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Comparative Analysis of the Mutation and Expression Profile of the Cytoprotective NRF2/KEAP1/P62/SQSTM1 Signaling Pathway in Different Glioma Subtypes: An *In Silico* Study

Farklı Glioma Alt Tiplerinde Sitoprotektif NRF2/KEAP1/P62/ SQSTM1 Sinyal Yolunun Mutasyon ve Ekspresyon Profilinin Karşılaştırmalı Analizi: Bir *In Silico* Çalışma

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ABSTRACT

Aim: The NRF2/KEAP1/p62/SQSTM1 pathway is the master regulator of antioxidant enzymes and detoxification proteins, both of which play a critical role in redox homeostasis. It shows that the this structurally active pathway has a crucial role in cancer as it inhibits tumorigenesis and metastatic processes and it induces pro-survival genes that promote chemoresistance. The relationship between the molecular mechanisms causing the pathway to malfunction and the development of brain tumors has yet to be fully clarified. The aim of this study is to analyze the genetic changes and expression level differences of the NRF2/KEAP1 pathway comparatively in low-grade gliomas (LGG) and glioblastoma multiforme (GBM) pathology.

Materials and Methods: Gene expression profiles and DNA sequences of GBM (n=591) and LGG (n=511) patients and healthy tissue were downloaded from the TCGA database. Not only were gene expression and mutation patterns determined in this study, but also the impacts of genes on survival were assessed. PolyPhen-2 and SNAP tools were used to estimate the pathogenic properties of the changes identified.

Results: A total of 16 mutations and gene amplification were identified in the *KEAP1*, *NRF2*, *p62/SQSTM1*, *HMOX-1*, and *MOAP1* for both cancer groups, and the mutation carrying frequency was 4.6%. IDH1 p.R132H and NRF2 p.S164* mutation association was determined in 1 patient with LGG. *KEAP1*, *NRF2*, and *HMOX1* expression levels for both LGG and GBM subtypes were determined to be high in patient samples compared to healthy samples ($p<0.05$).

Conclusion: By targeting the NRF2/KEAP1/p62/SQSTM1 pathway anomalies, new therapeutic approaches can be provided in the treatment of glioma, particularly for chemotherapy sensitivity.

Keywords: NRF2/KEAP1/p62/SQSTM1 signaling pathway, glioma, mutation, gene expression, oxidative stress

ÖZ

Amaç: NRF2/KEAP1/p62/SQSTM1 sinyalizasyon yolağı redoks homeostazında önemli rol oynayan antioksidan enzimlerin ve detoksifikasyon proteinlerinin ana düzenleyicisidir. Yapısal olarak aktif NRF2/KEAP1 yolağının, tümörigenezi ve metastatik süreçleri inhibe ettiği ve kemorezistansı teşvik eden hayatta kalma yanlısı genleri indüklediği için kanserde çok önemli bir role sahip olduğunu göstermektedir. Yolağın fonksiyonunun bozulduğu moleküler mekanizmalar ile beyin tümörleri gelişimi arasındaki ilişki tam olarak aydınlatılamamıştır. Bu çalışmanın amacı NRF2/KEAP1 yolağının genetik değişikliklerini ve ifade seviyesi farklılıklarını düşük dereceli glioma (LGG) ve glioblastoma multiform (GBM) patolojisinde karşılaştırmalı olarak analiz etmektir.

Gereç ve Yöntem: GBM ve LGG hastalarına ve sağlıklı doku örneklerine ait gen ekspresyon profilleri ve DNA dizileri, kanser genom atlas veri tabanından indirildi. *KEAP1*, *NRF2*, *p62/SQSTM1*, *HMOX-1* ve *MOAP1* genlerinin mutasyon ve ifade profilleri kapsamlı olarak analiz edildi. Çalışmada

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sadece gen ekspresyonu ve mutasyon paternlerinin tespiti değil, aynı zamanda hedef genlerin sağkalım üzerine olan etkileri de belirlendi. Ayrıca belirlenen değişikliklerin hastalık yapıcı patojenik özellikleri tahmini için PolyPhen-2 ve SNAP araçları kullanıldı.

Bulgular: Her iki kanser grubu için *KEAP1*, *NRF2*, *p62/SQSTM1*, *HMOX-1* ve *MOAP1* genlerinde toplam 16 (12 missense mutasyon, 1 nonsense mutasyon, 1 delesyon, 2 translokasyon) mutasyon ve gen amplifikasyonu belirlendi ve mutasyon taşıma sıklığı %4,6 idi. LGG'li 1 hastada IDH1 R132H ve NRF2 S164* mutasyon birlikteliği belirlendi. LGG ve GBM alt tiplerinin her ikisi için de *KEAP1*, *NRF2* ve *HMOX1* gen ekspresyon seviyeleri, hasta örneklerinde sağlıklı örneklere göre yüksek olarak belirlendi (p<0,05).

Sonuç: NRF2/KEAP1/p62/SQSTM1 sinyalizasyon yolağı anomalilerinin hedeflenmesi ile glioma tedavisinde özellikle kemoterapi duyarlılığı için yeni terapötik yaklaşımlar sağlanabilir.

Anahtar Kelimeler: NRF2/KEAP1/p62/SQSTM1 yolağı, glioma, mutasyon, gen ekspresyonu, oksidatif stress

INTRODUCTION

Brain tissue has limited antioxidant capacity and is highly indefensible to oxidative stress due to its higher energy requirement and higher content of lipids and iron, the auto-oxidation properties of neurotransmitters in an environment where free oxygen radicals are concentrated, and oxidized neurotransmitters also have the potential to cause more production of reactive oxygen species (ROS)[1-3]. This situation causes morphological and functional changes in the brain, as well as cognitive dysfunction and retardation. The NRF2/KEAP1/p62/SQSTM1 pathway responsible for the activation of the NRF2 transcription factor, which controls the transactivation of more than 500 cytoprotective genes, is a significant cellular component in protecting cells and tissues from electrophilic, oxidative, and xenobiotic stress[4-6]. NRF2 is bound to the KEAP1 in the cytoplasm under normal circumstances. As cells are subjected to oxidative stress, it has been found that NRF2 detaches from KEAP1, to which it is attached in the cytoplasm, and moves into the nucleus, where it binds to its target gene and stimulates transcription[7,8]. In addition to the KEAP1-dependent regulation of NRF2, various alternative KEAP1-independent mechanisms contribute to the regulation of NRF2. These include the cellular NRF2 protein and binding of the disrupting protein p62/SQSTM1 to KEAP1, which inhibits the interaction of NRF2 and KEAP1 causing an increase in its activity[7-9]. Abnormal NRF2/KEAP1 pathway causes the development of treatment resistance and provides cancer cells with a growth advantage due to the constitutive expression of cytoprotective genes[3,5-8]. Malignant cells are known to take advantage of their increased NRF2 pathway activity. This condition was first discovered in lung cancer and then in a variety of other types of cancer, such as ovarian, pancreatic, liver, pediatric leukemia, and bladder cancers[6-9].

