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Investigating the Association between Autophagy Markers LC3B, *SQSTM1/p62*, DRAM and Autophagy-Related Genes in Glioma

Farheen Danish [1], Muhammad Asif Qureshi [1,*], Talat Mirza [2], Wajiha Amin [3], Sufiyan Sufiyan [3], Sana Naeem [3], Fatima Arshad [1] and Nouman Mughal [3,*]

[1] Department of Pathology, Dow International Medical College, Dow University of Health Sciences, Karachi 75300, Sindh, Pakistan
[2] Departments of Research & Molecular Medicine, Ziauddin University, Karachi 75600, Sindh, Pakistan
[3] Departments of Biological and Biomedical Sciences, Agha University, Karachi 74800, Sindh, Pakistan
*   Correspondence: email2asifqureshi@gmail.com (M.A.Q.); noumanmughal2222@gmail.com (N.M.)

Abstract: High-grade gliomas are extremely fatal tumors, marked by severe hypoxia and therapeutic resistance. Autophagy is a cellular degradative process that can be activated by hypoxia, ultimately resulting in tumor advancement and chemo-resistance. Our study aimed to examine the link between autophagy markers expression in low (LGG) and high-grade gliomas (HGG). In 39 glioma cases, we assessed protein expression of autophagy markers LC3B, *SQSTM1/p62*, and DRAM by immunohistochemistry (IHC) and mRNA expression of autophagy genes; PTEN, PI3K, AKT, m-TOR, ULK1, ULK2, UVRAG, Beclin 1 and VPS34 using RT-qPCR. LC3B, *SQSTM1/p62*, and DRAM expression were positive in 64.1%, 51.3%, and 28.2% of glioma cases respectively. The expression of LC3B and *SQSTM1/p62* was notably higher in HGG compared to LGG. VPS34 exhibited a significant differential expression, displaying increased fold change in high-grade gliomas (HGG) compared to low-grade gliomas (LGG). Additionally, it exhibited robust positive associations with Beclin1 (rs=0.768), UVRAG (rs=0.802), and ULK2 (rs=0.786) in HGG. This underscores a potential association between autophagy and the progression of gliomas. We provide preliminary data for functional analysis of autophagy using cell culture model and to identify potential targets for therapeutic interventions.

Keywords: autophagy; autophagy related genes (ATG); high grade gliomas (HGG); low grade gliomas (LGG); *LC3B; SQSTM1/p62* & *DRAM*

1. Introduction

Central nervous system (CNS) cancers are rare and heterogeneous tumors with diverse biology and genetics and account for about 3% of all cancers in the world and are more common in men [1]. Gliomas constitute the most common type of brain tumors, comprising around 24.7% of all primary tumors in the brain and other CNS tumors and 74.6% of malignant cases [2]. The most prevalent malignant histology is glioblastoma making up 14.2% of all tumors and constituting 50.1% of all malignant CNS tumors [3]. New 2021 WHO CNS 5 classification has divided diffuse gliomas into adult type and pediatric type. The basic molecules for the integrated diagnosis of adult-diffuse gliomas are *IDH (*Isocitrate dehydrogenase*), p53, ATRX* (alpha-thalassemia/mental retardation, X-linked) & *1p/19q* co-deletion. Adult diffuse gliomas (IDH-mutant astrocytomas, IDH-mutant, 1p/19q codeleted oligodendroglioma and IDH-wildtype Glioblastoma) are diffusely infiltrating brain tumors [4]. High grade Gliomas predominantly wild type glioblastomas are extremely lethal neoplasms with a poor prognosis. Despite maximum neurosurgical resection and adjuvant therapy; temozolamide (TMZ), median survival barely extends to approximately 12 months [5]. Unfortunately, as with other solid tumors, chemo- resistance is one of the major challenges in this regard. Various mechanisms have been described for chemo resistance. Evasion of apoptosis is one of the mechanisms for tumor progression and chemo resistance in gliomas [6] probably through either deficiency in BAX or BAK or gain of Bcl2 or BclX [7]. Some studies have also revealed that the

resistance of cancer cells to chemotherapeutic drugs may be due to the up regulation of autophagy, thereby avoiding apoptosis [8,9].

Autophagy is a cellular degradation pathway for the breakdown and removal of impaired long lived proteins, as well as the elimination of organelles and pathogens[10].　Moreover, it has an important homeostatic role in maintaining the cell viability in stressed or nutritionally deprived states by recycling the cytoplasmic constituent [11]. Thus, it controls the quality and quantity of proteins and organelles. Dysfunctional autophagy contributes in various diseases, with cancer being one of them. Nevertheless, in cancer, role of autophagy is somewhat complicated and controversial. Autophagy is a double edge sword, has dual effects on cancer. On one hand, it promotes tumor cell survival, by breaking down macromolecules into smaller components like amino acids, fatty acids, and metabolic substances. Studies have suggested that autophagy is activated in glioblastoma (GBM) as a reaction to pathophysiological challenges like necrosis and an acidic milieu. Hypoxia, a characteristic feature of high-grade gliomas, is responsible for inducing autophagy in these tumors. As the tumor progresses and reacts to therapeutic interventions, cells must adjust their metabolism to endure in hypoxic and nutrient-deficient surroundings, which is commonly linked to chemotherapy and radiotherapy resistance. This process facilitates tumor growth and viability, contributing to the maintenance of intracellular metabolism. Under unfavorable hypoxic conditions, molecules such as hypoxia-induced factors (HIF2a and HIF2b), BECN1 (Beclin-1), and BNIP3(BCL2/adenovirus E1B 19KDa interacting protein 2) serve as survival mechanisms, promoting the progression of GBM and resistance to anticancer treatment in vivo [12–15]. Hypoxia causes BECN1 phosphorylation via the HIF-1a/BECN1 signaling pathway[16,17].Beclin-1 is a key regulatory protein in autophagosome formation, which binds to the class III PI–3 kinase Vps34, thus facilitating the progression of autophagic process [18,19]. Remarkably, in the presence of hypoxia, the initiation of autophagy through BNIP3/BNIP3L serves as a survival mechanism, fostering the progression of GBM and enhancing resistance to anticancer treatments in vivo [20]. A recent study showed that in U87 cells exposed to hypoxic conditions, the knockdown of HIF1A significantly reduced BNIP3 expression[21]. This implies that tissues with low oxygen levels are prone to show heightened autophagy, indicating that an increased autophagy flux may serve an adaptive function [15,22]. On the other hand, autophagy can also suppress tumor progression in development, as impaired autophagy causes oxidative stress, triggers DNA damage responses and results in genomic instability, a known cause for tumor initiation. Such dual effects on tumor behavior are most likely context dependent [23–26]. Moreover research indicated that prolonged hypoxic stress in vitro, up regulates a pro-apoptotic Bcl-2 protein (BNIP3), leading to hypoxia-dependent autophagic cell death (ACD) in GBM cells [27]. In terms of the mechanism, the elevated expression of BNIP3 leads to the release of BECN1 from complexes with Bcl-2 or Bcl-xL, thereby enabling BECN1 to activate autophagy[28]. Hence, autophagy has been found to show conflicting functions in tumor onset and advancement. Thus, manipulation of autophagy pathways as means of cell death in cancer has led to the exploration of both inhibitors and inducers.　Keeping in mind the contradictory results of autophagy in cancer, we investigated expression of autophagy markers and autophagy related genes (ATG) in our cohort of low and high grade gliomas.

