NDRG2 and NDRG4 Expression Is Altered in Glioblastoma and Influences Survival in Patients with *MGMT*-methylated Tumors

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Abstract. *Aim: The N-myc down-regulated gene (NDRG) family is a group of genes that have predominantly tumor-suppressive effects. The goal of this study was to investigate the expression of NDRG2 and NDRG4 in surgical specimens of human glioblastoma and in normal brain tissue, and to search for correlations with overall (OS) and progression-free survival (PFS). Materials and Methods: Samples from 44 patients (31 males, 13 females; mean age±SD=57.4±15.7 years) with primary (n=40) or recurrent glioblastoma (n=4) were analyzed by quantitative real-time polymerase chain reaction and immunohistochemistry, with dimensionless semiquantitative immunoreactivity score (IRS), ranging from 0-30] for expression of NDRG2 and NDRG4. Five non-tumorous autopsy brain specimens were used as controls. Results: On the protein level, expression of NDRG2 was significantly down-regulated in glioblastoma (IRS=3.5±3.0 vs. 8.8±3.3; p=0.001), while expression of NDRG4 was significantly up-regulated (IRS=5.4±3.7 vs. 0.75±0.4 vs, p<0.001). There was no statistically significant difference in PFS between a group of 15 patients with glioblastoma with MGMT methylation and enhanced expression of NDRG4 mRNA who were treated with adjuvant radiochemotherapy (temozolomide and 60 Gy) and a group of patients with low expression of NDRG4 mRNA [10 (range=5.5-14.2) months vs. 21 (range=10.7-31.3) months] (p=0.13). Conclusion: Expression of both NDRG2 and NDRG4 genes is significantly altered in glioblastomas. PFS among the patients with glioblastoma with MGMT methylation treated with radiochemotherapy differed significantly in high-expression groups compared to patients without MGMT methylation and without radiochemotherapy (p<0.05).*

Glioblastoma multiforme (GBM) is an aggressive infiltrating brain tumor, with average patient survival of less than 15 months (1). Hypermethylation of O^6-methylguanine DNA methyltransferase (*MGMT*) gene promoter is a recognized predictive biomarker of the response to temozolomide treatment as well as of overall survival time (OS) (2, 3). Patients with glioblastoma with hypermethylation of *MGMT* have a better response to temozolomide treatment in comparison to patients with unmethylated *MGMT*. Moreover, such patients have both a longer progression-free survival (PFS) and OS time. Glioblastoma is a heterogeneous tumor, classified into clinically relevant subtypes, according to DNA methylation profiles (4). Despite intensive molecular glioblastoma investigations, there are no molecular markers that can be used on daily basis in clinical practice for defining glioma malignancy grades with the competence to deliver reliable therapy.

Several molecular studies in glioblastoma and meningeoma pointed towards N-myc downstream-regulated gene 2 (*NDRG2*) and *NDRG4* genes as promising diagnostic markers involved in brain tumor pathology (5-9). *NDRG2* and *NDRG4* belong to the NDRG family, whose members share 53-65% identity at the amino-acid level (10, 11). NDRG2 is a cytoplasmatic protein involved in cell apoptosis, cell differentiation, cell growth, neuronal plasticity, and stress response (5). Due to its high expression in brain tissue, the importance of the *NDRG2* gene has been reported in different pathophysiological processes such as ischemia, and Alzheimer's disease (12, 13). The *NDRG2* gene is located at chromosome 14q11.2, and has been

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reported to be down-regulated in glioblastoma (6). Furthermore, *NDRG2* overexpression was found to repress glioblastoma cell proliferation *in vitro* (14). In numerous cell lines, *NDRG2* gene expression is hypoxia-inducible, and responsible for hypoxia-associated apoptosis, and is associated with hypoxia-induced resiststance of cancer cells to radiotherapy (14, 15).

Reduced *NDRG2* gene expression is associated with poor survival prognosis in patients with esophageal squamous cell carcinoma, clear cell renal cell carcinoma, liver, colorectal, gallbladder and lung cancer (16). In a recent study, the poor outcome of patients with glioblastoma correlated with *NDRG2* gene methylation, and reduced expression was reported (16). However, the main mechanism that underlines *NDRG2* silencing in glioma is still unknown.

In contrast to *NDRG2, NDRG4* expression has not been studied in glioblastoma so far. *NDRG4* expression is restricted to a small number of tissues including the heart and the brain, where it is expressed at high levels (5). This restricted expression pattern suggests that NDRG4 plays an important role in the human brain (10). It is known that NDRG4 is relevant in glioblastoma cell proliferation; after knock-down of *NDRG4*, cell-cycle arrest occurs and leads to apoptosis, based on the suppression of expression of cyclin D1, p27, X-linked inhibitor of apoptosis protein (XIAP) and survivin (5). Furthermore, it is reported that the function of NDRG2 and NDRG4 are different in GBM cells: NDRG2 overexpression reduces cell viability, whereas NDRG4 is required for G_1 progression and cell viability in a number of different GBM, and astrocyte model systems. The authors of one study concluded that NDRG4 presence in GBM cells is essential for continued progression through the cell cycle and finally for survival (5). NDRG4 can play a role as tumor suppressor in GBM, as demonstrated in the study of Ding *et al*. (17). NDRG4 is down-regulated in GBM compared to normal tissue and an overexpression of NDRG4 was found to inhibit proliferation of GBM cells.

The goal of this study was to determine the role of NDRG2 and NDRG4 in glioblastoma. We investigated the expression of NDRG2 and NDRG4 in tumor tissue from patients with GBM and correlated the expression of both to *MGMT* methylation status, treatment modalities and PFS.

