

Inhibition of N-Myc down regulated gene 1 in *in vitro* cultured human glioblastoma cells

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as a potent tool for artificially modulating gene expression of N-Myc down regulated gene 1 (*NDRG1*) gene induced under different physiological conditions (Normoxia and hypoxia) modulating NDRG1 transcription, mRNA stability and translation.

METHODS: A cell line established from a patient with glioblastoma multiforme. Plasmid DNA for transfections was prepared with the Endofree Plasmid Maxi kit. From plates containing 5×10^7 cells, nuclear extracts were prepared according to previous protocols. The pSUPER-NDRG1 vectors were designed, two sequences were selected from the human NDRG1 cDNA (5'-GCATTATTGGCATGGGAAC-3' and 5'-ATGCAGAGTAACGTGGAAG-3'. reverse transcription polymerase chain reaction was performed using primers designed using published information on β -actin and hypoxia-inducible factor (HIF)-1 α mRNA sequences in GenBank. NDRG1 mRNA and protein level expression results under different conditions of hypoxia or reoxygenation were compared to aerobic control conditions using the Mann-Whitney *U* test. Reoxygenation values were also compared to the NDRG1 levels after 24 h of hypoxia ($P < 0.05$ was considered significant).

RESULTS: 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 bound by nuclear HIF-1 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.

CONCLUSION: NDRG1 represents an additional diagnostic marker for brain tumor detection, due to the role of hypoxia in regulating this gene, and it can

Abstract

AIM: To study short dsRNA oligonucleotides (siRNA)

represent a potential target for tumor treatment in human glioblastoma. The siRNA method can represent an elegant alternative to modulate the expression of the hypoxia induced *NDRG1* gene and can help to monitor the development of the cancer disease treatment outcome through monitoring the expression of this gene in the patients undergoing the different therapeutic treatment alternatives available nowadays.

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Key words: N-Myc down regulated gene 1; Short dsRNA oligonucleotides; Human cancer diseases; Brain cancer; Radiotherapy

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INTRODUCTION

RNA interference (RNAi) or short dsRNA oligonucleotides (siRNA) approach represents a powerful tool for drug target discovery and validation in eukaryotic cell culture approaches, *in vitro*, as well as within *in vivo* systems, such as animal disease models and human therapeutics. siRNA is a potent tool for artificially modulating gene expression through the introduction of short interfering RNAs. These molecular mechanisms that the siRNA approach is consisted of are occurring naturally as a gene regulatory mechanism having a number of advantages over other gene/antisense therapies including specificity of inhibition, potency, the small size of the molecules and the diminished risk. The systems for stable and regulated expression of these molecules emerged as well. Selective gene inhibition *via* siRNA occurs *via* two methods: (1) siRNA cytoplasmic delivery mimicking an active endogenous RNAi mechanism intermediate; and (2) nuclear delivery of gene expression cassettes that express a short hairpin RNA, which mimics the micro interfering RNA active intermediate of a different endogenous RNAi mechanism. In contrast, screens of many siRNA sequences can be accomplished rapidly using synthetic oligos. The activity of siRNA in the cytoplasm may lower the barrier and thereby accelerate the successful development of therapeutics based on targeted non-viral delivery systems. Under hypoxia, hypoxia-inducible factor (HIF)-1 α is involved in the transcriptional regulation of the N-Myc down-regulated gene 1 (*NDRG1*) gene^[1,2] (Figure 1) together with other transcription factors. In this relation it is of interest to

investigate the expression of NDRG1 protein in human cancer^[3]. This gene is necessary for P53-mediated apoptosis and regulated by phosphatase and tensin homologue. In several cancers, it was suggested to be a tumour suppressor gene^[4].

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, United States) and GaMG, a cell line established from a patient with glioblastoma multiforme (Gade Institut of the University Bergen, Norway)^[5], were grown on glass Petri dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, non-essential amino acids, penicillin (100 IU/mL)/streptomycin (100 μ g/mL) and 2 mmol/L L-glutamine. Cells were treated with *in vitro* hypoxia for 1, 6 or 24 h at 5%, 1% or 0.1% O₂ as indicated in a Ruskinn Invivo₂ hypoxic workstation (Cincinnati, OH, United States) as previously described^[6,7]. 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% O₂, 5% CO₂) 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 5×10^7 cells, nuclear extracts were prepared according to previous protocols^[8] 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 (1 \times TBS, 1% Nonidet P-40) (Amresco, Vienna, Austria), 0.5% sodium deoxycholate, 0.1% SDS, protease inhibitors pepstatin A (1.4 μ mol/L), aprotinin (0.15 μ mol/L) and leupeptin (2.3 μ mol/L), and 100 μ mol/L phenylmethylsulfonyl fluoride (all were obtained from Sigma, St. Louis, MO, United States). 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 30 min incubation on ice. Cell lysates were cleared by centrifugation at $15\,000 \times g$ for 12 min at 4 °C. Twenty microgram of whole-cell lysates were separated onto SDS 8% polyacrylamide gel electrophoresis and transferred to a 0.45 μ mol/L nitrocellulose membrane (Protran BA 85; Schleicher and Schuell, Das-