2. Results

2.1. Clinicopathologic characteristics ofAdult Diffuse Glioma Patients

Demographic data of the studied cases showed that average age of the patients was 43.14 ± 13.25. Of the 39 adult diffuse glioma patients that were enrolled in this study, 23 (59%) were males and16 (41%) were females. Histological examination showed that *IDH-* mutated astrocytic tumors were identified as most frequently diagnosed subtype of adult diffuse gliomas (48.7%), followed by oligodendroglial tumors (30.8%) and Glioblastoma *IDH*-wild type (20.5%). When cases were stratified on the basis of histological grades, 15 (38.5%) cases were of grade 4, 13 (33.3%) were grade 3 and 11 (28.2%) were grade 2. Moreover, of the 39 cases, 25 (64.1%) showed *IDHR132* mutation, 16 (41%) showed *p53* mutation and 21 (53.8%) showed *ATRX* loss (mutation*)*, as shown in Table 1.

Table 1. Clinicopathologic characteristics of Adult diffuse glioma patients.

Variables		Values
Gender	Male	23 (59%)
	Female	16 (41%)
Age in years	Median (Range)	43 (47)
Histopathological Type	Astrocytoma (Grade 2-4)	19 (48.7%)
	Oligodendroglioma (2 and 3)	12 (30.8%)
	Glioblastoma (Grade 4)	08 (20.5%)
Histopathological grade	2	11 (28.2%)
	3	13 (33.3%)
	4	15 (38.5%)
Glioma Grade Group	Low Grade	11 (28.20%)
	High Grade	28 (71.8%)
IDH 1 **Mutant, N (%)**		25 (64.1%)
TP53, N (%) Mutant		16 (41%)
ATRX, N (%) Mutant		21 (53.8%)

2.2. Evaluation of autophagy status using immunohistochemical analysis of Molecular markers (LC3B, SQSTM1/p62 and DRAM)

Autophagy-related protein expression of *LC3B, SQSTM1/p62* and *DRAM* was performed in 39 LGG and HGG cases to assess the autophagy status in the current study. Diffuse cytoplasmic and nuclear expression of *LC3B* was found in all cases. Nonetheless, strong punctate cytoplasmic expression of *LC3B* and *SQSTM1/p62* was categorized as a positive result, and the presence of positive cytoplasmic staining for *DRAM* was also considered as a positive outcome. Our result showed a strong punctate cytoplasmic expression of *LC3B* and *SQSTM1/p62* and in HGG when compared with LGG and peritumoral non-glioma tissue. Additionally *DRAM* also exhibited positive and negative cytoplasmic staining (Figure 1).
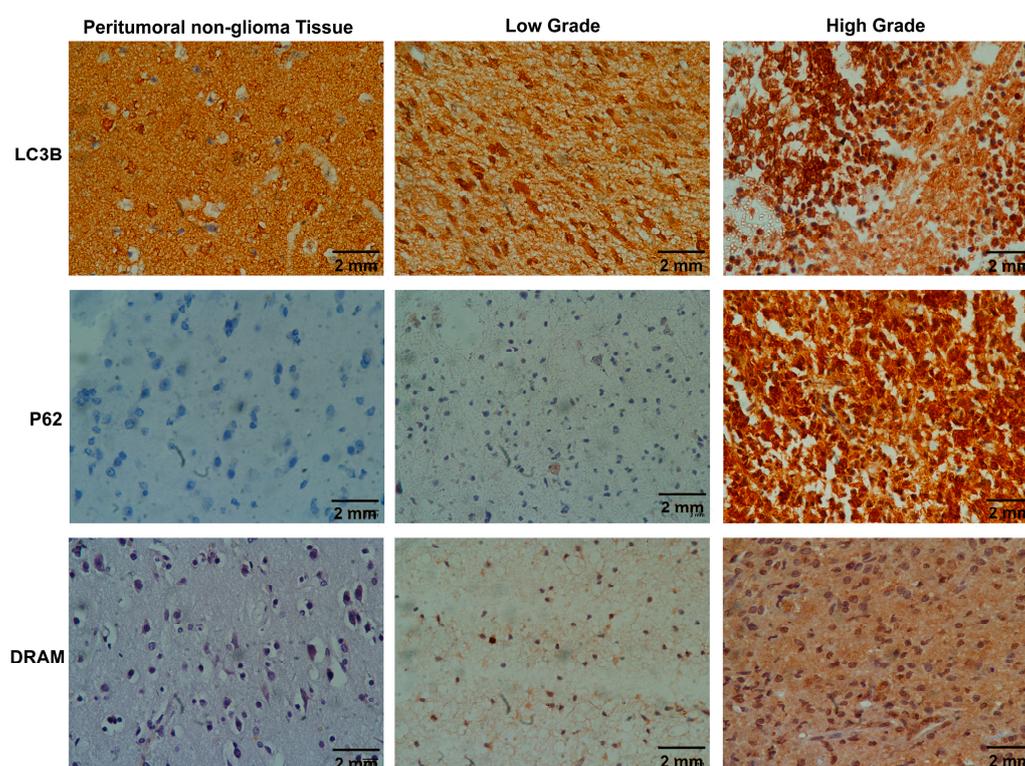

