PIK3CA Mutations in Diffuse Gliomas: An Update on Molecular Stratification, Prognosis, Recurrence, and Aggressiveness

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ABSTRACT

INTRODUCTION: *PIK3CA* is one of the most mutated oncogenes in solid tumors. In breast cancer (ER-positive, HER2-negative), these events represent a predictive biomarker of response to alpelisib. In glioblastomas (GBM), *PIK3CA* mutations were described as early constitutive events. Here, we investigated *PIK3CA* mutational profile across glioma molecular subgroups and its relevance during glioma recurrence. Furthermore, *PIK3CA* mutations' effect in PI3K pathway, prognosis, and response to therapy was also explored.

MATERIAL AND METHODS: Exons 10 and 21 of *PIK3CA* mutations were evaluated in 394 gliomas and 19 glioma recurrences from Instituto Português de Oncologia Lisboa Francisco Gentil (IPOLFG) and compared with The Cancer Genome Atlas (TCGA) data. TIMER2.0 and NetMHCpan4.1 were used to assess the immune-microenvironment contribution.

RESULTS: *PIK3CA* mutations were identified among all glioma subgroups, although with no impact on their stratification or prognosis. In both cohorts (IPOLFG and TCGA), *PIK3CA* mutation frequencies in *IDH*-mutant and *IDH*-wild-type GBM were similar (IPOLFG: 9% and 3%; TCGA: 8% and 2%). These mutations were not mutually exclusive with *PTEN* deletion and *EGFR* amplification. Despite their reduced frequency, we discovered *PIK3CA* mutations were maintained during glioma recurrence regardless of administered therapies. The immune microenvironment might not contribute to this phenotype as *PIK3CA* mutations did not influence immune cell infiltration.

CONCLUSIONS: Despite the absence of a predominant effect in glioma stratification, *PIK3CA* mutations were maintained during glioma recurrence, possibly contributing to glioma cell survival, representing promising therapeutic targets in recurrent glioma. Nevertheless, understanding the potential synergistic effects between *PIK3CA* mutations, *PTEN* deletion, and *EGFR* amplification is pivotal to targeted therapies' efficiency.

KEYWORDS: *PIK3CA* mutations, gliomas, molecular subgroups, progression, recurrence, immune cell infiltrates, PI3K-Akt pathway

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Introduction

Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (*PIK3CA*) has been indicated as one of the most frequently mutated genes in solid tumors.[1] This gene encodes the p110α catalytic subunit of class IA PI3K lipid kinases,[2] enzymes that participate in the PI3K/Akt signaling pathway, regulating cell proliferation, angiogenesis, growth, motility, and survival.[3] Using new high-throughput molecular methodologies, PI3K-Akt overactivation has become recognized as one of the most important driver mechanisms of aggressiveness acquired by tumor cells.[2] At present, it is known that PI3K-Akt pathway abnormal activation is triggered mainly by 3 molecular events: *PTEN* loss of function/inactivation and *EGFR* or *PIK3CA* overactivation.[4,5]

In the last decades, *PIK3CA* mutations have been identified mainly in patients with breast (~35%), endometrial (~36%), bladder (>18%), and colorectal cancer (18%-38%).[1,6-9] Exons 10 and 21 of *PIK3CA* are the main mutational hotspot regions found, with E542K, E545K, and H1047R being the most frequent mutations.[9-11] However, discrepant frequencies of these alterations have been reported in glioblastoma (GBM), ranging from 5% to 30%.[11-16] *PIK3CA* mutational analysis in less prevalent types of gliomas (oligodendrogliomas and astrocytomas) has been less explored.[14,17] Importantly, the data available

about the *PIK3CA* mutational profile in gliomas are based exclusively on histological criteria and could differ significantly when taking into account the 2016 World Health Organization (WHO) molecular classification of these tumors—GBM *IDH*-wildtype, GBM *IDH*-mutant, astrocytoma *IDH*-wildtype, astrocytoma *IDH*-mutant, and *IDH*-mutant and 1p/19q codeleted oligodendroglioma.[18-20]

In addition, *PIK3CA* activating mutations have been described as initial and clonal events found in all GBM surgical fragments, highlighting the potential relevance of these molecular alterations in glioma development.[16] Some studies have also stated that *PIK3CA* mutations might be associated with poor GBM patient outcomes[16,21] and even earlier recurrence.[21]

In fact, gliomas are the most common malignant brain tumors associated with a dismal prognosis, triggering several therapy resistance mechanisms that challenge patient treatment.[22] As the available therapeutic approaches for glioma are not efficient,[22] the potential inhibition of PI3K-Akt oncogenic signaling pathway should be further explored. Currently, the glioma molecular subgroup where *PIK3CA* mutations occur most frequently remains unclear, as well as their role in glioma stratification, prognosis, and aggressiveness.

PIK3CA has been highlighted as a promising therapeutic target in cancer.[23-25] Recently, in breast cancer, *PIK3CA* mutations were associated with an increased sensitivity to the selective p110α inhibitor alpelisib.[23,26] Consequently, alpelisib has been approved for treatment of advanced stage ER-positive, HER2-negative, and *PIK3CA*-mutated breast cancer, in combination with fulvestrant, leading to significantly prolonged progression-free survival (PFS).[23,26] Alpelisib treatment should also be considered for patients with glioma, but it is necessary to clarify which molecular subgroups would benefit the most from the administration of these inhibitors.

In this study, we investigated the frequency and clinical relevance of *PIK3CA* mutations in exons 10 and 21 in glioma molecular subgroups, the significance of these mutations in glioma recurrence, and their relationship with other molecular alterations of the PI3K-Akt pathway and the immune microenvironment.

