA-3' (sense) and 5'-TCA GCC CAA ACT GTT GAA GGA-3' (antisense); β -*ACTIN* 5'-GAT CAT TGC TCC TCC TGA GC-3' (sense) and 5'- TGT GGA CTT GGG AGA GGA CT- 3' (antisense).

Real-time quantitative PCR

Total RNA was extracted as previously described. The first strand cDNA was used as a template in the real-time quantitative PCR analysis. All measurements were performed in triplicate. β -*ACTIN* cDNA was used as the internal control and normalized for variances in input cDNA. The following primers were designed using the ABI Primer Express software: *NDRG2*-sense: 5'-GAGATATGCTCTTAACCAACCCG-3', *NDRG2*-antisense: 5'-GCT GCC CAA TCC ATC CAA-3'; β -

ACTIN -sense: 5'-ATC ATG TTT GAG ACC TTC AAC A-3', β *ACTIN*-antisense: 5'-CAT CTC TTG CTC GAA GTC CA-3'. *NDRG2* and β -*ACTIN* mRNA were detected using SYBR Green PCR Master Mix (Applied Biosystems, Brackley, UK) and an ABI PRISM 7500 Sequence Detection System (Applied Biosystems, UK) using the comparative CT Method for Relative Quantification. The PCR reaction consisted of 12.5 μ l of SYBR Green PCR Master Mix, 300 nM of forward or reverse primers, and 1.5 μ l template cDNA in a total volume of 25 μ l. The thermal cycling conditions were as follows: 95°C for 5 min followed by 50 cycles at 95°C for 30 s, 58°C for 30 s, and 72°C for 45 s. To verify that the used primer pair produced only a single product, a dissociation protocol was followed after thermocycling, thus determining dissociation of the PCR products from 65°C to 95°C.

Western blotting

Cells harvested from 60 mm culture dishes were lysed in 200 μ l of radioimmunoprecipitation assay (RIPA) buffer (0.05 M Tris-HCl, pH 7.4; 0.15 M NaCl; 0.25% deoxycholic acid; 1% NP-40; 1 mM EDTA; 1 mM PMSF; 1 μ g/ml aprotinin; and 1 μ g/ml leupeptin). Protein concentration was measured by bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL). Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to Hybond ECL nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ). Western blot analyses were performed using an anti-NdrG2 antibody, an anti-Caspase 3 antibody (Cell Signaling), anti-PARP antibody (Cell Signaling), an anti- β -Actin or anti- α -Tubulin antibody, followed by incubation in a species-matched horseradish peroxidase (HRP)-conjugated secondary antibody. The blot was developed with a chemiluminescence substrate solution (Pierce, Rockford, IL) and exposed to X-ray film.

Plasmid construction

A set of *NDRG2* promoter-driven luciferase reporter vectors were constructed by inserting the wide-type sequence (from -710 to +149 bp), 2 truncated sequences (from -254 to +149 bp and from -184 to +149 bp, respectively), 2 HRE1 mutants (GCGTCC mutated to GAAAAC and -189 to -184 bp were deleted), HRE2 and HRE3 mutants (motif GCGTGTGCGTG mutated to GCAAGTGCAAG) of the *NDRG2* promoter into a pGL3-basic vector (Promega, WI). The pCDNA3.0-*HIF-1 α* was a gift from Tang Hong (The Institute of Microbiology, Chinese Academy of Sciences, Beijing). The pGL3-5xHRE (*VEGF*) was a gift from Dr. Hua Han (Institute of Molecular Biology, The Fourth Military Medical University). The pSliencerTM3.1-H1neo was purchased from Ambion (Austin, TX). Four truncated *NDRG2* fragments, namely, CT1, CT2, CT3, and NT1 (Fig. 7D), were inserted into the pEGFP-C2 vector (Clontech, Palo Alto, CA).

