

# NRF2, DJ1 and SNRX1 and their prognostic impact in astrocytic gliomas

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**Summary.** Nuclear factor erythroid 2-related factor 2 (NRF2), DJ1 and sulfiredoxin 1 (SRXN1) are transcription factors which protect cells from the oxidative damage caused by reactive oxygen species and, on the other hand, are associated with resistance to cancer treatments. The immunohistochemical expression of NRF2, DJ1 and SRXN1 was assessed in human grade II-IV astrocytic gliomas. Their association to clinicopathologic and essential molecular factors was evaluated. The RNA expression levels and genetic alterations were analyzed from publicly available datasets. All studied molecules were commonly expressed. The cytoplasmic NRF2 expression was higher in tumors with a higher malignancy grade, whereas the nuclear and cytoplasmic DJ1 expression was associated with a lower grade. The presence of the isocitrate dehydrogenase 1 mutation (IDH1) was associated with an increasing cytoplasmic and nuclear expression of NRF2 and a nuclear DJ1 expression. When primary grade IV astrocytomas were compared to secondary glioblastomas, nuclear DJ1 was associated with secondary tumors. In grade II-IV tumors, the cytoplasmic NRF2 expression was associated with a poor prognosis, whereas nuclear NRF2 and both cytoplasmic and nuclear DJ1 were associated with a better patient prognosis. Recurrent homozygous deletions of DJ1 were observed, especially in the IDH

wild-type samples. When only the glioblastomas were evaluated, nuclear NRF2 and SRXN1 predicted better survival. As a conclusion, NRF2, DJ1 and SNXR1 can be used as prognosticators in gliomas.

**Key words:** NRF2, DJ1, SNXR1, Glioma, Prognosis

## Introduction

Nuclear factor erythroid 2-related factor 2 (NRF2) is a transcription factor which protects cells from the oxidative damage caused by reactive oxygen species (ROS). ROS are oxygen containing free radicals, which contain one or more reactive electrons on their outer orbital (Karihtala and Soini, 2007). They are harmful to cells and may react with proteins and the lipid components of the cells causing disturbances in the enzymatic activity of the cells or damage to cell membranes (Karihtala and Soini, 2007). They can also react with nucleic acids causing DNA damage and mutations, thus inducing a neoplastic transformation (Karihtala and Soini, 2007). Nrf2 protects cells from the ROS induced damage by activating antioxidative enzymes (AOEs). These neutralize the ROS to harmless molecules, mainly water. The target molecules of NRF2 include thioredoxin, thioredoxin reductases, peroxiredoxins, gamma glutamyl cysteine ligase, and glutathione-S-transferases, all of which participate in neutralizing reactive oxygen species. Even though the antioxidative enzymes protect normal cells from cellular damage, they are often induced in cancer cells protecting

them from the ROS damage induced by radiation or chemotherapy. They have also been shown to be active in astrocytic gliomas and affect the biological behavior and prognosis of these brain tumors (Haapasalo et al., 2003; Lau et al., 2008; Leinonen et al., 2014).

NRF2 prevents carcinogenesis by protecting cells from DNA mutations and it has been shown to act as a tumor suppressor (Kensler and Wakabayashi, 2010). On the other hand, the accumulation of NRF2 has been shown to increase chemo- and radiotherapy resistance, thus the accumulation of NRF2 promotes cancer aggressivity (Karihtala and Soini, 2007; Haves and McMahon, 2009). In the event that there is no oxidative or xenobiotic stress, NRF2 binds to KEAP1 (Kelch-like ECH-associated protein 1) in the cytoplasm of the cells and is then targeted to the proteasomal pathway and degraded. If there is oxidative or xenobiotic stress, NRF2 is released from KEAP1 and moves to the nucleus, where it attaches to the antioxidant/electrophile response element (ARE/EpRE) in the DNA and activates target genes, such as sulfiredoxin (Lau et al., 2008). Mice with a non-functioning NRF2 develop early onset emphysema and nutritional steatohepatitis due to a deficient antioxidative response. *Nrf2* knockout mice develop tumor foci in lungs after urethane exposure, which contributes to the initiation of lung carcinogenesis (Satoh et al., 2013). In humans, NRF2 mutations are detected in the esophageal, larynx, renal, hepatocellular, skin and lung carcinomas (Kim et al., 2010; Hartikainen et al., 2012; Cho et al., 2015). If NRF2 is not able to bind to KEAP1, NRF2 accumulates and increases chemoresistance in non-small cell lung cancer (Haves and McMahon, 2009). Moreover, an overexpression of NRF2 in lung, breast or neuroblastoma cell lines induces chemoresistance (Wang et al., 2008).

One of the target genes of NRF2 is sulfiredoxin 1 (SRXN1), the enzyme that belongs to the family of oxidoreductases. Peroxiredoxins (Prx) include six different types and in addition to affecting the redox balance of the cells, they also act as chaperones. Prxs are peroxidases in which cysteine serves as the primary site of oxidation during the reduction of peroxides. Prxs have been linked to several cancers, including astrocytomas (Nordfors et al., 2007; Järvelä et al., 2010). Oxidised peroxiredoxins are reduced by thioredoxin but in severe oxidative stress, Prxs may be hyperoxidized. A hyperoxidized form of Prx can be reduced by SRXN1, thus making it possible for these molecules to be further reduced by thioredoxin and to be returned back to a reduced, active state.

DJ1 is a multifunctional oxidative stress response protein defending cells from ROS and acting as cellular redox homeostasis. DJ1 acts as a transcriptional factor and its overexpression facilitates the activation of NRF2 (Wilhelmus et al., 2012). Moreover, it regulates the expression of SOD1 and SOD3 and harbors a cytoprotective effect on the brain (Wilhelmus et al., 2012). In a normal brain, DJ1 is expressed in neurons

and astrocytes (Wilhelmus et al., 2012). DJ1 has been linked to Parkinson's disease, Alzheimer's disease and multiple sclerosis and it is overexpressed in several cancers (Wilson et al., 2011; Wilhelmus et al., 2012).

