Targeting the NF-E2-related factor 2 pathway: A novel strategy for glioblastoma (Review)

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Abstract. Glioblastoma is the most common and malignant subtype among all brain tumors. Nuclear factor erythroid 2-related factor 2 (Nrf2) is an essential component of cellular defense against a variety of endogenous and exogenous stresses. A marked increase in research over the past few decades focusing on Nrf2 and its role in regulating glioblastoma has revealed the potential value of Nrf2 in the treatment of glioblastoma. In the present review, we discuss a novel framework of Nrf2 in the regulation of glioblastoma and the mechanisms regarding the downregulation of Nrf2 in treating glioblastoma. The candidate mechanisms include direct and indirect means. Direct mechanisms target tumor molecular pathways in order to overcome resistance to chemotherapy and radiotherapy, to inhibit proliferation, to block invasion and migration, to induce apoptosis, to promote differentiation, to enhance autophagy and to target glioblastoma stem cells. Indirect mechanisms target the reaction between glioblastoma cells and the surrounding microenvironment. Overall, the value of the Nrf2 pathway in glioblastoma provides a promising opportunity for new approaches by which to treat glioblastoma.

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1. Introduction

Glioma is one type of brain tumor that arises from glial cells and accounts for over 30% of all primary central nervous system tumors in the USA (1). Glioblastoma is the most common and malignant subtype of glioma, which is categorized as grade IV according to the classification of the World Health Organization (WHO). The median survival time of glioblastoma patients is approximately 14 months, in spite of aggressive surgery, radiation and chemotherapy (2).

Nuclear factor erythroid 2-related factor 2 (Nrf2) belongs to a subset of basic leucine-zipper (bZip) genes sharing a conserved structural domain (3). It is broadly expressed in tissues and can be activated in response to a range of oxidative and electrophilic stimulation. The activity of Nrf2 is primarily regulated by its inhibitor Kelch-like ECH-associated protein 1 (Keap1) (4). When uncoupled from the Nrf2/Keap1 complex, Nrf2 is transported into the nucleus and modulates the expression of antioxidant genes through interaction with the antioxidant response element (ARE) (5). An increasing body of literature has revealed alternative mechanisms of Nrf2 activation, including phosphorylation of Nrf2 by various protein kinases, interaction with other protein partners (p21, caveolin-1) and epigenetic factors (microRNA-144, -28 and -200a and promoter methylation) (6).

Recently, Nrf2 has been demonstrated as an important regulator in different types of cancer. A dramatic increase in research focusing on Nrf2 and the associated mechanisms in the regulation of primary malignant brain tumors such as glioblastoma has been carried out. High expression of Nrf2 in glioblastoma was found to protect it from the killing effects of antitumor therapies, and blocking of Nrf2 can inhibit glioblastoma. Thus, Nrf2 is a potential new target with which to treat glioblastoma. The mechanisms of the downregulation of Nrf2 in treating glioblastoma contain two main aspects: direct and indirect means. Direct mechanisms target tumor molecular pathways to overcome resistance to chemotherapy and radiotherapy, to inhibit proliferation, to block invasion and migration, to induce apoptosis, to promote differentiation, to enhance autophagy and to target glioblastoma stem cells (GSCs). Indirect mechanisms target the reaction between glioblastoma cells and the surrounding microenvironment, such as the perivascular, hypoxic and immune microenvironments.
In the present study, we review the function of Nrf2 in the regulation of glioblastoma, and the associated mechanisms concerning the downregulation of Nrf2 in treating glioblastoma.

2. Direct mechanisms (Table I)

**Overcoming resistance to chemotherapy and radiotherapy.**
Standard treatment of glioblastoma currently involves chemotherapy and radiotherapy. However, glioblastoma can easily develop resistance to chemotherapy and radiotherapy. It has been found that high expression of Nrf2 decreases the sensitivity of glioblastoma cells to chemotherapy and radiotherapy.