Materials and Methods

Tissue specimens. A total of 44 diagnostically confirmed specimens of GBM were retrieved as formalin-fixed, paraffin-embedded tissue blocks, and as cryopreserved tissue, between 2006 and 2012 from the Departments of Neurosurgery and Neuropathology, University of Giessen, Germany. Five brain autopsy specimens were used as reference for tumor-free brain tissue, and were provided by the Department of Neuropathology, University of Giessen, Germany. This study was approved by the local Ethical Committee (application number: AZ 07/09). All patients underwent macroscopic, total resection of the tumor.

RNA isolation, cDNA synthesis and quantitative real-time PCR. RNA isolation was performed from frozen specimens using the RNeasy Lipid Tissue Mini Kit® (QIAGEN GmbH, Hilden, Germany) following the manufacturer's instructions. RNA concentration was measured photometrically (NanoDrop® 1000 spectrophotometer; Thermo Fisher Scientific Inc., Waltham, USA). cDNA synthesis with the QuantiTect® Reverse Transcription Kit (QIAGEN GmbH, Hilden, Germany) using 1 μg of total RNA. Quantitative real-time PCR analysis was performed using Taqman® Gene Expression Master Mix and the following gene expression assays (all form Applied Biosystems, Darmstadt, Germany): human actin-β (*ACTB*; Hs99999903), human Importin 8 (*IPO8*) (Hs00914040), human TATA box-binding protein (*TBP*) (Hs00427620), human *NDRG2* (Hs01045115), and human *NDRG4*, (Hs01061225), Setup and cycling conditions adhered to the kit's manual and reactions were run in triplicate on a StepOnePlus instrument (Applied Biosystems).

Raw cycle threshold (Ct) data of qPCR experiments were processed by subtracting the mean Ct of all endogenous control genes (*ACTB, IPO8, TBP*) from the Ct of the according gene of interest (*NDRG2* or *NDRG4*). The relative expression was obtained from the resulting ΔCt value using the formula 2^(-(ΔCt)), taking into account the exponential nature of PCR methodology.

In preliminary experiments, we analyzed samples from individual's frontal, parietal, temporal and occipital lobes for NDRG2 and NDRG4 expression using qPCR. No significant differences in expression were found between these anatomical regions (data not shown). Therefore, we did not further match the control samples to the anatomical brain regions of the tumors.

Immunohistochemistry. Of all samples investigated using qPCR, a subset of 44 tumor samples and five non-tumorous brain tissue samples were available as paraffin-embedded tissue and used for immunohistochemistry. Immunohistochemical staining was performed using Cell Signaling Technology (Danvers, MA, USA) products and protocols as template, using antibodies against NDRG2 (rabbit monoclonal, #5667; dilution 1:400) and NDRG4 (rabbit monoclonal, #9039; dilution 1:400). The paraffin-embedded samples were cut into 3 μm sections, deparaffinized in xylene and rehydrated in graded alcohols. For antigen retrieval, the samples were heated in a steamer in citrate buffer (pH 6) for 10 min. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol. Non-specific binding sites were blocked for 60 min using the kits' blocking solution at room temperature, followed by incubation with the primary antibody for 90 min at room temperature. As a secondary antibody, an IgG Rabbit (#8112) specific ready to use detection reagent was applied for 30 min in a humidified chamber at room temperature. Visualization was carried out using diaminobenzidine (DAB). Slides were counterstained with hematoxylin.

Quantification of immunohistochemical staining. The sections were assessed by two investigators (MS and FS) who were blinded to the patient's characteristics and outcome. Immunoreactivity scores (IRS) were determined using staining intensity and number of positively stained cells. Staining intensity was determined on a scale of 0: no staining; 1: weak staining, light yellow; 2: moderate staining, yellowish brown; and 3: strong staining, brown. In addition, the percentage of positively stained cells was determined (0-100% in 10% steps). The IRS was calculated as the product of

Table I. *Baseline characteristic of patients*.

Characteristic				n	%
Patients		Total		44	100%
		Male		31	70.5%
		Female		13	29.5%
		Age at diagnosis, years	57.4±15.7		
Tumor entity	Glioblastoma	Primary		40	90.9%
		Recurrent		4	9.1%
Median survival (range), months	Unstratified patients		16 (10.6-21.4)	44	100%
	MGMT	Methylated	23 (14.8-29.2)	24	54.5%
		Unmethylated	11 (5.5-16.5)	19	43.2%
		Unavailable		1	2.3%
Therapy		Initial total resection		41	93.2%
		Missing data		3	6.8%
	Adjuvant treatment after first resection	Temozolomide		38	100%
		Radiation		38	100%
		Concomitant		36	94.7%
First recurrence	Total			24	54.54%

MGMT: O^6-Methylguanine DNA methyltransferase.

staining intensity and percentage, resulting in a value ranging between 0 and 30. Differences in assessment were discussed until consensus was reached.

Statistical analysis. Patients were divided into tumor groups and then into low- and high-expression groups. Low expression was defined as normalized gene expression levels at or below the mean expression of the respective tumor group, while all other patients of this group were classified as having high expression.

Statistical analysis was performed using SPSS 20 (IBM software, Ehningen, Germany) and in a second phase using Statistica [Version 12, StatSoft (Europe) GmbH, Hamburg, Germany]. The data distribution was preformed using Statistica program. The data distribution was normal except the data of NDRG2 IRS, hence parametric and non parametric tests were performed. Statistical analysis of gene expression was performed using the Mann–Whitney *U*-test, and checked with the Student t-test. Correlations of gene expression were calculated using Pearson's coefficient.

Survival analysis was performed by the Kaplan–Meier product-limit method for each tumor group, and the expression groups. Overall survival time was calculated from the date of the first surgery to death.

Comparisons of IRS values between tumor subgroups were computed using the Student *t*-test. *p*-Values of less than 0.05 were considered statistically significant.

