# Oxygen-dependent regulation of NDRG1 in human glioblastoma cells *in vitro* and *in vivo*

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**Abstract.** NDRG1 is a member of the N-myc downregulated gene (NDRG) family. Its induction occurs via diverse physiological and pathological conditions (hypoxia, cellular differentiation, heavy metal, N-myc, neoplasia) which modulate NDRG1 transcription, mRNA stability and translation. Hypoxia, among other factors, induces NDRG1 expression and plays an important role in its regulation of expression. To date, the complete detailed function of this protein in humans remains unknown. Hypoxia represents a common feature of solid tumors. In our study, differences in NDRG1 expression between different WHO grades of astrocytic tumors were comparatively examined *in vivo* in human low-grade astrocytoma (WHO grade 2) and glioblastoma (WHO grade 4) at both the protein and mRNA level by Western blot analysis and semi-quantitative RT-PCR, respectively. Furthermore, the same proteins were determined *in vitro* in U373, U251 and GaMG human glioblastoma cells using the same methods. HIF-1α protein and mRNA regulation under hypoxia was also determined *in vitro* in U251, U373 and GaMG cells. This regulation was shown at the same levels *in vivo* in human low-grade astrocytoma (WHO grade 2) and glioblastoma which showed a higher NDRG1 overexpression level in glioblastoma than in low-grade astrocytoma. siRNA- and iodoacetate (IAA)-mediated downregulation of NDRG1 mRNA and protein expression *in vitro* in human glioblastoma cell lines showed a nearly complete inhibition of NDRG1 expression when compared to the results obtained due to the inhibitory role of glycolysis inhibitor IAA. Hypoxia responsive elements (HREs) bound by nuclear HIF-1α in human glioblastoma cells *in vitro* under different oxygenation conditions and the clearly enhanced binding of nuclear extracts from glioblastoma cell samples exposed to extreme hypoxic conditions confirmed the HIF-1 Western blotting results. Due to its clear regulatory behavior under hypoxic condition in human tumor cells, NDRG1 represents an additional diagnostic marker for brain tumor detection, due to the role of hypoxia in regulating this gene, and it can represent a potential target for tumor treatment in human glioblastoma.

## Introduction

Hypoxia is a factor which plays an important role within the solid tumor microenvironment. It significantly influences the behavior of tumor cells via activation of genes encoding proteins involved in adaptation to hypoxic stress. Also, it plays an important role in tumor progression. It is selective for cells with enhanced glycolytic activity, causing production of large amounts of lactic acid, one of the most common features of tumor cells (Warburg effect). It has attracted much attention owing to its significant correlation with tumor progression, results of treatment and the overall prognosis of disease (1,2). Tumor tissue growth requires a sufficient supply of oxygen and nutrients. However, proliferating tumor cells quickly grow beyond the diffusion distance of oxygen from the nearest blood vessel (100-150 $\mu$m), leading to a tumor vasculature which is highly irregular and tortuous, with arteriovenous shunts, blind ends and incomplete endothelial linings. This has an effect on blood flow, which is less efficient than in normal

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tissues (3,4). Tumor expansion is characterized by rapid growth of cancer cells when tumors establish themselves in host tissues of organs. Rapid growth of tumors is accompanied by alterations in the cancer cell microenvironment, caused by an inability of local vasculature to supply enough oxygen and nutrients to the rapidly dividing tumor cells (5). This makes hypoxia one common feature of solid tumors (6). NDRG1 is a member of the N-myc downregulated gene (NDRG) family. *NDRG1* (also known as *Drg1*, *RTP*, *Rit42*, *PROXY-1* and *Cap43*) was identified as a gene upregulated during cellular differentiation (7-9). It is induced by hypoxia (10,11). HIF-1 as a transcription factor plays a major role in the regulation of hypoxia-responsive genes (12-14) and is also involved in the transcriptional regulation of the *NDRG1* gene (15,16) together with other transcription factors. Thus, it is important to investigate the expression of NDRG1 protein in human cancer (17). This gene is necessary for p53-mediated apoptosis and is regulated by PTEN (phosphatase and tensin homologue). In several types of cancers, it was suggested to be a tumor suppressor gene (18).

In this study, we showed for the first time that NDRG1 overexpression is tumor-type associated. In brain tumors, hypoxia plays an important role in its regulation in human astrocytic brain tumor cells both *in vitro* and *in vivo*. Further functional analysis showed the hypoxia-induced binding effect on HRE by HIF-1α within the nuclear portion which manifests its role as putative transcriptional enhancer for NDRG1. Experimental inhibition of NDRG1 expression in four glioblastoma cell lines *in vitro* by either siRNA technology or interference with tumor cell glycolysis might be a potential therapeutic tool in regulating the expression of this gene in glioblastoma. Further, *in vivo*, a clear association between brain tumor grade, hypoxia and overexpression of NDRG1 was observed at the mRNA as well as the protein level.

## Materials and methods

*Cell culture, hypoxia treatment and transfection of glioblastoma cell lines*. Early-passage U373, U251 and U87-MG human malignant glioblastoma from the American Type Culture Collection (ATCC, Rockville, MD, USA) and GaMG, a cell line established from a patient with glioblastoma multiforme (Gade Institut of the University Bergen, Norway) (19), were grown on glass Petri dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), non-essential amino acids, penicillin (100 IU/ml)/streptomycin (100 μg/ml) and 2 mM L-glutamine. Cells were treated with *in vitro* hypoxia for 1, 6, or 24 h at 5, 1 or 0.1% $O_2$ as indicated in a Ruskinn Invivo$_2$ hypoxic workstation (Cincinnatti, OH, USA) as previously described (20,21). For reoxygenation experiments, dishes were returned to the incubator following 24 h of hypoxia. Plasmid DNA for transfections was prepared with the Endofree Plasmid Maxi Kit (Qiagen, Hilden, Germany). Stable as well as transient transfections were performed using Fugene6 Transfection Reagent (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. The pSuper constructs transfected into U373, U251, U87-MG and GaMG glioblastoma cells lines were incubated for 8 h under standard normoxic conditions (21% $O_2$, 5% $CO_2$) post transfection with further incubation under hypoxic conditions (0.1%) for 24 h.