**Chemotherapy.** There are a variety of tumors that develop strong tolerance to chemotherapy, including glioblastoma (7). Recently, the role of Nrf2 in inducing chemotherapy resistance has been reported in several types of tumors (8). In glioblastoma, Nrf2 expression was found to be increased during drug resistance (8). Temozolomide (TMZ) is an alkylating agent which is commonly used for the treatment of glioblastoma (9-11). TMZ treatment was found to induce Nrf2 activation in the glioblastoma cell line U251 and downregulation of Nrf2 expression increased TMZ-induced cell death in U251 cells (12). In addition, the silencing of Nrf2 also increased cell necrosis induced by 5-fluorouracil (5-FU), cisplatin, etoposide (13-15), oxaliplatin (16) and doxorubicin (ADM) (17,18). Blocking Nrf2 activation is a potential method for enhancing chemotherapy sensitivity of glioblastoma cells (19).

Nrf2 may induce the chemoresistance of glioblastoma through stress response and a drug efflux mechanism (Fig. 1). The stress response mechanism implies that Nrf2 transcription upregulates endogenous phase II detoxifying enzymes, which may inactivate antitumor drugs by modifying their structures (20). In addition, activation of Nrf2 was also found to contribute to drug efflux pathways (21). ATP-binding cassette, subfamily G, member 2 (ABCG2) plays a crucial role in the efflux of xenobiotics and drugs, and Nrf2-mediated regulation of ABCG2 was found to increase the efflux of antitumor drugs and decrease the effect of chemotherapy (21). However, research suggests that Nrf2 is not an independent molecule in chemoresistance. The possible role of peroxiredoxin1 (Prx1) co-functioning with Nrf2 in chemoresistance has been suggested (22).

**Radiotherapy.** Radiotherapy is the foundation of therapy following maximal surgical resection of glioblastoma (23,24). However, glioblastoma displays high resistance to radiotherapy (25). Low-dose radiation induces Nrf2 activation reactively (12). The role of Nrf2 in radioresistance has been investigated. Using a genetically modified method to establish continuous activation of Nrf2, Nrf2 was found to protect glioblastoma against ionizing radiation toxicity, and Nrf2-inhibited tumor cells showed increased sensitivity to γ-irradiation (26).

The Nrf2/ARE pathway regulates the radioresistance of glioblastoma by modifying endogenous Nrf2 inhibitor and by upregulating the downstream signal of Nrf2 (27). Radioresistance may involve the loss-of-function mutations of

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**Table I. Direct mechanisms of the downregulation of Nrf2 in the treatment of glioblastoma.**

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<tr>
<th>Mechanism</th>
<th>Factors</th>
<th>Associated molecules</th>
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<td>Stress response mechanisms</td>
<td>Phase II detoxifying enzymes</td>
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<td>Overcoming resistance to radiotherapy</td>
<td>Drug efflux mechanisms</td>
<td>ABCG2</td>
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<td>Inhibiting proliferation</td>
<td>Endogenous Nrf2 inhibitors</td>
<td>Keap1</td>
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<td>Downstream molecules</td>
<td>HO-1</td>
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<tr>
<td>Blocking invasion and migration</td>
<td>Stress response mechanisms</td>
<td>HO-1</td>
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<td>Inducing apoptosis</td>
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<td>HO-1</td>
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<td>Promoting differentiation</td>
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<td>HO-1</td>
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<td>Enhancing autophagy</td>
<td>Downstream molecules</td>
<td>HO-1</td>
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<td></td>
<td>Matrix metalloproteinases</td>
<td>Keap1, GPx2, CXCR3-B</td>
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<td>Targeting glioma stem cells</td>
<td>Matrix metalloproteinases</td>
<td>HO-1</td>
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<td></td>
<td>Endoplasmic reticulum stress</td>
<td>Keap1, p62, LC3</td>
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<td></td>
<td>Downstream molecules</td>
<td>UPR</td>
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<td></td>
<td>Cross-linking</td>
<td>Bcl2, p53, MAPK, NF-xB</td>
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<td>Anti-redox molecules</td>
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<td></td>
<td>P62/SQSTM1 system</td>
<td>Keap1, p62, LC3</td>
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<td></td>
<td>Endoplasmic reticulum stress</td>
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<td></td>
<td>Downstream molecules</td>
<td>HO-1</td>
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<tr>
<td></td>
<td>Curcumin</td>
<td>EGFR, Ki-67, Kras, PI3K/Akt</td>
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<tr>
<td></td>
<td>Circulating cell-free DNA</td>
<td>HO-1</td>
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<td>HO-1</td>
<td>HO-1</td>
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</tbody>
</table>