Results

Patient collective. Of the 44 examined GBM samples, the mean patient age at diagnosis was 57.4±15.7 years. The PFS for the cohort was 16 (range=10.6-21.4) months. The mean OS was 23 (range=14.8-29.2) months for patients with *MGMT* methylation and 11 (range=5.5-16.5) months for patients without. Three of the patients had received chemotherapy or radiotherapy prior to first surgery. All patients underwent total resection of the tumor. Further details are listed in Table I.

Immunohistochemistry results. Immunohistochemistry was performed to investigate NDRG2 and NDRG4 expressions in five healthy brain tissue samples and 44 glioblastoma specimens. The k-statistics for the analyzed immunohistochemical slides gave a kappa value of 0.41 for NDRG2 and 0.30 for NDRG4, indicating a substantial level of inter-observer agreement.

In normal brain tissue, NDRG2, and NDRG4 expression was predominantly in the cytoplasm of glial cells (Figure 1 A and B). Normal brain tissue stained strongly positive for NDRG2 (IRS=8.8±3.3) and weakly for NDRG4 (IRS=5.4±3.7). The glioblastoma tissues exhibited heterogeneous cytoplasmatic staining with the NDRG2 (Figure 1 C and E) and NDRG4 antibody (Figure 1 D and F).

The IRS was 3.47 for NDRG2 and 5.4 for NDRG4 (p=0.003; *t*-test p=0.004) in glioblastoma (Figure 2). The IRS of NDRG4 in glioblastoma tissue (mean 5.53) was slightly higher compared to white matter in normal brain tissue (mean 5.39) (Figure 2).

qPCR results. The results of *NDRG2* mRNA expression analysis were similar to those from the immunhistochemical analysis: *NDRG2* gene expression was significantly down-regulated in glioblastoma (2.89) compared to the normal brain tissue (11.2) (p=0.001). Furthermore, in contrast to the results obtained from analysis of the protein level, *NDRG4* mRNA expression was significantly down-regulated in glioblastoma compared to the normal brain tissue (p=0.001) (Figure 2).

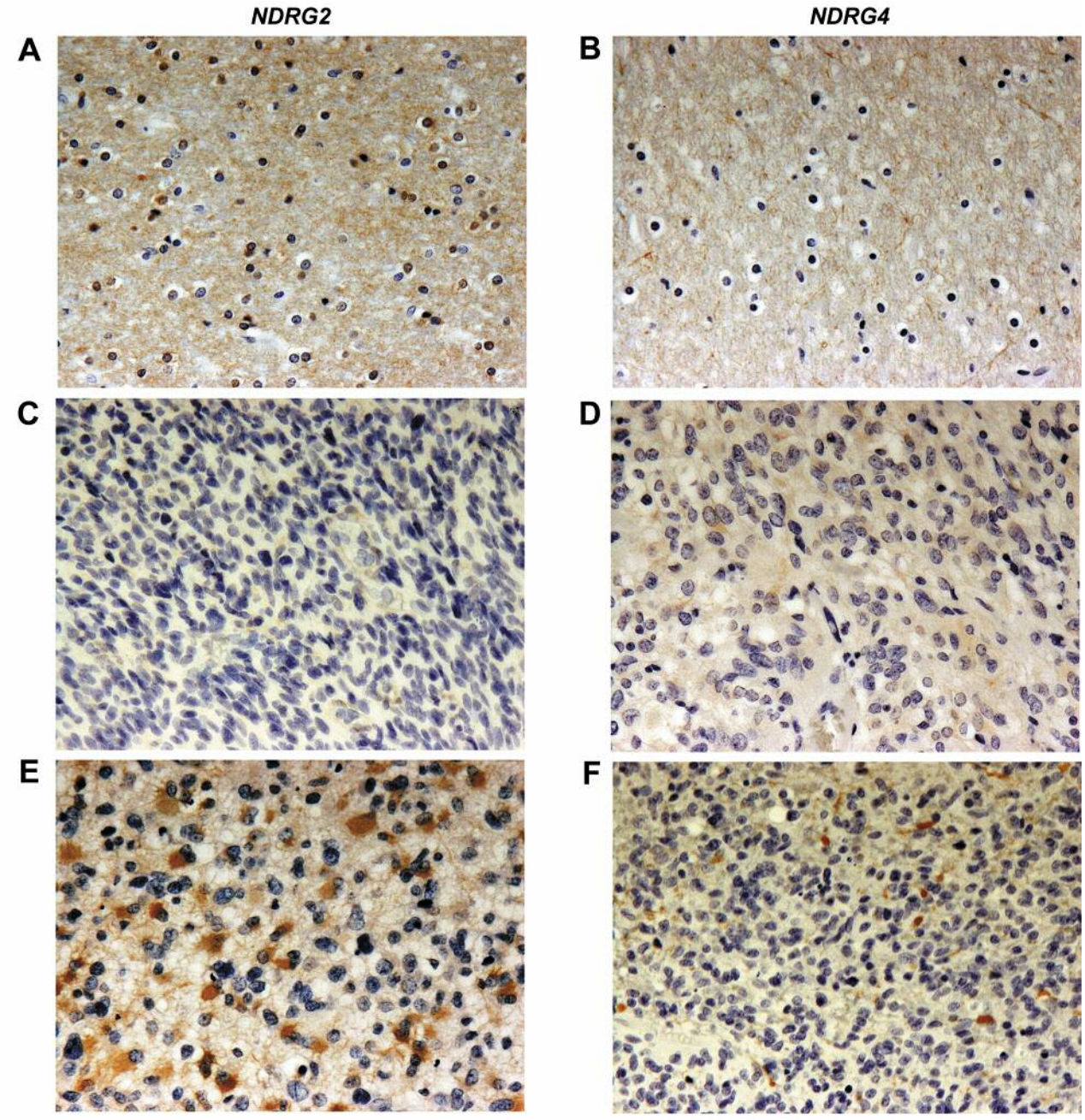

Figure 1. *Representative immunohistochemical staining of N-myc down-regulated gene 2 (NDRG2) (A), and NDRG4 (B) in non-tumoral tissue. Low and high expression of NDRG2 (C and E, respectively) and NDRG4 (D and F, respectively) in glioblastoma. Immunohistochemical staining of NDRG2 protein was lower in glioblastoma compared to normal brain tissue, while that of NDRG4 protein was higher.*