Materials and Methods

Study design and biological samples

A detailed workflow of our study design is shown in Figure 1. The Instituto Português de Oncologia Lisboa Francisco Gentil (IPOLFG) cohort used in this study contains 394 glioma samples, previously reclassified and characterized according to the 2016 WHO classification.[20] Simultaneously, a dataset with 19 cases of glioma recurrences, corresponding to 19 primary and 23 matched recurrence samples, was analyzed to identify *PIK3CA* mutations. Tumor samples were received as fresh or paraffin-preserved tissue for DNA extraction, performed using the methodologies already described.[20] This study was previously approved by the IPOLFG Ethical Board Committee (UIC/1203), and written informed consent was obtained from all living patients.

Genotyping

MGMT promoter methylation, *PTEN* deletion, and *EGFR* amplification were identified in diagnostic routine, as previously reported.[20] High-resolution chromosome comparative genome hybridization analysis was performed as previously described to evaluate *PIK3CA* copy number variations.[27]

According to a current update, exons 9 and 20 of *PIK3CA* were renamed as exons 10 and 21, based on Ensembl Transcript ID: ENST00000263967.4, RefSeq: NM_006218.4. Both *PIK3CA* exons and R172 *IDH2* hotspot mutations were evaluated by Sanger sequencing in the previously established glioma molecular subgroups.[20,28] The primers and conditions used for polymerase chain reaction (PCR) amplification are listed in Supplementary Table 1. An additional set of primers was used to target a pseudogene with more than 95% of homology with exon 10 of *PIK3CA*, found in chromosome 22.[29]

To determine the sequences of interest in *PIK3CA* and *IDH2*, ABI Prism 3130 Genetic Analyzer (Applied Biosystems, USA) was used following the protocol proposed by Big Dye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, USA), which can detect variants as low as 5% in a sample.

In silico analysis

In silico analysis was performed to predict the benign or pathogenic impact of new *PIK3CA* variants, not found in Ensembl, Catalogue of Somatic Mutations in Cancer (COSMIC), and the Human Gene Mutation Database (HGMB), using MutationTaster (http://www.mutationtaster.org), PolyPhen (http://genetics.bwh.harvard.edu/pph2), and Variant Effect Predictor (https://www.ensembl.org/info/docs/tools/vep/index.html).

In addition, the frequency of *PIK3CA* mutations in 567 GBM *IDH*-wild-type and 25 GBM *IDH*-mutant samples available in cBioPortal for Cancer Genomics (https://www.cbioportal.org/) was evaluated, hereafter referred to as The Cancer Genome Atlas (TCGA) cohort. Furthermore, the prognosis value of *PIK3CA* mutational status and the potential correlation between *PIK3CA* mutations, *EGFR*, and *PTEN* molecular alterations was determined.

The impact of *PIK3CA* expression, mutations, or amplification on the infiltration of distinct immune cell subsets in GBM and low-grade gliomas (LGG) was assessed using TIMER2.0.[30] As a complementary analysis, the correlation between *PIK3CA* expression or mutational status and the expression of various immune cell gene markers was also investigated using TIMER2.0. NetMHCpan4.1 algorithm was applied to determine whether *PIK3CA* mutations contribute to T-cell