Gliomas are the most malignant and aggressive form of brain tumors, and account for the majority of brain cancer-related deaths. Gliomas are the most common primary intracranial tumor, representing 81% of malignant brain tumors[10,11]. Recent data support that the concept "ROS is an indispensable participant in fostering proliferation, survival, and migration" in various cancer cell types including glioblastoma cells[2,3,11]. Overproduction of ROS is known to play a role in promoting these changes[2,3,10,12]. The goal of this study is to predict the functional effects of pathogenic mutations and expression level profiles in the NRF2/KEAP1/p62/SQSTM1 pathway genes in glioma subtypes, as well as to clarify the effects of these pathway elements on glioma pathogenesis and progression.

MATERIALS AND METHODS

Data Collection

The GBM and LGG data sets were obtained from the cbioPortal database and the demographic, clinical, and genetic data for our patient group are summarized in Table 1.

Mutation Profile Analysis

The cBio Cancer Genomics Portal (http://cbioportal.org) is an open-access bioinformatics tool that uses data from The Cancer Genome Atlas (TCGA) to provide mutation data[13]. In order to comprehensively examine the mutations found in KEAP1, NRF2, p62/SQSTM1, HMOX-1, and MOAP1 in GBM and LGG patient samples, GBM (n=591), LGG (n=511), it was selected as the cancer type of interest from the web interface. For this purpose, comprehensive mutation profile analyses were carried out through cBioportal using the features provided by the interface of the genes of interest.

In Silico Analysis of Mutation Impact

The probable pathogenicity of the mutations found in the KEAP1, NRF2, p62/SQSTM1, HMOX-1, and MOAP1 was determined using scores from the PolyPhen-2, SNAP, and the COSMIC databases. The PolyPhen-2 estimates the probability of the missense mutation damaging the protein and provides the user with this result (probably damaging, possibly damaging, benign or unknown) with a score[14]. The SNAP is an online tool that distinguishes between impact and neutral variants/non-synonym SNPs by considering various sequence and variant properties[15]. Furthermore, the detected mutations were scanned in the COSMIC database and their pathogenicity scores were determined[16]. Besides, evolutionary conservation analyses of the detected mutant amino acids were evaluated

Table 1. Demographic, clinical and genetic data of patients with GBM and LGG

Characteristic	GBM n=591 (%)	LGG n=511 (%)
Gender		
Male/Female/NA	175/122/294	285/226
Diagnosis age, years	59.6 (range, 21-89)	43 (range,19-87)
Race category		
White	252 (43.1)	474 (92.7)
Black or African American	31 (5.3)	21 (4.1)
Asian	4 (0.7)	7 (1.3)
NA	297 (50.2)	9 (1.7)
Sample type		
Primary	584 (98.8)	511 (100)
Recurrence	7 (1.2)	-
Overall survival status		
Living	103 (17.4)	388 (75.9)
Deceased	478 (80.8)	125 (24.4)
NA	10 (1.7)	-
Radiation therapy		
Yes	236 (39.9)	296 (57.9)
No	41 (6.9)	183 (35.8)
NA	314 (53.1)	34 (6.6)
Tumor disease anatomy		
CNS	-	511 (100)
Brain	298 (50.4)	-
NA	293 (49.5)	-
Tumor subtypes		
Glioblastoma multiforme	591 (100)	-
Astrocytoma	-	192 (37.5)
Oligodendroglioma	-	189 (36.9)
Oligoastrocytoma	-	130 (25.4)
Neoplasm histologic grade		
Grade I		-
Grade II		248
Grade III		263
Grade IV		-
NA		-
Genetic abnormalities	**Case (frequency %)**	
IDH1 mutation	25 (6.3)	394 (76.8)
IDH2 mutation	1 (0.3)	21 (3.5)
NFE2L2 mutation	1 (0.3)	1 (0.2)
SQSTM1 mutation	3 (0.8)	2 (0.4)
HMOX1 mutation	2 (0.5)	-
MOAP1 mutation	2 (0.5)	1 (0.2)
SQSTM1-NTRK2 fusion	-	1 (0.2)
KEAP1 amplification	7 (1.2)	12 (2.4)
NFE2L2 deep deletion	1 (0.2)	1 (0.2)
SQSTM1 amplification	1 (0.2)	2 (0.4)
SQSTM1 deep deletion	1 (0.2)	2 (0.4)
HMOX1 amplification	2 (0.5)	4 (0.8)
HMOX1 deep deletion	1 (0.3)	3 (0.6)
TXNRD2-HMOX1 fusion	1 (0.2)	-
MOAP1 deep deletion	1 (0.2)	-
1p status		
Gained	41 (6.9)	12 (2.34)
Lost	8 (1.3)	173 (33.8)
Not called	477 (80.7)	307 (60)
NA	65 (10.9)	19 (37.2)
19q status		
Gained	144 (24.3)	19 (3.7)
Lost	22 (3.7)	185 (36.2)
Not called	313 (52.9)	233 (45.5)
NA	112 (18.9)	74 (14.4)

NA: Not applicable, CNS: Central nervous system, LGG: Lower grade glioma, GBM: Glioblastoma multiforme

among different species via "Multiple sequence alignment" tool in the PolyPhen-2.

Gene Expression and Survival Analyses

GEPIA is an interactive tool developed to provide customizable analyses such as differential expression analysis in tumor or normal tissues, profiling according to cancer types or pathological stages, survival analysis, similar gene detection, and correlation analysis[17]. The profiles of KEAP1, NRF2, p62/SQSTM1, HMOX-1, and MOAP1 expressions were analyzed in box plot graphs created by the GEPIA using the data of the samples of GBM (n=163), LGG (n=518) obtained from TCGA data, and of 207 healthy tissues. Survival analyses (overall/disease free) of genes according to varying gene expression levels were conducted using GEPIA. Overall survival (OS) and disease free survival (DFS) analyses based on Log-rank test with 95% confidence interval were performed to create survival plots.