Correlation analysis of PFS and OS by MGMT methylation. An analysis of the impact of *MGMT* methylation on PFS and OS showed that patients with methylated *MGMT* had significantly longer PFS [median=14 (range=7.7-20.3) *vs.* 5 (range=0-10.5) months; p=0.007] and OS [median=23 (range=13.8-32.1) *vs.* 11 (range=5.5-16.5) months; p=0.001] than patients with unmethylated *MGMT* (Figure 3).

Correlation analysis of NDRG2 and NDRG4 expression levels with MGMT methylation. The qPCR analysis revealed a higher mean expression of *NDRG2* gene (3.4 *vs.* 1.4) as well as an increased mean *NDRG4* gene expression (3.0 *vs.* 1.7) in *MGMT* methylation-positive patients, that was not significant (Figure 4).

Immunohistochemistry showed a trend towards a lower expression of NDRG2 in methylated tumor tissues than in

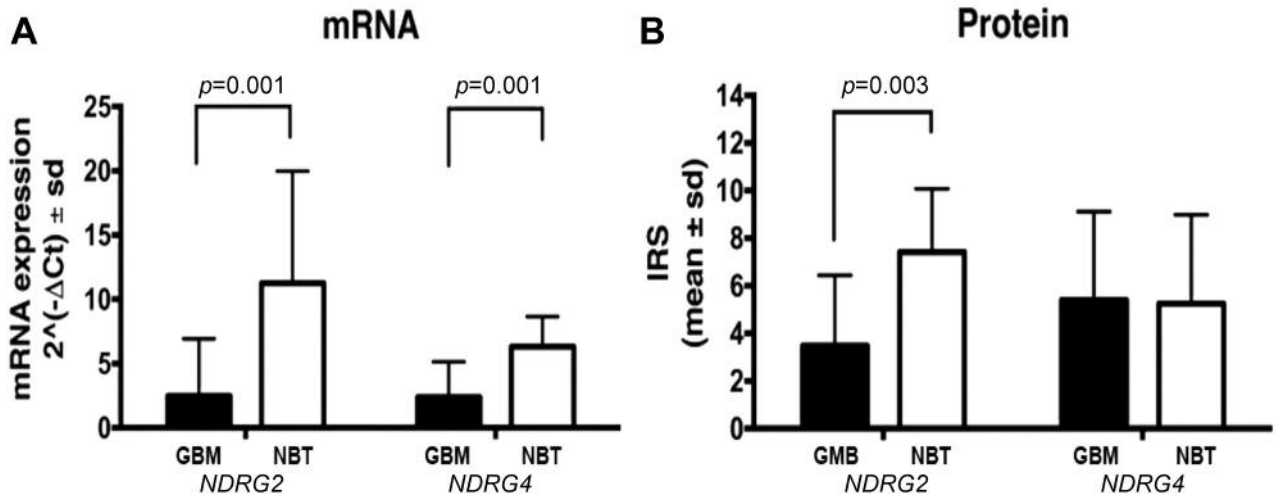

Figure 2. *Expression of N-myc down-regulated gene 2 (NDRG2) and NDRG4 mRNA (A) and protein (B) in normal brain tissue (NBT), and glioblastoma (GBM).*

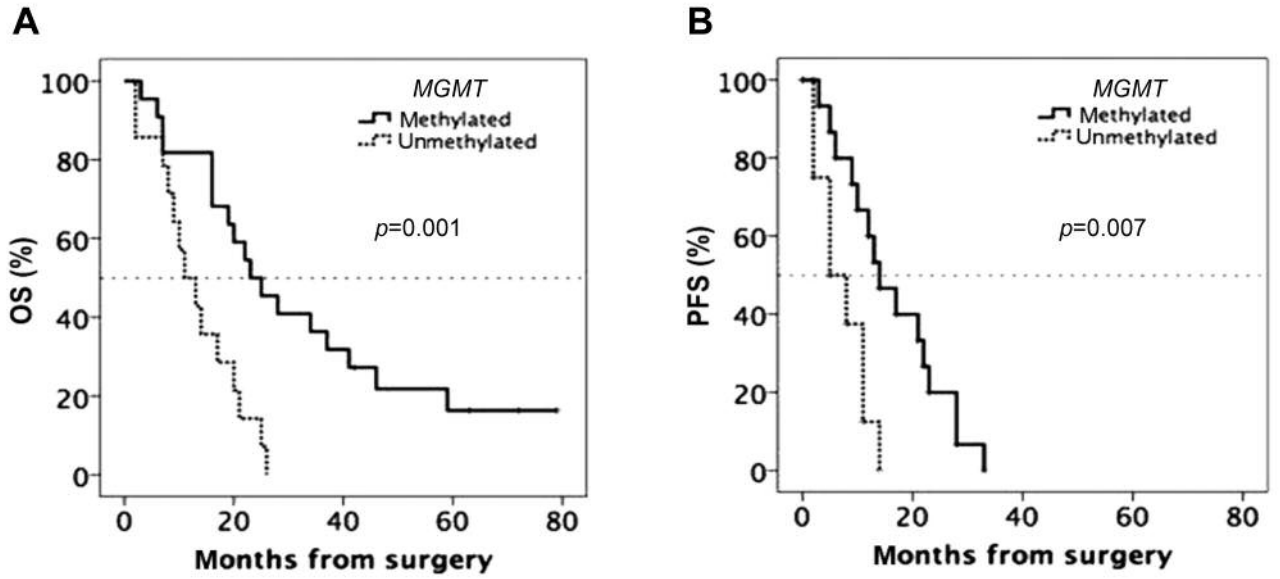

Figure 3. *Overall (OS) (A) and progression-free (PFS) (B) survival by O6-methylguanine DNA methyltransferase (MGMT) methylation status of patients with glioblastoma who initially underwent resection with curative intent, and who received adjuvant temozolomide and 60 Gy irradiation, but no neoadjuvant therapy.*

unmethylated samples (mean 3.1 *vs*. 3.9). However, the expression of NDRG4 by immunohistochemistry was similar to the results for mRNA and was higher in methylated tumor tissue than in unmethylated ones (5.9 *vs*. 4.6, not significant) (Figure 4).