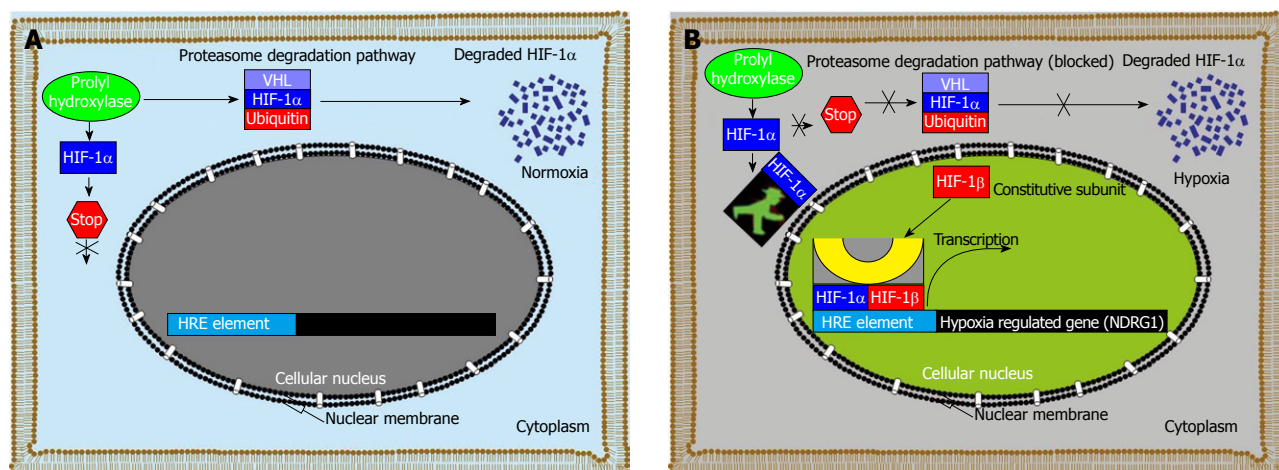


Figure 1 Hypoxia induced regulation of N-Myc down-regulated gene 1 in human brain cancer hypoxia-inducible factor-1 induced regulation of hypoxia induced N-Myc down-regulated gene 1 expression in human tumour cells. A: Under normoxic oxygenation conditions in the tumor cell microenvironment, hypoxia-inducible factor (HIF)-1 α is rapidly degraded via the von Hippel-Lindau tumour suppressor gene product (pVHL)-mediated ubiquitin proteasome pathway; B: When the tumor environment aeration conditions shifts from normoxic to hypoxic aeration conditions, HIF-1 α subunit becomes stable, translocates into the cellular nucleus and interacts with co-activators of which its transcription machinery is consisted such as p300/CBP to modulate the transcriptional activity of numerous hypoxia inducible genes, like N-Myc down-regulated gene 1 (NDRG1) in the case and about 61 other hypoxia induced genes^[28,40]. HRE: Hypoxia response element.

sel, 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, United Kingdom), diluted 1:1000 in 2.5% nonfat milk powder in TBS for 1h at room temperature or with followed by incubation with the M75 mouse monoclonal antibody against CA IX (Bayer Healthcare Co., diluted 1:7200) or with HIF-1 α 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 μ g/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 described in^[9].