*Preparation of nuclear extracts, whole-cell lysates and immunoblotting*. From plates containing 5x10$^7$ cells, nuclear extracts were prepared according to previous protocols (22) with minor modifications. Aliquots containing nuclear extracts were stored in aliquots at -80˚C. Whole-cell lysates were prepared with 0.1 ml RIPA buffer (1X TBS, 1% Nonidet P-40 (Amresco, Vienna, Austria), 0.5% sodium deoxycholate, 0.1% SDS, protease inhibitors pepstatin A (1.4 μM), aprotinin (0.15 μM) and leupeptin (2.3 μM) and 100 μM PMSF (all from Sigma, St. Louis, MO, USA). To inhibit protein dephosphorylation, phosphatase inhibitor mix (Sigma) was added. Using a syringe fitted with a 21-gauge needle to shear DNA, lysates were transferred to a pre-chilled microcentrifuge tube, followed by a 30-min incubation on ice. Subsequently, cell lysates were cleared by centrifugation at 15,000 x g for 12 min at 4˚C. Whole-cell lysates (20 μg) were separated onto SDS-8% polyacrylamide gel electrophoresis and transferred to a 0.45-μm nitrocellulose membrane (Protran BA 85; Schleicher & Schuell, Dassel, Germany). Nonspecific binding was blocked by 5% nonfat milk powder in TBS overnight at 4˚C followed by incubation with the NDRG1 primary antibody (ab8448, Abcam, Cambridge, UK), diluted 1:1000 in 2.5% nonfat milk powder in TBS for 1 h at room temperature or followed by incubation with the M75 mouse monoclonal antibody against CA IX (Bayer Healthcare Co., diluted 1:7200) or with HIF-1α monoclonal antibody (610959, BD Biosciences, dilution 1:500). Blots were washed twice in TBS/0.05% Tween-20 (Bio-Rad, Munich, Germany) and subsequently three times in TBS for 5-10 min each. The secondary antibody goat anti-rabbit-HRP (stock solution 400 μg/ml; DakoCytomation, Denmark) was incubated at a dilution of 1:2000 for one additional hour at room temperature followed by five wash steps as described above. Antibody detection and development was as previously described (23).

*Knock-down of endogenous NDRG1 by small interfering RNA and IAA*. Human glioblastoma cell lines U373, U251, U87-MG and GaMG were grown to 50% confluence on 10-cm plates in complete medium (RPMI-1640 medium or DMEM depending on the cell line) supplemented with 10% fetal calf serum, 100 μg/ml streptomycin and 100 units/ml penicillin. The small interfering RNA (siRNA) vector pSuper was a gift from R. Agami (The Netherlands Cancer Institute, Amsterdam, The Netherlands). The pSuper-NDRG1 vectors were designed as described previously (17,24). To establish pSuper-NDRG1, two sequences were selected from the human NDRG1 cDNA (5'-GCATTATTGGCATGGGAAC-3' (positions 398-416) and 5'-ATGCAGAGTAACGTGGAAG-3' (positions 601-619, relative to the start codon). All constructs were confirmed by sequencing. Also, cells were transfected with the empty vector pSuper (OligoEngine, Seattle, WA) and pSuper-NDRG1. Detection of reduced NDRG1 mRNA and protein levels was performed by Northern blotting as well as immunoblotting applying the goat polyclonal anti-NDRG1 antibody (Abcam ab 21727). Iodoacetate (IAA; 50 μM) was used as a glycolysis inhibitor and was added to the growth medium shortly before the respective hypoxia treatment.

*HRE binding assay*. The human NDRG1 promoter region sequence was assembled using public databases. The NDRG1 promoter nucleotide sequence was analysed using Genomatix Suite 3.0 software (25) (Genomatix, Munich, Germany) to find putative hypoxia responsive elements (HREs) within this promoter region. The analysis revealed the presence of three HREs located -1122, -1376 and -7503 bp upstream of the NDRG1 promoter region.

To determine the involvement of HIF-1α in NDRG1 regulation by nuclear HIF-1α binding on this putative binding site within the promoter region, this binding activity was determined via ELISA-based TransBinding Kit (TransBinding HIF Assay Kit, EK1020-PA, Biocat, Heidelberg, Germany) according to the manufacturer's instructions. Briefly, 20 μg of nuclear extracts obtained from glioblastoma cell lines (U373, U251, U87-MG and GaMG) cultured under hypoxic conditions of various duration and severity were assayed for their binding capacity to the HRE (in triplicate per experimental condition).