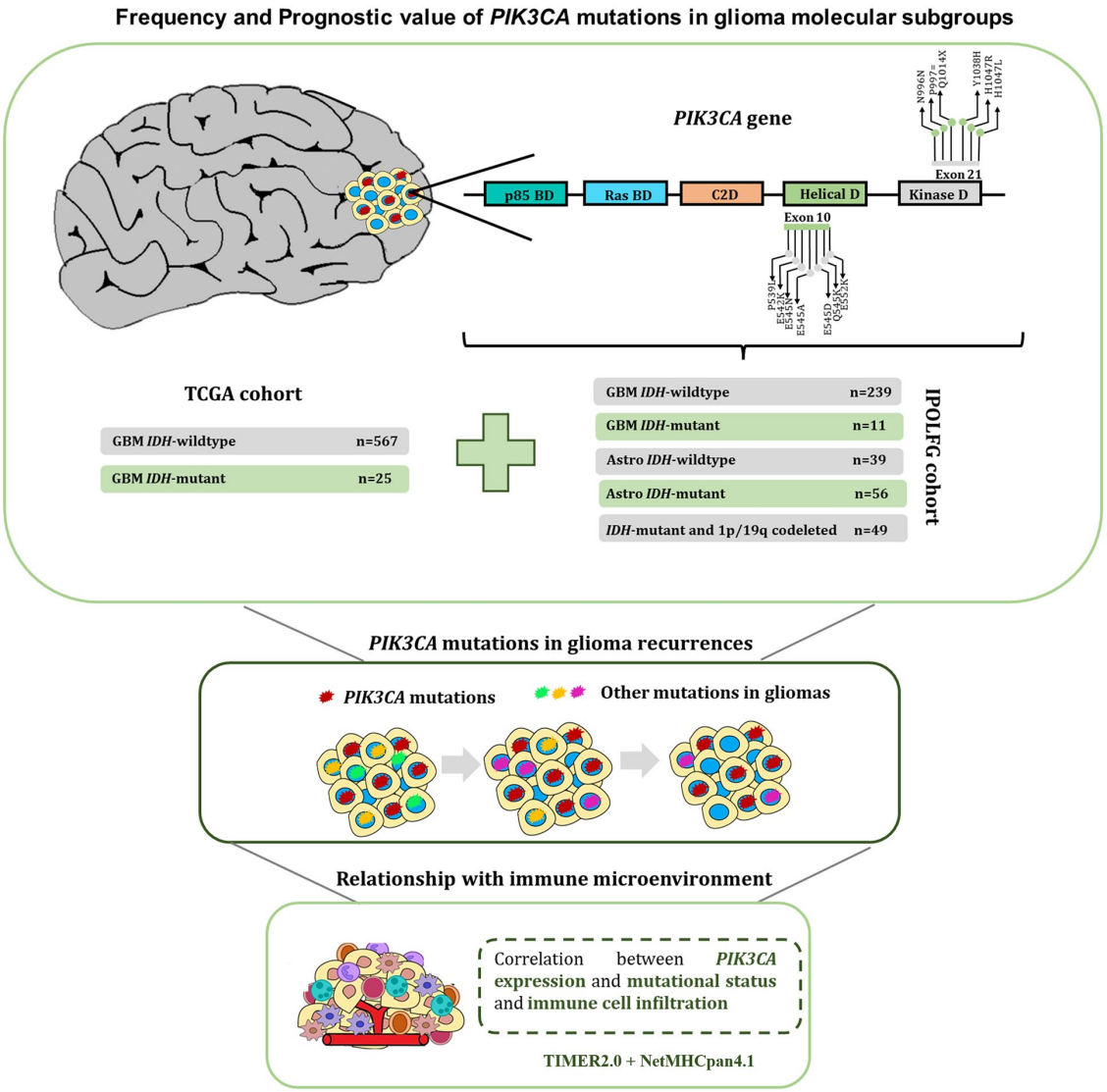


Figure 1. Study design and workflow used to investigate the frequency and relevance of *PIK3CA* mutations. The presence of *PIK3CA* mutations in exons 10 and 21 was assessed in 394 glioma samples from the IPOLFG cohort, classified according to the 2016 WHO molecular classification. Furthermore, *PIK3CA* mutational status was also assessed in 592 GBM samples from TCGA, divided into GBM *IDH*-wildtype and *IDH*-mutant. Then, 19 recurrence cases were evaluated to understand if *PIK3CA* mutations persisted throughout glioma progression. Finally, the relationship between *PIK3CA* expression and mutational status and the immune microenvironment was estimated using TIMER2.0 and NetMHCpan4.1.
GBM indicates glioblastomas; IPOLFG, Instituto Português de Oncologia de Lisboa Francisco Gentil; TCGA, The Cancer Genome Atlas; WHO, World Health Organization.

recruitment and activation, by estimating the binding affinity of wildtype and mutated 9 amino acid peptides to major histocompatibility complex (MHC) Class I molecules.[31]

Statistical analysis

The primary endpoint used was overall survival (OS), defined as the time from glioma diagnosis to patient death or last follow-up. Survival analysis was done using Kaplan-Meier estimator and the log rank test for group comparison. Multivariable analysis was determined using Cox regression proportional hazard model. Fisher exact test was used to compare the prevalence of *PIK3CA* mutations across glioma molecular subgroups. The chi-square statistical test was used to analyze putative associations between *PIK3CA* mutations and *PTEN* and *EGFR* molecular alterations.

The differences between immune cell infiltrates and the differential expression of immune cell gene markers in GBM and LGG under distinct *PIK3CA* mutational status were determined by Wilcoxon rank sum test. Kruskal-Wallis *H* test was used to compare the immune infiltration distribution in *PIK3CA*-amplified GBM and LGG samples. Spearman rank correlation coefficients with purity adjustment were obtained when comparing *PIK3CA* expression with immune cell infiltration or the expression of immune cell markers in GBM and LGG cohorts. To compare the differences between the binding affinity of mutated and corresponding wild-type p110α peptides with MHC class I complexes, 2-way analysis of variance

Table 1. *PIK3CA* mutations found in glioma molecular subgroups from the IPOLFG cohort.

GLIOMA MOLECULAR SUBGROUP	NO. OF SAMPLES	FREQUENCY OF *PIK3CA* MUTATIONS (%)	EXON	NUCLEOTIDE CHANGE	AMINO ACID CHANGE (NO. OF SAMPLES WITH MUTATION)
GBM, *IDH*-wildtype	239	6/239 (3)	21 10 10 21	c.3140A>G c.1633G>C c.1634A>C c.3112T>C[a]	H1047R (n=3) E545N (n=1) E545A (n=1) Y1038H (n=1)
GBM, *IDH*-mutant	11	1/11 (9)	21	c.3140A>G	H1047R (n=1)
IDH-mutant and 1p/19q codeleted gliomas	49	5/49 (10)	10 10 10 21 21	c.1616C>T c.1635G>T c.1656G>A c.2991C>T c.2988T>C[a]	P539L (n=1) E545D (n=1) E552K (n=1) L997L (n=1) N996N (n=1)
Astrocytomas, *IDH*-wildtype	39	4/39 (10)	21 21 10 10	c.3140A>G c.3140A>T c.1624G>A c.1636C>A	H1047R (n=1) H1047L (n=1) E542K (n=1) Q546K (n=1)
Astrocytomas, *IDH*-mutant	56	3/56 (5)	21 10 21	c.2965C>G c.1624G>A c.3040C>T[a]	L989V (n=1) E542K (n=1) Q1014X (n=1)

Abbreviations: GBM, glioblastomas; IPOLFG, Instituto Português de Oncologia de Lisboa Francisco Gentil.
[a]Unreported variant.

(ANOVA) was used followed by Bonferroni multiple comparisons test.

All tests were 2-sided, with a significance level of 5%. Statistical analysis and graphic representation were performed using IBM SPSS Statistics 21.0 and GraphPad Prism 8.4.3.