Figure 1. Representative images of autophagy markers, *LC3B, SQSTM1/p62 & DRAM* immunohistochemical stains in peritumoral non-glioma tissue, low grade glioma (LGG) and high

grade gliomas (HGG) at x400. Peritumoral non-glioma tissues and LGG showing negative staining for autophagy markers while HGG displaying strong dotted or punctate cytoplasmic staining for *LC3B* and *SQSTM1/p62* and diffuse cytoplasmic staining for *DRAM*.

2.3. Correlation of clinicopathological features and autophagy markers

Among the 39 glioma cases analyzed, 23 (59%) cases of HGG showed a positive expression of LC3B, whereas *SQSTM1/p62* demonstrated positive expression in all 20 (100%) HGG cases. DRAM overexpression was detected in the cytoplasm of 11 (28.2%) cases, encompassing both LGG and HGG (Table 2).

Table 2. Frequency of *LC3B, SQSTM1/p62 and DRAM* in 2, 3 & 4 WHO grades of Adult diffuse Gliomas.

		LC3B		P62		DRAM	
	Total	**Punctate**	**Diffuse**	**High**	**Low**	**Positive**	**Negative**
Cases	39	25 (64.1%)	14 (35.9%)	20 (51.3%)	19 (48.7%)	11 (28.2%)	28 (71.8%)
Grades	**G-2** (11)	02 (8%)	09 (64.2%)	00 (0%)	11 (58%)	04 (36.3%)	07 (25%)
	G-3 (13)	12 (48%)	01 (7.1%)	10 (50%)	03 (15.7%)	03 (27.2%)	10 (35.7%)
	G-4 (15)	11 (44%)	04 (28.5%)	10 (50%)	05 (26.3%)	04 (36.3%)	11 (39.2%)

Consequently, elevated levels of *LC3B* and *SQSTM1/p62* were frequently observed in HGG, with corresponding p-values of 0.001 and <0.001, respectively. However there was no significant correlation between *DRAM* overexpression and histologic grades (Table 3). Furthermore, autophagy status was considered to be positive in 19 (48.7%) cases, in this study population. A statistically significant correlation was detected when comparing the age groups with the autophagy markers. Significant correlations were found between age groups and the autophagy markers *LC3B* (p = 0.022) and *SQSTM1/p62* (p = 0.017). Moreover, when examining the correlation between autophagy markers and clinicopathologic factors such as gender, histological type, and molecular markers of glioma including *IDH, ATRX*, and *p53*, no statistically significant differences were observed (Table 4).

Table 3. Association of autophagy markers with histological grades.

Grade	LC3B		p-value	P62		p-value	DRAM		p-value
	Punctate	**Diffuse**		**High**	**Low**		**Positive**	**Negative**	
2	2(18%)	09(82%)		0(0%)	11(100%)		4(36%)	07(64%)	
3	12(92%)	1(7%)	**0.001***	10(77%)	3(23)	**0.001***	3(23%)	10(77%)	0.760
4	11(73%)	4(27%)		10(67%)	5(33%)		4(27%)	11(73%)	

Table 4. Correlation of autophagy markers with clinicopathologic parameters.

Clinicopathologic Parameters	*LC3B*		p-value	*P62*		p-value	*DRAM*		p-value
	Punctate	**Diffuse**		**High**	**Low**		**Present**	**Absent**	
Age <45	10(40%)	11(79%)	**0.022**	07(35%)	14(74%)	**0.017**	05(45%)	16(57%)	0.380
>45	15(60%)	03(21%)		13(65%)	05(26%)		06(55%)	12(43%)	
Gender									
Male	17(68%)	06(43%)	0.117	14(70%)	09(47%)	0.133	09(82%)	14(50%)	0.70
Female	08(32%)	08(57%)		06(30%)	10(53%)		02(18%)	14(50%)	

Histological type

Oligodendroglioma	09(75%)	3(25%)		09(75%)	03(25%)		04(33%)	08(67%)	
Astrocytoma	12(63%)	07(37%)	0.517	08(42%)	11(58%)	0.139	05(26%)	14(74%)	0.891
Glioblastoma	04(50%)	04(50%)		03(38%)	05(62%)		02(25%)	06(75%)	

Histological grade

2	02(18%)	09(82%)	**0.001**	0(0%)	11(100%)		04(36%)	07()64%)	
3	12(92%)	01(8%)		10(77%)	03(23%)	**<0.001**	03(23%)	10(77%)	0.760
4	11(73%)	04(27%)		10(67%)	05(33%)		04(27%)	11(73%)	

IDH1-R132 mut

Present	18(72%)	07(28%)	0.153	14(56%)	11(44%)	0.325	08(32%)	17(68%)	0.376
Absent	07(50%%)	07(50%)		06(43%)	08(57%)		03(21%)	11(79%)	

***ATRX* mut/Loss**

Present	12(57%)	09(43%)	0.261	07(33%)	14(67%)	0.17	05(24%)	16(76%)	0.380
Absent	13(72%)	05(28%)		13(72%)	05(28%)		06(33%)	12(67%)	

***Tp53* mut**

Present	09(56%)	07(44%)	0.303	06(38%)	10(62%0	0.133	06(38%)	10(62%)	0.237
Absent	16(70%)	07(30%)		14(61%)	09(39%)		05(22%)	18(78%)	