*Determination of NDRG1 mRNA expression in vitro in glioblastoma cell lines and human brain tissues in vivo by semi-quantitative RT-PCR*. Tissue biopsies were obtained surgically from two groups of patients: 15 patients with glioblastoma and 15 patients with low-grade astrocytoma (WHO grade 2). Samples were immediately stored at -80˚C in liquid nitrogen before further analysis. To compare the expression of the individual genes examined, RT-PCR was performed using primers designed using published information on ß-actin and HIF-1α mRNA sequences in GenBank (accession nos. NM_001101 for ß-actin, NM_001530.2 for HIF-1α and NM_006096 for NDRG1, respectively). An aliquot of 1-5 μg of total mRNA from human gliblastoma and astrocytoma tissue or glioblastoma cell lines was transcribed at 42˚C for 1 h in a 20-μl reaction mixture using 200 U RevertAid™ M-MuLV Reverse Transcriptase (RT), oligo(dT)18 primer and 40 U Ribonuclease inhibitor (all from Fermentas, Ontario, Canada). For PCR reactions, primers were designed in flanking exons with Primer3 software (available online http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) to produce a 593-bp amplification product of NDRG1: forward primer (F1) 5'-CTCTGTTCACGTCACGCTGT-3' and reverse primer (R1) 5'-CTCCACCATCTCAGGGTTGT-3'. To produce a 668-bp amplification product of ß-actin, the forward primer (F1) was 5'-CGTGCGTGACATTAAGG AGA'-3 (nucleotides 697-716) and reverse primer (R1) 5'-CACCTTCACCGTTCCAGTTT'-3 (nucleotides 1345-1364) and to produce a 233-bp amplification product of HIF-1α, the forward primer (F1) was 5'-TTACAGCAGCCAGA CGATCA-3' (nucleotides 2516–2535) and the reverse primer (R1) 5'-CCCTGCAGTAGGTTTCTGCT-3' (nucleo-ides 2729–2748). PCR was performed, and the PCR products were separated on agarose gels as previously described (26).

*Determination of the in vitro NDRG1 mRNA expression level after siRNA and IAA application via Northern blot analysis*. The yield of total RNA from ~2.5x10$^6$ cells was ~25 μg. It was isolated from cultured cells by direct lysis into guanidine thiocyanate. The integrity of total RNA was confirmed by testing 5 μg of each sample on an agarose gel. Total RNA (20 μg) was separated via electrophoresis on a 1% agarose gel containing 5.4% formaldehyde, transferred to nylon membranes (Hybond-N; Amersham Pharmacia, Braunschweig, Germany) by capillary blotting, and fixed with UV irradiation by a Biolink Crosslinker (Vilber Lourmat, France). The bound probe was removed during multiple hybridizations by incubating the membranes twice for 5 min in 0.1 M standard saline. In order to analyze the expression under *in vitro* hypoxia and reoxygenation in the human malignant glioma, the glioma cell lines U87-MG, U251, U373 and GaMG were treated in a similar way as described previously (27). Treatment with 100 μM DFO under aerobic conditions served as a positive control for hypoxia, and 50 μM IAA served as a glycolysis inhibitor. Probe labeling with $\alpha$-$^{32}$P-dCTP (Amersham) was accomplished via a radioactive labeling fit (Invitrogen); radioactive probe hybridization was conducted using Expresshyb Hybridization Solution (Clontech, Palo Alto, CA, USA) purchased from Amersham Pharmacia. All hybridization and the post hybridization washing steps were conducted as instructed by the supplier. NDRG1 cDNA probe was applied as previously described (17). Reactive bands were detected by X-ray film (Kodak, Rochester, NY, USA) via autoradiography at -80˚C. Film development was carried out with Kodak X–Omat 2000 processor after 3-14 days of exposure. The 18 S RNA band (1.9 kb) was used as a loading control for Northern blot experiments.

*Densitometric evaluation*. Densitometric evaluation of signal strengths in Western blotting or in semi-quantitative RT-PCR was performed with 1D Kodak Image Analysis Software. The amount of DNA or proteins gave signals that were measured in Kodak light units (KLU) and divided by the corresponding signals of the loading control ß-tubulin and ß-actin for Western blotting and semi-quantitative RT-PCR or 18 S RNA band for Northern blotting as previously described (21-23,26,27).

## Results

*Hypoxia leads to induction of nuclear HIF-1α in human glioblastoma cell lines in vitro*. Malignant glioma cell lines consistently displayed moderate (U251, U373, GaMG) to strong (U87-MG) HIF-1α protein expression under normoxic conditions (Fig. 1A-D). Incubation under severe hypoxic conditions (0.1% $O_2$) resulted in an early increase in HIF-1α protein expression after 1 h, which was most prominent in U251 and GaMG cells (Fig. 1B and C) and absent in U87-MG (Fig. 1D) due to high normoxic expression. HIF-1α protein expression nearly disappeared within 24-48 h of reoxygenation in U251, U373 and GaMG cells (Fig. 1A-C). An increase in $O_2$ concentration (1% $O_2$) resulted in an increase in HIF-1α protein expression after 1 h, which was most prominent in U251, GaMG and U373 cells (Fig. 1A-C) and absent in U87-MG (Fig. 1D) due to high normoxic expression. HIF-1α protein expression decreased but kept stable and did not disappear during 24 h of reoxygenation in U251, U373 and GaMG cells. An increase in $O_2$ concentration (5% $O_2$) resulted as above in an early increase in HIF-1α