Influence of radiochemotherapy on mRNA and protein expression of NDRG2 and NDRG4. The analysis of the mean *NDRG2* and *NDRG4* mRNA (2.50 and 2.40, respectively) and protein (3.48 and 5.40, respectively) expression in patients with primary glioblastoma without radiochemotherapy treatment compared to patients with recurrent tumor after neoadjuvant treatment showed that with this therapy mRNA and protein expression was not significantly lower than without this treatment. Marker expression was not significantly altered in the previously treated tumors compared

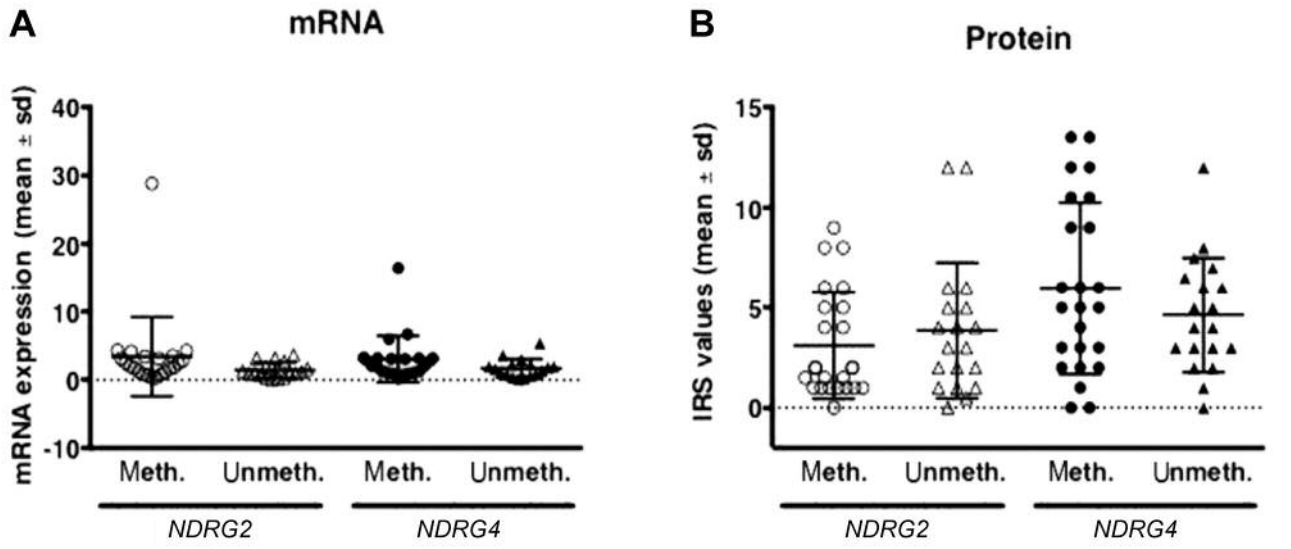

Figure 4. *Expression of N-myc down-regulated gene 2 (NDRG2) and NDRG4 mRNA (A) and protein (B) in all glioblastoma samples by O^6-methylguanine DNA methyltransferase (MGMT) promoter methylation status. Data points show individual mRNA (2^−dCt) and protein immunoreactivity scores (IRS) (intensity of staining × percentage of positively stained cells). Bars indicate mean expression within the group and standard deviation. Marker expression was not significantly altered in MGMT-methylated vs. unmethylated tumors (p>0.05).*