Treatment of cells with siRNA for *HIF-1 α* and *NDRG2*

Three siRNA motifs were selected from the human *HIF-1 α* or *NDRG2* cDNA sequence according to the AA-N19 rule (www.ambion.com/techlib/resources/RNAi). The 3 target sequences for *HIF-1 α* were localized at positions 145, 377, and

1257 bp downstream from the start codon of *HIF-1 α* . An inverted sequence (which does not target any gene) was used as a control (sense, 5'-GAT CCA GTT CAA CGA CCA GTA GTC TTC AAG AGA GACT TAC TGG TCG TTG AAC TTT TTT TGG AAA-3' and antisense, 5'-AGC TTT TCC AAA AAA GTT CAACGACCAAGTA GTC TCT CTT GAAGAC TAC TGG TCG TTG AAC TG-3' [16-18]. The 3 target sequences for *NDRG2* were localized at positions 363, 604, and 612 bp downstream from the start codon of *NDRG2*. The inverted sequence or 3 different siRNA oligonucleotides were annealed and cloned into a pSliencerTM3.1-H1neo vector. The transfection of the siRNA expression vector was performed using LipofectamineTM 2000 as recommended by the manufacturer.

Luciferase assays

1 $\times$ 10⁵ cells (HEK 293) were seeded to each well (of a 24-well plate) and cultured overnight in a medium containing 10% fetal bovine serum. Twenty-four hours after reporter gene vector transfection, cells were lysed with 100 μ l of passive lysis buffer. Ten microliters of the extract was taken for a firefly luciferase assay and Renilla luciferase assay by a multiwell plate luminometer. The ratio of firefly luciferase activity (expressed from the reporter construct) to Renilla luciferase activity (expressed from pRL-TK) in each sample was served as a measure of normalized luciferase activity.

Chromatin Immunoprecipitation (ChIP) Assay

ChIP analysis was performed on 6 $\times$ 10⁶ A549 cells transiently transfected with pCDNA-*HIF-1 α* , or with the 1mM NiCl₂ treatment for 24 h. In brief, cells were cross-linked with 1% formaldehyde for 10 min at 37°C. The cells were then washed, lysed, and sonicated to generate 200 to 500 bp DNA fragments according to the procedure outlined in the ChIP Assay kit (Upstate Biotechnology, Charlottesville, VA). The samples were pre-cleared with 60 μ l of salmon sperm DNA-protein A-agarose (Upstate Biotechnology, Charlottesville, VA) and subsequently incubated with either 5 μ g of anti-Hif-1 α antibody or without an antibody as a control, overnight with rotation at 4°C. Immunocomplexes were recovered with salmon sperm DNA-protein A-agarose (Upstate Biotechnology, Charlottesville, VA), washed extensively, and eluted with ChIP elution buffer (Upstate Biotechnology, Charlottesville, VA). Following the reversal of cross-links at 65°C for 4 h, samples were extracted with phenol/chloroform and precipitated by ethanol, and amplified by PCR using primers flanking the HRE region or control region: *NDRG2* HRE-1 (sense primer, 5'-GTT AGC GGC GAA GCC ACA GG-3' (-235 bp/-215 bp); antisense primer, 5'-TGG GGT CAA TGC CTC AAG GG-3' (-82 bp/-62 bp)); *NDRG2*-negative control (sense primer, 5'-CAA AGG CTG AGG CTC CAA GAG-3' (+889 bp/+910 bp); antisense primer, 5'-TCG GGAAGG GAT GGG TAG G-3' (+1063 bp/+1082 bp). To ensure that PCR amplification was performed in the linear range, template DNA was amplified for a maximum of 30 cycles. PCR products generated from the ChIP template were sequenced to verify the identity of the amplified DNA.

NDRG2 subcellular localization analysis

The treated cells were fixed (4% paraformaldehyde in PBS,

Fig.1. Increase of NDRG2 mRNA caused by hypoxia and its mimetic. (A) Analysis of NDRG2 and NDRG1 transcript in A549 cells by semiquantitative RT-PCR during hypoxia (2% O₂). These results are representative of three independent experiments. (B) Total RNA was isolated from A549 cells treated with NiCl₂ and examined for NDRG2 transcript by semiquantitative RT-PCR. NDRG1 transcript was used as a positive control and β -ACTIN transcript was used as an internal standard. The results are representative of four independent experiments. (C) Measurement of NDRG2 mRNA in A549 cells by Real-time PCR upon treatment of NiCl₂. A549 cells (5 $\times$ 10⁵) were seeded in 6 cm plates and incubated with NiCl₂ for 0 h, 3 h, 6 h, 9 h, 12 h and 24 h. Total RNA was harvested and tested for NDRG2 mRNA expression by real-time PCR, as described in "Materials and Methods." Results are expressed as fold increase relative to NDRG2 mRNA levels in the absence of NiCl₂ treatment (equal to 1). Results presented are the mean $\pm$ S.D. of three experiments.