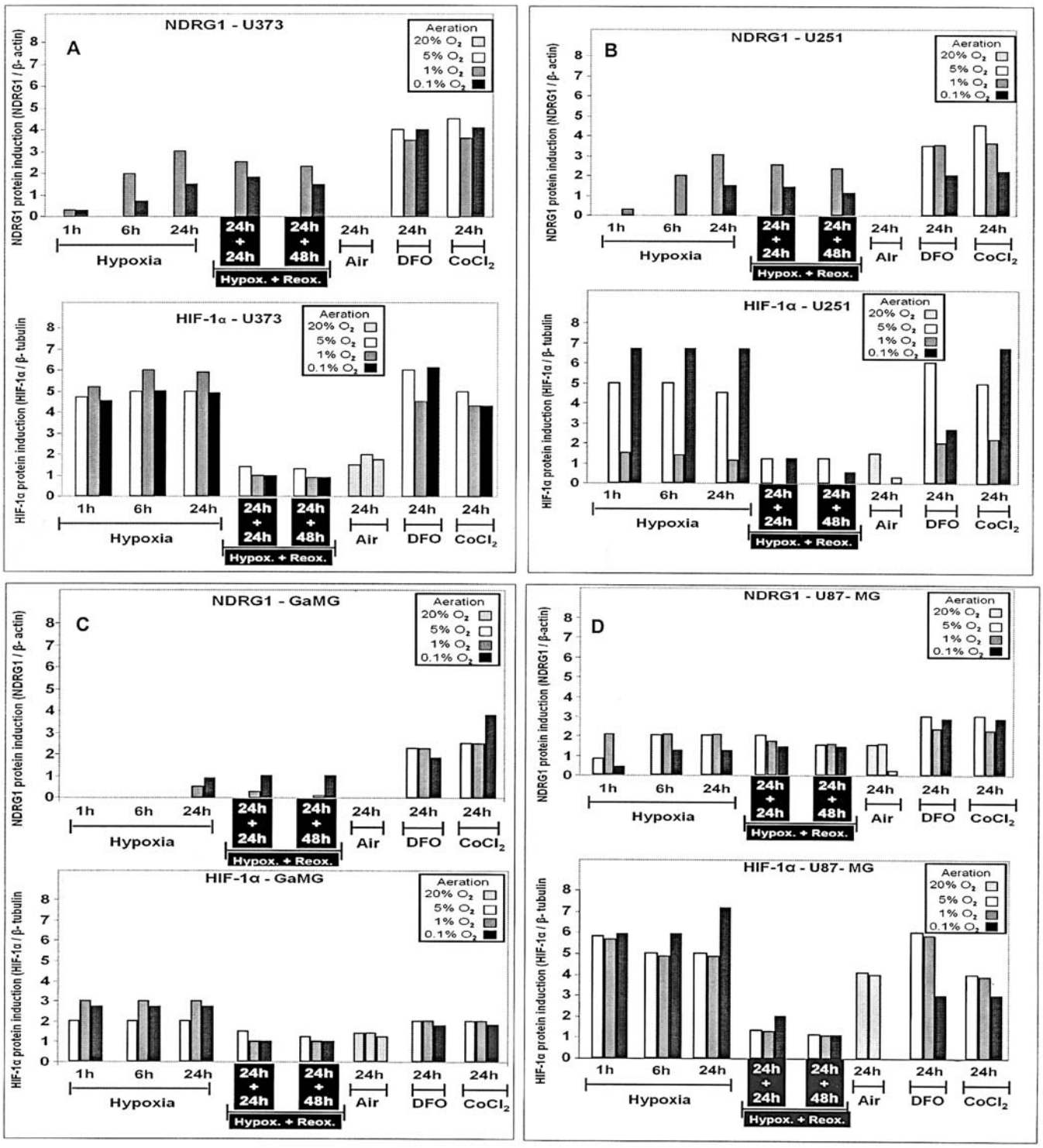


Figure 1. Comparative Western blot analysis of NDRG1 protein expression under *in vitro* hypoxia and reoxygenation in whole-cell lysates and HIF-1α in nuclear extracts of human malignant glioma (A) U373, (B) U251, (C) GaMG and (D) U87-MG cell lines.

protein expression after 1 h, which was present in U251, GaMG and U373 cells (Fig. 1A-C) and absent in U87-MG cells (Fig. 1D) due to high HIF-1α protein expression level under normoxic conditions.

*NDRG1 protein expression under different oxygenation conditions in human glioblastoma in vitro*. NDRG1 was induced and expressed in human glioblastoma cells, *in vitro*, in a different, cell-dependent manner as a consequence to alternating $O_2$ concentrations. While under moderate hypoxic conditions (5% $O_2$) NDRG1 was only expressed in U87-MG cells (Fig. 1D) with a minimal degradative effect of reoxygenation. NDRG1 expression was increasing at 1% $O_2$, where the strongest degree of NDRG1 expression was displayed at this $O_2$ concentration. Due to a decrease in $O_2$ concentration of the tumor environment, NDRG1 expression

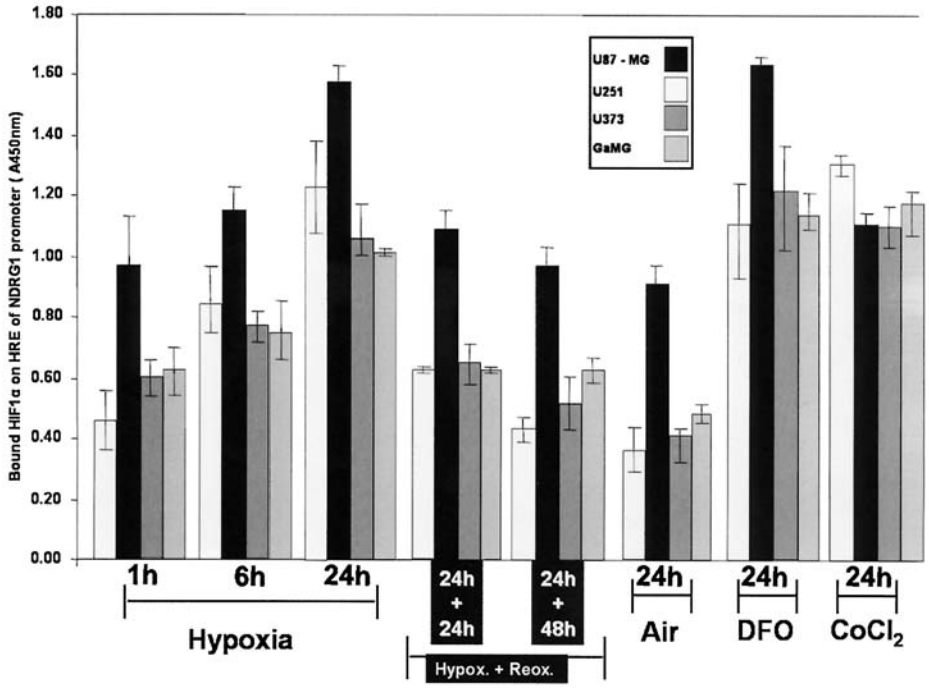


Figure 2. Determination of HIF-1α binding on HREs of the NDRG1 promoter region via ELISA.