2.4. Analysis of Autophagy related genes (ATG) expression in High grade and Low grade gliomas

Following the immunohistochemical analysis of well-established autophagy markers, we selected nine autophagy related genes including the master regulators and core autophagy genes such as *PTEN, PI3K, AKT, mTOR, ULK1, ULK2, UVRAG, Beclin 1* and *VPS34.* The difference in relative mRNA expression of autophagy related genes was compared in different grades of gliomas in 39 cases. We found a highly significant statistical difference in *VPS34* mRNA relative expressions among grade 2 and 3 (p=0.01) and grade 2 and grade 4 (p=0.01). Nevertheless, there was no notable difference in expression of other genes associated with the essential autophagy machinery between low and high grade gliomas. Comparisons of *PI3k* (p=0.495), *AKT* (p=0.85), *PTEN* (p=0.78), *Beclin1* (p=0.217), *ULK1* (p=0.785), *ULK2* (p=0.524), and *UVRAG* (p=0.387) between grade 2 and grade 4 showed statistically insignificant differences. Similarly, the negative autophagy regulator *mTOR* was not significantly differentially expressed between the two glioma grades (p = 0.07), but higher expression was noticed in higher grade.
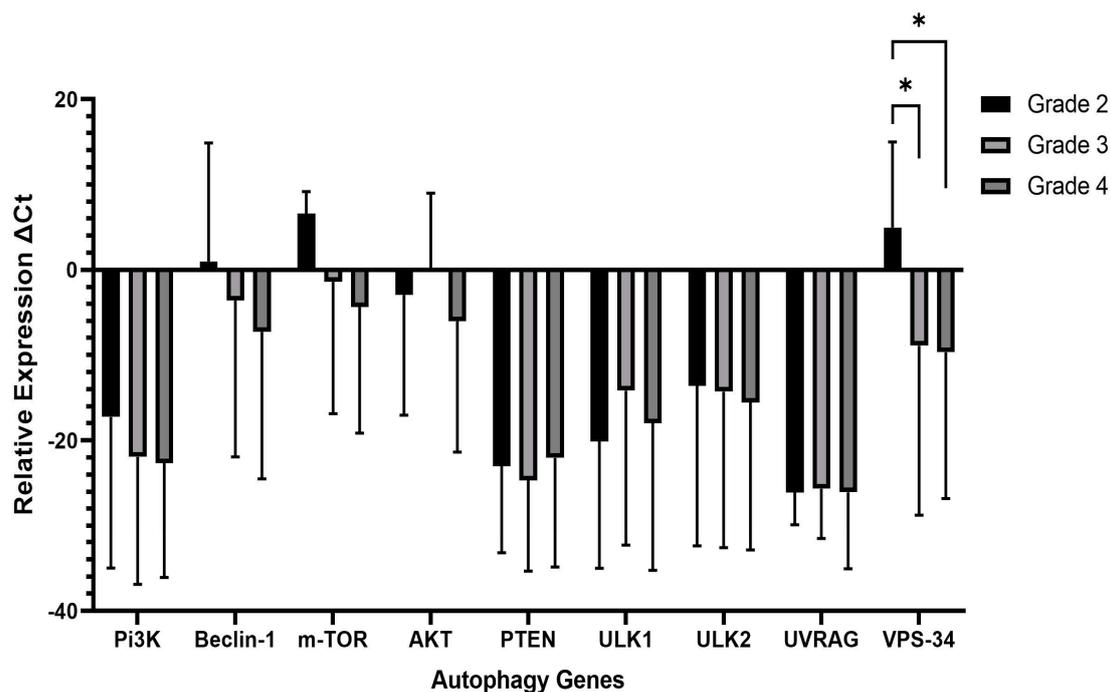
Figure 2. Expression of autophagy-related genes (ATG) in 2, 3 & 4 WHO grades of diffuse gliomas in 39 patients using housekeeping gene Beta-actin. The Y-axis shows the relative expression of each ATG gene tested. The line with the asterisk sign shows a significant difference (*$p < 0.01$) in the expression of VPS34 between the various grades of gliomas.

Next, log2 fold change (FC) was executed using grade 2 gliomas as reference group. Two genes were selected for log2 fold change (FC) ≥ 2. We found expression of *VPS34* to be significantly increased in higher grade gliomas (p=0.01). Expression of mTOR, which is a negative regulator of autophagy, is increased in grade 3 and grade 4. However it didn't reach statistical significance (p=0.07).
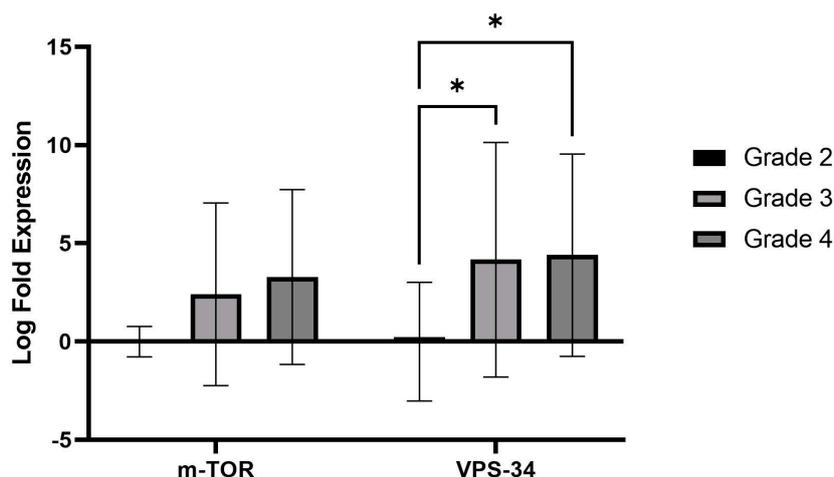

Figure 3. The graph depicts the fold change in the expression of VPS34 and mTOR in 2, 3 & 4 WHO grades of gliomas. The Y-axis represents the logarithmic fold expression. The lines marked with asterisks indicate a notable difference (**p < 0.01) in VPS34 expression between low and high-grade cases.

Furthermore, we applied Spearman correlation test to examine the correlation between ATG genes in the WHO grade 2, 3 and 4 gliomas. In WHO grade 2, Significant strong positive correlation was observed between *PI3K and PTEN, ULK2; mTOR and PTEN, ULK1; PTEN and PI3K, m-TOR, ULK1, ULK 2, UVRAG; ULK1 and mTOR; ULK 2 and PI3K, PTEN, ULK 1; UVRAG and PTEN.* Significant moderate positive correlation was observed between *BECLIN 1* and *mTOR, PTEN* and *BECLIN 1, ULK2 and UVRAG, VPS34; UVRAG and ULK2; VPS34* and *ULK 2* (Table 5).