Nrf2, nuclear factor erythroid 2-related factor 2; ABCG2, ATP-binding cassette, subfamily G, member 2; Keap1, Kelch-like ECH-associated protein 1; HO-1, heme oxygenase-1; GPx2, glutathione peroxidase-2; EGFR, epidermal growth factor receptor; MMP-9, matrix metalloproteinase 9; Bcl2, B-cell lymphoma 2; MAPK, p38/mitogen-activated protein kinase; NF-xB, nuclear factor-xB; SQSTM1, sequestosome 1; UPR, unfolded protein response; cirDNA, circulating cell-free DNA.
the Nrf2 inhibitor Keap1, which allows Nrf2 to be continuously transported to the nucleus (28). Other research has demonstrated that Nrf2 induces radioresistance by regulating the function of the major downstream molecule heme oxygenase-1 (HO-1) (29). Downstream activation of Nrf2-ARE-dependent HO-1 was found to be important in the maintenance of resistance to irradiation (12).

Inhibition of proliferation. Glioblastoma cells usually maintain a high rate of proliferation. High expression of Nrf2 gives glioblastoma an advantage for growth, and knockdown of Nrf2 was found to inhibit the proliferation and growth of human glioblastoma cells (20,30,31).

The candidate mechanisms of Nrf2 in the regulation of proliferation mainly include three means: i) upregulation of downstream molecules of Nrf2; ii) cross-talk with other signaling pathways; iii) post-transcriptional regulation. Nrf2 can induce the growth of tumor cells by increasing the expression of HO-1, glutathione peroxidase-2 (GPx2) (32,33) and CXCR3-B (34), which are downstream molecules of Nrf2 and are important in the regulation of the growth and proliferation of glioblastoma. The growth rate of cancer cells is inhibited by downregulation of these molecules. Nrf2 is also involved in regulating a variety of other signal transduction pathways. Recently, studies have demonstrated that Nrf2 can enhance cell proliferation by regulating epidermal growth factor receptor (EGFR), Ki-67, Kras, and phosphoinositide-3-kinase (PI3K)/Akt pathway, which are necessary for maintaining the proliferation of glioblastoma (35-38). Finally, Nrf2 may improve the accumulation of various proliferation-related proteins by regulating the associated small interfering RNA fraction. Recent studies have identified several microRNAs (miRs) as post-translational targets of Nrf2 to regulate proliferation. Studies have shown that NADPH and ribose are essential for the cell proliferation in tumors (39,40), and loss of Nrf2 was found to decrease the expression of the redox-sensitive histone deacetylase HDAC4, resulting in increased expression of miR-1, miR-200a and miR-206, which markedly impaired NADPH production and ribose synthesis (41,42).

Blocking of invasion and migration. Glioblastoma can easily invade and migrate to surrounding brain tissue. Nrf2 may facilitate the remodeling of the tumor microenvironment making it advantageous for the autonomic invasion and migration of cancer cells (43). Nrf2 acts as a master switch in these processes by upregulating the expression of various invasion and migration-related proteins (44).

The Nrf2/ARE pathway may regulate glioblastoma invasion and migration through matrix metalloproteinases (MMPs) and oxidative stress-related molecules. MMP activation could improve the degradation of intercellular connections, which enables glioblastoma cells to easily invade and migrate (45). Downregulation of the expression of Nrf2 in the U251 glioblastoma cell line was found to inactivate matrix metalloproteinase-9 (MMP-9) and to decrease the invasion and migration of glioma (44). Oxidative stress is another important mechanism involved in the invasion and migration of glioblastoma. HO-1 is the downstream molecule of Nrf2, which is important in regulating oxidative stress. Inhibition of HO-1 can weaken the invasive and migratory abilities of glioblastoma (46,47).