30 min, RT), rinsed, and permeabilized with 0.1% Triton X-100 in PBS (15 min). Permeabilized cells were then incubated with horse serum in PBS to block nonspecific binding. After thorough rinsing with PBS, the cells were incubated overnight at 4°C with an anti-NdrG2 antibody (1:100), rinsed with PBS, and incubated with fluorescent isothiocyanate (FITC)-coupled anti-goat antibody (1:1500) (1 h, 37°C). Dual-color detection was performed by confocal laser scan microscopy after treatment with 50 μ g of propidium iodide (PI) for 5 min to label nuclear DNA.

Cell fraction assay

After A549 cells were exposed to NiCl₂ for 6 or 12 h, the nuclear extracts were prepared as described in the protocol of NE-PER nuclear and Cytoplasmic Extraction Reagents (Pierce, Rockford, IL). Briefly, 1 $\times$ 10⁷ cells were washed twice with ice-cold phosphate-buffered saline and added to 200 μ l of ice-cold cytoplasmic extraction reagent I, incubated on ice for 10 min, added to 11 μ l of ice-cold cytoplasmic extraction reagent II, and incubated on ice for 1 min; the tube was centrifuged for 5 min at maximum speed in a microcentrifuge (16,000 g). The supernatant fraction (cytoplasmic extract) was immediately transferred to a clean pre-chilled tube and 100 μ l of ice-cold nuclear extraction reagent was added into the insoluble fraction by vortexing for 15 s every 10 min for a total of 40 min. The tube was centrifuged at maximum speed in a microcentrifuge for 10 min. The nuclear extract fraction was moved to a clean pre-chilled tube. All extracts were analyzed by western blot.

Transfection

All transfections were performed using lipofectamineTM 2000, according to the manufacturer's instructions. The psilencer-X and psilencer-NDRG2 were transfected into A549 cells respectively. In all cases, single colonies were obtained in the presence of the G418 (500 μ g/ml) selection after 4 weeks. All clones were analyzed by western blotting for NDRG2 expression. Two separate stable clones were used in a further experiment to exclude clone artifacts.

Cell growth assays

The cell growth was monitored by cell counting and MTT assay, respectively. MTT assay is based on the activity of mitochondrial dehydrogenases in live cells, which oxidize the tetrazolium salt (MTT, Sigma) to a blue formazan salt. The cells were seeded into 96-well plates at an initial density of 10³ cells/well in triplicate. The cells were then washed and incubated with MTT (100 μ g/ml) at 37°C for 4 h. The supernatant was moved, and 150 μ l DMSO was added to each well. The absorbance of the reaction solution at 570 nm was recorded. Each experiment was performed in quadruplicate, and the values are reported as mean $\pm$ S.D.

Flow Cytometry

For the flow cytometric analysis of cell cycles, cells were harvested by trypsinization, washed with PBS, and fixed and stored in 75% ethanol at 4°C before DNA analysis. After removal of the ethanol by centrifugation, cells were incubated with a

Fig. 2. The augments of NdrG2 protein levels upon hypoxia and its mimetics. The cell lysate was resolved by SDS-PAGE and NdrG2 and α -Tubulin were assessed by Western blot analysis. Representative experiments from the 3 were performed with each cell type are shown. (A) A549 cells were exposed to hypoxia (2% O₂) for the indicated time periods. (B) A549 cells were exposed to hypoxia (5% O₂) for the indicated time periods. (C) A549 cells were exposed to 10% O₂ for the indicated time periods. (D) HepG2 and SKBR cells were exposed to hypoxia (2% O₂) for 12 h and 24 h respectively. (E) A549 cells were exposed to NiCl₂ or CoCl₂. NdrG2 and Hif-1 α were immunoidentified. The α -Tubulin bands in the bottom panel show loading. These results are representative of three independent experiments.

phosphate-citric acid buffer at room temperature for 45 min. After centrifugation, the cells were then stained with a propidium iodide solution for 24 h. Stained nuclei were analyzed for DNA-propidium iodide fluorescence using a Becton Dicking FACScan flow cytometer (Becton Dickinson, San Jose, CA). The resulting DNA distributions were analyzed by Modifit (Verity Software House, Inc.) to examine the proportion of cells in sub- G₀, G₁, S, and G₂-M phases of the cell cycle. Cell death was determined by a two-color analysis of fluorescein isothiocyanate-labeled annexin V (Roche Applied Science).