Table 5. Correlation between autophagy related genes in grade 2 Gliomas.

	PI3K	BECLIN1	mTOR	AKT	PTEN	ULKI	ULK2	UVRAG	VPS34
PI3K	-	0.495	0.863	0.505	<u>0.824</u>	0.995	<u>0.731</u>	0.978	0.560
BECLIN1	0.495	-	<u>0.615</u>	0.110	<u>0.670</u>	0.505	0.462	0.495	0.275
m-TOR	0.863	<u>0.615</u>	-	0.357	<u>0.835</u>	<u>0.846</u>	<u>0.604</u>	0.896	0.473
AKT	0.505	0.110	0.357	-	0.434	0.500	0.247	0.489	0.055
PTEN	<u>0.824</u>	<u>0.670</u>	<u>0.835</u>	0.434	-	0.802	<u>0.714</u>	<u>0.846</u>	0.577
ULK1	0.995	0.505	<u>0.846</u>	0.500	<u>0.802</u>	-	<u>0.753</u>	0.956	0.516
ULK2	<u>0.731</u>	0.462	0.604	0.247	<u>0.714</u>	<u>0.753</u>	-	<u>0.670</u>	<u>0.626</u>
UVRAG	0.978	0.495	0.896	0.489	<u>0.846</u>	0.956	<u>0.670</u>	-	0.599
VPS34	0.560	0.275	0.473	0.055	0.577	0.516	<u>0.626</u>	0.599	-

The table displays the correlation coefficient (r) value for each pair of genes, with underlined values indicating statistically significant ($p<0.05$) correlations between gene pairs.

In grade 3 gliomas, significant strong positive correlation was observed between PI3K and mTOR, ULK2, UVRAG; mTOR and PTEN, ULK1; PTEN and PI3K, m-TOR, ULK1, ULK 2, UVRAG; mTOR and PI3K, PTEN and mTOR, ULK2, UVRAG; ULK1 and ULK2, UVRAG; ULK 2 and PI3K, PTEN, ULK 1, UVRAG, VPS34; UVRAG and PI3K, PTEN, ULK1, ULK2,VPS34. Significant moderate positive correlation was observed between PI3K and ULK1; BECLIN 1 and mTOR; mTOR and VPS34; PTEN and ULK1; ULK1 and PI3K, VPS34 (Table 6).

Table 6. Correlation between autophagy related genes in grade 3 Gliomas.

	PI3K	BECLIN1	mTOR	AKT	PTEN	ULKI	ULK2	UVRAG	VPS34
PI3K	-	0.866	<u>0.748</u>	-.094	0.988	<u>0.674</u>	<u>0.781</u>	<u>0.747</u>	0.192
BECLIN1	0.886	--	<u>0.659</u>	0.143	0.852	<u>0.566</u>	0.549	0.567	0.875
mTOR	<u>0.748</u>	<u>0.659</u>	-	-.088	<u>0.714</u>	0.544	0.527	0.396	<u>0.627</u>
AKT	-.o94	0.143	-.088	-	-.104	-.346	-.538	-.380	-.204
PTEN	0.988	0.852	0.714	-.104	-	<u>0.621</u>	0.753	<u>0.735</u>	0.889
ULK1	<u>0.674</u>	0.566	0.544	-.346	<u>0.621</u>	-	0.742	<u>0.784</u>	0.652
ULK2	<u>0.781</u>	0.549	0.527	-.538	<u>0.753</u>	<u>0.742</u>	-	<u>0.814</u>	0.715
UVRAG	<u>0.747</u>	0.567	0.396	-.380	<u>0.735</u>	<u>0.784</u>	0.814	-	0.802
VPS34	0.912	0.875	<u>0.627</u>	-.204	0.899	<u>0.652</u>	0.715	<u>0.802</u>	-

The table displays the correlation coefficient (r) value for each pair of genes, with underlined values indicating statistically significant ($p<0.05$) correlations between gene pairs.

In WHO grade 4 diffuse gliomas, significant strong positive correlation was observed between *PI3K* and *BECLIN 1, ULK2; BECLIN 1* and *ULK1, ULK2, VPS34; ULK2* and *ULK1; UVRAG* and *ULK2; VPS34* and *ULK2.* Significant moderate positive correlation was observed between *PI3K* and *PTEN* and *VPS34; ULK 1* and *VPS34* (Table 7).

Table 7. Correlation between autophagy related genes in grade 4 Gliomas.

	PI3K	BECLIN1	mTOR	AKT	PTEN	ULKI	ULK2	UVRAG	VPS34
PI3K	-	<u>0.721</u>	0.461	0.479	0.646	0.943	<u>0.782</u>	0.893	0.600
BECLIN1	<u>0.721</u>	-	0.575	0.514	0.382	<u>0.707</u>	<u>0.714</u>	0.579	<u>0.768</u>
mTOR	0.461	0.575	-	0.504	0.557	0.496	0.386	0.432	0.411
AKT	0.479	0.514	0.504	-	0.443	0.571	0.486	0.361	0.464
PTEN	<u>0.646</u>	0.382	0.577	0.443	-	0.568	0.375	0.504	0.257

ULK1	0.943	<u>0.707</u>	0.496	0.571	0.568	-	<u>0.811</u>	0.886	<u>0.643</u>
ULK2	<u>0.782</u>	<u>0.714</u>	0.386	0.486	0.375	0.811	-	<u>0.729</u>	<u>0.786</u>
UVRAG	0.893	0.579	0.432	0.386	0.574	0.886	0.729	-	0.514
VPS34	<u>0.600</u>	<u>0.768</u>	0.411	0.464	0.257	<u>0.643</u>	0.786	0.514	-

The table displays the correlation coefficient (r) value for each pair of genes, with underlined values indicating statistically significant ($p<0.05$) correlations between gene pairs.