Results

Frequency and prognostic impact of PIK3CA mutations in glioma molecular subgroups

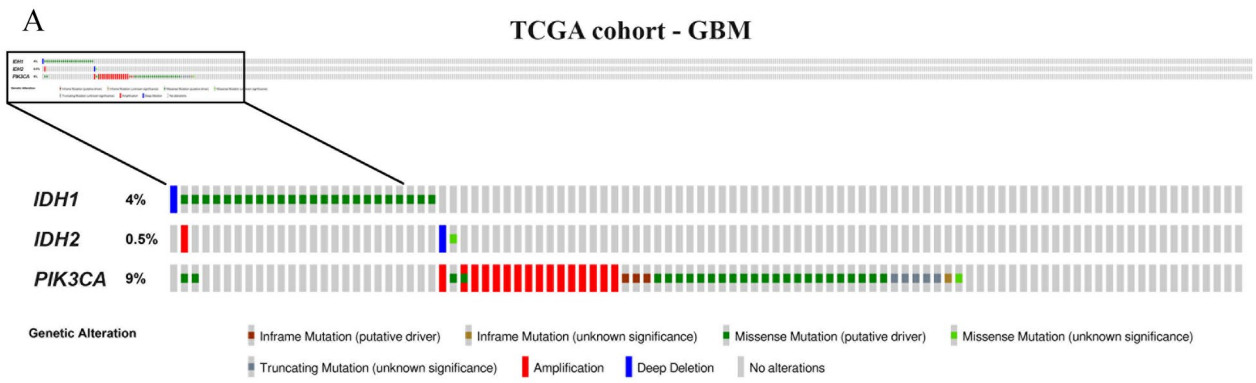
Considering the lack of data about the frequency of *PIK3CA* mutations in exons 10 and 21 in glioma molecular subgroups, we evaluated the presence of these alterations in 394 diffuse glioma samples. A detailed workflow of this study is shown in Figure 1. PIK3CA mutations were most frequent in *IDH*-mutant and 1p/19q codeleted oligodendrogliomas (10%) and astrocytomas *IDH*-wildtype (10%) (Table 1). In contrast, the GBM *IDH*-wild-type subgroup, the most lethal, presented the lowest mutational frequency (3%).

The frequency of *PIK3CA* mutations was higher in GBM *IDH*-mutant (9%) than GBM *IDH*-wildtype (3%) (Table 1), but without statistical significance ($P = .273$; Supplementary Table 2). Nevertheless, similar results were obtained in TCGA cohort when evaluating only *PIK3CA* hotspot exons: 8% in GBM *IDH*-mutant and 2% in GBM *IDH*-wildtype (Figure 2). Furthermore, when we analyzed the entire *PIK3CA* gene, an analysis that includes mutations and amplifications regardless of pathogenicity confirmation (described in Supplementary Table 3), these frequencies increased to 12% and 9%, respectively (Figure 1). Copy number variation analysis of TCGA data demonstrated that 16 GBM cases harbor a *PIK3CA* amplification, whereas in the IPOLFG cohort, no amplifications were detected (Supplementary Table 3 and Figure 2). This difference may be explained by the reduced sampling size of IPOLFG cohort and even by the distinct techniques used to assess copy number variations.

Overall, in the IPOLFG cohort, we detected 19 *PIK3CA* mutations in the hotspot exons—5% of all glioma samples (Table 1). Although H1047R and E542K were the most common *PIK3CA* mutations detected, during this analysis, we also found 3 distinct undescribed variants (Table 1). Variant N996N was found 55 base pairs from the splice site, in *IDH*-mutant and 1p/19q codeleted oligodendrogliomas, and was estimated as pathogenic, likely to induce splice site changes, leading to the loss of amino acid sequences belonging to the helix. Unreported variant Y1038H in exon 21 was present in GBM *IDH*-wildtype subgroup and predicted to be pathogenic, with moderate impact on the protein encoded. Finally, Q1014X was found in the astrocytoma *IDH*-mutant subgroup and estimated as pathogenic due to a premature stop codon in position 3042 instead of 3207, resulting in a truncated p110 catalytic subunit lacking 55 amino acids.

Then, we determined that *PIK3CA* mutations did not have prognostic value in the most representative molecular subgroup of the IPOLFG cohort, GBM *IDH*-wildtype ($P = .956$; Figure 3A). Nevertheless, the presence of *PIK3CA* mutations slightly decreased the median OS (10 months vs 9 months). This trend was confirmed using TCGA data, where we included all *PIK3CA* molecular alterations (Supplementary Table 3). According to the univariable analysis, these alterations were