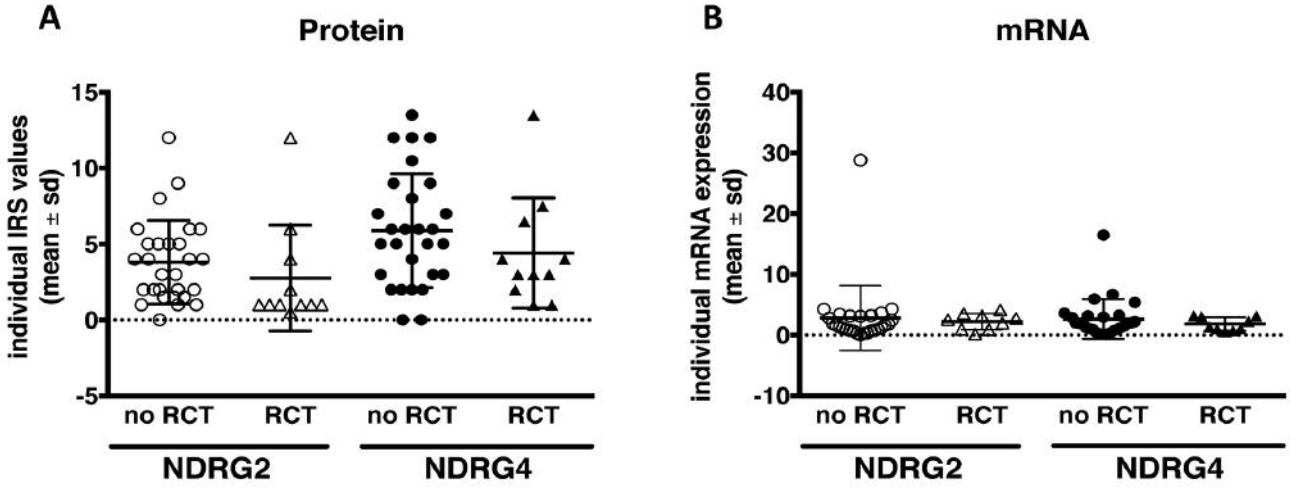

Figure 5. *Expression of N-myc down-regulated gene 2 (NDRG2) and NDRG4 mRNA (A) and protein (B) in patients presenting with primary glioblastoma (without neoadjuvant treatment) and those with relapsing glioblastoma (previously resected and adjuvantly treated, RCT). Individual mRNA expression levels (2^-dCt) and individual immunoreactivity scores (IRS) for protein expression (intensity of staining × percentage of positively stained cells) are shown. Bars indicate mean expression and standard deviation. Marker expression was not significantly altered in the previously treated tumors compared to the primary tumors (p>0.05).*

to the primary tumors ($p>0.05$) (Figure 5). Analysis of PFS by stratifying for *MGMT* methylation status and *NDRG2/4* mRNA and protein expression. In the following, we considered PFS. We included 23 patients with primary glioblastoma after the total initial resection of tumor issue, with non-neoadjuvant treatment and adjuvant therapy with 60 Gy and temozolomide. The PFS was reviewed by *MGMT* methylation status.

The analysis of PFS for these patients depending on *NDRG2* mRNA expression is shown in Figure 6 A and B. Patients with *MGMT* methylation and low *NDRG2* expression (≤1.650) had median PFS of 14 (range=8.8-19.1) months compared with 13 (range=0-29.6) months for those with *MGMT* methylation and high *NDRG2* mRNA expression (p=0.43; t-test p=0.41). Patients without *MGMT* methylation with low *NDRG2* mRNA expression had a PFS of 8 (range=1.6-14.4) months compared to 2 (range=0.6-28.1) months for those with high *NDRG2* expression, although again not significantly different (Figure 6).

Low protein expression of NDRG2 (≤2) with *MGMT* methylation was significantly associated with poor PFS of 10 months (range=5.4-14.6) compared to 22 (range=11.0-32.7) months for those with high protein expression of NDRG2 and *MGMT* methylation (p=0.08; t-test p=0.061). Patients with the combination of low protein expression of NDRG2 and unmethylated *MGMT* had a median OS of 5 (range=0-5) months in our data. The observed PFS for those with high NDRG2 protein expression and unmethylated *MGMT* was 8 (range=0.3-15.7) months (p=0.52; t-test p=0.49) (Figure 6 C and D).

Patients with *MGMT* methylation and low mRNA expression of *NDRG4* (≤1.88) had a PFS of 10 (range=5.8-14.2) months compared to 21 (range=10.7-31.3) months for those with high *NDRG4* expression and *MGMT* methylation (p=0.14; t-test p=0.13). Patients with unmethylated *MGMT* gene promoter and low *NDRG4* mRNA expression had a PFS of 8 (range=3.2-12.8) months compared to 5 (range=0-11.4) months for those with high *NDRG4* mRNA (p=0.94; t-test p=0.71) (Figure 6E and F).

Patients with low NDRG4 protein expression (≤5.0) and *MGMT* methylation had PFS of 12 (range=6.9-17.1) months compared with 17 (range=7.3-26.7) months for those with high NDRG4 protein expression (>5.0) and *MGMT* methylation (p=0.38; t-test p=0.37). The low NDRG4 protein expression in combination with unmethylated *MGMT* was associated with a mean PFS of 8 months (range: 1.6-14.4). *vs*. 5 (range=0.2-9.8) months for those with high protein NDRG4 expression group with unmethylated *MGMT* (p=0.76; t-test p=0.45) (Figure 6G and H).

Patients with methylation of *MGMT*, high mRNA expression of *NDRG2* and high mRNA and IRS expression of *NDRG4* after radiochemotherapy had significantly longer PFS in comparison to the patients without *MGMT* methylation and without therapy (p<0.05) (Figure 7).

Confirmation of qPCR gene expression levels using immunohistochemistry. A subset of the samples analyzed using qPCR was also available for immunohistochemical staining for NDRG2 and NDRG4 by one antibody. We first determined whether the qPCR expression levels correlated with the IRS in all samples, tumors and normal tissue. The mRNA expression data for NDRG2, and NDRG4 did not correlate (Pearson correlation r=0.154, p>0.05). In contrast, we observed a linear correlation tendency between NDRG2 and NDRG4 IRS expression levels (Pearson correlation r=0.334, p<0.027).

Discussion

To the best of our knowledge, this is the first report that investigates NDRG2 and NDRG4 expression and *MGMT* methylation status in relation to PFS and OS in patients with GBM. The analysis of *NDRG2* gene expression in such patients and its comparison to the expression in patients treated with radiochemotherapy, in combination with *MGMT* methylation status and survival time could help understand the role of this gene. Investigation of NDRG2 and NDRG4 expressions (mRNA and IRS) showed significantly longer PFS in those patients, who underwent radiochemotherapy and had methylation of *MGMT* (Figure 7).

Investigation of *NDRG2* expression showed that unusual promoter methylation of *NDRG2* appears to be the major molecular mechanism for the down-regulation of NDRG2 expression in glioblastomas. This epigenetic inactivation of NDRG2 occurs in primary glioblastoma but is rare in secondary GBM (6).

Furthermore, we found that the expression of *NDRG2* and *NDRG4* genes at the protein or mRNA level changes in response to radiochemotherapy. We hypothesize that radioresistance of tumor cells overexpressing *NDRG2* gene is associated with down-regulation of NDRG2 after the neoadjuvant therapy (Figure 5).