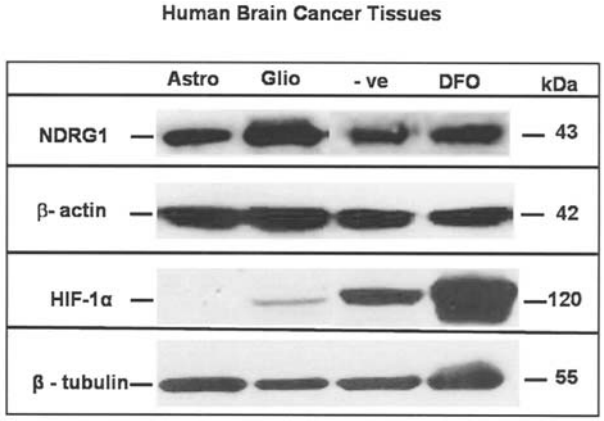


Figure 3. Expression of NDRG1 protein in human brain tissue *in vivo*. NDRG1 expression was three times higher in glioblastoma (Glio) when compared to low-grade astrocytoma (Astro). HIF-1α was weakly expressed only in glioblastoma. -ve, negative control.

was present in U373, U251 and GaMG cells (Fig. 1A-C) with the lowest expression rate in GaMG and with a stability over 48 h reoxygenation after 24 h hypoxia. During reoxygenation NDRG1 was relatively stable in all 4 tumor cell lines with the lowest expression in GaMG cells. Under extreme hypoxic conditions, a high expression level of NDRG1 was exhibited with a comparative high expression level during reoxygenation. NDRG1 increased in GaMG and U373 cells after 24 h at 0.1% $O_2$, as shown in the Western blot analysis (Fig. 1A-D) with a weak stability over 48 h reoxygenation after 24 h hypoxia. All cell lines did not show NDRG1 expression at 5% $O_2$ except in U87-MG cells, where there was always a strong degree of NDRG1 expression present regardless of oxygenation conditions.

With the exception of U87-MG cells, where NDRG1 was strongly expressed independently of $O_2$ concentration, no expression of NDRG1 was displayed in a normoxic environment in all the other cell lines and at 5% $O_2$. A high degree of NDRG1 expression, with a maximum at 0.1% and relative stability during reoxygenation, emerged at 1-0.1% $O_2$. HIF-1α was moderately to strongly expressed after 1 h at 0.1% $O_2$, and stable up to 48 h reoxygenation after 24 h at 0.1% $O_2$.

*Hypoxia responsive element binding by nuclear HIF-1α in human glioblastoma cells in vitro under different oxygenation conditions*. Oxygen- and time-dependent elevation of nuclear HIF-1α binding on HRE was displayed. Relative stability upon reoxygenation for 24 and 48 h after 24 h hypoxia was shown (Fig. 2). The strongest binding capacity was in U87-MG, followed by GaMG and U373 cells. The weakest HRE binding occurred in U251 cells, reflecting the lowest level of activated nuclear HIF-1α in this cell line under the conditions examined. Nuclear extracts from glioblastoma cells treated with 100 $\mu$M DFO and 50 $\mu$M $CoCl_2$ served as positive controls.

*Regulation of NDRG1 and HIF-1α protein expression in vivo in human glioblastoma and low-grade astrocytoma tumor tissue*. Western blot analysis of representative tumor samples revealed an elevated expression pattern of NDRG1 protein expression in glioblastoma compared to low-grade astrocytoma (Fig. 3). HIF-1α protein was only rather weakly expressed in glioblastoma.

*NDRG1 mRNA expression in vitro and in vivo*. Semi-quantitative RT-PCR showed a clearer $O_2$-dependent NDRG1 expession in U87-MG than in U373 cells under hypoxia and reoxygenation conditions examined. Bar graphs (Fig. 4)

