The association between NRF2 transcriptional gene dysregulation and IDH mutation in Grade 4 astrocytoma

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Abstract

Objectives: Nuclear factor erythroid-2-related factor 2 (NRF2), a transcriptional gene factor related to nuclear factor erythroid 2, plays a role in the development of gliomas with isocitrate dehydrogenase (IDH) mutation. Its impact on tumour recurrence has seldom been investigated.

Methods: A group of 34 patients diagnosed with Grade 4 astrocytoma was included in a retrospective cohort. NRF2 protein and gene expressions were assessed using different profiling assays. The association between IDH mutation, NRF2 expression, and tumor recurrence was investigated.

Results: The mean patients’ age in this study was 50 years. Out of the total number of tumors analyzed, 21 of them had IDH mutation. NRF2 protein expression was found to be overexpressed in 27 tumors and reduced in 7 tumors. Additionally, NRF2 gene was upregulated in 24 tumors and downregulated in 10 tumors. Insignificant statistical difference was observed in recurrence-free interval (RFI) between patients with upregulated or downregulated NRF2 gene or protein expressions (p-value>0.05). However, this relationship is distinctive when NRF2 expression was compared to IDH mutation. Tumors with different levels of NRF2 expressions and IDH mutations showed significant statistical variation in RFI (p-value=0.001). There was insignificant impact on RFI among patients receiving different chemotherapies (TMZ or TMZ plus) who had abnormal NRF2 gene activities (p-value=0.97).

Conclusions: WHO-Grade 4 astrocytoma with IDH mutation and altered NRF2 expression showed a delayed tumor recurrence compared to IDH-wildtype glioblastoma.

Keywords: Grade 4 astrocytoma; glioblastoma; NRF2; IDH; recurrence

Introduction

The most severe form of primary malignant brain tumor in adults is Grade-4 astrocytoma. Despite decades of treatment options available after surgical resection, the overall survival (OS) remains unchanged [1]. Thus, many biomarkers have been investigated to prevent disease progression [2]. Although various genetic and epigenetic changes have been linked to Grade-4 astrocytoma, exploring a key mechanism for treatment resistance continues to be a difficult therapeutic hurdle. As other methods have yielded inconclusive outcomes, the analysis of gene expression has become crucial in formulating effective treatment plans for malignant brain tumors [3].

Nuclear Factor Erythroid 2-Related Factor 2 (NRF2) is a transcriptional gene that has an anti-apoptotic,
anti-oxidative and anti-cancer effects due to its interaction with different signaling regulatory pathways [4]. NRF2 is inactive in normally oxygenated environment; however, it migrates to the nucleus during oxidative stress to promote hemostasis [5]. NRF2 has the ability to counteract oxidative stress by facilitating the release of enzymes that are associated with nicotinamide adenine dinucleotide phosphate (NADPH), including isocitrate dehydrogenase (IDH) and other metabolic regulators. This pathway leads to the production of NADPH as a way to address oxidative stress (Figure 1) [6].

Based on the old reports, tumour degree was found to be associated with NRF2 activity. Nonetheless, patients with benign tumor do not exhibit elevated NRF2 levels, whereas the levels of NRF2 are variably regulated in patients with high-grade tumours such as glioblastoma [5–9]. Although this finding has not been broadly and specifically correlated with IDH mutation, it has been observed that some gliomas with IDH mutations demonstrate atypical NRF2 expression [7–9].

Because NRF2 plays a critical role in cell cycle regulation, its pathways have been identified as potential targets for treating high-grade gliomas. Multiple studies are currently targeting NRF2 molecular pathways to overcome treatment resistance, inhibit the proliferation of glioma cells, and promote apoptosis [10]. NRF2 has a dual characteristic that has been identified, which brings up some concerns when examining it as a potential treatment option [11]. Upon detection of harmful substances, the cell initiates the activation of NRF2, which subsequently transcribes defensive genes and regulates cytoprotective mechanisms. The cell will keep producing NRF2 until it reaches a certain threshold. Once the threshold is crossed, the role of NRF2 shifts from being cytoprotective to promoting apoptosis. For this reason, NRF2 overexpression has been observed to safeguard against antitumor therapies in glioblastoma, while suppressing NRF2 could impede the progression of the disease [9].