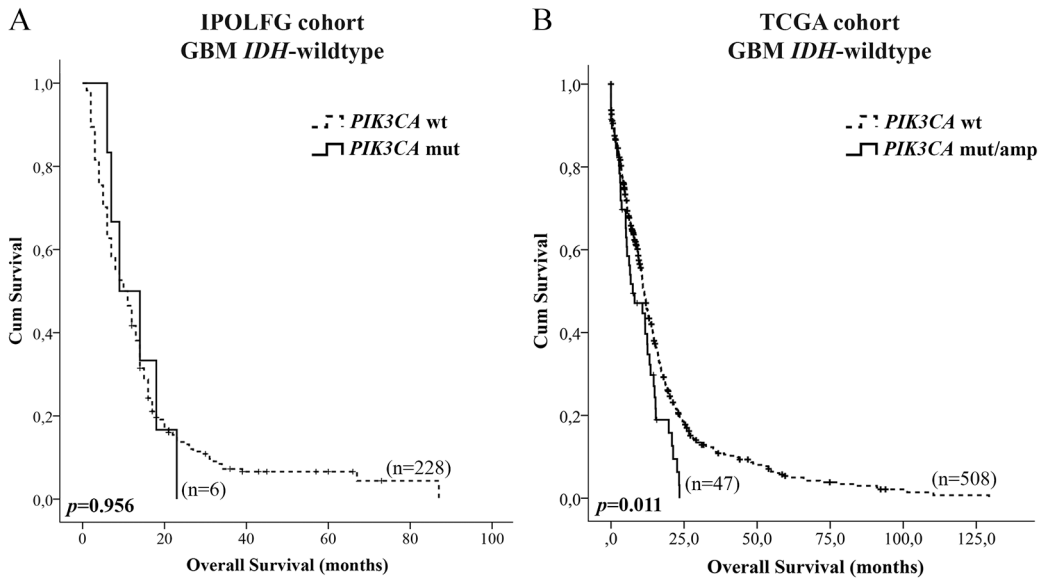
A



B

Glioma Molecular Subgroup	Frequency of *PIK3CA* mut (%)	Frequency of *PIK3CA* mut/amp in whole gene (%)
GBM, *IDH*-wildtype – TCGA	10/567 (2%)	50/567 (9%)
GBM, *IDH*-mutant – TCGA	2/25 (8%)	3/25 (12%)
GBM, *IDH*-wildtype – IPOLFG	6/239 (3%)	NA
GBM, *IDH*-mutant – IPOLFG	1/11 (9%)	NA

Figure 2. Frequency of *PIK3CA* mutations and copy number variations in GBM molecular subgroups from TCGA cohort. (A) *PIK3CA, IDH1*, and *IDH2* molecular alterations in GBM samples. Whole *PIK3CA* gene analysis showed that 9% of GBM cases contain molecular alterations. (B) Similarity between the frequency of *PIK3CA* mutations in exons 10 and 21 in GBM molecular subgroups from TCGA and IPOLFG cohorts. When considering *PIK3CA* mutations and amplifications throughout the whole gene, frequencies vary slightly in TCGA cohort.
GBM indicates glioblastomas; IPOLFG, Instituto Português de Oncologia de Lisboa Francisco Gentil; TCGA, The Cancer Genome Atlas.



Confounder variables	Multivariate Analysis		
	Hazard ratio	95% CI	*p*-value
PIK3CA molecular alterations	1.169	0.84-1.62	0.348
Age	0.994	0.99-1.00	0.070
Gender	0.888	0.74-1.10	0.215
Type of treatment	1.103	0.92-1.30	0.293

Figure 3. Impact of *PIK3CA* molecular alterations in the overall survival of GBM *IDH*-wild-type patients. (A) Kaplan-Meier curve estimates the impact of *PIK3CA* mutations in the prognosis of patients included in the IPOLFG cohort. (B) Prognosis value of *PIK3CA* molecular alterations found in all exons was estimated using data from GBM *IDH*-wild-type patients available in TCGA cohort. Survival data were only available for 47 of the 53 cases containing *PIK3CA* molecular alterations. Multivariable analysis included 555 GBM *IDH*-wild-type samples.
GBM indicates glioblastomas; IPOLFG, Instituto Português de Oncologia de Lisboa Francisco Gentil; TCGA, The Cancer Genome Atlas.

Table 2. *PIK3CA* mutational analysis in 19 recurrent glioma cases from the IPOLFG cohort.

	INITIAL DIAGNOSIS	FIRST RECURRENCE DIAGNOSIS	SECOND RECURRENCE DIAGNOSIS	*PIK3CA* MUTATIONAL ANALYSIS		
				PRIMARY TUMOR	FIRST RECURRENCE	SECOND RECURRENCE
Case 1 (n=2)	Astrocytoma *IDH*-wildtype	GBM *IDH*-wildtype	NA	Wildtype	Wildtype	NA
Case 2 (n=2)	Astrocytoma *IDH*-mutant	GBM *IDH*-mutant	NA	Mutated (c.2965C>G)	Mutated (c.2965C>G)	NA
Case 3 (n=2)	Astrocytoma *IDH*-mutant	GBM *IDH*-mutant	NA	Wildtype	Wildtype	NA
Case 4 (n=3)	Astrocytoma *IDH*-mutant	GBM *IDH*-mutant	GBM *IDH*-mutant	Wildtype	Wildtype	Wildtype
Case 5 (n=2)	Astrocytoma *IDH*-wildtype	GBM *IDH*-wildtype	NA	Wildtype	Wildtype	NA
Case 6 (n=2)	Astrocytoma *IDH*-wildtype	GBM *IDH*-wildtype	NA	Wildtype	Wildtype	NA
Case 7 (n=2)	Astrocytoma *IDH*-mutant	GBM *IDH*-mutant	NA	Wildtype	Wildtype	NA
Case 8 (n=2)	Astrocytoma *IDH*-wildtype	GBM *IDH*-wildtype	NA	Wildtype	Wildtype	NA
Case 9 (n=3)	GBM *IDH*-mutant	GBM *IDH*-mutant	GBM *IDH*-mutant	Mutated (c.1633G>A)	Mutated (c.1633G>A)	Mutated (c.1633G>A)
Case 10 (n=2)	GBM *IDH*-wildtype	GBM *IDH*-wildtype	NA	Wildtype	Wildtype	NA
Case 11 (n=2)	Astrocytoma *IDH*-wildtype	GBM *IDH*-wildtype	NA	Wildtype	Wildtype	NA
Case 12 (n=2)	Astrocytoma *IDH*-mutant	GBM *IDH*-mutant	NA	Wildtype	Wildtype	NA
Case 13 (n=2)	Astrocytoma *IDH*-mutant	GBM *IDH*-mutant	NA	Wildtype	Wildtype	NA
Case 14 (n=2)	Astrocytoma *IDH*-mutant	GBM *IDH*-mutant	NA	Wildtype	Wildtype	NA
Case 15 (n=2)	Astrocytoma *IDH*-mutant	GBM *IDH*-mutant	NA	Wildtype	Wildtype	NA
Case 16 (n=2)	Astrocytoma *IDH*-wildtype	GBM *IDH*-wildtype	NA	Wildtype	Wildtype	NA
Case 17 (n=2)	Astrocytoma *IDH*-wildtype	GBM *IDH*-wildtype	NA	Wildtype	Wildtype	NA
Case 18 (n=3)	Astrocytoma *IDH*-wildtype	Astrocytoma *IDH*-wildtype	GBM *IDH*-wildtype	Wildtype	Wildtype	Wildtype
Case 19 (n=3)	Astrocytoma *IDH*-mutant	Astrocytoma *IDH*-mutant	GBM *IDH*-mutant	Wildtype	Wildtype	Wildtype