There are no large studies on diffusely infiltrating astrocytomas which describe the expression of NRF2, SNRX1 or DJ1 and their relationship with one another. In these highly malignant tumors, surgical treatment is often inadequate and the 5-year patient survival of glioblastomas has been reported to be 9.8% with the latest therapeutical methods (Stupp et al., 2009). Here, we evaluate NRF2, SRNX1 and DJ1 in astrocytic gliomas, assess their reciprocal associations and correlate their expression to important clinicopathologic and molecular factors.

### Materials and methods

The study material included 273 diffusely infiltrating astrocytoma samples. These were obtained from the patients operated on the Unit of Neurosurgery, Tampere University Hospital, Tampere, Finland, during 1983-2001. The brain tumor specimens were fixed in 4% phosphate-buffered formaldehyde and processed into paraffin blocks. An experienced neuropathologist (H. Haapasalo) evaluated the tumors according to the WHO 2007 criteria on the basis of H&E-stained slides (Louis et al., 2007). These criteria divide diffusely infiltrating astrocytomas into three grades (2-4) according to the presence of atypia, mitotic activity, necrosis, and endothelial proliferation. The neuropathologist pinpointed one histologically representative tumor region in each sample specimen and this specimen was included to tumor microarrays (TMAs). TMAs were constructed with a custom-built instrument (Beecher Instruments, Silver Spring, MD) and the sample diameter of the tissue cores was 600  $\mu\text{mol/L}$ . TMAs were composed of 273 astrocytic tumors [grade 2 (33), grade 3 (41), and grade 4 (199)] and consisted of 213 primary tumors and 60 recurrences. The age of patients with primary tumors varied from 29 to 91 (median  $\pm$  SD, 66 $\pm$ 14) and recurrent tumors from 15 to 90 (median  $\pm$  SD, 56 $\pm$ 13). Overall survival was known for 213 patients [grade 2 (24), grade 3 (28), and grade 4 (161)]. The median follow-up time for 12 patients who survived was 15 years. The tumors were radically resected if possible and most patients with a high-grade glioma also received radiotherapy and/or chemotherapy (but no temozolomide because of the study period). Therapy modalities were known among 209 patients (presence or absence of radiotherapy or chemotherapy).

Four-micrometer-thick tissue sections were cut from the paraffin-embedded blocks. After deparaffinization and rehydration, the sections were heated in a microwave oven for 2 $\times$ 5 minutes in trisaminomethane-ethylenediaminetetraacetic acid (Tris-EDTA) buffer (pH 9.0), incubated in a Tris-EDTA buffer for 20 min and washed twice for 5 minutes in phosphate-buffered saline

(PBS). Hydrogen peroxide (5%, 5 min) was used to block endogenous peroxidase. Nonspecific binding was blocked with 1.5% normal serum in PBS for 35 min at room temperature. The primary antibodies used were NRF2 (Santa Cruz Biotechnology Inc., Santa Cruz, CA), DJ-1 (Abcam Inc., Cambridge, MA), and SRX1 (Proteintech Group Inc., Chicago, IL). The sections were incubated overnight at 4°C with the mouse monoclonal anti-NRF2, SRNX1, and DJ1 antibodies (dilutions 1:1000, 1:500, and 1:500, respectively). The slides were then incubated with a biotinylated secondary antibody and avidin-biotin-peroxidase complex (ABC Vectastain Elite Kit, Vector Laboratories, Burlingame, CA). Careful rinses were performed with PBS in each step of the immunostaining procedure. The color was developed with diaminobenzidine tetrahydrochloride (DAB) (Sigma, St Louis, MO). The slides were counterstained with Mayer hematoxylin, washed, dehydrated, cleared, and mounted with Depex (BDH, Poole, UK). In the negative controls, the primary antibody was omitted. The immunostaining was quantitatively divided into 4 groups according to the percentage of cell positivity: negative (<5%), faint staining (5-25 %) moderate staining (25-50%) and strong staining ( $\geq 50\%$ ). This evaluation was made in the area with the greatest tumor cell density. In addition, we analyzed the expression, according to location, as either nuclear or cytoplasmic.

The R132H point mutation specific mouse monoclonal antibody (Dianova GmbH, Hamburg, Germany) was used to detect IDH1-R132H specific gene mutations. Fully automated immunostaining was performed by a Bondmax immunostainer (Leica Biosystems Newcastle Ltd, Newcastle upon Tyne, United Kingdom). A Bond Dewax Solution (catalogue No. AR9222) was used for deparaffinization. For epitope retrieval, an RTU Epitope Retrieval Solution 1, pH 5.9-6.1 (catalogue No. AR9961) was used for 30 min at 100°C. The slides were incubated for 30 minutes at room temperature with the IDH1-R132H point mutation specific antibody (dilution 1:50). The staining kit used was the Bond Refine Detection kit. The slides were rinsed between steps with the Bond Wash Solution (catalogue No. AR9590).

Proliferation by Ki-67 (MIB-1), p53 immunohistochemistry, apoptosis, and EGFR amplification detection by chromogenic in situ hybridization were done as previously described (Haapasalo et al., 1999; Nordfors et al., 2015).

#### Statistical analysis

All statistical analyses were done using IBM SPSS Statistics 20.0 or Graphpad Prism. The analyses were performed by means of the  $\chi^2$  test, Mann-Whitney test, t-test or Kruskal-Wallis test. The log rank test, Kaplan-Meier curves, and Cox multivariate regression analysis were used in the survival analysis. The statistical significance of associations was declared at the traditional threshold ( $p=0.05$ ).

#### Analysis of publicly available datasets

The RNA expression levels in grade II-IV diffuse gliomas were visualized from the REMBRANDT data using a freely available analysis tool ([www.betastasis.com](http://www.betastasis.com)). The survival association analysis was also performed using the same tool. Genetic variants were extracted from The Cancer Genome Atlas (TCGA) grade low-grade glioma (grade II-III diffuse gliomas) and glioblastoma exome sequencing datasets using cBioPortal (<http://www.cbioportal.org/>).