Statistical Analysis

All statistical analyses that were used in the evaluation of the study data were performed on the GEPIA database. The one-way ANOVA test was used to measure differential expression. GEPIA performs the analysis of OS or DFS, also called relapse-free survival, based on gene expression. GEPIA uses Log-rank test, the Mantel-Cox test, for hypothesis test. To compare low and high expression groups, the log-rank test was used. In all tests, the statistically significant value was accepted as $p<0.05$.

RESULTS

Results of Mutation Profile Analysis

The cBioPortal interface was used to analyze the genome sequencing data of 1106 patients in order to identify genetic changes in the KEAP1, NRF2, p62/SQSTM1, HMOX-1, and MOAP1 in GBM and LGG patient samples. Of GBM and LGG patients, 4.6% were found to have at least one genetic change in the KEAP1, NRF2, p62/SQSTM1, HMOX-1, and MOAP1. A total of 16 genetic changes (12 missense, 1 nonsense, 1 deletion, 2 translocations) were detected in all study genes (Table 2). When the frequency of changes among the analyzed genes was examined, it was determined that KEAP1 was the gene with more mutations among the patient groups (1.7%), and NRF2 was determined to be the gene with less mutation (0.4%) (Figure 1). While no nucleotide changes in KEAP1 were detected, amplification of the gene was found in both glioma subtypes. In GBM and LGG patient samples, the localization of mutations detected on the domains of proteins belonging to the study genes is shown in Figure 2 as a representation.

Results of Mutation Profile Analysis in LGG Patients

The frequency of carrying LGG genetic anomalies was determined to be 6.1%. Six mutations (NRF2, p.S164*; SQSTM1, p.R107Q and p.A2V; MOAP1, p.K164N and p.R204T) were detected in the LGG group. The nonsense p.S164* mutation found in the *NRF2* gene was thought to cause early termination of the NRF2 polypeptide at the 164. amino acid, resulting in the formation of a truncated protein, according to our findings. There was one patient in Astrocytoma subtype with the coexistence of NRF2 p.S164*and the IDH1 p.R132H missense mutation characteristic for glioma.

SQSTM1, p.R107Q and p.A2V missense mutations were identified in two different patients with Astrocytoma subtype SQSTM1. It was determined that the patient carrying the p.A2V mutation also carried the IDH1 p.R132H mutation. The p62/SQSTM1 t (5;9) (q35;q21) fusion gene with NTRK2, which is a tyrosine kinase responsible for neural development, was identified in a patient of the Oligodendroglioma subtype. Apoptosis modulator 1 (MOAP-1), a BH3-like protein that plays a key role in apoptosis, was identified with p.K164N and p.R204T mutations on the PNMA domain in two different patients in Astrocytoma subtype carrying IDH1 p.R132H missense mutation.

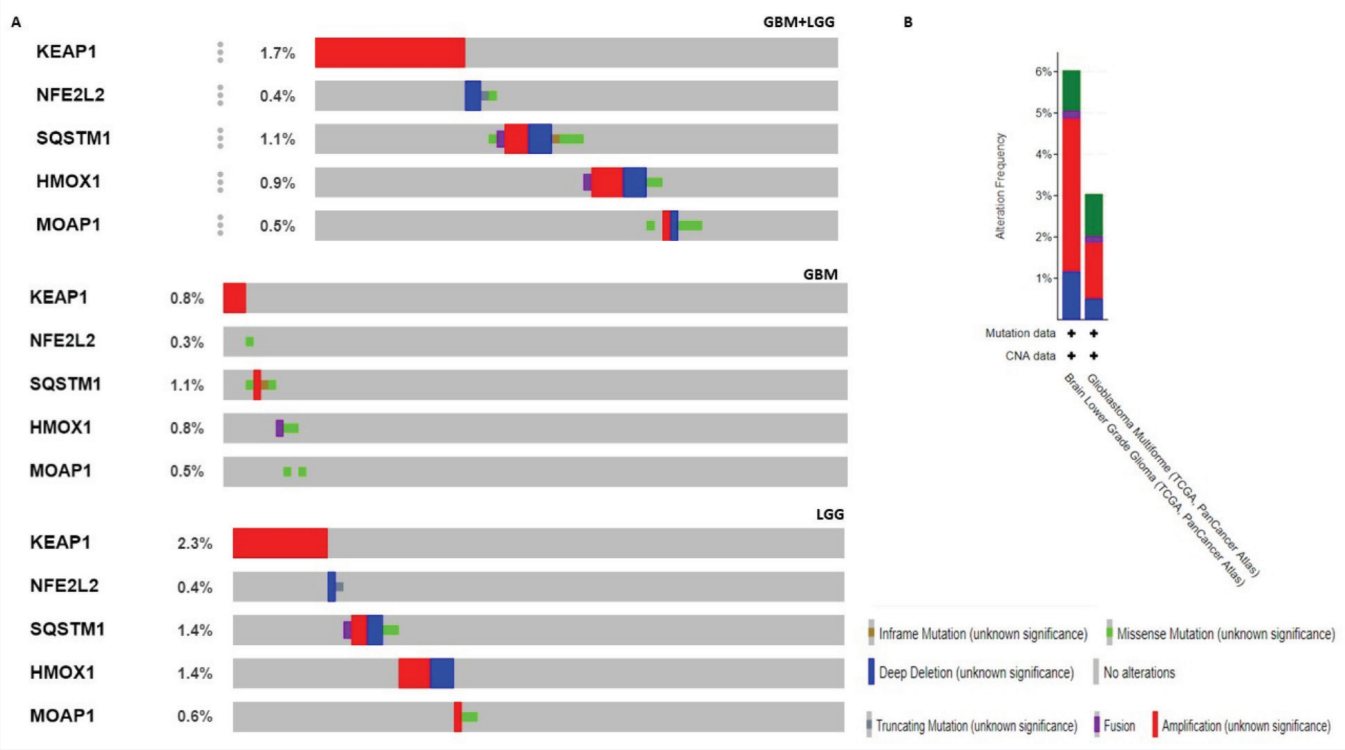

Figure 1. Distribution of mutations in *KEAP1*, *NRF2*, *p62/SQSTM1*, *HMOX-1*, and *MOAP-1* genes in GBM and LGG cancer patient group (A, B)