Knock-down of endogenous NDRG1 by siRNA and iodoacetate

Human glioblastoma cell lines U373, U251, U87-MG and GaMG were grown up 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 μ g/mL streptomycin, and 100 units/mL penicillin). The pSUPER-NDRG1 vectors were designed as mentioned before^[10-17]. 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 to 619), relative to the start codon). All constructs were confirmed by sequencing. Transient transfection of siRNA constructs into the glioblastoma brain tumor cell lines exposed to extreme hypoxic aeration condition was *via* Fugene6 solution (Roche, Germany) according to the manufactur-

ers suggested instructions. Also, cells were transfected with the empty vector pSUPER (Oligo-Engine, Seattle) and pSUPER-NDRG1. Detection of reduced NDRG1 mRNA and protein levels was performed by Northern blotting and as well as immunoblotting, applying the Goat polyclonal anti-NDRG1 antibody (Abcam ab 21727). Iodoacetate (IAA; 50 μ mol/L) was used as a glycolysis inhibitor and was added to the growth medium shortly before the respective hypoxia treatment.

Tissue biopsies were obtained surgically from two groups of patients: 15 patients with glioblastoma multiforme (GBM) and 15 patients with low-grade astrocytoma (LGA; WHO grade 2). Samples were immediately frozen at -80 °C and stored in liquid nitrogen before further analysis. To compare the expression of the individual genes examined, reverse transcription polymerase chain reaction was performed using primers designed using published information on β -actin and HIF-1 α mRNA sequences in GenBank (accession numbers NM_001101 for β -actin, NM_001530.2 for HIF-1 α , and NM_006096 for NDRG1, respectively). An aliquot of (1-5) μ g of total mRNA from human glioblastoma and astrocytoma tissue or glioblastoma cell lines was transcribed at 42 °C for 1 h in a 20 μ L reaction mixture using 200 U RevertAidTM M-MuLV RT, oligo(dT)18 primer and 40 U Ribonuclease inhibitor (all from Fermentas, Ontario, Canada). For polymerase chain reaction (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, the forward primer (F1) was 5'-CTCTGTTTCACGTCACGCTGT-3' and the reverse primer (R1) 5'-CTCCACCATCTCAGGGTTGT-3'. To produce a 668 bp amplification product of β -actin, the forward primer (F1) was 5'-CGT-GCGTGACATTAAGGAGA-3' (nucleotides 697-716)

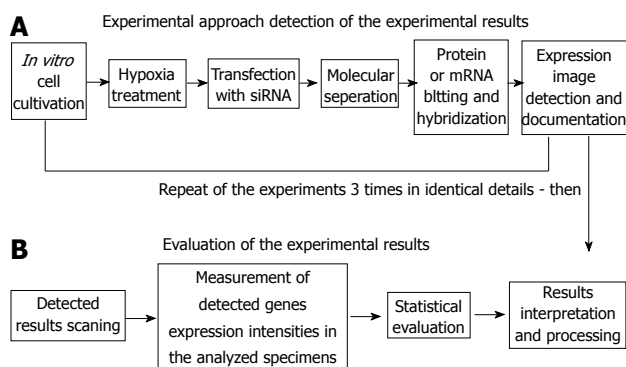


Figure 2 Detection and experimental monitoring of hypoxia induced N-Myc down-regulated gene 1 in specimens of human brain cancer. The experimental monitoring approach includes (A) experimental approach detection of the experimental results. Here, the tumor cell lines are first cultivated *in vitro* and subsequently transfected with the N-Myc down-regulated gene 1 (*NDRG1*) short dsRNA oligonucleotides (siRNA) construct and treated with fixed O_2 concentration in the hypoxia chamber followed by the specimens extraction, quantification, quality control and molecular separation of tumor cells specimens. Further protein or mRNA blotting and Hybridization with subsequent *NDRG1* expression image detection and documentation take place. The experimental approach with these different stages are repeated at least three times to have statistical significant results that are necessary for evaluation of the experimental results (B), where first the films with the detected results are scanned followed by the detection of the genes expression (which is in the case *NDRG1* gene and the house keeping genes or loading controls (β -actin and 18s RNA, respectively) measurement of intensities in the analyzed specimens, statistical analysis and evaluation of the obtained results.

and the reverse primer (R1) 5'-CACCTTCACCGTTC-CAGTTT-3' (nucleotides 1345-1364) and to produce an 233 bp amplification product of HIF-1 α , the forward primer (F1) was 5'-TTACAGCAGCCAGACGATCA-3' (nucleotides 2516-2535) and the reverse primer (R1) 5'-CCCTGCAGTAGGTTTCTGCT-3' (nucleotides 2729-2748). The PCR was performed and PCR products were separated on agarose gels as mentioned previously.