Statistical analysis

Data were analyzed by Student's *t*-test. Results were expressed as the means $\pm$ standard deviations (SD). Differences were considered significant when the *p* value was less than 0.05.

Results

Hypoxia and its mimetics induce the up-regulation of NDRG2

After exposing A549 cells to hypoxia (2% O₂), or similar conditions, an increase of NDRG2 mRNA expression (2.9 $\pm$ 0.1-fold increase of integrated band density compared with the control, *p*<0.01) was observed. NDRG2 mRNA expression gradually increased after 6 h of exposure to hypoxia (Fig. 1A). In addition, the mRNA amount of NDRG2 and NDRG1 were also increased upon treatment with NiCl₂ (Fig. 1B). For a more accurate quantitative analysis, real-time PCR was used to confirm the change in the NDRG2 mRNA level upon NiCl₂-

Fig. 3. The regulation of Hif-1 α on NDRG2 gene expression. (A) A549 cells were transiently transfected with HIF-1 α cDNA containing plasmid for different time periods. Cells were lysed and NdrG2 and Hif-1 α examined by Western blot. The expression of NdrG2 or Hif-1 α was quantified and the relative intensities were normalized to that of β -Actin. Each bar represents the average of triplicate determinations $\pm$ S.E. (B) HEK293 cells were divided into three groups: control (no plasmid was transfected); transfected with mixture of three HIF-1 α target siRNA vectors or transfected with pSilence3.1-x, which does not target any gene. Twenty-four hours after transfection, the cells were exposed to hypoxic conditions for another 24 h. NdrG2 and Hif-1 α expression were examined by Western blot. β -Actin bands in the bottom panel show loading. These results are representative of three independent experiments.

exposure. The dissociation curve of the NDRG2 product showed a single peak at different times, and PCR products had the same single expected DNA bands in agarose gel analysis (data not shown). The result of real-time PCR is in accordance with those of semiquantitative RT-PCR (Fig. 1C).

NdrG2 concentration in A549 cells exposed to hypoxia (2%, 5% and 10% oxygen) or treated with NiCl₂ or CoCl₂ was analyzed by western blot. There was a gradual time-dependent increase of NDRG2 expression in 2% oxygen (Fig. 2A). No significant changes in NDRG2 expression were noted in either 5% or 10% oxygen (Fig. 2B and C). In other cell lines, including HepG2 and SKBR-3, hypoxia (2% O₂) also induced up-regulation of NDRG2 expression (Fig. 2D). Similar results were also found after A549 cells were exposed to NiCl₂ or CoCl₂ (Fig. 2E). Under the same conditions, expression of HIF-1 α was also observed.

Hif-1 α is responsible for up-regulation of NDRG2 expression

To determine whether NDRG2 expression is up-regulated by exogenous Hif-1 α , A549 cells were

transfected with the wild-type HIF-1 α gene; the effect of Hif-1 α on NDRG2 expression was investigated at different time points. As Fig. 3A illustrates, the over-expression of Hif-1 α resulted in a significant up-regulation of NDRG2 expression. NDRG2 expression was increased at 6 h and peaked at 24 h.

In order to clarify whether Hif-1 α is necessary for NDRG2 induction, we used RNA interference to block endogenous HIF-1 α expression in A549 cells. The influence of Hif-1 α on NDRG2 expression was then observed under hypoxic conditions (Fig. 3B). The data suggest that the reduction of endogenous Hif-1 α resulted in the abolishment ($p < 0.01$) of a hypoxia-induced increase of NdrG2.