Abbreviations: GBM, glioblastomas; IPOLFG, Instituto Português de Oncologia de Lisboa Francisco Gentil; NA, not available.

associated with shorter OS of GBM *IDH*-wild-type patients ($P = .011$; Figure 3B), with median OS being reduced from 11 to 7 months. However, multivariable analysis showed that *PIK3CA* molecular alterations were not independent prognostic factors ($P = .348$).

In addition, we also evaluated the correlation between *PIK3CA* mutations in exons 10 and 21 and molecular alterations in other important players of the PI3K/Akt pathway, *EGFR* and *PTEN*, in both cohorts. *EGFR* amplification and *PTEN* deletion coexist with *PIK3CA* mutations in glioma molecular subgroups, although no significant associations were observed (Supplementary Tables 4 and 5). Furthermore, no significant association was found with *MGMT* methylation, a well-studied epigenetic alteration found in gliomas.

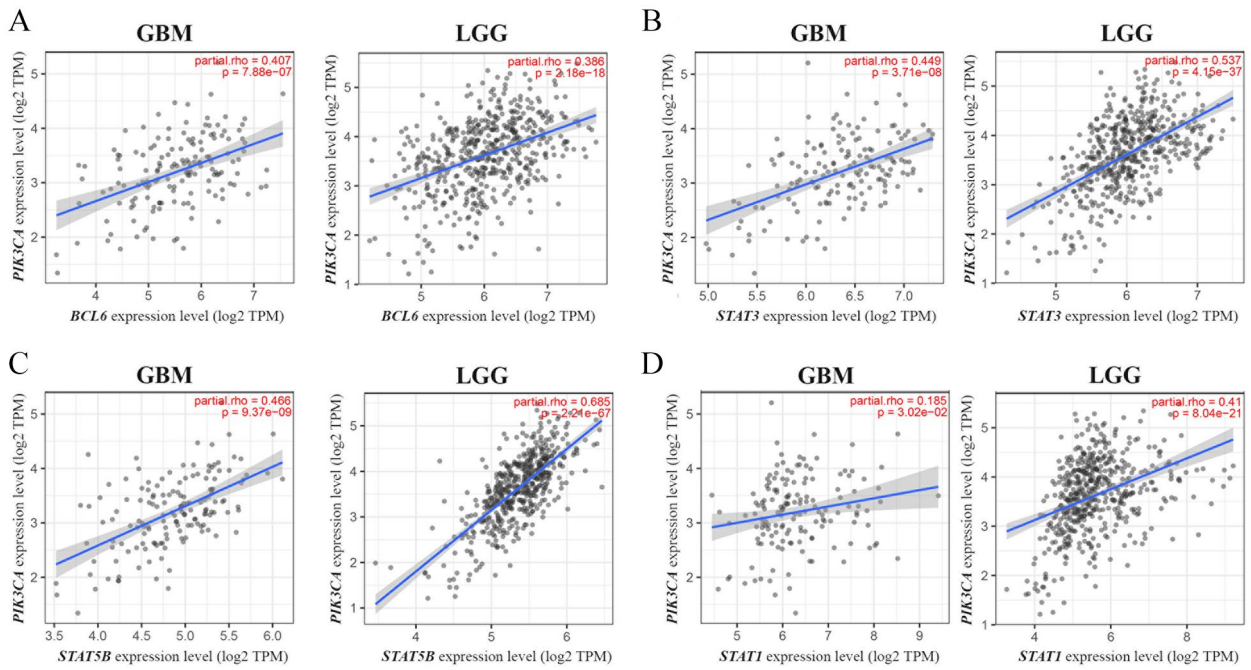


Figure 4. Correlation between *PIK3CA* expression and the expression of the immune cell gene markers *BCL6* (A), *STAT3* (B), *STAT5B* (C), and *STAT1* (D), in GBM and LGG samples from TCGA. Data were obtained from the TIMER2.0 resource, including *P* values and Spearman's rank correlation coefficients with purity adjustment (partial rho). GBM indicates glioblastomas; LGG, low-grade gliomas; TCGA, The Cancer Genome Atlas.

New PIK3CA polymorphism in glioma molecular subgroups

During *PIK3CA* mutational analysis, we detected a single-nucleotide polymorphism (SNP)—Rs45455192 (C>T), 55 nucleotides from the beginning of exon 10—that has never been described in gliomas. This SNP was detected in heterozygosity in the IPOLFG cohort in 18% of GBM *IDH*-wildtype, 27% of GBM *IDH*-mutant, 25% of *IDH*-mutant and 1p/19q codeleted oligodendrogliomas, 21% of astrocytomas *IDH*-wildtype, and 18% of astrocytomas *IDH*-mutant (Supplementary Table 6). For all glioma molecular subgroups analyzed, Rs45455192 was not associated with glioma patient prognosis (Supplementary Figure 1).