3. Discussion

Autophagy is primarily a stress response process, and most of body tissues need autophagy to eliminate accumulated damaged organelles and unfolded proteins to maintain normal homeostatic milieu. In cancer, autophagy plays both tumor suppressing and tumor promoting effects depending on the specific context, thus could influence on the prognosis, either favorably or adversely. We investigated whether autophagy plays anti-tumoral or pro-tumoral functions in various grades of gliomas.

In this current research, we assessed the immunohistochemical expression of autophagy markers as well as mRNA levels of ATG in different grades of adult-type diffuse gliomas, while also assessing their correlation with clinicopathological parameters. Our investigation revealed a significant connection between the immunohistochemical expression of *LC3B, SQSTM1/p62, DRAM*, and the overall autophagy status, particularly in relation to tumor grades. Notably, among the 25 positive cases of *LC3B*, only two were observed in the LGG. Similarly, out of the 20 strongly positive cases of *SQSTM1/p62*, none was found in the LGG category, and this difference achieved statistical significance (as indicated in Table 3). These results were in accordance with other studies which reported that *LC3B* and *SQSTM1/p62* was highly expressed in high grade gliomas [29–31]. Remarkably there is statistically significant association between the autophagy markers *LC3B* and *SQSTM1/p62* (p=0.000), highlighting the interaction among each other.   These results may be attributed to the active participation of *LC3B* and *SQSTM1/p62* in the process of autophagy, given their roles as essential structural components of the autophagosome[32] and recent literature also revealed that that *SQSTM1/p62* overexpression is capable of promoting mitochondrial and classical macroautophagy [33], which promote tumor progression and chemo-resistances. Furthermore increased expression of *LC3B* and *SQSTM1/p62* in HGG compared with LGG may be in response to confer stress tolerance which is definitely more in HGG, serves to maintain tumor cell survival [34]. Hypoxia, which is major hallmark of Glioblastoma, is known to induce autophagy in these tumors, which eventually produce survival mechanism facilitating the breakdown of various cellular components to generate ATP and metabolic precursors as a means to cope up with stress. This highlights the crucial role of autophagy in protecting cells against stressful conditions[35]. In a study conducted by Deng et al; it was reported that both mRNA and protein levels of *SQSTM1/p62* were elevated in human glioma tissues, and the suppression of *SQSTM1/p62* had an anti-tumor effect on glioma cells [34]. These findings may support that high levels of *LC3B* and *SQSTM1/p62 e*xpression or prompted autophagy correlated with advanced tumor grade and aggressiveness with the potential role of autophagy as tumor enhancer, thus supporting our results.

We also looked another important autophagy modulator *DRAM1* in our study and found its overexpression in 28.2% of the cases, although it was not significantly associated with tumor grades but higher expression of *DRAM1* was present in high grade gliomas, when compared with LGG. *DRAM 1* primarily resides in lysosomes and is frequently down regulated in various human cancers[36]. *DRAM1* enhances lysosomal acidification and facilitates the fusion of lysosomes with autophagosomes, thus triggers the autophagy. Moreover *DRAM1* plays pivotal role in governing the association of *SQSTM1/p62* with autophagosomes and its subsequent degradation through autophagy. Therefore, reduced *DRAM1* expression might be associated with decreased *SQSTM1/p62* localization within autophagosome, highlighting the role of *DRAM1* in *SQSTM1/p62* -mediated autophagy. Our findings align with the study by Geng et al; which also reported reduced *DRAM1* expression in non-small cell lung carcinoma, correlating with an unfavorable prognosis [37]. However, another study has shown high expression of both *DRAM1* and *SQSTM1/p62* in

Glioblastoma, where they regulate cell migration and invasion, and are associated with shorter or poor overall survival [38]. These conflicting results could arise from variations in demographics and sample sizes. Thus utilizing the expression levels of *LC3B, SQSTM1/p62* and *DRAM1* to assess autophagy status in glioma patients could be potential predictive markers.

While investigating the autophagy gene expression patterns in both low (grade 2) and high (grade 3 and 4) grades of tumors, we noticed a remarkable and statistically significant up-regulation in autophagy critical kinase, vacuolar protein sorting 34 (*Vps34*) gene expression thus indicating a tumor promoter role of *VPS34* gene in autophagy. Previous research has shown that *VPS34* is involved in initiating autophagy through its interaction with Vps15/Atg14/UVRAG/Beclin1[39]. Consequently, this has gathered considerable attention as a potential target for inhibiting autophagy [40,41] .

We also evaluated the autophagy upstream pathway, *PI3K/AKT/mTOR* and recorded higher expression of *mTOR* transcripts in HGG as compared to LGG, which is a negative regulator of autophagy but it didn't reach significant value($p = 0.07$). *PI3K/Akt/mTOR* signaling pathway is a frequently disrupted pathway across different cancer types [42,43] and abnormal activation of this pathway has been associated with tumor development, progression, invasion and metastasis [44] and is indeed activated in glioma cells [45]. Nonetheless the *PTEN, PI3K, AKT, mTOR, Beclin-1, UVRAG, ULK1* and *ULK2* genes were remained unchanged when compared in both grades. The significant increase in transcriptional expression of VPS34 implies an increased initiation of autophagy, as VPS34 plays a crucial role in the nucleation of autophagosomes. Conversely, the absence of substantial transcriptional alterations in other genes suggests that these components may not be profoundly influenced at the transcriptional level in high-grade gliomas. Nevertheless, it is important to consider the potential involvement of post-transcriptional, post-translational, or alternative regulatory mechanisms. The specific modulation of autophagy in high-grade gliomas appears to be contingent on the unique context of these tumors. Gliomas exhibit heterogeneity, and the signaling pathways governing autophagy may be subject to the diverse genetic and epigenetic profiles inherent to individual tumors.