However, Thangasamy et al found that the Nrf2 inducer sulforaphane (SFN) can inhibit the expression of tyrosine kinase receptor, receptor d’origine nantais (RON), which can...
mediate the invasion of carcinoma cells (48), indicating that Nrf2 may play a dual role in regulating the invasiveness of tumors.

**Induction of apoptosis.** In most glioblastoma cells, apoptosis is inhibited (49,50). It has been suggested that Nrf2 can block the apoptotic death of cancer cells (51). Overexpression of Nrf2 was found to significantly diminish apoptosis (52). Inhibition of the Nrf2 transcription factor rendered cancer cells more susceptible to apoptosis (53).

The Nrf2/ARE pathway may regulate apoptosis by cross-linking with the B-cell lymphoma 2 (Bcl2), p53, p38/mitogen-activated protein kinase (MAPK) and nuclear factor-κB (NF-κB) pathways. Bcl2 is an important gene in tumor genesis and in the anti-apoptosis process (54,55). Following increased expression of Nrf2, the expression of caspases 3 was decreased and the apoptosis rate was reduced, accompanied by the upregulated expression of Bcl-2/Bax. This indicates that Nrf2 regulates apoptosis through the Bcl2-related pathway (56,57). p53 is important due to its anticancer function, and plays an essential role in tumor apoptosis (58). Nrf2 also regulates the tumor-suppressor p53 by influencing the degradation of p53. The Nrf2 downstream molecule NQO1 interacts with p53 and induces its degradation by the proteasome in a ubiquitin-independent manner (59). In addition, Nrf2 also attenuates the effect of the apoptosis inducer diamide in glioblastoma by upregulating the activity of p38/MAPK and inhibiting the NF-κB pathway (60,61).

**Promotion of differentiation.** Glioblastoma cells are usually in a poor stage of differentiation and exhibit low maturity (62-64), and differentiation therapy is required as a therapeutic strategy for malignant tumors (65,66). Nrf2 induces the suppression of differentiation by inhibiting a powerful differentiation inducer 1α, 25-dihydroxyvitamin D3 (1,25 D3) (67,68), suggesting that Nrf2 plays an important role in the cooperative suppression of cancer cell differentiation.

Nrf2 may regulate the differentiation of glioblastoma through cross-talk with the Notch pathway and upregulation of anti-redox molecules. The Notch pathway is important for cell-cell communication, which involves genetic regulatory mechanisms that control the cell differentiation process (69). Nrf2 adaptive response pathway could directly activate the Notch signal through recruitment of the Notch intracellular domain (NICD) transcriptome and restrain glioblastoma cells in a low state of differentiation (70). In addition, high accumulation of reactive oxygen species (ROS) can induce the differentiation of cells (71). Nrf2 was found to upregulate the anti-redox molecule GST to eliminate ROS and reverse the differentiation induced by ROS (71,72). It has been reported that neuronal differentiation inducer retinoic acid (RA) increased Nrf2 expression reactively (73,74), and down-regulation of Nrf2 improves the efficiency of RA in inducing differentiation (73,74).

**Enhancement of autophagy.** Autophagy is a lysosomal degradation process. Autophagy principally plays an adaptive role to protect organisms against diverse pathological conditions (75,76). Many studies have shed light on the importance of autophagy in glioblastoma (77). Knockdown of Nrf2 was found to regulate the autophagy induced by TMZ in the U251 glioblastoma cell line (78).

Nrf2 may regulate autophagy by altering the P62/SQSTM1 system and endoplasmic reticulum (ER) stress reaction (Fig. 2).
The protein of p62, also known as sequestosome 1 (SQSTM1), is one of the adaptors of autophagy. It has been found to play a critical role in the formation of cytoplasmic proteinaceous inclusion. Keap1 uncoupled from the complex with Nrf2 can bind to the autophagy-adaptor protein p62, and then interacts with LC3 and transports the ubiquitin conjugate to the autophagosome for degradation (79-81). ER stress is a cellular stress response which is activated in response to an accumulation of the unfolded protein response (UPR). High expression of Nrf2 can also induce autophagy by increasing ER stress and by increasing ER-associated degradation (82).