In line with earlier findings of Deng *et al*., we showed that NDRG2 is down-regulated in GBM at both RNA and protein levels in comparison to normal tissue (18). *NDRG2* is also down-regulated in several other tumor types such as thyroid carcinoma, colon cancer, renal cancer (11, 19, 20). Furthermore, Deng *et al*. showed that NDRG2 overexpression could inhibit glioblastoma cell proliferation. NDRG2 has been reported to suppress cellular proliferation, invasion and metastasis and be obligatory for apoptotic pathways containing FAS-mediated cell death and p53-mediated apoptosis (5, 15, 21, 22).

Schilling *et al*. (5) and Ding *et al*. (18) reported on the function of *NDRG4* gene in glioblastoma. In this study, we analyzed the expression of *NDRG4* gene and compared it to the methylation status of *MGMT* gene and survival time. Additionally, we compared our results with the results of Schilling *et al*. and Deng *et al*., who have an opposite opinion on the impact of *NDRG4* gene expression in GBM.

Other than in the case of expression of *NDRG2* and, *NDRG4* genes seems to be expressed in a different way at the RNA level from that at the protein level. Immunohistochemical analysis showed similar results compared to the findings of Schilling *et al*. (5), with a minimal overexpression of *NDRG4*

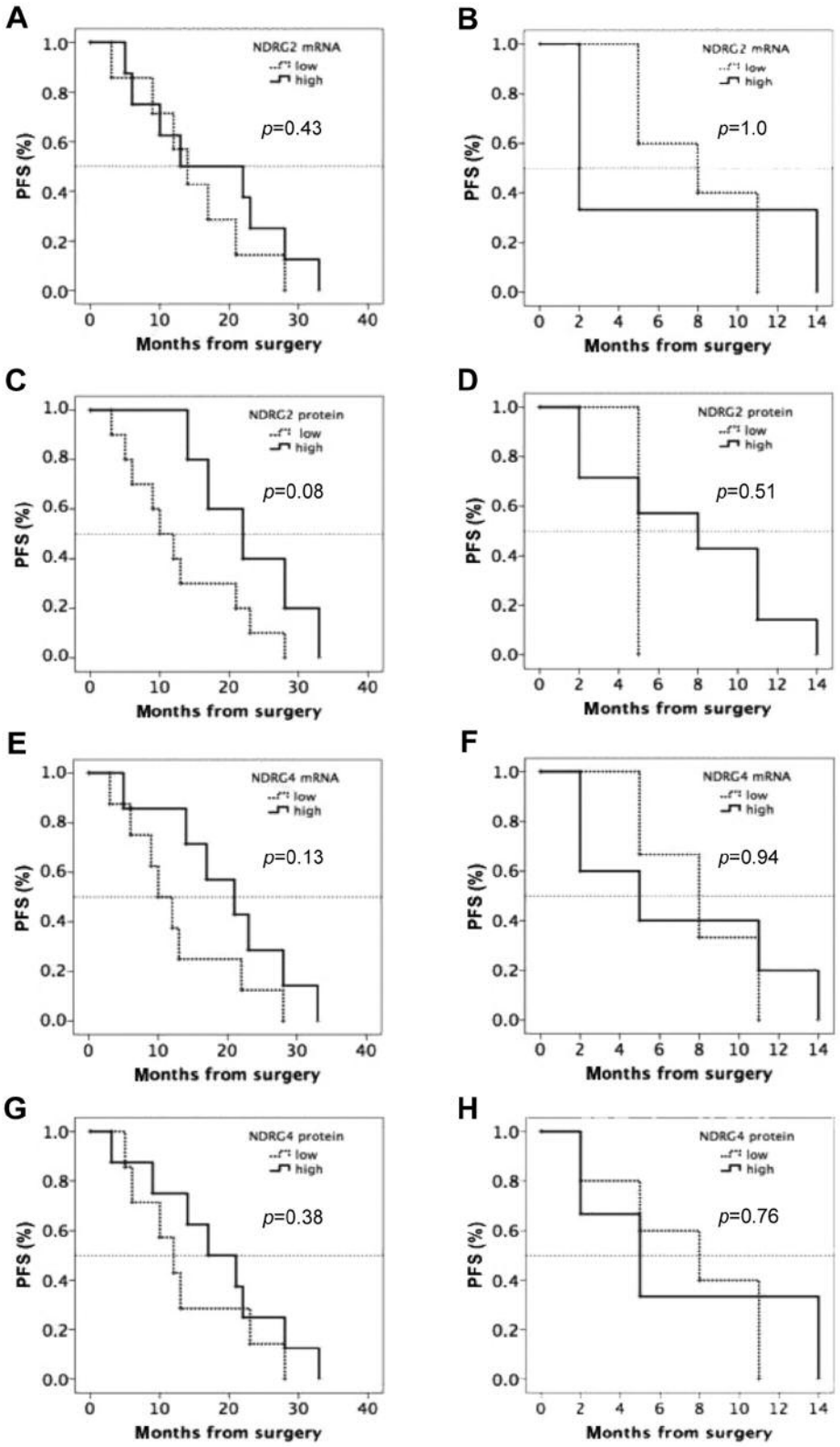

Figure 6. *Progression-free survival of patients with glioblastoma who underwent resection and received adjuvant temozolomide and 60 Gy irradiation, but initially received no neoadjuvant therapy. Patients are stratified by O^6-methylguanine DNA methyltransferase (MGMT) methylation status (A, C, E, G: methylated; B, D, F, H: unmethylated). Curves show progression-free survival depending on NDRG2 and NDRG4 mRNA and protein expression.*