Visualisation, expression level evaluation and analysis

The data presented here are representative for 3 similar experiments. Densitometric evaluation of Northern blots was performed with 1D Kodak Image Analysis Software. Signals were measured in Kodak light units and divided by the corresponding signals of the house keeping gene β -actin or 18s RNA for the northern blot results. *NDRG1* mRNA and Protein level expression results under different conditions of hypoxia or reoxygenation were compared to aerobic control conditions using the Mann-Whitney *U* test. Reoxygenation values were also compared to the *NDRG1* levels after 24 h of hypoxia ($P < 0.05$ was considered significant). Further details are outlined in (Figure 2).

RESULTS

Hypoxia induced *NDRG1* mRNA in human glioblastoma detection via Western blotting

In all four glioblastoma cell lines examined including the U373 cell line (Figure 3, upper panel), expression of

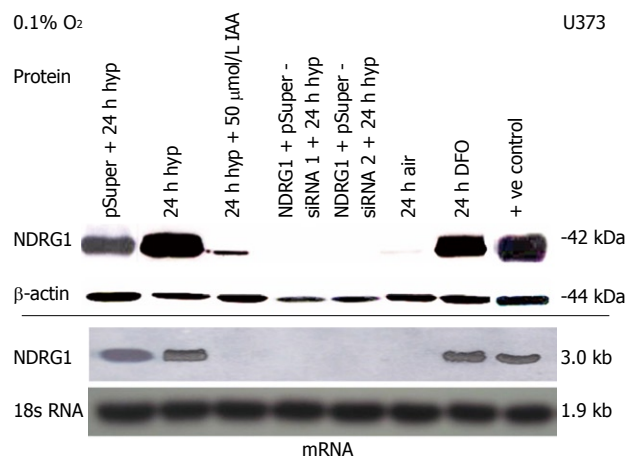


Figure 3 Inhibition of N-Myc down-regulated gene 1 protein and mRNA expression in U373 human glioblastoma cell line *in vitro* via short dsRNA oligonucleotides- and iodoacetate-mediated interference into human tumor cellular glycolysis process. Upper panel: Western blotting analysis result diagram related to the specific inhibition of hypoxia induced expression in human brain tumor cells (U373 Glioblastoma cell line as an example) on protein level via Western blotting analysis. Clear complete inhibition of N-Myc down-regulated gene 1 (*NDRG1*) induced by extreme hypoxic conditions in the tumor microenvironment ($0.1\% O_2$ for 24 h) was present upon transfection with the one of the two variants of *NDRG1* short dsRNA oligonucleotides (siRNA) construct applied in these experiments when compared to the nearly complete inhibition via inhibitive interaction with the tumor cell glycolysis pathway. β -actin served as a loading control. Figure shows one representative experiment out of three experiments; Lower panel: Northern blotting analysis displaying the specific inhibition of hypoxia induced *NDRG1* mRNA expression in human brain tumor cells (U373 Glioblastoma cell line as an example) on mRNA level. Complete inhibition of *NDRG1* induced by extreme hypoxic conditions in the tumor microenvironment ($0.1\% O_2$ for 24 h) was could be achieved upon transfection with the one of the two variants of *NDRG1* siRNA construct applied in these experiments. Inhibitive interaction into the glycolysis pathway due to the parallel treatment with $50 \mu\text{mol/L}$ with iodoacetate for 24 h showed a similar functional effect. The 18s RNA fragment with a molecular weight of 1.9 kb served as a loading control. This is one representative experiment out of three experiments.

NDRG1 was either reduced or inhibited upon application of one of the two siRNA constructs, each separately showing that the inhibition of *NDRG1* was 100% of its basal expression level under normoxic conditions and 97% from its expression level after hypoxic treatment ($0.1\% O_2$) for 24 h. Also, when $50 \mu\text{mol/L}$ of the glycolysis inhibitor IAA was applied, *in vitro*, for 24 h with 0.1% hypoxia, on protein level there was an inhibition of 85% of the expression level.

Hypoxia induced *NDRG1* mRNA in human glioblastoma detection via northern blotting

On mRNA level (Figure 3, lower panel), there was a complete inhibition of *NDRG1* mRNA expression also when $50 \mu\text{mol/L}$ glycolysis inhibitor IAA was applied, *in vitro*, for 24 h with 0.1% hypoxia treatment that was applied to all glioblastoma cell lines exposed to $0.1\% O_2$ examined *in vitro*, showing that the inhibition level on the mRNA level is more effective and not depending on the nature of the option applied (Chemical treatment with $50 \mu\text{mol/L}$ IAA or the transfection with either *NDRG1* siRNA construct) for *NDRG1* down regulation with final inhibition of expressed *NDRG1* in glioblastoma