#### Results

Cytoplasmic NRF2 immunopositivity was detected in the tumor tissue sections as follows: 60 of 262 (23%) were strongly, 100 (38%) moderately, 88 (34%) weakly stained, and 14 (5%) were negative. A strong nuclear staining for *nrf2* was observed in 86 cases (33%), moderate in 59 (23%), weak in 65 (25%), and 52 (20%) were negative. When the DJ1 cytoplasmic immunopositivity was assessed, 14 of 273 cases (5%) were strongly, 46 (17%) were moderately, 82 (30%) were weakly stained, and 131 (48%) were negative. No strong nuclear staining for DJ1 was detected, whereas 6 (2%) cases were moderately, 48 (18%) weakly stained, and 219 (80%) were negative. The nuclear immunohistological SRNX1 expression was detected as follows: in 62 of 271 (23%) was strong, 99 (37%) moderate, 94 (35%) weak, and 16 (6%) remained negative. No cytoplasmic expression was observed for SRNX1. Representative staining images are shown in Fig. 1.

When the reciprocal associations of the studied molecules were evaluated, the cytoplasmic DJ1 expression correlated significantly with the cytoplasmic and nuclear NRF2 expression ( $p<0.001$ ,  $p=0.048$ , respectively, chi-square test) and SNRX1 expression ( $p=0.012$ , chi-square test). Furthermore, cytoplasmic and nuclear NRF2 positivity were associated to an elevated SNRX1 immunopositivity ( $p<0.001$ ,  $p=0.003$ , respectively, chi-square test).

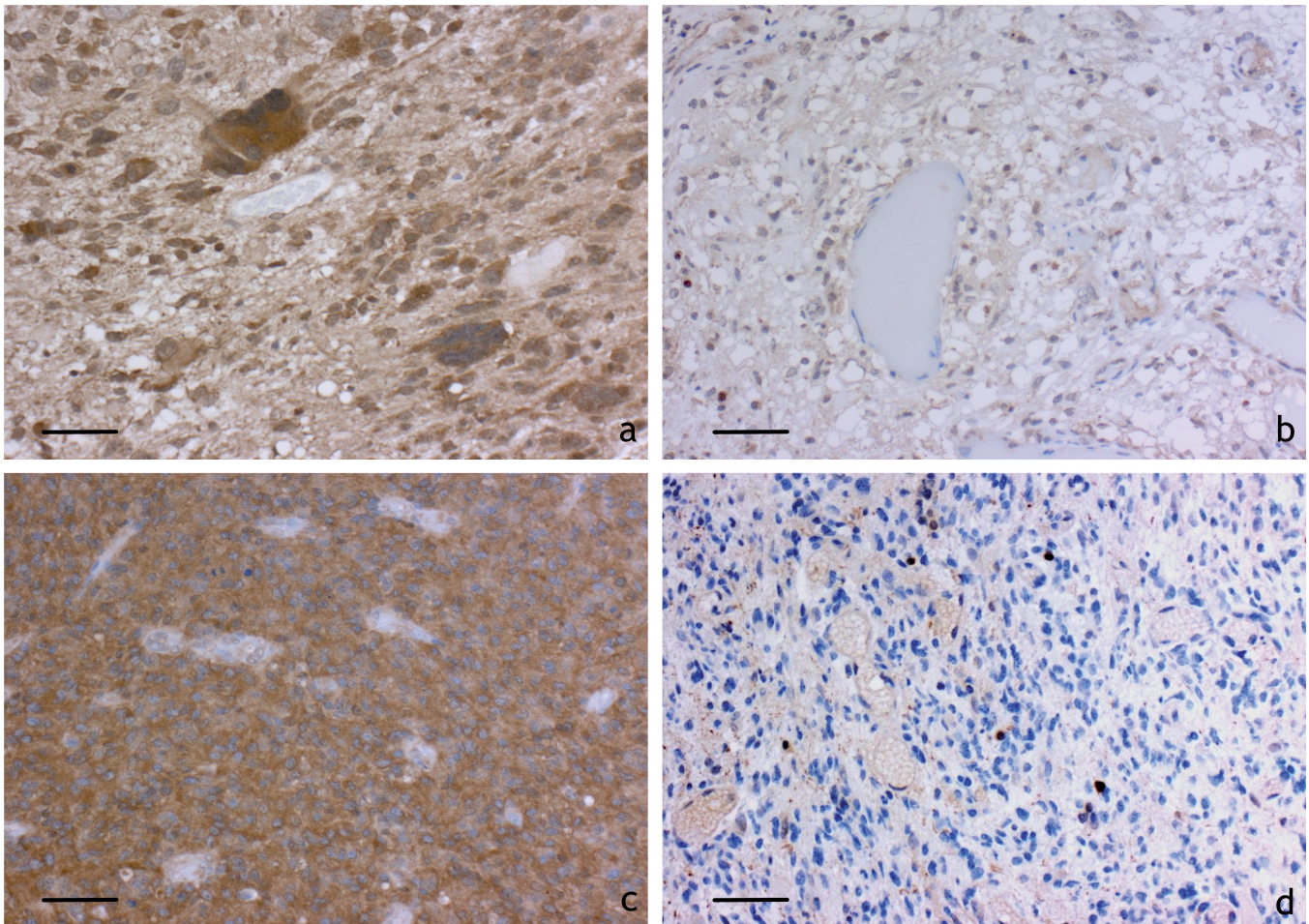
The statistical comparison of the IHC expression and WHO tumor grade revealed significant associations: both cytoplasmic and nuclear DJ1 expressions were associated with a lower grade ( $p=0.032$ ,  $p<0.001$ , respectively, chi-square test). On the contrary, the cytoplasmic NRF2 expression was higher in tumors with a higher malignancy grade ( $p<0.001$ , chi-square test), whereas there was no correlation between the nuclear NRF2 expression and grade. Also, there was a nearly significant association between the increasing SRNX1 expression and the lower grade ( $p=0.051$ , chi-square test).