PIK3CA mutations in recurrent glioma

Recently, *PIK3CA* mutations were associated with earlier recurrence in patients with GBM[21] and reported as constitutive events shared by all GBM tumor mass.[16] However, these mutations were never investigated throughout glioma progression. Thus, we evaluated whether *PIK3CA* mutations persist throughout glioma recurrence or whether they are important events in glioma initiation that are lost during tumor recurrence.

We assessed 19 recurrent glioma cases. L989V and E545K, 2 reported mutations, were detected in 2 cases out of 19 (cases 2 and 9 depicted in Table 2), both in the primary tumor and in the recurrence samples. Clinical data indicate that both patients were treated with radiotherapy (RT) and/or chemotherapy (CT) (Supplementary Table 7), which might suggest that *PIK3CA* mutations are maintained throughout glioma recurrences regardless of the therapy administered.

The relationship between immune cell infiltration and PIK3CA in diffuse gliomas

It is known that distinct glioma molecular profiles are associated with the recruitment of different immune subsets, which can justify variations in tumor aggressiveness and therapy resistance.[32-35] Knowing that *PIK3CA* mutations seem to be maintained throughout glioma progression, we decided to explore the relationship between the immune microenvironment and the *PIK3CA* gene to understand whether this interplay can influence glioma aggressiveness.

Infiltration levels for various types of immune cells were assessed according with *PIK3CA* expression and mutational and amplification status in GBM and LGG samples, using TIMER2.0 (Supplementary Tables 8, 9, and 10). The results obtained show no significant associations with the infiltration of the immune subsets analyzed. We also explored the interaction of *PIK3CA* expression and mutational status with the expression of various immune cell markers, in GBM and LGG (Supplementary Tables 11 and 12). Only *BCL6, STAT3, STAT5B*, and *STAT1* showed a significant positive correlation with *PIK3CA* expression levels, the latter only in LGG samples (Figure 4). Finally, no differences were detected between the binding affinity of wildtype and mutated p110α peptides to MHC Class I molecules (Supplementary Figure 2), according to the NetMHCpan4.1 algorithm.

These results seem to indicate that *PIK3CA* mutational status is not associated with immune cell infiltration, although a

possible crosstalk between PI3K/Akt and JAK-STAT pathways was suggested.

Discussion

PIK3CA somatic activating mutations have been found in various cancer types, but their impact in patient prognosis and response to therapy is still under investigation.[1] Considering that *PIK3CA* mutations are described as predictive biomarkers of a better response to alpelisib treatment in breast cancer,[23] their potential as biomarkers should be further explored in other contexts. Currently, there is an urgent need for new biomarkers to improve the clinical management of patients with glioma, as these neoplasms are highly invasive and lethal, and effective therapies are nonexistent.[22] To understand if *PIK3CA* mutations could constitute good biomarkers in glioma, we evaluated, for the first time, the frequency and clinical relevance of mutations in exons 10 and 21 of *PIK3CA* in 5 well-characterized molecular subgroups of glioma previously defined.[20,36]

The overall frequency of *PIK3CA* mutations in the IPOLFG glioma cohort was low (5%), with the highest percentage belonging to the *IDH*-mutant and 1p/19q codeleted glioma and astrocytoma *IDH*-wild-type subgroups (10%). However, these frequencies might be slightly underestimated, as only exons 10 and 21 of *PIK3CA* were analyzed and not the entire gene. Overall, *PIK3CA* mutations were distributed among all glioma molecular subgroups, suggesting that their individual presence is not associated with the differences in subgroup aggressiveness. Therefore, these events alone cannot help to stratify patients with glioma according to their diagnosis. Only one other study has calculated the frequency of *PIK3CA* mutations in *IDH*-mutant 1p/19q codeleted gliomas.[17] TCGA Research Network[17] analyzed the entire *PIK3CA* gene in a more robust cohort and determined that 20% (17/84) of *IDH*-mutant 1p/19q codeleted LGG harbored *PIK3CA* mutations. These results highlight the importance of *PIK3CA* mutations in this subgroup.

In addition, *PIK3CA* mutations were not considered independent prognostic factors of OS in the IPOLFG or the TCGA cohorts. A recent study published by Tanaka et al[21] reported that *PIK3CA* hotspot mutations are associated with shorter PFS in patients with GBM *IDH*-wildtype independently of confounder variables, but they found no significant association with OS, similar to the results obtained in our study. Further research in other cohorts is therefore crucial to better understand the role of *PIK3CA* mutations in the prognosis of patients with glioma.