GBM: Glioblastoma multiforme, LGG: Low-grade gliomas

Results of Mutation Profile Analysis in GBM Patients

The carrying prevalence of GBM genetic abnormalities was detected to be 3,5%. Ten mutations *NRF2*, p.E564K; p62/SQSTM1 p.R96Q; p.E280del, p.F193L; p.R183P, *HMOX-1*, p.A194T; p.F33L, *TXNRD2-HMOX1* fusion gene; *MOAP-1*, p.P45L; p.A111V were detected in the GBM group. The frequency of carrying mutation with co-occurrence in NRF2-p62/SQSTM1; HMOX1-MOAP1 was statistically significant for the GBM patient group (p=0.037 and p=0.055, respectively). Mutations identified on the p62/SQSTM1 were on the PBI, LIR, and TBS domains. The p.R183P mutation was on the TBS Domain. The mutation p.F193L in the same domain and p.R96Q in the PBI domain was identified in a 23-year-old female patient carrying the IDH2 p.G383V and p.K251N missense mutations. It was also determined that same patient had NRF2 on the DNA binding domain p.E564K missense mutation. SOSTM1 p.E280del frame shift deletion was on the LIR domain. In addition, the fusion gene t(5;9)(q35;q21) was identified with p62/SQSTM1 and NTRK2, a tyrosine kinase responsible for neural development. HMOX1 mutations were identified only in the GBM patient group. All missense mutations detected in the HMOX-1 were located on the Heme oxygenease Domain. Two missense mutations (p.P45L and p.A111V) in the MOAP1 were identified on the PNMA domain.

Results of *In Silico* Analysis of Mutation Impact

According to the analysis results of PolyPhen-2, SNAP Database, and COSMIC programs, it is determined that among the mutations detected in our study, especially the pathogenic scores of the NRF2 p.E564K, SQSTM1 p.R96Q, and HMOX1 p.F33L mutations may be pathogenic due to the fact that they are close to 1 and "affected" and they are predicted to have disease-causing properties. Especially in Table 2, it is seen that all of the mutations found in the GBM patient group are pathogenic. As a result of multiple sequence alignment analysis, it was discovered that 10 of the 12 mutations detected modified their amino acids located at the critical point that was preserved among different species. In addition, p.E564K, p.S164*; SQSTM1 p.R96Q, p.R183P, and p.R107Q;

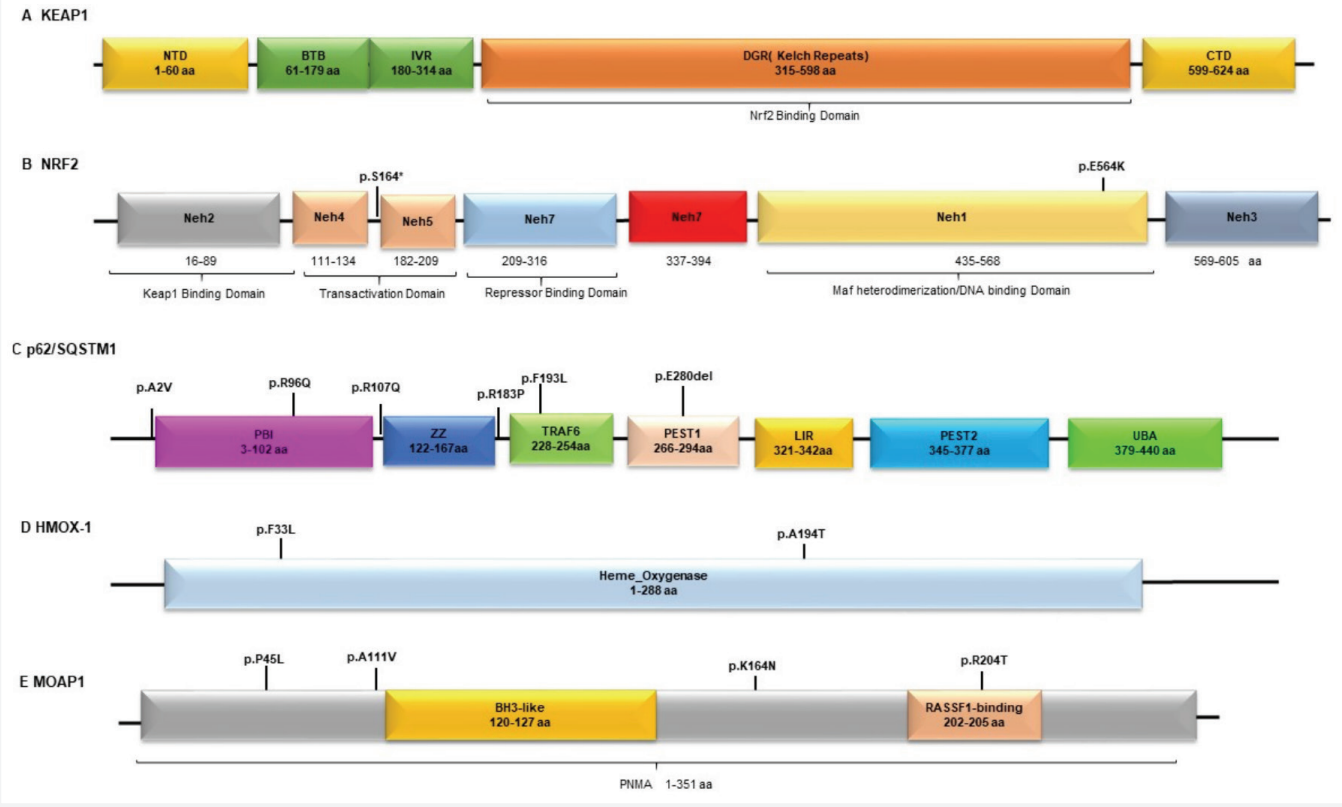

Figure 2. Schematic representation of domain architecture of the KEAP1, NRF2, p62/SQSTM1, HMOX-1, and MOAP1 proteins and mutations detected in patients with GBM and LGG (A) Human Keap1 is a polypeptide comprising 624 amino acids. (B) Human NRF2 is a polypeptide comprising 605 amino acids, which contains seven Neh domains. (C) Human p62/SQSTM1 is a polypeptide comprising 440 amino acids. (D) Human HMOX-1 is a polypeptide comprising 288 amino acids. (E) Human MOAP1 is a polypeptide comprising 351 amino acids

GBM: Glioblastoma multiforme, LGG: Low-grade gliomas

Table 2. Mutations of the *NRF2*, *p62/SQSTM1*, *HMOX-1* and *MOAP1* genes in LGG and GBM patients