When the IHC results were correlated with the typical molecular pathological features of the astrocytomas, there were no associations between the NRF2 expressions and proliferation by Ki-67/MIB-1 (neither whole material nor primary tumors), whereas

the increasing cytoplasmic and nuclear DJ1 expression was associated with a decreasing proliferation in the whole tumor material ( $p=0.025$ ,  $p=0.006$ , respectively, Kruskal-Wallis test), as well as for nuclear DJ1 in primary tumors ( $p=0.037$ , Kruskal-Wallis test). SRNX1 was not associated with proliferation. None of the studied molecules were associated with the p53 status or EGFR-CISH ( $p=n.s.$ , chi-square test). An increasing cytoplasmic NRF2 extent correlated significantly with the presence of apoptosis in the whole material ( $p=0.039$ , chi-square test, apoptosis: negative vs. positive). Importantly, IDH1 mutation was associated with increasing cytoplasmic and nuclear expression of NRF2 in primary tumors ( $p=0.011$ ,  $p=0.032$ , respectively, chi-square test) and also in the whole tumor material (cytoplasmic NRF2;  $p=0.001$ , nuclear NRF2;  $p=0.086$  near. sig). IDH1 mutation correlated significantly with an increasing nuclear DJ1 expression ( $p<0.001$ , chi-square test). When primary grade IV

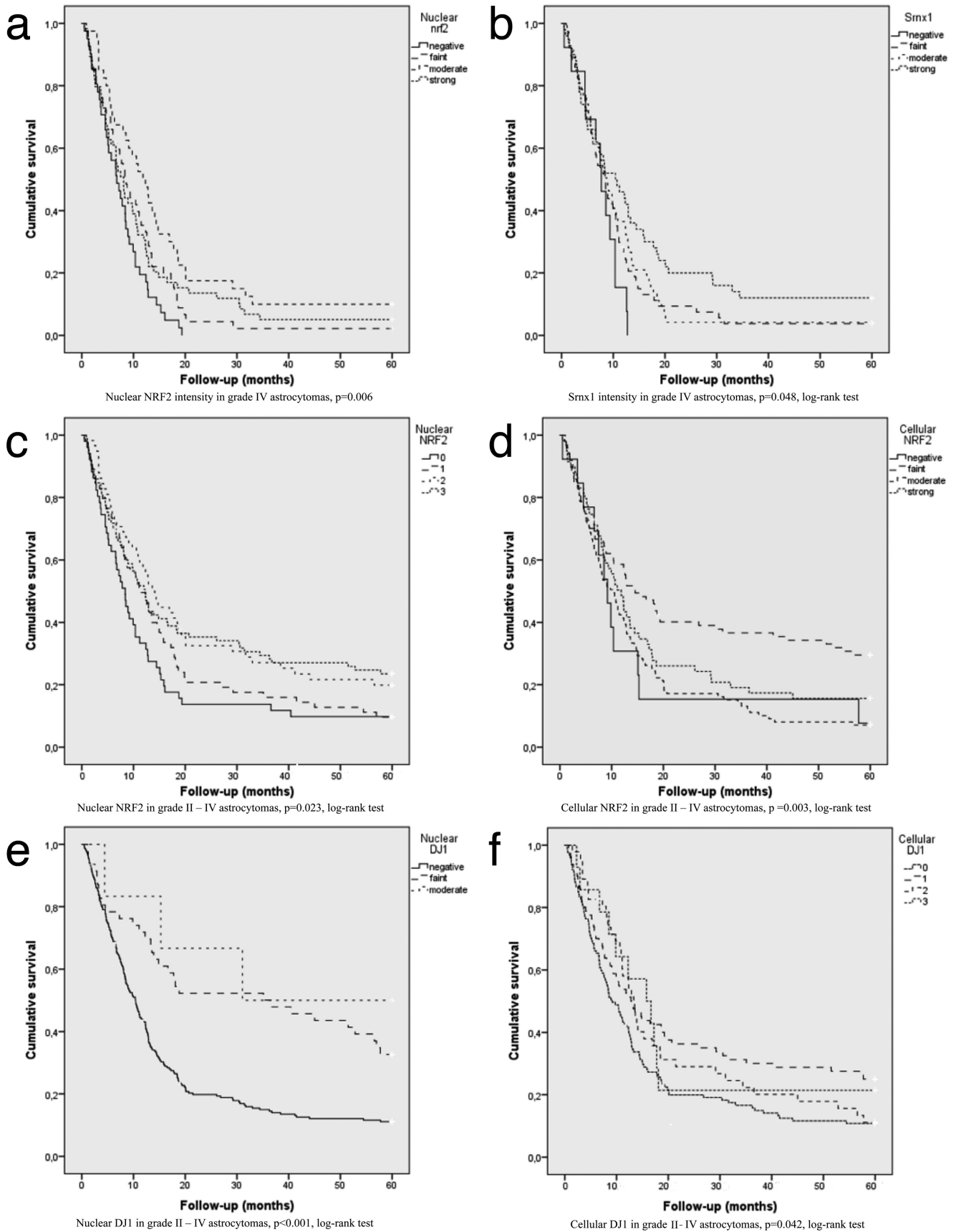
astrocytomas were compared to secondary GBMs, the nuclear DJ1 expression was associated to secondary GBMs ( $p=0.023$ , chi-square test). When all studied molecules were correlated to the HIF-1 $\alpha$  and VEGF expression, no statistical associations were found ( $p=n.s.$ , chi-square test), suggesting that the expression of these molecules is not caused by hypoxia.

We also wanted to study the associations between different anti oxidative enzymes (AOEs) and DJ1, SRNX1 and NRF2. When Prx proteins (Prx1- Prx4) were correlated, the nuclear DJ1 expression was associated to the Prx1 and Prx2 expression ( $p<0.001$ ,  $p=0.021$ , respectively, chi-square test) and cytoplasmic NRF2 was associated with Prx4 and Thioredoxin (Trx) expression ( $p=0.004$ ,  $p=0.029$ , respectively, chi-square test). The nuclear NRF2 extent correlated with Prx 4 positivity ( $p=0.026$ , chi-square test). The SRNX1 expression correlated with the Trx expression ( $p=0.013$ , chi-square test), whereas none of the studied molecules



**Fig. 1.** a. Strong cytoplasmic and moderate nuclear NRF2-staining in a giant cell glioblastoma. b. Faint nuclear but no cytoplasmic NRF2-staining in a grade II diffuse astrocytoma. c. Strong cytoplasmic but negative nuclear DJ1-staining in a grade III anaplastic astrocytoma. d. Weak nuclear but negative cytoplasmic SRNX1-staining in a glioblastoma. Scale bars: 50  $\mu$ m.