NRF2 expression aids glioma cell growth by up-regulating Hypoxia Inducible Factor-1α (HIF-1α) and Vascular Endothelial Growth Factor (VEGF) [12]. X Ji et al., discovered that NRF2 signaling is linked to HIF-1α in glioma angiogenesis [13]. Additionally, it was found that inhibiting NRF2 in gliomas can decrease the activation of HIF-1α and VEGF signaling, possibly by reducing oxygen consumption [13, 14]. Similarly, NRF2 gene downregulation was shown to increase glioblastoma sensitivity to chemotherapeutic agents [4]. It also regulates the expression of antioxidant enzymes for tumor cells against oxidative stressors such as chemotherapeutic drugs and radiation [15]. Zhang et al., has demonstrated that the combination of Temozolomide (TMZ) and radiotherapy has been shown to induce resistance via the NRF2 pathway. Consequently, the inhibition of NRF2 activity can potentially restore the effectiveness of therapy [16]. Although there is no concrete evidence to support the efficacy of the compounds currently being tested in clinical trials, there is a potential for combination therapies targeting several NRF2-mediated pathways. The molecular interactions associated with NRF2 in malignant gliomas are still being actively investigated and multiple new compounds have been identified.

The aims of our study are to examine the correlation between NRF2 gene expression and IDH mutation and the impact of this association on tumour recurrence in Grade 4 astrocytoma.

Materials and methods

Selection of patients

This study has been granted approval by a combined ethical agreement between King Faisal Specialist Hospital and King Abdulaziz University (agreement # CA-2020-06), to utilize patient tissue for our study. The study involved a cohort of 34 patients who were diagnosed with Grade 4 astrocytoma following gross resection (Table 1). The pathological diagnosis followed the 5th edition of WHO classification of Central Nervous System (CNS) tumours [1, 17].

Radiation was given to all patients and TMZ was given only to 29 patients at a rate of 150–200 mg/m² up to 12 cycles (Table 1) [18]. All patients enrolled in this project are unfortunately deceased. The data collected from the patients encompassed their age, gender, IDH mutation status, treatment history, and Recurrence-Free Interval (RFI). RFI was determined by estimating the period from the date of gross tumor resection to the first day of tumor recurrence.

Tissue processing

A 4-μm section from each 34 formalin-fixed paraffin-embedding (FFPE) tissue block was obtained for expression assays. The thick section slides were employed for the protein expression, utilizing Immunohistochemistry (IHC) with an anti-NRF2 antibody. On the other hand, the tissue rolls were used in RNA extraction to assess NRF2 gene expression using Real Time-Polymerase Chain Reaction (RT-qPCR).

Anti-NRF2 protein expression assay

Anti-NRF2 antibody (Rabbit polyclonal, Elabscience, Cat# E-AB-64023, Houston, Texas, USA) was targeted on 34 tissue sections using IHC assay. The Ultra-View detection Kit from Ventana (Tucson, AZ, USA) was employed on automated auto-stainer for IHC processing. The protocol involved deparaffinization using EZ Prep and followed by heat pre-treatment. Subsequently, the antibody was adjusted to a dilution of 1:100 and incubated for 20 min at 75°C. The tumor
Figure 1: A schematic diagram illustrating NRF2 pathway during oxidative stress condition, and how it influences WHO-Grade 4 astrocytoma. It also shows the potential therapeutic targets. NRF2 is a protein that is regulated by KEAP1 to maintain its degradation. When there is oxidative stress, KEAP1 is uncoupled from NRF2, allowing NRF2 to move to the nucleus and bind to other proteins to form a complex. This complex regulates the expression of genes involved in antioxidation and metabolism. Mutations in the NRF2 gene can affect this process. KEAP1, Kelch-like ECH-associated protein 1; ROS, reactive oxygen species.
Table 1: The biological data of 34 tumours enrolled in our study.