No	Gene	Nt alteration	Rs number	Alteration type	Localization	AA position	Coexistence with IDH1/2 mutation	Cancer type	Tumor type	Clinical significance		
										PolyPhen-2 (score)	SNAP (score)	COSMIC prediction
C-1	*NRF2*	c.1687G>A	COSV67960917	Missense mutation	Neh1 DNA binding Domain	p.E564K	p.G383V p.K251N	GBM	Primary	Probably Damaging (1.00)	Effect (51)	Pathogenic (0.94)
C-2	*NRF2*	c.491C>G	COSV67962580	Nonsense mutation	Neh5 Domain	p.S164*	p.R132H	LGG	Primary	NA	NA	Pathogenic (0.91)
C-3	*SQSTM1*	c.287G>A	COSV62435109	Missense mutation	PBI Domain	p.R96Q	p.G383V p.K251N	GBM	Primary	Probably Damaging (1.00)	Effect (60)	Pathogenic (0.99)
C-4	*SQSTM1*	-	-	Deletion	LIR Domain	p.E280del	-	GBM	Primary	NA	NA	UNK
C-5	*SQSTM1*	c.579C>T	COSV100657686	Missense mutation	TBS Domain	p.F193L	p.G383V p.K251N	GBM	Primary	Benign (0.12)	Effect (30)	Neutral (0.02)
C-6	*SQSTM1*	c.548G>C	COSV100657714	Missense mutation	TBS Domain	p.R183P	-	GBM	Primary	Benign (0.22)	Effect (38)	Pathogenic (0.97)
C-7	*SQSTM1*	c.320G>A	COSV62434138	Missense mutation	-	p.R107Q	-	LGG	Primary	Possibly Damaging (0.65)	Neutral (-10)	Pathogenic (0.90)
C-8	*SQSTM1*	c.5C>T	COSV62435509	Missense mutation	PBI Domain	p.A2V	p.R132H	LGG	Primary	Benign (0.33)	Neutral (-94)	UNK
C-9	*SQSTM1*	t(5;9)(q35;q21)	-	Fusion gene	-	SQSTM1-NTRK2	-	LGG	Primary	NA	NA	UNK
C-10	*HMOX1*	c.580G>A	COSV53340335	Missense mutation	Heme oxygenase Domain	p.A194T	p.R132H	GBM	Primary	Benign (0.02)	Neutral (-83)	Pathogenic (0.76)
C-11	*HMOX1*	c.99C>A	COSV99330761	Missense mutation	Heme oxygenase Domain	p.F33L	-	GBM	Primary	Possibly Damaging (0.86)	Effect (30)	Pathogenic (0.96)
C-12	*HMOX1*	-	-	Fusion gene	Heme oxygenase Domain	TXNRD2-HMOX1	-	GBM	Primary	NA	NA	UNK
C-13	*MOAP1*	c.134C>T	COSV52092948	Missense mutation	PNMA Domain	p.P45L	-	GBM	Primary	Probably Damaging (0.98)	Effect (66)	Neutral (0.03)
C-14	*MOAP1*	c.332C>G	COSV52092285	Missense mutation	PNMA Domain	p.A111V	-	GBM	Primary	Possibly Damaging (0.61)	Neutral (-83)	Neutral (0.05)
C-15	*MOAP1*	c.492G>C	COSV99365609	Missense mutation	PNMA Domain	p.K164N	p.R132H	LGG	Primary	Probably Damaging (0.99)	Effect (17)	Neutral (0.46)
C-16	*MOAP1*	c.614G>A	COSV52093035	Missense mutation	PNMA Domain	p.R204T	p.R132H	LGG	Primary	Probably Damaging (0.96)	Effect (55)	Neutral (e 0.04)

UNK: Unknown, NA: Not available, SNP: Single nucleotide polymorphism, C: Change, UTR: Untranslated region, LGG: Lower grade glioma; GBM: Glioblastoma multiforme

HMOX1 p.A194T, p.F33L mutations found in NRF2 are available as somatic mutations in the COSMIC database, and they are specifically noted for different types of solid cancers.

Gene Expression and Survival Analysis Results

The gene expression profiles of KEAP1, NRF2, p62/SQSTM1, HMOX-1, and MOAP1 were determined as a result of the comparison of GBM (n=163), LGG (n=518) patients with respect to the healthy group. According to the analysis results, KEAP1, NRF2, and HMOX1 expression levels were determined to be statistically significant in patient samples compared to healthy samples and higher in both cancer groups, while MOAP1 expression was found to be lower in patient samples compared to the healthy group ($p<0.005$) (Figure 3). The p value for NRF2 in the LGG group was found to be significant based on the OS analysis findings. Those with low levels of NRF2 expression were found to have a statistically significant longer OS time than those with high levels ($p=0.00027$). However, according to our DFS analysis results, high NRF2 expression in LGG patient group was statistically significant compared to individuals with low NRF2 expression ($p=0.0011$). Individuals with low gene expression of the HMOX1 have a statistically significant longer OS period than those with high gene expression ($p=0.025$) in the LGG patient group. Individuals with low levels of MOAP1 expression have longer OS than those with high levels of expression ($p=0.008$, Figure 4). Individuals with low gene expression of the SQSTM1 have a statistically significant longer DFS period than those with high gene expression in the GBM patient group ($p=0.0043$, Figure 5). Besides, individuals with high levels of expression in all other genes and both cancer subtypes, except for high levels of HMOX1 expression, have long OS. As a result of comparing the m-RNA levels of individuals with and without mutations for each gene, no statistical difference was found between the groups, the results are presented in Figure 5.