*NRF2, DJ1 and SNRX1 in astrocytic gliomas*



**Fig. 2.** Survival curves (log-rank test) for NRF2, DJ1 and SRNX1.

*NRF2, DJ1 and SNRX1 in astrocytic gliomas*

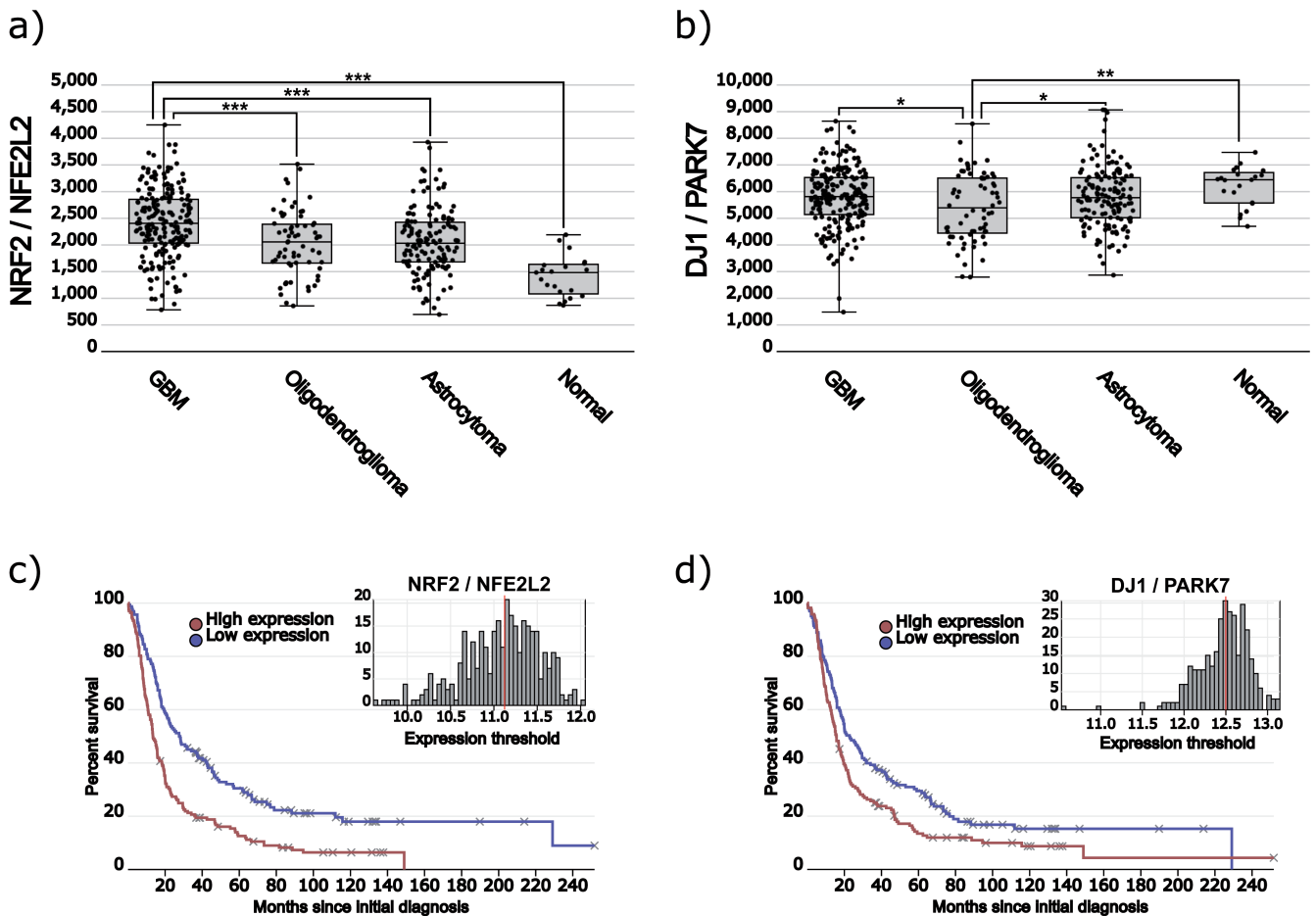
correlated with the thioredoxin reductase (TrxR). Furthermore, the cytoplasmic NRF2 expression was associated with the Prx1 and Prx4 expression ( $p=0.028$  and  $p=0.003$ , respectively, chi-square test), whereas the nuclear NRF2 positivity was associated with the Prx4 and Prx6 expression ( $p=0.018$ ,  $p=0.035$ , respectively, chi-square test).

*Survival analysis*

The overall survival data were known for 213 patients and survival was tested by means of a log-rank test in relation to IHC. Interestingly, in the univariate analysis, the expression of cytoplasmic and nuclear DJ1 predicted better patient prognosis ( $p=0.042$ ,  $p=0.001$ , respectively, log-rank test). Similar results were observed for nuclear NRF2 ( $p=0.023$ , log-rank test), whereas cases with weak cytoplasmic NRF2 staining had better prognosis than negatively, moderately or

strongly stained cases ( $p=0.003$ , respectively, log-rank test). The nuclear NRF2 expression was a predictor of better survival also among grade IV glioblastomas ( $p=0.006$ , log-rank test). SRNX1 was not associated with patient prognosis in the whole tumor material, but when grade IV tumors were analyzed separately, SRNX1 predicted better survival ( $p=0.048$ , log-rank test). The survival graphs are shown in Fig. 2.