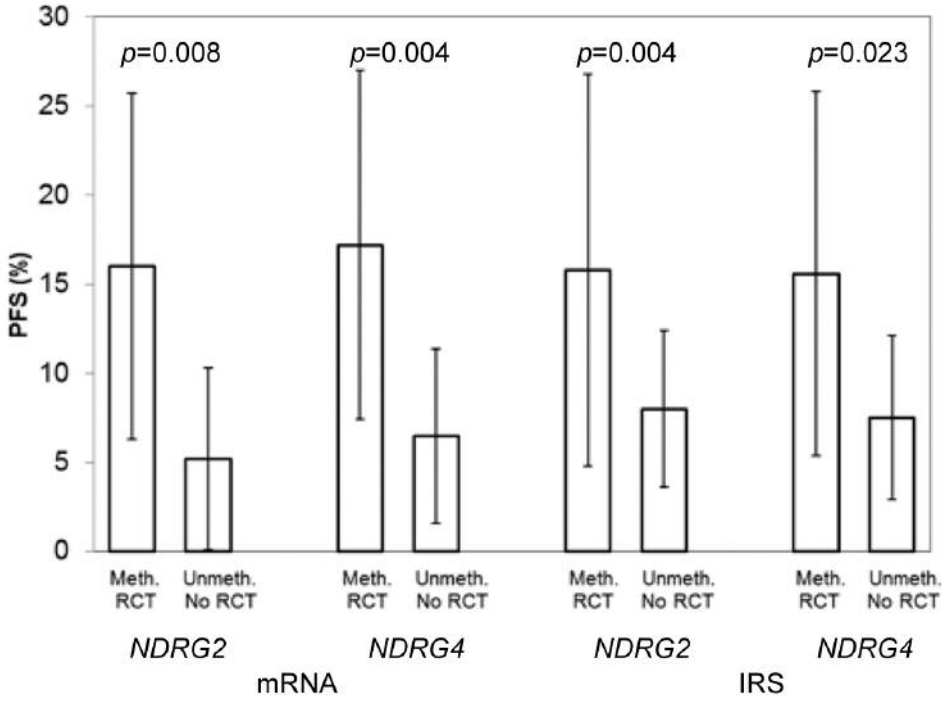

Figure 7. *Progression-free survival of patients with glioblastoma who underwent resection and received adjuvant temozolomide and 60 Gy irradiation. Patients are stratified by O^6-methylguanine DNA methyltransferase (MGMT) methylation status, mRNA and protein expression. Curves show progression-free survival depending on NDRG2 and NDRG4 mRNA and protein expression by immunoreactivity score (IRS). Data show significant differences in expression. RCT: Radiochemotherapy; unmeth.: unmethylated gene; meth: methylated gene.*

gene in glioblastoma cells compared to normal brain tissue. Schilling *et al*. also showed that *NDRG4* gene is up-regulated in GBM compared to human cortex tissue, and that knocking-down *NDRG4* reduced viability of GBM cells. This led to tumor progression and ultimately changed OS. Both Schilling *et al*. (5) and Ding *et al*. (17) also used analyses by western blot. It should be noted that we analyzed expression of genes in GBM and in normal brain tissue. Schilling *et al*. performed their analysis in cultured cells derived from three human GBM xenografts. In contrast to their study, the sample size in our study was greater (n=44). In addition, we found that expression of both *NRDG2* and *NRDG4* gene changes under radiochemotherapy (Figure 7).

The expression analysis at the mRNA level showed inconsistent results in comparison to the previous immunohistochemical analysis. We obtained results similar to those by Ding *et al*. *NDRG4* gene was down-regulated in GBM cells as compared to normal brain tissue. There was a correlation between expression of NDRG4 with PFS (17). We conclude that down-regulation of NDRG4 at the protein as well as at the mRNA level in cases with unmethylated *MGMT* is associated with longer PFS. In the case of *MGMT* methylation, overexpression of NDRG4 could play a role in PFS.

These results are similar to those obtained by Ding *et al*. who reported that NDRG4 expression was down-regulated at both RNA and protein levels in GBM tissue compared to normal brain tissue. At the protein level (western blot), these authors found *NDRG4* down-regulation in GBM tissue compared to normal brain tissue. They also described results similar to our findings and to the results obtained by Schilling *et al*., that is NDRG4 expression in the IHC-analysis was higher expression in GBM samples compared to normal brain tissue (5, 17).

We propose the larger NDRG4 expression at the protein level as being due to the non-specific staining of the antibody (17).

Ding *et al*. used GBM samples and normal brain tissue comparable to those in our report. In our opinion, the lack of discrepancy between the results obtained by our teams at the mRNA level in contrast to the study of Schilling *et al*. is due to the similar approach and sample size (n=49 *versus* n=44, respectively) (5, 17). Melotte *et al*. also described the role of *NDRG4* gene in colorectal carcinoma as a tumor-suppressive one (17, 21).

We found that in the case of patients with methylation of *MGMT* promoter gene and *NDRG4* overexpression, PFS is longer compared to patients with unmethylated glioblastomas with low *NDRG4* expression.

Our data suggest that *NDRG4* could play a role as tumor-suppressor gene in GBM similarly as was demonstrated by Melotte *et al*. for colorectal cancer (21).

However, some limitations of our study should be emphasized. Owing to the retrospective character and the heterogeneous patient population, a selection bias cannot be excluded.

In summary, our study indicates that the NDRG gene family seems to play a role in human GBM prognosis. According to our data, PFS depends not only on *MGMT* methylation, but also on expression of *NDRG2* and *NDRG4* genes. The low expression of *NDRG2* at the mRNA level leads to a longer PFS independent of methylation status. *NDRG2* gene might act as an oncogene if overexpressed in glioblastoma cells.

NDRG4 gene in *MGMT*-methylated cells is, in our opinion, a putative tumor-suppressor gene and *NDRG4* occurs as an oncogene in cells with unmethylated *MGMT*. This hypothesis might be helpful in creating novel strategies for glioma therapy and prevention.

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