DISCUSSION

In our study, the mutation profiles and gene expression patterns of the *KEAP1*, *NRF2*, *p62/SQSTM1*, *HMOX-1*, and *MOAP1*, which are the key actors in the cytoprotective

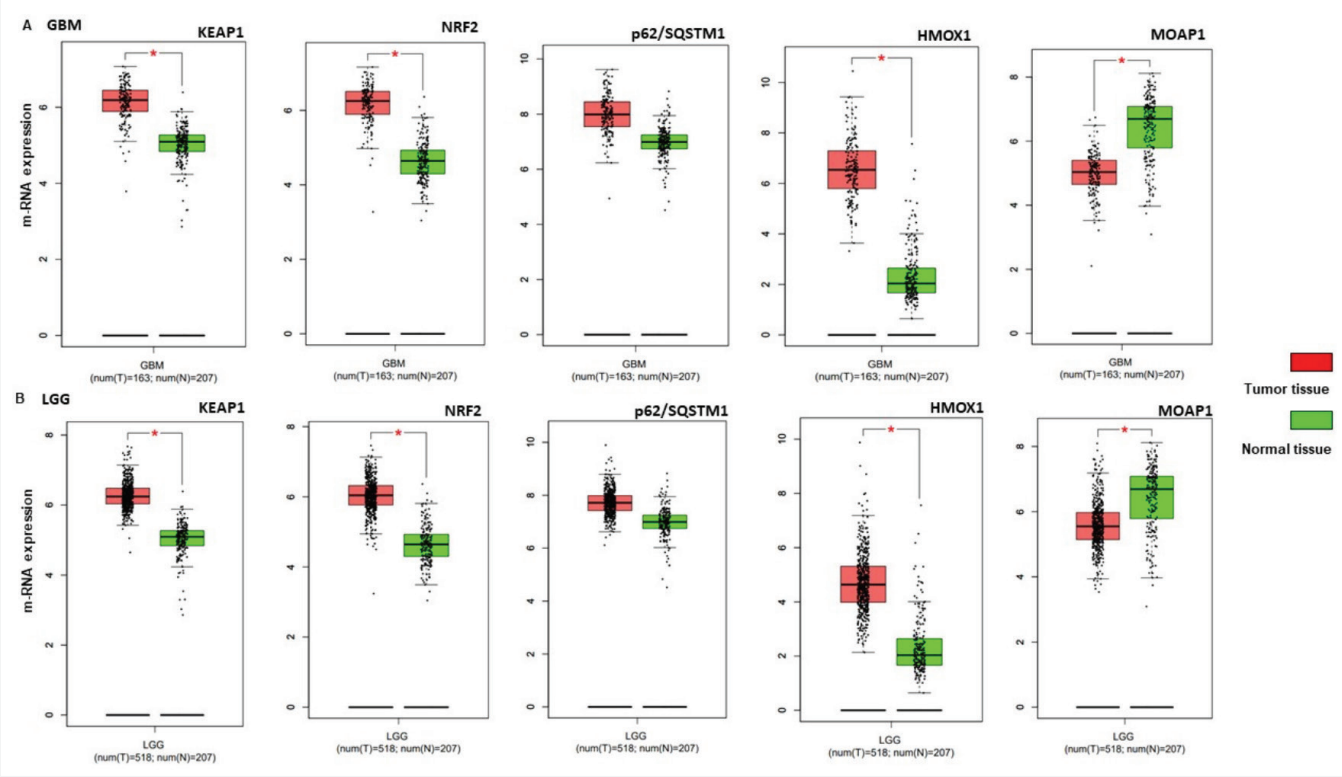

Figure 3. Comparative analysis of the tissue-specific differential expression of *NRF2*, *p62/SQSTM1*, *HMOX-1*, and *MOAP1* genes in brain tissues using GEPIA in GBM (A) and LGG (B) patients. The m-RNA expression of *NRF2*, *p62/SQSTM1*, *HMOX-1*, and *MOAP1* genes between tumor and normal tissues. TPM (Transcripts Per Million) were used to measure gene expression levels. The expression data are first log2 (TPM+1) transformed for differential analysis and the log2FC is defined as median (Tumor) – median (Normal). Genes with higher [log2FC] values and lower q values than pre-set thresholds are considered differentially expressed genes. Statistically significant value was considered as $p<0.05$. Log2(TPM + 1) was used for log-scale

GBM: Glioblastoma multiforme, LGG: Low-grade gliomas

pathway, were evaluated and compared in GBM and LGG patient samples. Primarily, among the genome sequencing results of a total of 1102 GBM and LGG patients that are available in TGCA data sets, the mutation profiles of the genes of interest KEAP1, NRF2, p62/SQSTM1, HMOX-1, and MOAP1 have been comprehensively analyzed. In our study, 12 of the 16 mutations detected in the NRF2/KEAP1 signaling pathway in GBM and LGG patients were found to be missense mutations, 1 was non-sense mutation, 1 was deletion, and 2 were translocations. It was determined that patient groups carried 6.1% mutations for LGG and 3.5% for GBM, and the gene carrying the most genetic abnormalities was KEAP1 1.7% for both tumor types. Mutations have been detected in study genes, particularly in sequences encoding important domains of the genes. It was determined that the NRF2 p.E564K, p62/SQSTM1 p.R96Q, and HMOX1 p.F33L missense mutations that we detected in our study affected the critical amino acids conserved in the evolutionary process according to the results of the inter-species evolutionary analysis. According to the results of the evolutionary analysis, it is thought that the missense mutations in question may be effective in the development of glioma due to their effect on amino acids that have been conserved throughout the evolutionary process and their domain regions, and their possible pathogenic properties obtained as a result of functional pathogenic effect analyses and the possibility of altering the expression of antioxidant response genes.

There are seven highly conserved domains known as NRF2-ECH homology domains[6,8,9,18]. The p.E564K mutation that we defined in NRF2 is located on the Maf heterodimerization/DNA binding domain/Neh1 of the protein. Neh1 contains a well-preserved CNC-bZIP region, which is required for DNA binding and heterodimer formation with NRF2 dimerization partners, sMaf proteins, as well as a nuclear localization signal required for NRF2 nuclear translocation. Furthermore, Neh1 is able to interact with UbcM2, the E2 ubiquitin-conjugation enzyme, to regulate NRF2 protein stability[5,6,8,9,18]. Considering that NRF2 is a transcription factor, the p.E564K