The prognostic significance of all studied molecules was evaluated in the Cox stepwise regression analysis with the important clinicopathological factors, such as patient age in years (from 17 to 54, 55-69,  $\geq 70$ ), WHO grade (2-4), and IDH1 status (mutated or non-mutated) as explanatory factors in the analysis. Interestingly, cytoplasmic NRF2, IDH1 status, WHO grade and patient age were independent prognosticators (cytoplasmic NRF2:  $p=0.022$ , odds ratio 0.782, 95% CI 0.635-0.965; IDH1  $p<0.001$ , odds ratio 0.263, 95% CI 0.159-0.435; WHO grade  $p<0.001$ , odds ratio 1.749, 95% CI 1.247-



**Fig. 3.** a. NRF2/NFE2L2 RNA expression levels are highest in glioblastoma. The expression was analyzed from the REMBRANDT dataset. \*\*\*:  $p<0.001$ , student's t-test. b. DJ1/PARK7 RNA expression levels are lower in II-III oligodendroglioma than in other diffuse glioma subtypes. The expression was analyzed from the REMBRANDT dataset. \*\*:  $p<0.01$ , student's t-test; \*:  $p<0.05$ , student's t-test. c. High RNA expression of NRF2/NFE2L2 ( $p=$ , log-rank test) is associated with poor survival in diffuse glioma. d. High RNA expression of DJ1/PARK7 ( $p=$ , log-rank test) is associated with poor survival in diffuse glioma. The REMBRANDT dataset was used for the survival association analysis.

## NRF2, DJ1 and SNRX1 in astrocytic gliomas

2.453; patient age  $p=0.009$ , odds ratio 1.455, 95% CI 1.097-1.930).

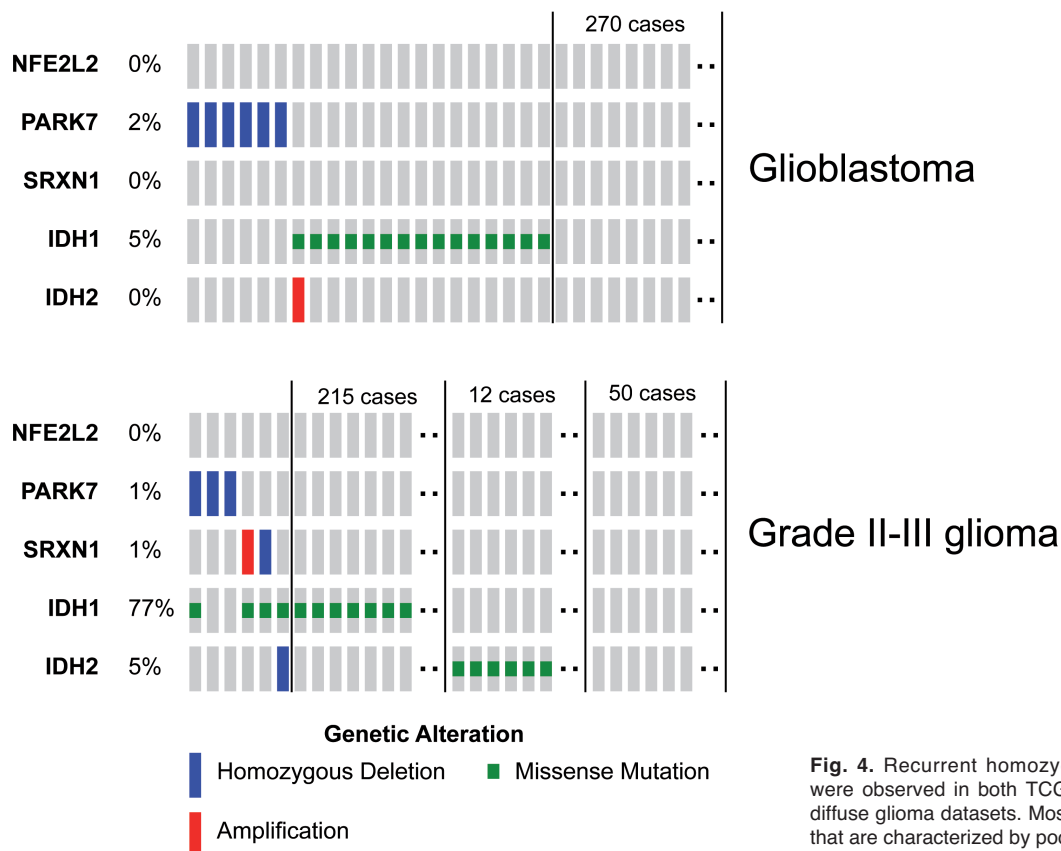
### RNA expression levels and survival analysis

In the REMBRANDT diffuse glioma dataset, NRF2 transcript levels were significantly higher in glioblastoma than in other diffuse glioma subgroups (grade II-III oligodendroglioma or grade II-III astrocytoma) ( $p<0.001$ , student's t-test) or in normal controls ( $p<0.001$ , student's t-test) (Fig. 3a). A high NRF2 RNA expression was also associated with poor prognosis in grade II-IV gliomas ( $p=3.16 \times 10^{-7}$ , log-rank test) (Fig. 3c), which is consistent with a better prognosis that was associated with weak cytoplasmic NRF2 staining in our staining cohort (Fig. 2). A similar survival association was also observed when only grade II-III oligodendrogliomas ( $p=0.020$ , log-rank test) or astrocytomas ( $p=0.014$ , log-rank test) were included into the analysis (Fig. 5a-b). No survival associations were observed among grade IV glioblastomas ( $p=n.s.$ , log-rank test) (Fig. 5c). The transcript levels of DJ1/PARK7 were lower in oligodendrogliomas than in other diffuse glioma groups ( $p<0.05$ , Student's t-test) or normal controls ( $p<0.01$ , Student's t-test) (Fig. 3b). Furthermore, a high DJ1 RNA expression was also

associated with poor prognosis in grade II-IV diffuse gliomas ( $p=0.003$ , log-rank test) (Fig. 3d) and in grade II-III oligodendrogliomas ( $p=0.044$ , log-rank test), but not among glioblastomas or grade II-III astrocytomas ( $p=n.s.$ , log-rank test) (Fig. 5d-f). SRXN1 was not included into the gene expression arrays that were used in the REMBRANDT dataset.