Identification of a specific binding sequence for Hif-1 α in the NDRG2 gene

To further explore whether Hif-1 α directly regulates NDRG2 transcription, luciferase reporter constructs containing varied lengths of NDRG2 promoter or mutated HRE were constructed (Fig. 4A), and the effects of Hif-1 α on their activity were tested. As shown in Fig. 4B, after HEK293 cells were transiently co-transfected with

Fig. 4. NDRG2 promotor luciferase reporter assays. (A) Schematic diagram of wild-type, truncated or mutated NDRG2 promoter with three consensus HREs constructs are shown. (B) Relative luciferase activity of the NDRG2 promoter report gene. HEK293 cells were transfected with 20 ng of reporter constructs and 1 μ g of pCDNA3.0-HIF-1 α expression vector or pCDNA3.0-Lac Z vector for 24 h. To correct for transfection efficiency variation, cells were co-transfected with 0.2 ng of pRL-TK vector. The pGL3-5xHREs (VEGF) were used as a positive control. The luciferase results were reported as relative light units of firefly luciferase activity normalized with respect to the Renilla luciferase activity. The mean values from three independent experiments are shown. (C) Hif-1 α protein binds to the NDRG2-HRE-1 site in vivo. ChIP assay of a genomic fragment (nt position -235 bp to -62 bp, where +1 represents the transcription site containing the NDRG2-HRE 1 site in A549 cells with overexpression of exogenous Hif-1 α or with the NiCl₂ (1mM) treatment. Immunoprecipitation was performed using a gene-specific antibody against Hif-1 α , followed by PCR amplification. Input chromatin (input), which represents a portion of the sonicated chromatin before immunoprecipitation, and genomic DNA were used as positive controls. Immunoprecipitation without an antibody served as a negative control. PCR amplification revealed that the NDRG2-HRE1 is present in Hif-1 complexes extracted from immunoprecipitation. The control sequence was amplified in the chromatin input and genomic DNA but not in the samples immunoprecipitated with an antibody against Hif-1 α .

the NDRG2 wild-type promoter vector (from -710 to +149 bp) and HIF-1 α encoding cDNA, the luciferase activity increased by 5.3 ± 0.3 -fold compared with the controls ($p < 0.01$). It was shown that the HRE2 and HRE3 truncation or mutation in the NDRG2 promoter could reduce their luciferase activity as compared with the NDRG2 wild-type promoter (36.4% and 24.8%). However, the HRE1 deletion or mutation in the NDRG2 promoter could more significantly reduce their luciferase activity as compared with the NDRG2 wild-type promoter (51.5% and 50.4%). In addition, truncation of all HREs in the NDRG2 promoter resulted in a significant loss of luciferase activity (80.9%) as compared with the activity of the wild-type NDRG2 promoter. Next, we performed in vivo ChIP assays to examine whether Hif-1 α can

associate with the HRE site in NDRG2 promoter. As shown in Fig. 4C, Hif-1 α -antibody could precipitate a DNA fragment containing the HRE 1 site of NDRG2 promoter, and no specific PCR products were amplified using primers around the region (+889bp/+1082bp) in the NDRG2 intron 1. We could not detect the interaction of Hif-1 α with the HRE-2 or HRE3 site in the NDRG2 promoter (date not shown).

NDRG2 sensitizes A549 cells to hypoxia-induced apoptosis

The up-regulation of NDRG2 upon hypoxia may affect the cell susceptibility to hypoxia. To further investigate the functional role of NdrG2 under hypoxia conditions, the cells were infected with an adenovirus

Fig. 5. Impact of enforced expression of NDRG2 on hypoxia-induced apoptosis in A549 cells. A549 cells were infected with Ad-NDRG2 or Ad-lac Z for 12 h, then the cells were exposed to hypoxia (1% O₂) for 48 h, after which time the death of these cells were determined by two-color analysis of annexin V and propidium iodide (PI) using a Becton Dickinson FACSCalibur by flow cytometry. The percentage of apoptotic cells were quantified, and the results are the means $\pm$ S.D. of three independent experiments.