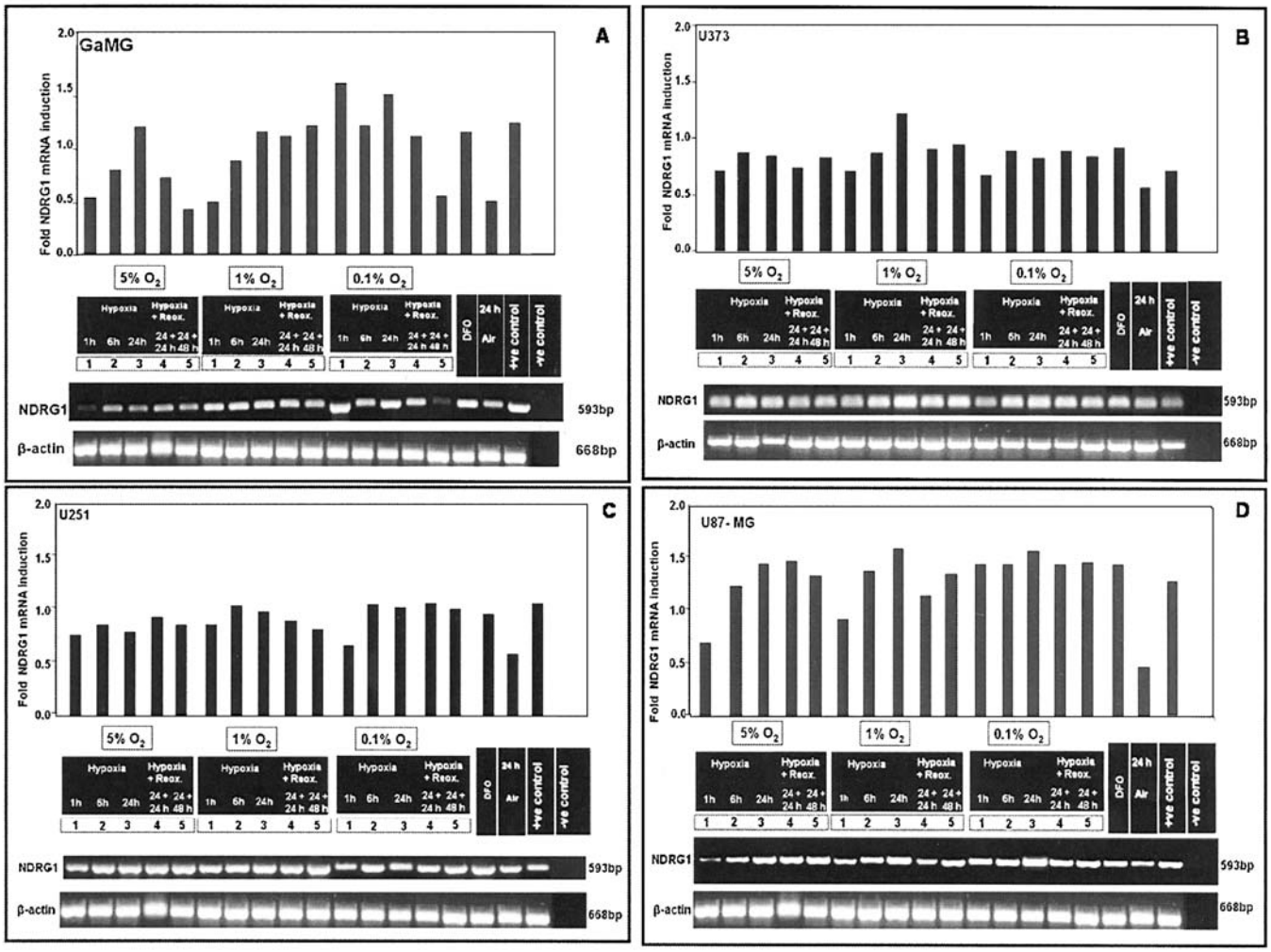


Figure 4. NDRG1 mRNA expression in human glioblastoma (A) GaMG, (B) U373, (C) U251 and (D) U87-MG cell lines after *in vitro* application of different hypoxic conditions.

show the band intensities after densitometric evaluation and normalization to ß-actin expression. *In vitro*, mRNA expression of HIF-1α was similar to mRNA of all cell lines examined (results not shown).

*In vivo*, mRNA expression of HIF-1α was similar in tumor specimens from patients with low-grade astrocytoma or glioblastoma (results not shown). A tumor-grade association with NDRG1 mRNA expression was exhibited *in vivo*. No increase in NDRG1 expression was shown in low-grade astrocytoma while an increase of at least 2-fold in NDRG1 expression was shown in 10/15 patients in glioblastoma (Fig. 5).

*siRNA- and glycolysis inhibitor IAA-mediated down-ation of NDRG1 protein expression in vitro*. Under severe hypoxia (0.1% $O_2$) NDRG1 was strongly inhibited after transfection with anti-NDRG1 siRNA to an expression level comparable to that obtained after inhibition via IAA (Fig. 6).

*siRNA- and IAA-mediated downregulation of NDRG1 mRNA expression in vitro in human glioblastoma cell lines*. In all four glioblastoma cell lines, expression of NDRG1 was either reduced or inhibited upon application of one of the two siRNA constructs, each separately, or when 50 $\mu$M glycolysis inhibitor IAA was applied, *in vitro*, for 24 h with 0.1% hypoxia (Fig. 6). At the mRNA level there was a complete inhibition of NDRG1 mRNA expression when 50 $\mu$M was applied to glioblastoma cell lines exposed to 0.1% $O_2$.

## Discussion

NDRG1 protein expression has been described to be present in normal brain or brain tumor tissue (28). NDRG1 was suggested to be a prognostic marker for hypoxic regions within a tumor mass because of its stability as a protein (2,29,30) and because it is highly expressed in malignant tumor tissues compared to normal tissue of the same origin (29-31).

The differences between different types of brain tumors and brain tumor cell lines regarding the NDRG1 response to hypoxia and reoxygenation which was observed here can, in part, be explained by the genetic background of the cell lines investigated which may indirectly influence their degree of expression (Table I). For example, p53 downregulates NDRG1 expression (32), and an increase in NDRG1 expression in malignant brain tumors could be related to the loss of expression control due to a p53 mutation. Mutations