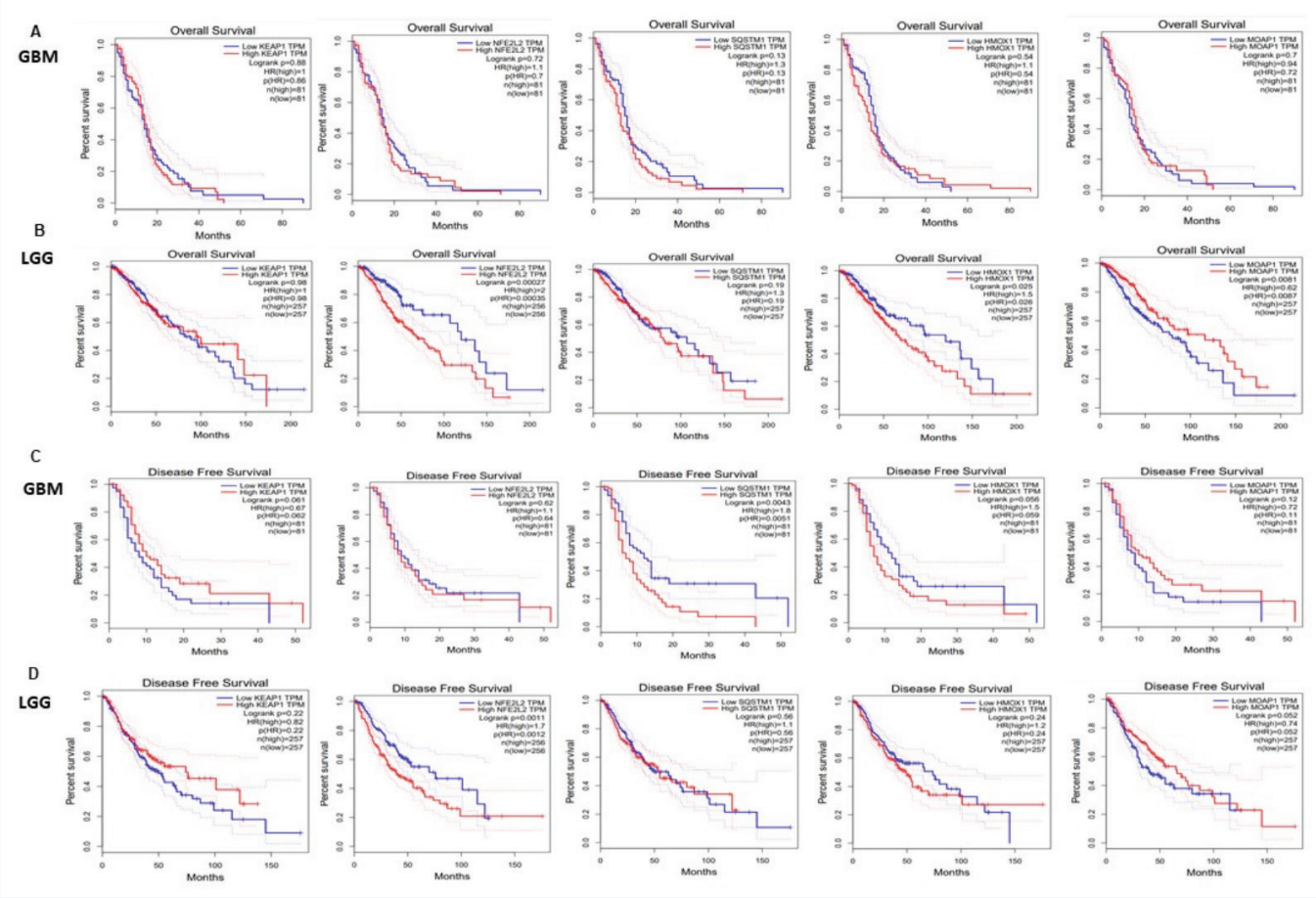

Figure 4. Kaplan–Meier analysis of OS (A, B) and DFS (C, D) of the GBM and LGG patients according to different NRF2, p62/SQSTM1, HMOX-1, and MOAP1 levels

GBM: Glioblastoma multiforme, LGG: Low-grade gliomas, DFS: Disease free survival, OS: Overall survival

mutation is in a position to affect the activation/constitutive expression of the genes responsible for detoxification and may pose a potential problem for the localization of the protein as the same mutation is located on the domain containing NLS signaling. The NRF2 p.S164* nonsense mutation is truncating mutation and is expected to cause the formation of the stop codon as early as the 164. codon before NRF2 protein synthesis is complete. IDH1 is involved in energy metabolism with the production of NADPH in the cytoplasm and peroxisomes by catalyzing the conversion of isocitrate to alpha-ketoglutarata (α-KG). Numerous studies have reported that the IDH1 mutation can cause a decline in NADPH and the accumulation of ROS in cells[19,20]. However, the relationship between the biological significance of IDH1 mutations and cellular redox homeostasis is not completely known. In particular, it has been determined that cells overexpressing IDH1 p.R132H are more sensitive to the chemotherapeutic temozolomide and expression of NRF2 in these cells is significantly decreased[20]. NRF2 is known to be associated with treatment resistance and poor prognosis in glioma[3,4,20]. In our study, there are 2 individuals carrying both IDH1; NRF2 (p.R132H; p.S164*) mutations in LGG subtype and mutations in GBM subtype (p.G383V; p.K251N; p.E564K). When it was examined in the patients by the cBioPortal, it was determined that the NRF2 expression level decreased at the cellular level. The association of p.R132H mutation in LGG and NRF2 p.S164* mutation, which causes early termination of the polypeptide, may cause chemotherapy sensitivity.

The most well-known mechanism of the noncanonical pathway is NRF2 activation through the p62/SQSTM1 protein. p62/SQSTM1, a multi-domain and multi-functional protein, protects cells from stress by autophagy pathway and NRF2 activation. p62/SQSTM1, which encodes the p62 protein, is an adapter protein involved in a variety of fundamental cellular processes, including OS response, apoptosis, and cell differentiation[4,21]. p62/SQSTM1 facilitates the degradation of unwanted molecules by fixing ubiquitin proteins to the autophagosome membrane. Furthermore, p62/SQSTM1 acts as a signaling hub for multiple pathways associated with neurodegeneration and is seen as a potential therapeutic target in the treatment of neurodegenerative diseases[4,12,21]. The p.R96Q mutation, shown in Figure 2, affects the PB1 domain, which is a modulator for protein-protein interactions. It is located in the ZZ-type zincfinger domain, which is also involved in p.F193L and p.R183P protein-protein interactions. p.E280del is also on the PEST domain, and the frameshift is in a position to cause mutation, leading to nonfunctional polypeptide production. It is known in the literature that full-length p62/SQSTM1 regulates NRF2 activation through a positive feedback loop[4,12,21].