Fig. 6. Impact of NDRG2 knock down on hypoxia-induced apoptosis in A549 cells. The stable clone of A549 cells transfected with psilencer-X or psilencer-NDRG2 were used here. (A) The two kinds of cells were exposed to hypoxia (1% O₂) for different periods. The effect on cell growth was assayed using the MTT assay as described under “Material and Methods.” The results are the means $\pm$ S.D of three independent experiments. (B). These cells were exposed to hypoxia (1% O₂) for 48 h, then the cells were lysed and NdrG2, activated Caspase 3, cleaved PARP and Actin were examined by Western blot, with Actin as the loading control. (C) These cells were harvested after they were exposed to hypoxia (1% O₂) for 0 h, 24 h and 48 h separately, and then the death of these cells was determined by two-color analysis of annexin V and propidium iodide (PI) using a Becton Dickinson FACSCalibur by flow cytometry. The percentage of apoptotic cells were quantified, and the results are the means $\pm$ S.D of three independent experiments.

carrying NDRG2 cDNA (Ad-NDRG2) or the control virus Ad-LacZ at 25 MOI (multiplicity of infection) before exposing them to a hypoxia culture. After being exposed to 1% O₂ for 48 h, the cells with NDRG2 over-expression exhibited an increase in cell apoptosis (Fig. 5) ($p < 0.01$). The over-expression of NDRG2 by Ad-NDRG2 infection was confirmed by western blot (Fig. 5, in box).

These results suggest that NDRG2 can sensitize A549 cells to hypoxia-induced apoptosis.

To further confirm the role of NDRG2 in cell susceptibility to hypoxia, we conducted a NDRG2 gene knock-down by using the siRNA expression vector Psilencer-NDRG2 or the control vector Psilencer-X transfection and analyzed cell death upon hypoxia.

Fig. 7. NdrG2 nuclear translocation induced by exposing cells to hypoxia or NiCl_2 . The location of NdrG2 in A549 cells was observed by confocal laser scan microscopy before or after exposure of the cells to (A) NiCl_2 (1mM) or (B) hypoxia (2% O_2). NdrG2 was labeled with FITC (green) and the nuclei were counterstained with PI (red). The gain signal was adjusted using a normal goat Ig-G stained slide as a control. A representative experiment of three performed is shown. (C) Distribution of NdrG2 in A549 cells after exposure to NiCl_2 . The distribution of NdrG2 in different cell fractions was observed by anti-NdrG2 antibody before or after exposure to NiCl_2 (1mM). (C) C, Cytoplasmic extract; N, nuclear extract. A representative experiment of the three performed is shown. (D) Schematic diagram of different NdrG2 truncated segment, which are named separately as NT1, NT2, NT3 and CT1. (E) Four fragments were constructed into a pEGFP vector and transfected into A549 cell lines respectively, after exposure to hypoxia for 12 h. The phenomena of translocation was observed by fluorescent microscope.

The Psilencer-NDRG2 transfected A549 cells showed a higher surviving rate ($p < 0.01$) than the control cells, after being exposed to 1% O_2 (Fig. 6A). The efficient silencing of NDRG2 is shown in Fig. 6B. The cell apoptosis was also confirmed by proteolytic cleavages of caspase 3 and poly ADP-ribose polymerase (PARP), which are commonly used as markers for cells undergoing apoptosis. The A549 cells with Psilencer-X exhibited a higher degree of cleavage of endogenous Caspase 3 and PARP when exposed to hypoxia (1% O_2) for 48 h, while the cells with

Psilencer-NDRG2 are resistant to hypoxia (Fig. 6B). To quantitatively illustrate the role of NdrG2 in hypoxia-induced apoptosis, we performed flow cytometry analysis on A549 cells that were stably transfected with Psilencer-X or Psilencer-NDRG2 and then exposed the cells to hypoxia (1% O_2) for an indicated time. As shown in Fig. 6C, the A549 cells with a normal NDRG2 level went into rapid and extensive apoptosis, whereas the percentage of apoptotic cells was obviously decreased in A549 cells with NDRG2 gene silencing ($p < 0.01$).