Targeting GSCs. The glioma stem cell (GSC) hypothesis suggests that neoplastic clones are maintained exclusively by a rare fraction of cells with stem cell properties (83). The identification of brain tumor-initiating cells established a new cellular target for more effective therapies (84-86). Over the past decades, Nrf2 was found to be pivotal in the maintenance of the stemness of human GSCs. Knockdown of Nrf2 was found to inhibit the proliferation of GSCs, and significantly reduce the expression of self-renewal-related factors Bmi1, Sox2 and cyclin E (87).

Nrf2 may maintain the stemness of GSCs by cross-linking with MAPK and p53 pathway, regulating HO-1 and circulating cell-free DNA (cirDNA) (Fig. 3) (88). High expression of Nrf2 can regulate the expression of MAPK and p53 in stem cells, which plays a critical role in the self-renewal of GSCs, indicating that Nrf2 may regulate self-renewal through MAPK and p53 pathway (89). Nrf2 downstream compound HO-1 is important in maintaining the high proliferation of stem cells. The HO-1 inducer cobalt protoporphyrin (CoPP) markedly improved stem cell proliferation (90). Nrf2 also plays an important role in regulating the reaction of stem cells to cirDNA, which is a small fraction of DNA in the plasma and has been found to be important in inhibiting the apoptosis of stem cells. (91).

Table II. Indirect mechanisms of the downregulation of Nrf2 in the treatment of glioblastoma.

<table>
<thead>
<tr>
<th>Mechanisms</th>
<th>Factors and associated molecules</th>
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<tbody>
<tr>
<td>Microenvironment</td>
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<tr>
<td>Perivascular</td>
<td>HIF-1α, VEGF</td>
</tr>
<tr>
<td>Hypoxic</td>
<td>HIF-1α, HO-1</td>
</tr>
<tr>
<td>Immune</td>
<td>Cytokines: IFN-γ, IL-4, IL-5, IL-13</td>
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<tr>
<td></td>
<td>Immune cells: Th, microglia</td>
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</tbody>
</table>

Nrf2, nuclear factor erythroid 2-related factor 2; HIF-1α, hypoxia-inducible factor α; VEGF, vascular endothelial growth factor; HO-1, heme oxygenase-1; IFN-γ, interferon-γ; Th, T helper cell.

3. Indirect mechanisms

The microenvironment is a functional unit enabling complex and dynamic interactions with tumor cells (92). Glioblastoma cells are influenced by non-malignant cells of the tumor microenvironment such as vascular endothelial cells, fibroblasts and immune cells (93). The microenvironment serves as the basis for indirect mechanisms of Nrf2 in the treatment of glioblastoma. Indirect mechanisms include three main aspects of the microenvironment: i) perivascular, ii) hypoxic and iii) immune microenvironment (Table II).

Perivascular microenvironment. Angiogenesis plays a key role in glioblastoma in order to provide energy and maintain the development and progression of glioblastoma. Glioblastoma cells develop a framework to induce the angiogenesis around them (94,95). Recent studies have begun to explore the role of Nrf2 in tumor angiogenesis (96,97). In human glioblastoma cell line U251, knockdown of Nrf2 was found to significantly decrease microvessel density (MVD) and expression of small vessel marker CD31 (38).

Nrf2 may regulate angiogenesis through hypoxia-inducible factor 1α (HIF-1α) and vascular endothelial growth factors (VEGFs). As a main downstream molecule of Nrf2, HIF-1α is one of the master regulators that orchestrate cellular responses to hypoxia. Activation of HIF-1α can lead to the activation of numerous perivascular compounds, such as angiopeitien, endothelin-1, inducible nitric oxide synthase (iNOS), adrenomedullin and erythropoietin. Blocking HIF-1α can inhibit the angiogenesis effect of Nrf2 (98). Another important inducer of vessels is VEGF. Nrf2 elevates VEGF expression and improves the growth of the vascular endothelia in tumors. Through a positive feedback loop, VEGF can also activate Nrf2 in an ERK1/2-dependent manner and induce the production of antioxidative enzymes (99). Anti-angiogenesis effects of Nrf2 knockdown were documented in chick chorioallantoic membrane assays and endothelial tube formation assays (100).