<table>
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<th>IHC grading</th>
<th>NRF2 Ct</th>
<th>NRF2 RE</th>
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IDH, isocitrate dehydrogenase; IHC, immunohistochemistry; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NRF2 nuclear factor erythroid 2-related factor 2; RE, relative expression; CTX, chemotherapy; RFI: recurrence free-interval.

Microenvironment was assessed for the presence of tumor cells (TC) using anti-NRF2 antibody. Tissue sections were initially examined at ×10 magnification to identify a focal living area exhibiting anti-NRF2 expression. Subsequently, this area was further examined at ×25 magnification. Cells displaying anti-NRF2 expression were classified as NRF2 positive, while the total cell population demonstrated both NRF2-expressing cells and non-stained cells. The labelling index (LI) of NRF2 expression in each selected area was assessed using the following equation:

Labelling index (%) = \( \frac{\text{NRF2} + \text{TC}}{\text{Total cells}} \times 100 \)

Three expression arrays were estimated: Grade [0]: <10% expression; Grade [I]: 10–50% expression; Grade [II]: >50% expression (Figure 2). Expression with Grade [0] and [I] was categorized as “low expression, while expression with Grade [II] was categorized as “high expression”.

RNA extraction and cDNA synthesis

RNA was isolated from a total of 34 tumor samples and two control samples using the RNeasy FFPE Kit (QIAGEN). Each FFPE sample was transferred to RNase-free Eppendorf tube after being mixed with m-Xylene for deparaffinization. The samples were then centrifuged to collect the capsule, followed by a wash with ethanol and another round of centrifugation. The ethanol was subsequently evaporated in a 37°C dry bath and all samples were incubated for 15 min followed by a short cooling. A mixture of Buffer RBC and the sample was vortexed, and then 100% ethanol was added and mixed was then centrifuged. The eluates-containing RNA was used for spectrophotometric analysis. The High-Capacity cDNA Reverse Transcription Kit (Applied Biosystem™, Waltham, MA, USA) was utilized for cDNA synthesis. A master mix was prepared by a mixture of Reverse Transcriptase (RT) Buffer, dNTP Mix, Random Primers, and MultiScribe™ RT. This mixture was then combined with 80 ng of RNA, and the final volume was adjusted to 10 µL.
Gene expression profiling using RT-qPCR

The primers for NRF2 gene and one reference gene, Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH), were designed. The following primer sequences for NRF2 have been used in the processing: forward 5′-TCCATTCCTGAGTTACAGTGTCT-3′, Reverse 5′-CATGGTAGTCTCAACAGC-3′. The RT-qPCR analysis was conducted using the EverGreen Master Mix (Haven Scientific, KAUST, KSA), following the manufacturer’s instructions in triplicate reactions. In brief, the previously synthesized cDNAs were combined with the master mix and enough amount of oligo in order to achieve optimum PCR reactions. The reactions were performed in semi-skirted plates which were preserved with the RT-qPCR Adhesive Seal (Haven Scientific, KAUST, KSA). An initial denaturation step at 95°C for 3 min was performed and followed by 40 cycles of denaturation for 15 seconds and 60 s annealing process.

Two replicates of threshold cycle (C_T) values were used for both the targeting gene and the reference gene. The mean C_T and standard deviation for the reference gene (GAPDH) and the target gene (NRF2) were calculated from the RT-PCR data. Relative quantification (RQ), fold change (FC) and ΔC_T values were also assessed for each sample. The results are summarized in Table 1.