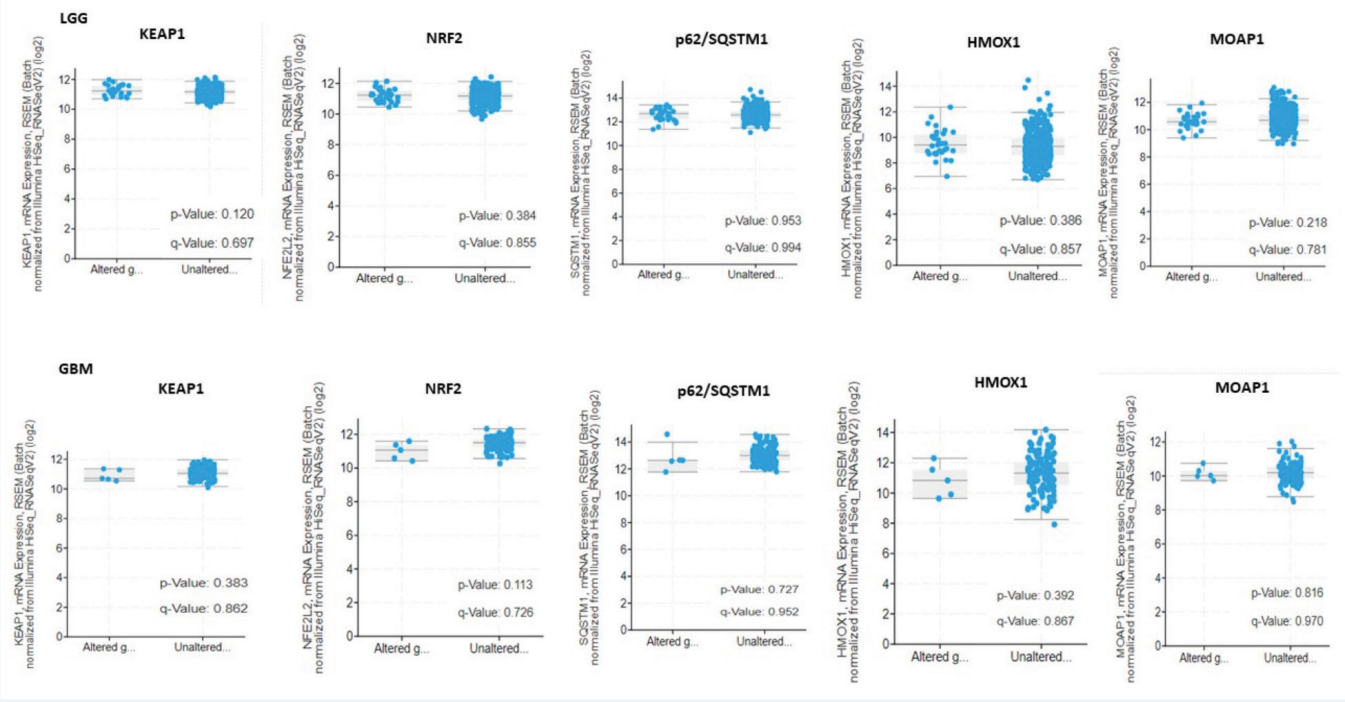

Figure 5. Mutational status for each gene is shown and the fold change indicates expression levels in the altered groups normalized to the expression levels in the unaltered groups (A) LGG (B) GBM

GBM: Glioblastoma multiforme, LGG: Low-grade gliomas

Gene fusions including NTRK1, 2, and 3 cause structural activation or overexpression of TRK receptors, potentially causing oncogenesis[22]. In the p62/SQSTM1-NTRK2 fusion protein, we detected in our study, SQSTM1, a multifunctional signal adapter involved in autophagy, fused to the 16-20 exons of NTRK2 with exon 1-5, resulting in the formation of a reading frame, which links the aminoterminal part of p62/SQSTM1 to the kinase domain of the TrkB. This, in turn, can lead to uncontrolled activation of p62/SQSTM1[4,12,21,22]. It has only been recently reported in the literature that Bax binding protein MOAP1 regulates the p62/SQSTM1-KEAP1-NRF2 signal via p62 degradation. MOAP1 interacts with the PB1-ZZ domains of p62 and disintegrates the p62 by interfering with its own oligomerization and liquid-liquid phase separation[23]. Since the p.R96Q mutation is located on the PBI domain, it may have the property to affect the MOAP1-p62/SQSTM1 interaction. HMOX-1, which expression is regulated by the NRF2 protein, is considered a cytoprotective agent and its modulation of expression activity levels offers therapeutic potential. HMOX-1 has been recognized as an antioxidant, anti-inflammatory, anti-apoptotic factor and is known to form one of the defense mechanisms against tissue damage caused by OS. In human glioma tumors, HMOX-1 is known to be overexpressed in comparison to normal brain tissues and during tumor progression, but the molecular mechanisms underlying how HMOX-1 affects glioblastoma tumor progression remain unclear[24,25]. The p.F33L and p.A96T mutations detected in our study are in the Heme oxygenease domain. We determined that p.F33L mutation was preserved among species throughout the evolutionary process. Pölönen et al.[12] reported that NRF2/p62 activation could be a prognostic marker for the mesenchymal subtype of GBM. We think that the missense mutations (C-1, C-3, C-4, C-5, and C-6) in the NRF2 and p62 in the GBM subgroup in present study may be responsible for the development of the mesenchymal subtype.

Study Limitations

We have performed comprehensive molecular profile analyses of the genes responsible for glioma pathogenesis. We are mindful that our study has certain limitations. This is because this study was carried out with a limited experimental design using bioinformatics tools. Therefore, a wet laboratory study and a larger sample group are needed to clarify the effect of KEAP1, NRF2, p62/SQSTM1, HMOX-1, and MOAP1 on glioma pathogenesis.

CONCLUSION

Gene expression analyses were also performed according to healthy patient samples in two tumor groups formed from the same patient population. As a result of our analysis of gene expression profiles, this pathway was found to be upregulated in both gliomas when compared to healthy samples of NRF2, KEAP1, and HMOX-1. The expression level of the MOAP1 is statistically significantly lower than the healthy sample group ($p<0.05$). However, we did not detect any significant changes in the level of expression of SQSTM1. Our study has results that can be useful in the development of new therapeutic approaches by determining the molecular differences and expression profiles between GBM and LGG. This study is important in terms of understanding the frequency and molecular features of the NRF2/KEAP1/p62/SQSTM pathway mutations detected in gliomas.

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Ethics

Ethics Committee Approval and Informed Consent: It is not required as it is analyzed *in silico* for bioinformatics.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: D.F.A., Design: D.F.A., Data Collection or Processing: D.F.A., S.H.A., Analysis or Interpretation: D.F.A., S.H.A., Literature Search: D.F.A., S.H.A., Writing: D.F.A.

Conflict of Interest: No conflict of interest was declared by the authors.

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