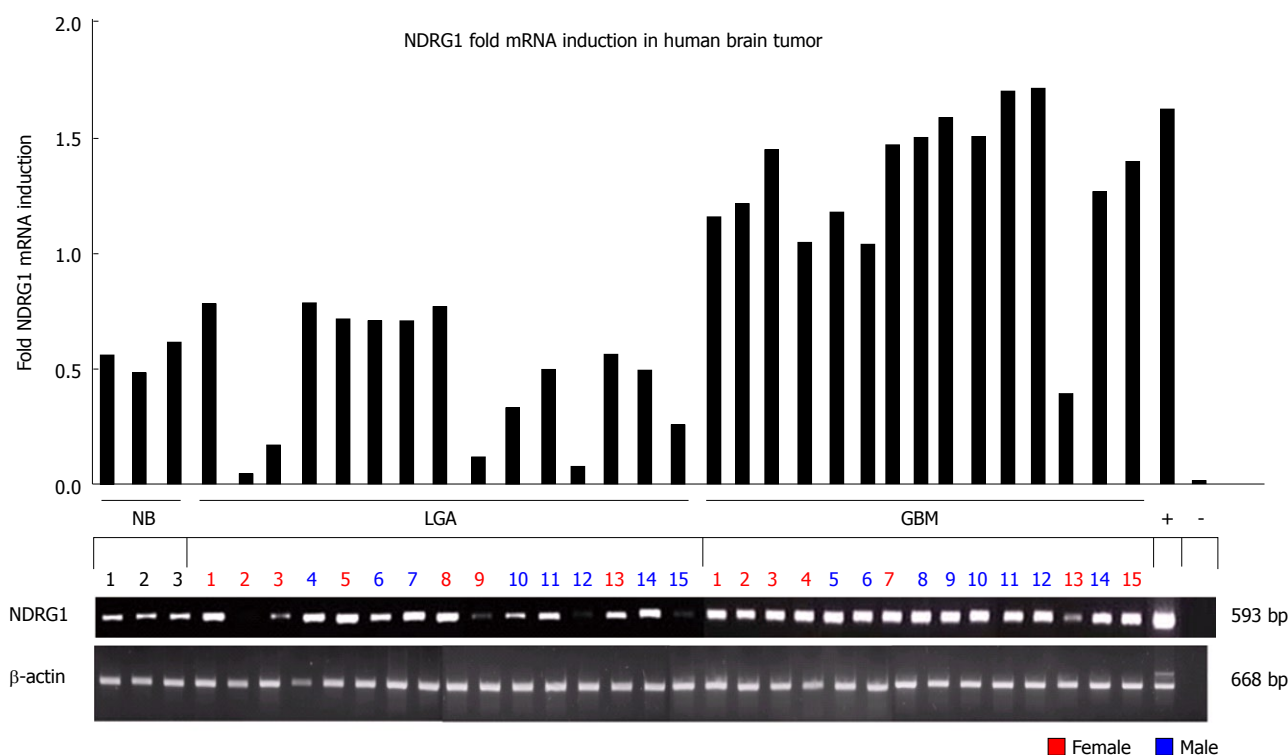


Figure 4 Expression of N-Myc down-regulated gene 1 mRNA in human brain cancer tissue *in vivo*. NDRG1: N-Myc down-regulated gene 1; NB: Nonneoplastic brain; LGA: Low-grade astrocytoma; GBM: Glioblastoma.

cells (Figure 3, lower panel).

Hypoxia induced NDRG1 expression in 2 groups of human brain tumour specimens

In vivo, mRNA expression of HIF-1 α 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 GBM in no patient with LGA as seen in (Figure 4).

DISCUSSION

NDRG1 protein expression has been described to be present in normal brain or brain tumor tissue^[11] as well as being an important gene that is playing an active role in the regulation of a broad spectrum of human cancer diseases like human gastric cancer, squamous cell carcinomas, breast cancer, human hepatocellular carcinoma, brain tumors and leukemia^[12-22]. NDRG1 was suggested to be a prognostic marker for hypoxic regions within a tumor mass because of its stability as a protein^[23-25] and because it is highly expressed in malignant tumor tissues compared to normal tissue of the same origin^[26].

During the different sets of experiments within our study, we observed, a brain tumor-type-dependent increase in NDRG1 mRNA expression level. NDRG1 protein and NDRG1 mRNA were generally up-regulated in response to prolonged to severe (0.1% O₂) *in vitro*

hypoxia, although the effect was undetectable at the protein level in one cell line with a strong constitutive, normoxic NDRG1 expression.