Statistical analysis

To assess the correlation between NRF2 gene and protein expression, a sensitivity and specificity analysis was conducted using the McNemar test. The distribution of RFI in relation to other variables was compared using Kaplan-Meier curves (KMC). Fisher’s Exact test was also employed to investigate the association between NRF2 gene expression and IDH mutation. Statistical significance was determined at a p-value of less than 0.05.

Results

The average age of the patients was 50 years (SD: 17.3). IDH was mutant in 21 tumors. NRF2 protein was overexpressed in 27 (79.4 %) tumors and reduced in 7 (20.6 %) tumors. NRF2 gene expression was upregulated in 24 (70.6 %) tumors and downregulated in 10 (29.4 %) tumors. Tumors with low levels of NRF2 protein expression as detected through IHC showed NRF2 gene downregulation in RT-qPCR. Conversely, 24 tumors with high levels of NRF2 expression as detected by IHC demonstrated NRF2 gene upregulation in RT-qPCR. The specificity and sensitivity of protein expression to gene expression were 100 and 70 %, respectively. The overall diagnostic accuracy between the two testing methods was 91 % (Figure 3).

There was no significant statistical distinction observed in RFI between tumors exhibiting upregulated or downregulated NRF2 gene or protein expressions (p-value >0.05) (Figure 4a and b). This relationship is distinctive when NRF2 expressions were compared to IDH mutation. There was a significant correlation found between abnormal NRF2 gene expression and IDH mutation. Tumors with different levels of NRF2 gene expression and IDH mutations showed significant statistical variation in RFI (p-value=0.001) (Figure 5a). Out of the 14 IDH-mutant tumors with upregulated NRF2, 10 were wildtype for IDH. Nonetheless, IDH-mutant tumors with either upregulated or downregulated NRF2 expressions exhibited a delayed tumor recurrence compared to tumors with IDH wildtype, regardless of NRF2 abnormality, highlighting the greater impact of IDH mutation on RFI. Furthermore, there was no significant statistical impact observed on patients receiving TMZ or TMZ and other chemotherapeutic agents with abnormal NRF2 gene activities (p-value=0.97) (Figure 5b). However, an interesting finding was noted among patients with NRF2 downregulation who received TMZ treatment, as they experienced late tumor recurrence.

Discussion

The activation of NRF2 gene signaling pathways provides a novel therapeutic strategy in many cancers including brain.
Figure 3: Matching consistency of NRF2 expression between IHC and RT-PCR.

Figure 4: (a and b) The impact of NRF2 gene and protein expressions on RFI. RFI, Recurrence-free interval.
tumours. NRF2 has been found to be upregulated in malignant astrocytoma, mainly Grade 4 type, which can contribute to uncontrolled tumour growth and resistance to chemotherapy. By promoting antioxidant defense, reducing inflammation, and enhancing DNA repair, NRF2 inhibition could potentially help to overcome the resistance of malignant astrocytoma to conventional treatments. Currently, the exact process that causes NRF2 upregulation in malignant astrocytoma remains unclear. However, one of the commonly linked factors is IDH mutation. Studies indicate that IDH mutation significantly impacts the regulation of the NRF2 gene, suppressing its expression [4]. This can cause less cellular oxidative stress [9, 15].

While NRF2 is frequently present in IDH-mutant WHO-Grade 4 astrocytoma, the association between these two factors has not been scientifically considered as a cause for delayed tumor recurrence. Our study did not reveal a substantial correlation between IDH mutation and NRF2 upregulation. Out of the 14 IDH-mutant tumors with upregulated NRF2, 10 cases with upregulated NRF2 expression were IDH wildtype. Nonetheless, IDH mutation with upregulated or downregulated NRF2 exhibited a delayed tumor recurrence compared to IDH-wildtype tumors. This indicates that the IDH drivers could potentially halt NRF2 activity through unknown mechanisms.