Hypoxic microenvironment. Hypoxia and tumor genesis are closely related (101). Glioblastoma has extensive areas of hypoxia and displays high tolerance to a low concentration of oxygen (102,103). Nrf2 has been identified as a regulator of
several genes involved in the hypoxic defense response, such as HIF-1α (104). In human glioblastoma, high expression of Nrf2 was significantly correlated with high tolerance to a low concentration of oxygen, less tumor necrosis on MRI and lower 1-year survival of patients (105).

It is believed that Nrf2 regulates the hypoxia resistance by HIF-1α and HO-1. HIF-1α is a downstream molecule of Nrf2 and is one of the master regulators of hypoxia (98). In a CoCl₂-induced hypoxia model, blockage of Nrf2 suppressed the expression of HIF-1α, and suppressed the migration and invasion of tumors in a hypoxic microenvironment (106). HO-1 is another important molecule for resistance to hypoxia. In a 6-hydroxydopamine (6-OHDA)-induced hypoxic model, Nrf2 activation induced upregulation of HO-1, and mediated the cellular adaptive survival response to a hypoxic microenvironment (107).

Immune microenvironment. Glioblastoma can escape from tumor immunosurveillance and inactivate the reaction between tumors and immune cells. The immune microenvironment surrounding glioblastoma plays an important role in these processes (108). In addition, Nrf2 was also found to be a critical regulator of the immune reaction (109).

The Nrf2/ARE pathway may regulate tumor immunosurveillance through regulation of the secretion of cytokines and the function of immune cells. Nrf2 regulates the secretion of many types of cytokines. Activation of Nrf2 was found to suppress the production of interferon-γ (IFN-γ), while inducing the production of T helper cells 2 (Th2), cytokines IL-4, IL-5, and IL-13 (110). Nrf2 also regulates the function of immune cells. In glioblastoma, Th helper cells (Th) play an important role in the adaptive immune system. Th helps the activation of other immune cells by releasing T cell cytokines. Nrf2 is a regulator of Th and activates CD4(+) T cells from differentiating towards Th2, representing a novel regulatory mechanism in CD4(+) T cells (111). Microglia act as the main form of active immune defense in the central nervous system (CNS). Nrf2 also mediates immunoresistance by modifying the function of microglia. Activation of the Nrf2/HO-1 pathway was found to suppress BV2 microglial cells and immunology in the brain (112). Upregulation of Nrf2 suppressed innate immune microglial cells in the CNS. Various small activators of Nrf2 (113) such as carnosol, supercurcumin and dimethyl fumarate are effective modulators of microglial-related immune responses (112).

4. Conclusion

In the past decades, a marked increasing in research has been carried out focusing on Nrf2 and its role in regulating glioblastoma and the possibilities of the downregulation of Nrf2 for treating glioblastoma. Nrf2 plays an extensively role in the regulation of glioblastoma; hence, downregulation of Nrf2 can interfere with a variety of behaviors of glioblastoma and actions of the microenvironment surrounding glioblastoma. Thus Nrf2 has promising value as a therapeutic target for glioblastoma. However, Nrf2 downregulation in most studies was obtained through RNA interference or knockdown technology, rather than pharmaceutical compounds, making targeted Nrf2 therapy somewhat difficult and less appealing at this time from a translational perspective. Recently, biochemists have identified the small molecule, ochratoxin A, as an inhibitor of Nrf2 (113). Although it is a toxin produced by Aspergillus ochraceus, the single compound is a potential new strategy with which to inhibit Nrf2 in glioblastoma. For these reasons, future studies should focus on regulatory methods of Nrf2, which can be easily translated to the clinical setting and be used safely.

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References