Hypoxia induces the nuclear translocation of Ndr g2

The subcellular localization of Ndr g2 in A549 cells exposed to hypoxia or NiCl_2 was determined by Ndr g2 protein fluorescent staining visualizing under confocal laser scanning fluorescence microscopy. The dual-color detection of Ndr g2 and PI demonstrated that Ndr g2 was faintly localized to the cytoplasm before exposure to hypoxia. Protein staining increased and the protein translocated from the cytoplasm to the nucleus after A549 cells had been treated with NiCl_2 (Fig. 7A) or exposed to hypoxia for 12 h (Fig. 7B). To support the evidence of nuclear translocation of Ndr g2 upon NiCl_2 -exposure, a cell fraction assay was performed. As can be seen in Fig. 7C, Ndr g2 indeed translocated from the cytoplasm to the nucleus fraction after A549 cells were exposed to NiCl_2 for 6 h and 12 h. In order to find the segment contributing to the translocation of Ndr g2 protein, 4 different truncated *NDRG2* segments were constructed into a fluorescent vector (Fig. 7D). When transiently transfected into A549 cell lines, all the segments except NT2 were also translocated from cytoplasm to nucleus in hypoxia conditions after approximately 12 h (Fig. 7E). It is indicated that the segment from 101 to 178 amino acid of Ndr g2 is responsible for translocation.

Discussion

NDRG2 was first discovered by our laboratory in 1999. We speculated that *NDRG2* played a role in the stress response because it was closely related to *NDRG1*, which itself is linked to stress responses [19, 20]. Hypoxia, one of the stress responses, was chosen to examine the possible involvement of the *NDRG2* gene in tumor cells. The data here suggests that the level of expression of *NDRG2* mRNA was up-regulated in some tumor cell lines. Under similar conditions, the expression of Ndr g2 protein was also up-regulated. It was found that the Ndr g2 protein was gradually increased after exposing cells to hypoxic conditions for 6 h in A549 cells. A similar result was also observed in the case of Ndr g1 [3]. In addition, it was clear that Ndr g2 up-regulation was related to O_2 concentration. The difference of *NDRG2* expression was only noted when cells were exposed to 2% O_2 , suggesting that Ndr g2 might be more active in lower hypoxic environments.

Transcription factor Hif-1 α is a key determinant of oxygen-dependent gene regulation. It is exclusively

activated in hypoxia and activates target genes, which influence energy metabolism [21], cell proliferation [22], hematopoiesis [23] and vascular development [24]. Hif-1 α can specifically bind to cis-acting HRE in the promoter or enhancer region of various hypoxia-inducible genes during hypoxia. Sequence analysis of the human *NDRG2* gene promoter revealed three putative HRE motifs. HRE-1 is at the position from -188 to -183 bp; HRE-2 is from -371 to -367 bp and HRE-3 from -377 to -373 bp. These HRE motifs showed to some extent homology with the classical Hif-1 binding consensus sequence 5'-RCGTG-3' (R=A/G), as well as another alternative consensus sequence BACGTSSK (B=G/C/T, S=G/C, and K=G/T) [25]. The luciferase reporter constructs were used to verify the hypoxia-responsive region of the *NDRG2* promoter that may associate with Hif-1 α . The results showed that truncation of all HRE in *NDRG2* promoters resulted in a significant loss (80.9 %) of luciferase activity compared to the wild-type *NDRG2* promoter. This suggests that HRE-1, HRE-2 and HRE-3 in the *NDRG2* promoter are closely related with regulation of Hif-1 α . HRE-1 is more important than HRE-2 and HRE-3 because the deletion or mutation of HRE-1 in the *NDRG2* promoter resulted in a dramatic decrease of luciferase activity, whereas the deletion or mutation of HRE-2 and HRE-3 only resulted in a slight decrease of luciferase activity. The interaction between Hif-1 α and HRE 1, HRE 2 or HRE 3 was analyzed by chromatin immunoprecipitation assay. The result indicated that Hif-1 α could specifically bind the HRE-1 site in the *NDRG2* promoter, but not HRE-2 or HRE-3 in vivo, suggesting that Hif-1 α can only interact directly with the *NDRG2* promoter through HRE-1 to regulate the transcription of *NDRG2*. On the other hand, the data suggest that exogenous Hif-1 α could be expressed after it was transfected into the A549 cell line, and expression levels of Hif-1 α gradually increased during a given time period under hypoxic conditions. Hif-1 α over-expression, in turn, resulted in a significant up-regulation of *NDRG2* expression. To further clarify whether the up-regulation of *NDRG2* expression was related to endogenous Hif-1 α , the expression of the latter by siRNA was blocked. The result showed that Ndr g2 could be markedly reduced by blockade of Hif-1 α . It strongly suggests that *NDRG2* is a downstream target of the Hif-1 α protein that can bind to the HRE-1 site in the *NDRG2* promoter and induce the *NDRG2* expression in response to hypoxia. It was observed that *HIF-1 α* siRNA transfection or HRE1 deletion reduced the hypoxic response only to 60% or 80%, respectively. It indicated