Two studies have shown that IDH1 and IDH2 mutations lead to increased levels of reactive oxygen species (ROS), which in turn activate NRF2 [19, 20]. IDH-mutant gliomas were shown to increase levels of NRF2 and their downstream targets. These findings suggest that there is a link
between IDH mutation and NRF2 upregulation, which could have important implications for cancer treatment [19]. Tonelli et al. found that IDH-mutant gliomas cause excessive release of Nicotinamide Adenine Dinucleotide Phosphate (NADP) oxidative coenzyme which in turn uncouples NRF2 inside the nucleus [20]. Keap1, a component of the E3-ubiquitin ligase complex, binds to NRF2 and keeps it continually ubiquitinated and degraded. However, when the body is under stress, Keap1’s cysteine residues get oxidized by ROS, causing it to separate from NRF2. NRF2 can then travel to the nucleus and forms a complex with musculoaponeurotic fibrosarcoma (Maf) oncogene homolog known as the NRF2-Maf complex. This complex binds to antioxidant response elements and helps in regulating cellular processes during stress (Figure 1) [20].

Because NRF2 and IDH both share similar chromosomal location, their functions are concurrently related. The tricarboxylic acid cycle (TCA cycle) is a center for cellular energy production and the precursor to metabolic pathways. Hence, NRF2 regulates TCA cycle genes including IDH (Reactome pathway ID: R-HSA-9755514.4) which cause excessive production of D2-hydroxyglutarate (D2-HG). Elevated levels of D2-HG are found in IDH mutant tumors as shown in (Figure 6). The mechanism by which the NRF2 protein is involved with other related mutant genes to develop glioma is described in the Supplementary file.

Several drugs have been found to affect NRF2 function either directly or indirectly. PDI inhibitor, corilagin, valproic acid, melatonin, and trans-retinoic acids are examples of drugs that indirectly impact NRF2 signaling in TMZ-resistant glioblastoma by inducing apoptosis and inhibiting its activity [10, 21, 22]. In our study, patients with NRF2 downregulation who had TMZ treatment experienced delayed tumor recurrence. Nevertheless, there was an insignificant impact on tumor recurrence among patients receiving TMZ and TMZ plus, regardless NRF2 gene alteration. Studies have shown that the use of a compound similar to sphingosine-1-phosphate can decrease the presence of NRF2 in glioblastoma cells, thus increasing their sensitivity to TMZ. Another method of impeding tumor growth is by targeting vascular and growth factor receptors. By controlling NRF2 expression, angiogenesis can be reduced, and tumor growth becomes inhibited [23].

Although the sample size in our study was limited, it is crucial to recognize that our research is the initial endeavor to establish a connection between NRF2, IDH, and tumor recurrence, and explore the underlying mechanism.

**Conclusions**

Our study concluded that WHO-Grade 4 astrocytoma with IDH mutation and altered NRF2 expression exhibits a delayed tumor recurrence compared to IDH-wildtype glioblastoma. This explains that the IDH driver could potentially halt NRF2 activity through unknown mechanisms. It also indicates that NRF2 is not a direct prognostic indicator.

**Acknowledgments:** Special thanks to Deanship of Scientific Research at King Abdulaziz University for their support.

**Research ethics:** The local Institutional Review Board deemed the study exempt from review. This study was performed in line with the principles of the Declaration of
Helsinki. The approval was granted by Biomedical Ethics Committee at King Faisal Specialist Hospital and Research Center [CA-2020-06] and King Abdulaziz University [HA-02-J-008] to authorize using Patient Samples in Research.

Informed consent: Informed consent was obtained from all individual participants included in the study.

Author contributions: All authors have accepted responsibility for the entire content of this manuscript and approved its submission.

Competing interests: Authors state no conflict of interest.

Research funding: No funding.

Data availability: The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

References


Supplementary Material: This article contains supplementary material (https://doi.org/10.1515/oncologie-2023-0262).