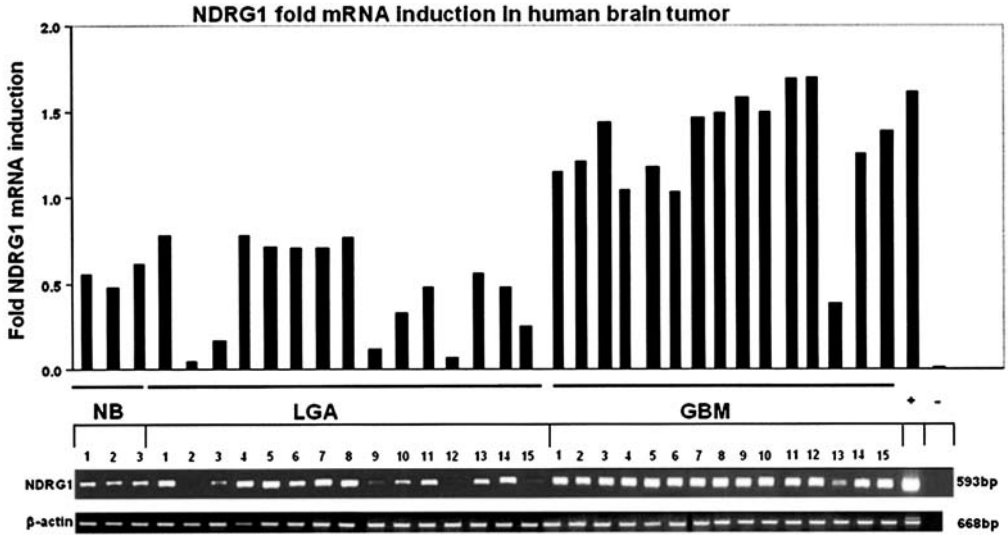


Figure 5. Expression of NDRG1 mRNA in human brain cancer tissue *in vivo*. LGA, low-grade astrocytoma; GBM, glioblastoma.

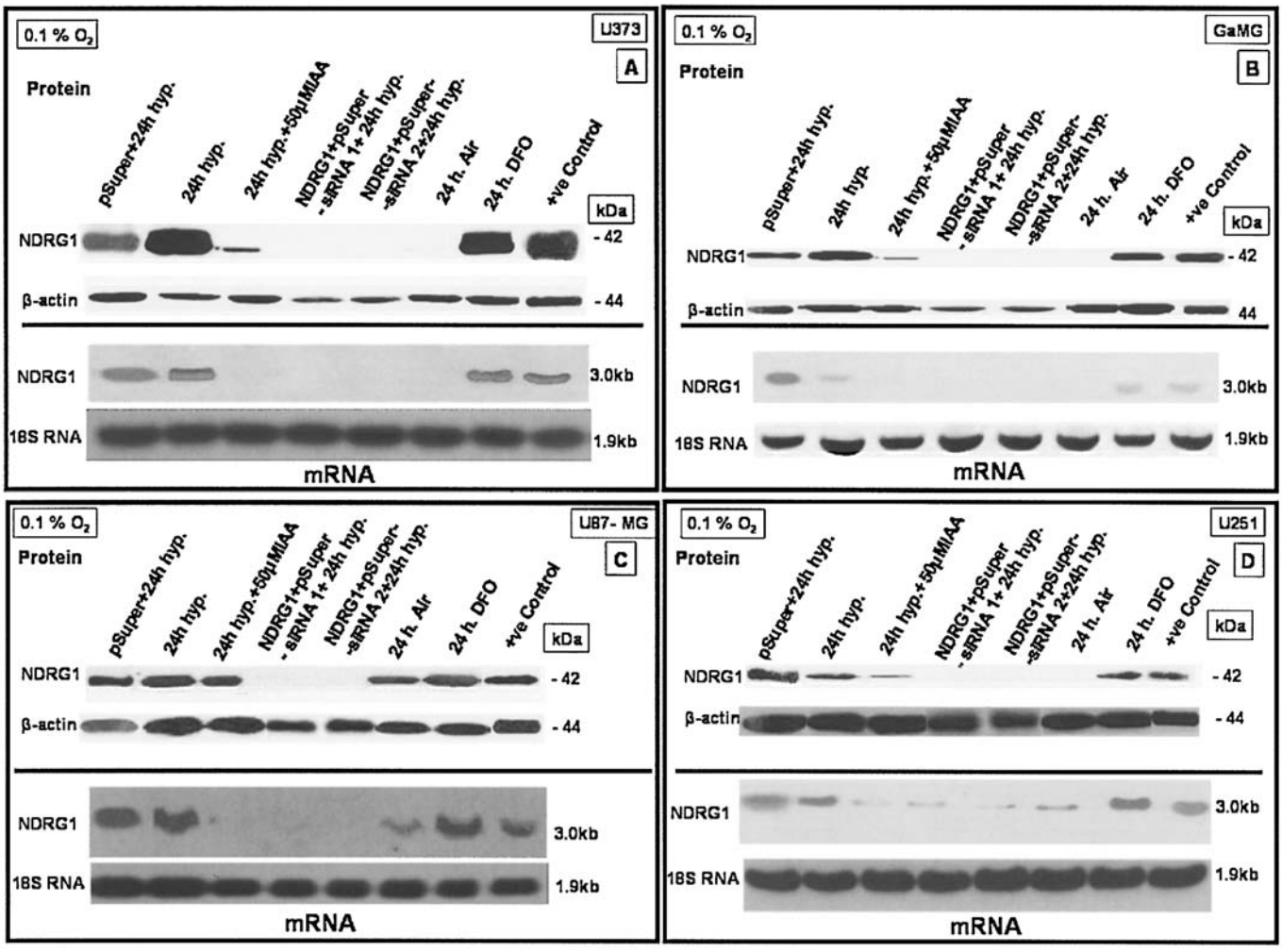


Figure 6. Inhibition of NDRG1 protein and mRNA expression in 4 different human glioblastoma (A) U373, (B) GaMG, (C) U87-MG and (D) U261 cell lines *in vitro* via siRNA- and IAA-mediated interference by glycolysis.