### DJ1 homozygously deleted in a subpopulation of patients

TCGA exome sequencing data was used to analyze genetic alterations (homozygous deletions, amplifications and missense mutations) in the NRF2/NFE2L2, DJ1/PARK7, and SRXN1 genes both in glioblastomas and in grade II-III diffuse gliomas. Recurrent homozygous DJ1 deletions were observed in a subpopulation of cases in both cohorts (Fig. 4). Eight out of nine cases were IDH wild-type and one oligodendroglioma case carried a typical IDH1 p.R132H mutation in addition to DJ1 deletion. Among the IDH wild-type samples, 2.9% of glioblastomas and 3.8% of grade II-III diffuse gliomas carried a homozygous deletion in DJ1, further suggesting its role as a tumor suppressor in diffuse gliomas. SRXN1 was homozygously deleted in one patient and amplified in another. No alterations were observed in NRF2.



**Fig. 4.** Recurrent homozygous deletions of DJ1/PARK7 were observed in both TCGA glioblastoma and grade II-III diffuse glioma datasets. Most of the cases are IDH wild-type that are characterized by poor prognosis.

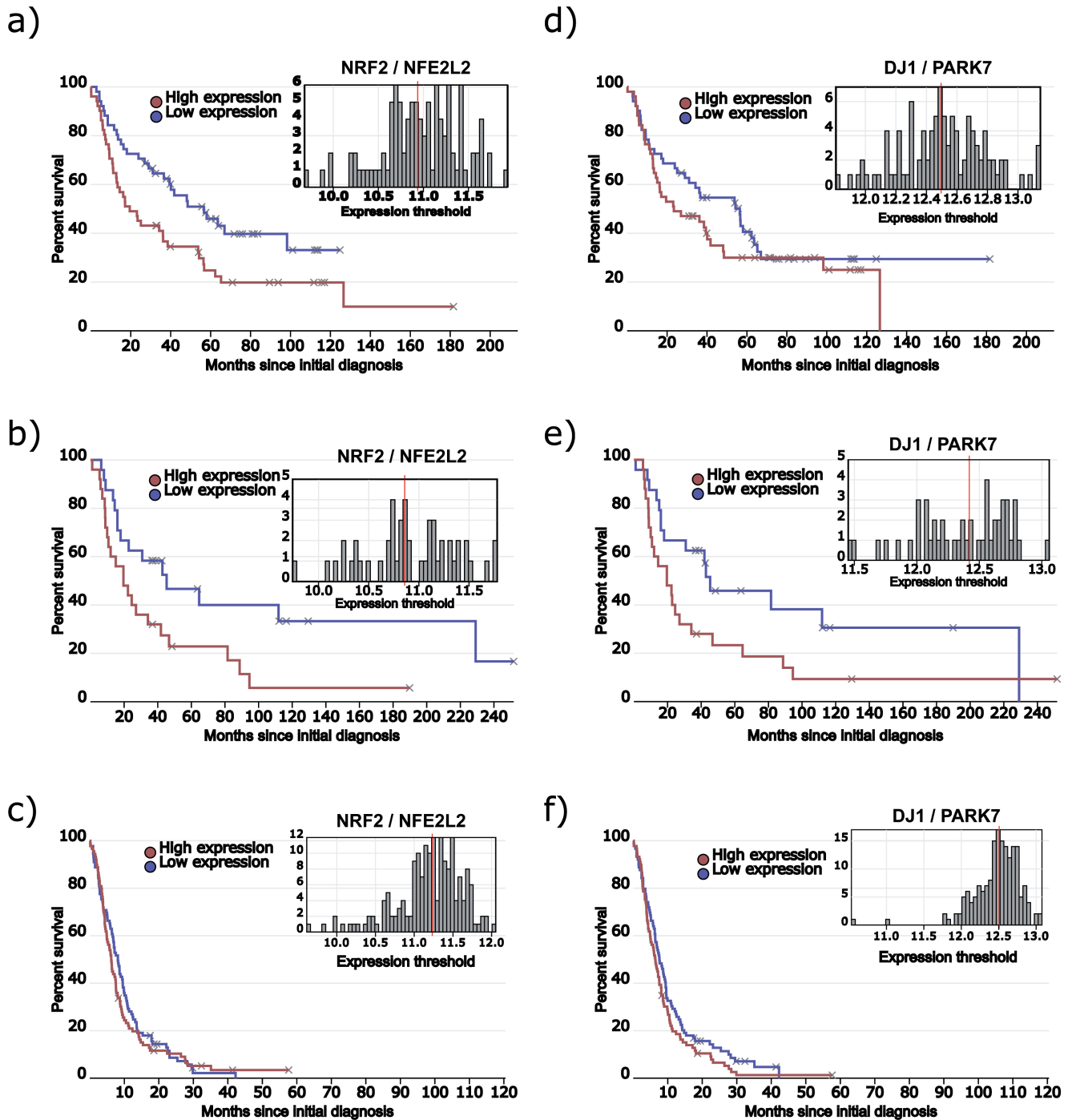


Fig. 5. Survival association analysis in grade II-III astrocytomas (a, d), grade II-III oligodendrogliomas (b, e), and grade IV glioblastomas (c, f). a. High RNA expression of NRF2 was significantly associated with poor survival in grade II-III astrocytomas ( $p=0.014$ , log-rank test). b. High RNA expression of NRF2 was significantly associated with poor survival in grade II-III oligodendrogliomas ( $p=0.020$ , log-rank test). c. NRF2 expression was not associated with survival in glioblastoma ( $p=0.42$ , log-rank test). d. DJ1 expression was not significantly associated with survival in grade II-III astrocytomas ( $p=0.26$ , log-rank test). e. High RNA expression of DJ1 was significantly associated with poor survival in grade II-III oligodendrogliomas ( $p=0.044$ , log-rank test). f. DJ1 expression was not associated with survival in glioblastoma ( $p=0.13$ , log-rank test).



## Discussion

According to our results, the NRF2, SRNX1 and DJ1 expression was common in diffusely infiltrating astrocytomas. Cytoplasmic NRF2 expression was associated with a high WHO tumor grade and poor patient prognosis. In both cytoplasmic and nuclear locations, SRNX1 and DJ1 were associated with a lower WHO grade, and furthermore, DJ1 predicted a better prognosis.