that some other factors may also be involved in the *NDRG2* up-regulation process. The analysis of the *NDRG2* promoter (using T fsitescan software, <http://www.ifti.org/cgi-bin/ifti/Tfsitescan.pl>) revealed that the other transcription factor binding sites, such as p53, NF- κ B, SP1 and Ap-2 etc, exist upstream of the *NDRG2* gene or the *NDRG2* intron 1. Consequently, the response of Hif-1 may not be the sole response.

Evidence indicates that hypoxia induces proteome and genome changes [1] which lead to cell cycle arrest, differentiation, apoptosis and necrosis [26, 27]. During a stress response, a series of cellular metabolic and biosynthetic events occur to adapt to the hypoxic environment. The significant increase in the expression of some genes involved in hypoxia offer a good example, in particular, of genes associated with cell growth [27, 28]. *NDRG2* generally showed the down-regulated expression in tumor tissues and the growth inhibition in tumor cells [4, 6]. It suggested that enhanced expression of *NDRG2* may play a role in controlling cell growth as well as its sensitivity to hypoxia. The intention is to find the significance of *NDRG2* gene up-regulation under hypoxia conditions. There was an enhanced resistance of A549 cells to hypoxia induced apoptosis if *NDRG2* gene was knocked down by siRNA. It was noticeable that the ectopic expression of *NDRG2* only enhanced the apoptosis of A549 cells upon hypoxia, but not under normal culture status (data not shown). It is similar that the silencing of the *NDRG2* gene only slightly affects the growth of A549 cells in normal conditions. However, the enforced expression of *NDRG2* in A549 cells did suppress the growth of the cells through inducing G1 phase arrest, but did not have an effect on cell death. It demonstrates that *NDRG2* is an important molecule for the hypoxia-induced apoptosis in A549 cells.

It was found that NdrG2 was translocated from the cytoplasm to the nucleus after the tumor cells were exposed to hypoxia or NiCl₂ for 9 h. To our knowledge, no nuclear localization signal (NLS) sequence has been identified in NdrG2 protein. Although NLSs are the most common type of nuclear import elements, other sequences are often important for targeting certain proteins to the nucleus. For example, adenomatous polyposis coli (APC) and breast cancer 1 (BRCA1) were recently shown to enter the nucleus via pathways that are quite independent of their NLSs [29]. It is speculated that NdrG2 may have its own motif that is responsible directly or indirectly for

its nuclear translocation. To map the corresponding motif for NdrG2 translocation, a series of truncated *NDRG2* vectors were made and it was found the segment (101 to 178 amino acids) of NdrG2 are responsible for its translocation. This result provided a valuable clue for further studies in the novel translocation mechanism and detailed function of NdrG2.

In summary, the data suggests that the expression of *NDRG2* in tumor cell lines was up-regulated under hypoxia conditions with concomitant translocation of NdrG2 from the cytoplasm to the nucleus. The induction of *NDRG2* expression under hypoxia was dependent on the Hif-1 transcription factor, which can directly bind to the HRE1 of *NDRG2* promoter and regulate the expression of *NDRG2*. Also important is a novel function of NdrG2 was discovered. *NDRG2* may be a gene that is responsible for the hypoxia-induced apoptosis in A549 cells. Consequently, *NDRG2* might be a potential target gene in the treatment of certain cancers which escape apoptosis and are resistant to anticancer treatment under hypoxia conditions.

Abbreviations

BSA (bovine serum albumin); ChIP (chromatin immunoprecipitation); DMEM (Dulbecco's modified Eagle's Medium); FITC (fluorescein isothiocyanate); HIF-1 (Hypoxia-inducible factor 1); HRE (Hypoxia reaction element); HEK 293 (human embryonic kidney 293); MOI (multiplicity of infection); NDRG (N-myc downstream-regulated gene); PBS (phosphate buffered saline); PCR (polymerase chain reaction); PARP (poly ADP-ribose polymerase); PI (propidium iodide; kDa, kilodalton).

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