The spearman's correlation between autophagy genes was examined in grades 2, 3 and 4 glioma cases and we found significant positive correlation among different genes. It's essential to highlight that these observed correlations were statistically significant, but whether they have a biological basis or not remains uncertain. For instance in grade 2 gliomas *PTEN* showed a strong positive correlation with autophagy genes in line with a study by Errafiy Rajaa [46]. However the absence of *PTEN* correlation in grade 4 in current study highlights loss of *PTEN* which is a hallmark of GBM, could be due to mutation or promoter methylation of *PTEN* gene [47,48].

Similarly in HGG the significant positive correlation between *VPS34* and *ULK1, ULK2, UVRAG, Beclin1, PI3K* genes can also be justified biologically, as literature prevailed that *VPS34* kinase forms a stable complex with Beclin1 and p150 which serves as binding partner for *ATG14L, UVRAG and AMBRA* [49] which are responsible for promotion of autophagy. Thus significant positive correlation indicates that expression of autophagy genes tends to change consistently together, providing insights into the regulatory connections within the autophagic pathways. This understanding may have implications for comprehending the underlying mechanisms influencing tumor progression.

Nevertheless, a limitation of our study is the absence of autophagy flux assessment in formalin fixed paraffin embedded tissue blocks (FFPE) samples. Elevated expression levels of *LC3B and SQSTM1/p62* do not consistently correlate with an overall augmentation in autophagy and may be attributed to a potential hindrance in autophagy at the later stages of autophagosome processing. Thus need for a more comprehensive assessment of autophagy, particularly considering the dynamic nature of the process. Moreover, ATG5, ATG12, ATG 7 and ATG 4 genes are crucial for two conjugation systems (Atg8-Atg4 and Atg12-Atg5) involved in autophagy and would provide valuable information, as these processes contribute to the formation and elongation of autophagosomes, facilitating the degradation and recycling of cellular components. Unfortunately, due to financial constraints, we were unable to conduct this analysis. However, future comprehensive studies utilizing diverse techniques will be invaluable in addressing these limitations.

4. Materials and Methods

4.1. Patients Selection

Patients of adult –diffuse gliomas were enrolled from Histopathology department of Dow Diagnostic Reference and Research Lab (DDRRL), Dow University of Health Sciences (DUHS). The research protocol received approval from the Institutional Review board at Dow university of health sciences [Ref: IRB-1150/DUHS/Approval/2018]. Clinical information was recorded. The inclusion criteria required the inclusion of patients diagnosed with adult diffuse gliomas of all grades. Nevertheless, patients diagnosed with pediatric-type diffuse gliomas and astrocytomas with circumscribed morphology were excluded from the study.   Initially, there were 50 prospective cases of adult diffuse gliomas that were included in the study. Nonetheless, due to the use of multiple immunohistochemical stains and the extraction of nucleic acids from tissue samples, some cases ran out of available tissue. Consequently, 39 patients were finally included in the study. The mRNA and protein expression of autophagy-related genes (ATG) were determined using Quantitative real-time PCR (qPCR) and IHC.

4.2. Tissue processing for histopathological analysis

Biopsy specimens of brain tumors were received in histopathology department from different hospitals and centers of city through its collection point service. For histopathological examination, every specimen was placed in an automated tissue processor (Thermo scientific, model EXCELSIOR AS) for 12 hours, where they were exposed to graded series of alcohol as 70%, 95% 100% followed by xylene, and then paraffin. The paraffin blocks obtained were sliced into sections measuring 4-5µm thickness for subsequent hematoxylin and eosin staining (H&E). Before staining slides were deparaffinized and sections were treated with xylene, followed by hydration through decreasing concentration of ethanol, as 100%, 70% and 50%.   Slides were then cleaned with water before being submerged in H & E for staining. To eliminate excess water from the slide after staining, the sections were submerged in 60%, 80%, and 100% ethanol. Next, slides were mounted by coverslip with Enthelan® after being submerged in xylene (Merck, Darmstadt, Germany). Histopathological examination of the H &E-stained slides at a microscopic level was conducted utilizing the Nikon Eclipse E200 optical microscope (Nikon Instruments Inc. in Tokyo, Japan) and the tumor characterized as adult diffuse gliomas were enrolled in the study. The cases of diffuse gliomas were categorized according to CNS5 classification into distinct subtypes and malignancy grades using histopathological features such as cellularity, atypia, necrosis, micro-vascular proliferation and mitosis.

4.3. Tissue processing for immunohistochemical examination

IHC was carried out using Autostainer Link 48 (Dako North America Inc., USA, S no AS3006D1307), on formalin-fixed paraffin embedded (FFPE) tissue blocks for *LC3B, p62* and *DRAM* antibodies. Sections were sliced into 3-4µm and affixed on charged slides (EnVision FLEX visualization systems) and dried for 60 minutes at 60-70-ºC. Following deparaffinization with xylene rinsed in decreasing concentration of ethanol and then rehydrated in distilled H2O. To facilitate antigen unmasking, retrieval solution (EnVision Flex Target Retrieval solution, pH 9.0, TRIS HCL) was used for 20 minutes in a preheated water bath. To quench endogenous peroxidase activity, slides were immersed in peroxidase blocking solution (EnVision Flex Peroxidase blocking reagent, RTU) for 10 minutes. TBST (Tris Buffer saline with Tween 20, EnVision Flex wash Buffer) was used for washing then sections were incubated for 30-60 minutes at room temperature with primary antibodies LC3B ((ABCAM, ab51520: 1: 800), p62 ((Invitrogen, clone: SOSTM1: 1: 25) and DRAM (Invitrogen, 1; 25). Subsequently, the sections were subjected to a 30-minute treatment at room temperature with a secondary antibody (EnVision Flex/HRP, RTU), after washing with PBS buffer. DAB solution 3, 3-diaminobenzidine tetrahydrochloride solution (EnVision Flex DAB+ chromogen) was applied for 10 minutes to the sections on the slides to reveal the color of antibody staining and

counterstained with hematoxylin. Slides were washed for 10 minutes in running water. Sections were then dehydrated (in graded alcohol as 80%, 90%, and 100%), cleaned and cover-slipped using DPX mounting solution.