As for malignant gliomas, we show here for the first time in a large patient series, that there is a logical association in the case of the DJ1-NRF2-SRX1 axis, as proposed in a cell model and animal model studies. These reciprocal associations, and most importantly, the finding that all these molecules were associated with patient prognosis highlights their importance and special nature in the tumorigenesis of gliomas.

The complexity of antioxidant enzyme functions and associations to different cancer types has been investigated widely. Recent studies on brain tumors have reported various findings. Kanamori et al. (2015) found that anaplastic gliomas with mutated IDH1/2, NRF2 and its target genes were downregulated. However, in minor cases of IDH1/2-mutant anaplastic gliomas, with an increased expression of NRF2 target genes, the clinical outcomes were poor. They did not find a significant prognostic correlation within GBMs or the whole tumor material. Similarly, we found a correlation between the IDH1 mutation and NRF2 or DJ1, underlining the role of the IDH-mutation in the glioma tumorigenesis. The IDH mutation enhances DNA methylation and epigenetic remodeling, which stalls cell differentiation and increases malignancy (Lu et al., 2012; Turcan et al., 2012). In the experimental rat model, the IDH1 mutation decreases the proliferation of glioma cells, decreases GSH levels and increases the level of ROS in tumor cells (Shi et al., 2013). Furthermore, the IDH1 mutation sensitizes glioma cells to radiation and oxidative stress (Li et al., 2013; Mohrenz et al., 2013), which is partly the reason for a better treatment response and prognosis in IDH-mutated cases. It is possible that DJ1 and NRF2 are mediating the increased ROS-induced signaling in IDH-mutated tumors.

Ji et al. (2014) suggested that NRF2 should be considered as a critical transcription factor for controlling glioblastoma angiogenesis by HIF1- $\alpha$ . We did not find any association with NRF2 and HIF1- $\alpha$  or VEGF in our material and could thus not verify the existence of such association in vivo in malignant astrocytomas. In human ovarian cancer cells, NRF2 inhibition by shRNA has been shown to increase p53 signaling to enhance cell death in response to hydrogen peroxide treatment (You et al., 2011). However, we did not find any associations between NRF2 and the p53 status. Both hypoxia and tumor suppressor p53 play major roles in tumorigenesis. Nevertheless, the connection between p53 and the regulation of hypoxia in gliomas remains mostly unclear and requires further

studies.

Tsai et al. (2016) reported that NRF2 overexpression positively correlated with the WHO grade in gliomas and meningiomas, but no significant prognostic correlates were found, and the authors claim that this might be due to a limited number of patients. Discrepantly to our finding, Ji et al. (2013) reported that the NRF2 expression was a predictor of the poor survival of glioblastoma. It seems that the location of NRF2 immunopositivity is essential and it should be evaluated in detail (cytoplasmic vs. nuclear), which has not been done previously. Surprisingly, in our analysis, the NRF2 RNA expression and cytoplasmic protein expression predicted poor prognosis, whereas nuclear NRF2 was associated with a better prognosis. This suggests that active (e.g. nuclear) NRF2 acts as a tumor suppressor whereas the regulation of its expression is associated with tumorigenic pathways and thus worse survival. It is known that NRF2 is tightly regulated at protein level, which has a dramatic effect on its activity and downstream effects.

Miyajima et al. (2010) found a significant correlation between the nuclear DJ1 expression and longer patient survival in the series of grade II - IV astrocytomas. However, they did not find a statistical correlation between the cytoplasmic DJ1 expression and better survival presented in this study. Again, the evaluation of the IHC staining location seems to be essential in predicting patient prognosis. Furthermore, the RNA expression of DJ1 appears to have an inverse survival association, as a high expression predicted a worse outcome in diffuse gliomas. This discrepancy suggests that RNA and protein levels do not positively correlate in diffuse gliomas. It is known that RNA and protein levels do not necessarily correlate, and this might be due to different reasons, such as altered regulation of protein translation or changes in protein stability (Vogel manuscript <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3654667/>). Interestingly, the higher nuclear DJ1 expression was significantly associated with IDH1 mutation. In concordance, we found a correlation between nuclear DJ1 and secondary glioblastomas, as well as a correlation of both nuclear and cytoplasmic DJ1 expression to lower grade tumors. It seems that DJ1 prevents the harmful effects of ROS and prevents malignant progression in diffuse glioma. The tumor suppressive role of DJ1 is further supported by the fact that it is homozygously deleted in the subpopulation of diffuse glioma patients (Fig. 4), most of which do not carry any typical IDH mutations and are thus characterized by a poor prognosis. Recurrent homozygous deletions are typically a good indication of a tumor suppressor, and are often observed for example in CDKN2A/p16 gene in glioblastoma (Brennan et al., 2013).

It has been previously shown that the targets of NRF2, namely Prx1 and Prx2, predict better survival in astrocytic gliomas (Järvelä et al., 2010). These results are consistent with our results that associated better

patient prognosis with high nuclear NRF2 protein staining. Interestingly, Prx II expression levels correlated with resistance to radiation therapy or certain anti-cancer drugs in gliomas (Smith-Pearson et al., 2008). Overall, in our material, no associations were found between the patient cohorts with different treatment modalities (radiotherapy and/or cytostatic drugs) and NRF2, SRN1 or DJ1 expression (data not shown). However, the tumor material consisted of patients treated before the temozolomide era, thus it is not possible to assess the molecular biology with modern cytostatic drugs in this study. In addition, the results presented here indicate that a tentative attitude to clinical conclusions should be maintained when interpreting the results from genetic and experimental studies. Clinical trials are needed to evaluate the NRF2 functions in detail.

As a conclusion, NRF2, DJ1 and SRNX1 are commonly expressed in diffusely infiltrating astrocytomas and they can be used in predicting patient prognosis, although their biology is complex and still poorly understood. Their expression may be related to the special nature of gliomas as tumors with no evident exogenous factors in carcinogenesis and the abundance of IDH1 mutations affecting the ROS balance in the tumor cells. Moreover, NRF2 immunohistochemistry should be evaluated in detail, separating the cellular and nuclear immunopositivity. Further trials are required to determine their role in clinical setting, e.g. in development of therapeutic interventions.

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