Even though *PIK3CA* mutations did not significantly impact glioma diagnosis and prognosis, we are concerned about the harmful effect that these mutations could have when combined with other molecular alterations that dysregulate the PI3K-Akt pathway. Class I PI3K is responsible for the phosphorylation of phosphatidylinositol 4,5 bisphosphate (PIP2) to phosphatidylinositol-3,4,5-triphosphate (PIP3) when activated by upstream receptor tyrosine kinases (RTKs). PIP3 accumulates in the plasma membrane and activates protein kinase B (PKB; also known as Akt) through phosphatidylinositol dependent kinase (PDK) recruitment.[37] Akt plays a central role as mediator of this signaling pathway, being responsible for the phosphorylation of several downstream targets. Mechanistic target of rapamycin (mTOR) is one of the most relevant downstream targets of this signaling pathway, triggering cell death inhibition and promoting cell survival, growth, and angiogenesis.[2,38] Phosphatase and tensin homologue deleted on chromosome 10 (*PTEN*) antagonizes PI3K activity by dephosphorylating PIP3 back to PIP2, making it a strict negative regulator of PI3K activity.[37] Considering that in diffuse large B-cell lymphoma, *PTEN* deletion and *PIK3CA* mutations are mutually exclusive events,[39] we analyzed whether there is some correlation between *PIK3CA* mutations, *EGFR* amplification, and *PTEN* deletion in gliomas. None of these events are mutually exclusive in glioma subgroups. Despite not being significantly correlated, it would be interesting to evaluate whether there is a cooperative impact of these molecular alterations in glioma aggressiveness and response to therapy. This cooperative effect may compromise the individual targeting of p110α, PTEN, or EGFR, due to compensatory mechanisms potentiated by molecular alterations present in other genes belonging to this pathway. For example, in lung adenocarcinomas, cumulative *PIK3CA* mutations and *EGFR* mutations are associated with poorer outcomes compared with tumors with *EGFR* mutations, suggesting that the mutual effect between these molecular alterations promotes tumor aggressiveness.[40] Furthermore, concomitant alterations in *PIK3CA* and *PTEN* genes were correlated with an additive or synergistic effect in PI3K activation in a model of endometrial cancer.[6] Therefore, the putative mutual effect between these molecular alterations should be further assessed in gliomas.

We also found a SNP, described for the first time in molecular subgroups of glioma in this study, that did not demonstrate any correlation with patient prognosis. This polymorphism was described in human oral squamous carcinoma[41] and also in a sample of pancreaticobiliary adenocarcinoma.[42] In the future, it could be interesting to analyze the association between this polymorphism and glioma risk to understand whether it is a germinal or somatic alteration.

Another important question addressed in this study was the role of *PIK3CA* mutations among glioma progression. Here, for the first time, we detected that, when present, *PIK3CA* variants are in the primary tumor and in the respective recurrences. As these patients were submitted to RT and/or CT, these variants seem to be maintained during glioma recurrences independently of the therapy administered. However, we have only reported 2 *PIK3CA* mutated recurrence cases, and thus, further studies are needed to validate whether *PIK3CA* mutations provide any adaptative tumor advantage, contributing to therapy resistance. Recently, *PIK3CA* mutations were proposed as a biomarker to monitor CT resistance in colorectal cancer.[43] They have also been associated with paclitaxel, anthracycline,

and anti-HER2 therapy resistance in patients with breast cancer.[44-46] In cervical cancer cells, E545K mutation confers radio resistance through β-catenin signaling pathway activation.[47] Therefore, it is of the utmost importance to clarify the role of *PIK3CA* mutations in glioma progression and response to therapy.

Recently, some studies have highlighted the importance of exploring the relationship between specific glioma molecular profiles and immune cell infiltration, which can have a considerable impact on tumor aggressiveness.[32-35] However, no study thus far has examined the association between *PIK3CA* mutations and the remodeling of glioma's immune microenvironment. Hence, we sought to better understand this relationship and how it might affect glioma aggressiveness and therapy resistance. Overall, our results suggest that *PIK3CA* mutations do not heavily influence glioma immunogenicity. It is known that, when compared with other tumors, central nervous system tumors usually display lower infiltration of immune cells.[48] Similar to our results, a recent study found that, in triple negative breast cancer, *PIK3CA* mutations were not associated with the density of tumor-infiltrating lymphocytes.[49] However, other studies seem to highlight an association between PI3K/Akt activation and immune suppression in breast, prostate, and bladder cancers.[50-52] Further research should be performed to better understand these dynamics in glioma.

We did find a positive correlation between *PIK3CA* expression and distinct markers for different immune cell populations, *BCL6, STAT3, STAT5B*, and *STAT1*, in glioma. These results indicate a strong crosstalk between the PI3K/Akt and the JAK-STAT pathways in glioma, independent from their roles in immune cell function. Constitutive activation of STATs is thought to contribute to oncogenesis and has been found in gliomas.[53] STAT3 overactivation, which can be induced by phosphorylation via EGFR, has been linked with shorter OS in patients with GBM.[54] Likewise, STAT5B was indicated as a putative therapeutic target for patients with GBM, being an inductor of GBM cell growth, cell cycle progression, invasion, and migration.[55] In the future, it could be interesting to explore if alterations in both the PI3K/Akt and the JAK-STAT pathway could have a synergistic effect in increasing glioma aggressiveness.

Conclusions

Overall, *PIK3CA* mutations were identified among all glioma molecular subgroups, with higher prevalence in *IDH*-mutant and 1p/19q codeleted oligodendrogliomas and astrocytomas *IDH*-wildtype. In addition, *PIK3CA* molecular alterations were not independent prognostic factors in GBM *IDH*-wildtype patients and were not mutually exclusive with *EGFR* amplification or *PTEN* deletion. Despite low frequency of these mutations in glioma molecular subgroups, they constitute early events maintained during tumor progression regardless of the therapy administered, possibly hinting at a role in glioma cell survival. Thus, even though *PIK3CA* mutations alone might not be a useful biomarker in glioma patient stratification, these results highlight *PIK3CA* as a potential promising therapeutic target in glioma recurrent cases.

In the future, clarifying not only the impact of *PIK3CA* mutations in response to alpelisib treatment in cell models but also the potential role of other molecular alterations might have on the efficacy of p110α targeted therapies will be essential for their success in glioma.

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Author Contributions

MP and LR were responsible for concept and design. CB, AT, AA, and MM were responsible for experiments and procedures. Data analysis was done by CB, AT, SE, MP, and LR. CB, AT, and MP were responsible for writing the article. All authors have sufficiently contributed to the work performed. All authors read and approved the final manuscript.

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Supplemental Material

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