In a previous approach were human tumor specimens from patients suffering from LGA or GBM where analyzed showed that tumor specimens with GBM displayed a higher level of NDRG1 than low-grade astrocytoma both at the protein and mRNA level^[27-29].

As known by previous contributions that induction of NDRG1 sequence-specific posttranscriptional gene silencing in different glioblastoma cell lines, *in vitro*, by RNA interference^[30,31] resulted in a strong inhibitory activity of NDRG1 expression, both on mRNA and protein level. This approach when compared to glycolysis inhibition *via* IAA application, which has previously been shown to possess HIF-1-inhibitory functions^[32-36], or HIF-1 α and hypoxia induced genes like NDRG1 inhibitory functions can represent one innovative option with a high potential in the monitoring of human cancer disease like brain cancer as shown by the results of this series of research experiments, since hypoxia-tolerant human glioma cells reduce their oxygen consumption rate in response to oxygen deficit, a defense mechanism that contributes to survival under moderate hypoxic conditions^[37]. Overcoming the metabolic restrictions of hypoxia may allow for the progression of lower-grade tumors to GBM.

An alternative of this level when used within a framework of an integrated detection of monitoring system as shown or as it can be seen by the results of other experimental approaches used to detect different sets of cancer

disease related genes that are hypoxia induced^[11,27-29,35-42] 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.

Experimental inhibition of NDRG1 expression in four glioblastoma cell lines *in vitro* by either siRNA technology or interference into tumor cell glycolysis might be a potential therapeutic tool in regulating the expression of this gene in glioblastoma. Furthermore, successful inhibition of tumor cell growth by RNAi aimed at oncogenes *in vitro* and *in vivo* may represent alternative therapeutic applications for these diseases. RNAi is a molecular biology tool with a big potential as therapeutic agent of cancer in human.

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COMMENTS

Background

N-Myc down-regulated gene 1 (*NDRG1*) is a member of the 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.

Research frontiers

Up to this date, the complete detailed function of this protein in humans remains unknown. Hypoxia represents a common feature of solid tumors. Hypoxia-inducible factor-1 (HIF-1) is a key regulator of tumor cell hypoxia. It regulates the expression of several genes related to oxygen homeostasis in response to hypoxic stress. NDRG1 has been shown to possess more specific characteristics for clinical analysis and identification purposes and has been found to be a stable marker of acute tumor hypoxia.

Innovations and breakthroughs

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.

Applications

Direct inhibition of NDRG1 *via* siRNA or indirect inhibition through interfering with the cancer cell glycolytic activities *via* application of iodoacetate might be a potential therapeutic tool for regulating the expression of this gene in glioblastoma.

Terminology

Hypoxia is a pathological condition where organ tissues are deprived of adequate oxygen supply due to the failure to deliver oxygen to target tissues. The difference between normal oxygen supply and the demand at the cellular level may result in an oxygenation hypoxic condition. Normoxia normal oxygen concentration as a result of the normal or adequate oxygen supply on the cellular level, which is typically 20%-21% O₂; Glioblastoma multiforme: Highly invasive brain tumors. It is the last stage of human brain tumor where patients suffering from this type of brain tumor have a maximum life expectancy of maximum 2-6 mo. Human glioblastoma multiforme (GBM) cells vary in their ability to survive under hypoxic conditions. HIF-1 α subunits are highly inducible by different oxygenation conditions in human GBM cells, HIF-1 acts as a master regulator of numerous hypoxia inducible genes related to angiogenesis, cell proliferation/survival, and glucose/iron metabolism. Tumor therapy is referring to the approaches applied against various cancer diseases in human. They include application radiation therapy, surgical removal of cancer tissue, drugs or other substances that block the cancer growth and spread by interfering with specific molecules involved in tumor growth and progression including medical agents that interfere with cell growth signalling or tumor blood vessel development, cancer cells specific death promotion, stimulating the immune system to de-

stroy specific cancer cells, and cancer cells toxic chemical agents delivery into cancer cells as well as gene therapeutic modalities; Tumor microenvironment: Extracellular environment present in a very small region of a solid tumor. It must be mentioned that cells in different areas of solid tumors will have markedly different microenvironments; Angiogenesis: The formation of new blood vessels in human body tissues.

Peer review

Due to its clear regulatory behaviour 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, it can represent a potential target for tumor treatment in human glioblastoma.

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