of the p53 gene are the most frequent genetic alterations in gliomas (33). We must take into consideration the other hypothesis which states that hypoxic regulation of NDRG1 can occur via hypoxia-inducible factor 1 (HIF-1)-dependent as well as HIF-1-independent pathways; its expression is therefore not limited to tissues expressing HIF-1 (15),

Table I. Overview of known genomic mutations in human malignant glioma cell lines and brain tumor tissues with potential effects on the hypoxia-regulated expression of NDRG1 or HIF-1α.

| Tumor cell line/tumor tissue | Genetic mutations (+/-) | Reference | NDRG1 expression normoxia | NDRG1 expression hypoxia |
|---|---|---|---|---|
| GaMG malignant glioma cells | No mutation | (44) (50) | (-/+) | + |
| U251 malignant glioma cells | Mutated p53 | (45) | (-/+) | + |
| | P14ARE/p16 deletion | (46) | | |
| | No mutations associated with samples examined | | | |
| U373-MG malignant glioma cells | Apoptosis resistant mutant p53 | (44) (50) | (-/+) | + |
| | Peroxisome proliferator-activated receptor-γ | (51) | | |
| | PTEN mutation | | | |
| U87-MG malignant glioma cells | P14ARE/p16 deletion | (45) | (-/+) | + |
| | PTEN mutation | (50) | | |
| Normal brain tissue | No mutation known in healthy brain | - | (-/+) | + |
| Astrocytoma brain tumor tissue | Hypermethylation of p14ARF | (33) | (-/+) | + |
| | Hypermethylation of O6 methylguanine-DNA methyltransferase genes | (33) | | |
| Glioblastoma brain tumor tissue | Mutated p53 | (3) | (-/+) | + |

(-/+), no change; -, decrease; +, increase.

although it is well known that NDRG1 is under control of a vHL tumor suppressor gene, and the loss of this control may result in the upregulation of HIF-1α and NDRG1 in affected cells.

Although it has been shown that NDRG1 is expressed at the necessary minimal level in the brain (30), we observed, in our study, a brain tumor-type-dependent increase in NDRG1 mRNA expression level. *In vivo*, glioblastoma displayed a higher level of NDRG1 than low-grade astrocytoma both at the protein and mRNA level. NDRG1 protein and NDRG1 mRNA were generally up-regulated in response to prolonged moderate (1% $O_2$) to severe (0.1% $O_2$) *in vitro* hypoxia, although the effect was undetectable at the protein level in one cell line with a strong constitutive, normoxic NDRG1 expression.

The observed differences in expression between NDRG1 protein and corresponding NDRG1 mRNA may be explained by different post-transcriptional processing (31) or a post-transcriptional regulation of NDRG1 mRNA by NDRG1 protein levels (34-36). High basal levels of HIF-1α and NDRG1 may be an adaptive response in cells with increased metabolic demands or altered signal transduction pathways unrelated to hypoxia but which govern HIF-1 cellular activity.

In this study, the overexpression of NDRG1 in patient tumor samples was not directly proven to be caused specifically by hypoxia. Our *in vitro* results (pattern of hypoxia-mimetic $CoCl_2$- and DFO-induced upregulation of HIF-1α and NDRG1) suggest that HIF-1α and NDRG1 are expressed in a hypoxia-related fashion specifically in GBM. These results are in concordance with previous findings describing HIF-1-dependent regulation of NDRG1 (13,27) and are supported by previous immunohistochemical studies describing a predominant expression pattern of HIF-1α protein consistent with chronic hypoxia (37,38), in addition to our results on the hypoxia-induced gene expression in similar tumor tissues (21). NDRG1 overexpression can represent an indicator of poor prognosis of patients with brain tumors although an independent prognostic role has not been established in the limited patient cohort now studied. Hypoxia-induced HIF-1α plays an active role by regulating NDRG1 expression together with Egr-1 and p53 (39,40).

Due to HRE positioning in the distal promoter region, HIF-1 is acting as a transcriptional enhancer, as hypothesized in a similar gene expression model (41). Clear involvement of glucose availability in hypoxic HIF-1α and NDRG1 expression in malignant glioma cells was demonstrated since application of the glycolysis inhibitor iodoacetate led to a sharply reduced accumulation of HIF-1α via a translational or post-translational effect (27,38). Therefore, iodoacetate-induced interference with glycolysis in malignant gliomas may represent a therapeutic approach in targeting HIF-1α or NDRG1 in this tissue.

Induction of NDRG1 sequence-specific posttranscriptional gene silencing in different glioblastoma cell lines, *in vitro*, by RNA interference (42,43) resulted in a strong inhibitory activity of NDRG1 expression, both at the mRNA and protein level. This approach, when compared to glycolysis inhibition via IAA application, which has previously been shown to be HIF-1-inhibitory (44-49), can present a therapeutic strategy targeting hypoxia-induced NDRG1. However, the

success of such approaches still awaits the development of an efficient delivery system that can affect a large number of tumor cells.

In conclusion, the measurement of the activity of specific transcription factor HIF-1α in nuclear extracts via ELISA approaches can be an alternative method for the indirect rapid detection of tumor oxygenation status, particularly when the nuclear extract yield is not high enough for detection via Western blotting. Also, the high stability of NDRG1 protein in human glioblastoma cells *in vitro* depends to a certain degree on $O_2$ concentration and is tumor cell specific. Direct inhibition of NDRG1 via siRNA or indirect inhibition through interfering with the cancer cell glycoltic activities via application of IAA might be a potential therapeutic tool for regulating the expression of this gene in glioblastoma. Detailed understanding of how hypoxia regulates transcription of the *NDRG1* gene increase knowledge of the cellular responses of normal and cancer cells towards low oxygen tension.

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