4.4. Immunohistochemical evaluation of autophagy markers

LC3B and *SQSTM1/p62* punctate/dotted cytoplasmic staining was considered as positive [50,51]. The immunopositivity was scored on the basis of strong intensity and percentage positive glioma cells. Immunopositivity of >50% of tumor cells was considered as positive for *LC3B*, whereas for *SQSTM1/p62* it was considered positive when it exceeded >30%[52]. The intensity and percentage scores were multiplied to find the *DRAM* overexpression. The intensity of *DRAM1* staining was scored as follows: 0 (no staining), 1 (weak staining), 2 (moderate staining), and 3 (strong staining). Percentage scores were assigned as follows: 1 (0–25%), 2 (26–50%), 3 (51–75%), and 4 (71–100%). Every sample was assigned a score, which was multiplied to get a total value that ranged from 0 to 12. Score of ≥4 was defined as *DRAM1* overexpression, and score of < 4 was categorized as weak or negative expression [53]. The immunohistochemical staining was independently scored by two pathologists at 40x objective magnification and then discrepancies were discussed on a multi head microscope and final scores were determined. Autophagy status was considered as positive when two out of the three autophagy-associated proteins were detected in each sample[54].

4.5. RNA extraction and cDNA synthesis

For the RT-qPCR analysis, total RNA was isolated from FFPE blocks via Pure Link FFPE, total isolation (Invitrogen; Thermo Fisher Scientific, Inc) according to the manufacturer's protocols [55]. Subsequently DNase treatment was carried out for any DNA contamination. This involved combining 1μg of RNA template with 1μl of reaction buffer containing MgCl2, 1μl of DNase-I, RNase-free (Thermo Fisher Scientific, Cat. No. EN0521), and nuclease-free water in a 0.2 ml tube. The resultant mixture was incubated in the Master cycler X50a (Eppendorf, Germany) for 30 min at 37 °C. To prevent RNA degradation, following the DNase-I treatment, we introduced 1μl of 50 mM EDTA and the samples were incubated at 65 °C for 10 minutes. The RNA integrity was evaluated with a nanodrop, and subsequently, cDNA was generated according to the manufacturer's instructions using the Revert-Aid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Cat. No. K1612). The resulting cDNA was stored at -20°C for future applications.

4.6. Gene expression analysis via quantitative real time (qPCR)

qPCR was conducted to assess the genes expression levels of *ULK1, ULK2, Beclin 1, UVRAG, VPS34, PTEN, PI3K, AKT,* and *mTOR*, using PCR kit (PowerUp™ SYBR™ Green Master Mix (Thermo Fisher Scientific, Cat. No A25742), and primers (Eurofins, USA). β-actin, a housekeeping gene was used for result normalization in a qPCR assay, utilizing the corresponding primer sets. To perform qPCR analysis, cDNA samples were used and 10uL reaction mixture was prepared. Mixture comprised of 2 microliter cDNA, 1μl primers of both forward and reverse primers, and 5μl of PowerUp™ SYBR™ Green Master Mix and 1-2 microliter of nuclease free water. The thermal cycling conditions for the reaction were as follows: an initial 2 min hold at 50°C, another 2-minute hold at 95°C, and then 40 cycles of denaturation for 15 sec at 95°C followed by annealing at 60°C for 1 minute. 2-ΔΔCt (Livak's method) was used to analyze the relative changes in gene expression and multivariate Anova test was performed for statistical significance. Following the differential expression of ATG genes, the log2 fold change was calculated. Grade 2 gliomas, which are considered low grade, were chosen as the baseline group for computing the log2 fold change (FC). Table 8 shows the list of primers that were utilized.

Table 8. Names of target genes of and their corresponding primer employed for mRNA quantification.

Genes	Forward primer	Reverse primer
Beclin-1	5′ -AATGACTTTTTTCCTTAGGGGG-3′	5′ -GTGGCTTTTGTGGATTTTTTCT-3′
m-TOR	5′ -TGGGACAGCATGGAAGAATA-3′	5′- TGTTGTGCCAAGGAGAAGAG-3′
UVRAG	5′- CTGTTGCCCTTGGTTATACTGC -3′	5′- GATGATTTCTTCTGCTTGCTCC -3′
VPS34	5′-GCT GTC CTG GAA GAC CCA AT-3′	5′-TTC TCA CTG GCA AGG CCA AA-3′
PTEN	5′-CCAAGCTTATGACAGCCATCATC-3′	5′-CGCGGATCCTCAGACTTTTGTAA-3′
ULK1	5′-GGACACCATCAGGCTCTTCC-3′	5′-GAAGCCGAAGTCAGCGATCT-3′
ULK2	5′-TTCCTGCTCTAAGGGTTTGCTT-3′	5′-CCAGCGAGGGAGAACAACTG-3′
PI3K	5′ - ATGCAAATTCAGTGCAAAGG-3′	5′ - CGTGTAAACAGGTCAATGGC-3′
AKT	5′ -GCAGCACGTGTACGAGAAGA-3′	5′ -GGTGTCAGTCTCCGACGTG-3′

4.7. Statistical Analysis

Descriptive statistics were used to express as means with standard deviation. Pearson's Chi-square test was executed for the association of demographics, clinical pathologic parameters, and molecular markers with tumor type and grade. Multivariate Anova test was performed test to identify any notable difference in the relative gene expression among all the examined ATG genes in 2, 3 & 4 WHO grades of diffuse gliomas and $p < 0.05$ was considered as significant. Spearman's correlation test was executed to determine the correlation between autophagy genes in grade 2, 3 and 4. We performed all the analyses using IBM SPSS version 24, and used a significance threshold of $p < 0.05$ to determine statistical associations.

5. Conclusions

In summary, the significantly high expression of autophagic proteins *LC3B* and *SQSTM1/p62*, coupled with increased mRNA expression levels of *VPS34* in high-grade glioma, underscores the connection between autophagy and the advancement of gliomas. Moreover, the assessment of autophagy status through *LC3B* and *SQSTM1/p62* expression could serve as a promising prognostic tool for glioma patients. We provide preliminary data for functional analysis of autophagy using cell culture model and to identify potential targets for therapeutic interventions.

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Informed Consent Statement: Written informed consent was obtained from all patients involved in this study.

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