Molecular profile and clinical features of patients with gliomas using a broad targeted next generation-sequencing panel

OURANIA ROMANIDOU[1*], PARASKEVI APOSTOLOU[2*], KYRIAKOS KOUVELAKIS[3], KYRIAKOS TSANGARAS[4], ALEXIA ELIADES[4], ACHILLEAS ACHILLEOS[4], CHARALAMBOS LOIZIDES[4], CHRISTOS LEMESIOS[4], MARIOS IOANNIDES[4], ELENA KYPRI[4], GEORGE KOUMBARIS[4], KYRIAKI PAPADOPOULOU[5], ATHANASIOS PAPATHANASIOU[2], GEORGIOS RIGAKOS[6], IOANNIS XANTHAKIS[7], FLORENTIA FOSTIRA[2], VASSILIKI KOTOULA[8], GEORGE FOUNTZILAS[5,9,10] and PHILIPPOS C. PATSALIS[4,11]

[1]Department of Medicine, Medical Oncology Unit, Giannitsa General Hospital, 58100 Giannitsa; [2]Molecular Diagnostics Laboratory, InRASTES, National Centre for Scientific Research Demokritos, 15341 Athens; [3]Section of Biostatistics, Hellenic Cooperative Oncology Group, Data Office, 11526 Athens, Greece; [4]NIPD Genetics Ltd., 2409 Nicosia, Republic of Cyprus; [5]Laboratory of Molecular Oncology, Hellenic Foundation for Cancer Research/Aristotle University of Thessaloniki, 54006 Thessaloniki; [6]Third Department of Medical Oncology, Hygeia Hospital, 15123 Athens; [7]Oncology Department, European Interbalkan Medical Center, 55535 Thessaloniki; [8]Department of Pathology, School of Health Sciences, Faculty of Medicine, Aristotle University of Thessaloniki, 54006 Thessaloniki; [9]Aristotle University of Thessaloniki, 54006 Thessaloniki, Greece; [10]Department of Medical Oncology, German Oncology Center, 4108 Limassol; [11]School of Medicine, University of Nicosia Medical School, 2417 Nicosia, Republic of Cyprus

Received September 13, 2022; Accepted November 2, 2022

DOI: 10.3892/ol.2022.13624

Abstract. Gliomas are the most common malignant primary brain tumors characterized by poor prognosis. The genotyping of tumors using next generation sequencing (NGS) platforms enables the identification of genetic alterations that constitute diagnostic, prognostic and predictive biomarkers. The present study investigated the molecular profile of 32 tumor samples from 32 patients with high-grade gliomas by implementing a broad 80-gene targeted NGS panel while reporting their clinico-pathological characteristics and outcomes. Subsequently, 14 of 32 tumor specimens were also genotyped using a 55-gene NGS panel to validate the diagnostic accuracy and clinical utility of the extended panel. The median follow-up was 19.2 months. In total, 129 genetic alterations including 33 structural variants were identified in 38 distinct genes. Among 96 variants (single nucleotide variants and insertions and deletions), 38 were pathogenic and 58 variants of unknown clinical significance. *TP53* was the most frequently mutated gene, followed by *PTEN* and *IDH1* genes. Glioma patients with *IDH1* mutant tumors were younger and had significantly longer overall survival compared to patients with wild-type *IDH1* tumors. Similarly, tumors with *TP53* mutations were more likely observed in younger patients with glioma. Subsequently, a comparison of mutational profiles of samples analyzed by both panels was also performed. Implementation of the comprehensive pan-cancer and the MOL panels resulted in the identification of 37 and 15 variants, respectively. Of those, 13 were common. Comprehensive pan-cancer panel identified 24 additional variants, 22 of which were located in regions that were not targeted by the MOL panel. By contrast, the MOL panel identified two additional variants. Overall, the present study demonstrated that using an extended tumor profile assay instead of a glioma-specific tumor profile panel identified additional genetic changes that may be taken into consideration as potential therapeutic targets for glioma diagnosis and molecular classification.

Correspondence to: Professor Philippos C. Patsalis, NIPD Genetics Ltd., 31 Neas Engomis, Engomi, 2409 Nicosia, Republic of Cyprus
E-mail: p.patsalis@nipd.com

*Contributed equally

Abbreviations: CNAs, copy number alterations; CNS, central nervous system; FFPE, formalin-fixed, paraffin-embedded; HeCOG, Hellenic cooperative oncology group; InDels, insertions and deletions; MOL, laboratory of molecular oncology; NGS, next generation sequencing; OS, overall survival; P/LP, pathogenic/likely pathogenic; SNVs, single nucleotide variants; SVs, structural variants; VEP, variant effect predictor; VUS, variants of unknown clinical significance; WHO, World Health Organization

Key words: gliomas, tumor, targeted next generation sequencing, genetic variants, somatic, molecular profiles, prognostic biomarkers, molecular diagnosis, precision medicine

Introduction

Tumors of the brain and Central Nervous System (CNS) are uncommon. Of these tumors, gliomas represent approximately

one-third of all primary CNS tumors and the majority of malignant CNS tumors with a yearly incidence of about 6/100000 (1). Patients with gliomas have a varying 5-year survival rate, a high mortality rate, and a poor prognosis depending on histology and grading. The poorest prognosis is for glioblastoma, which has a 5-year relative survival rate of 6.8 (2,3).

Traditionally, the classification of tumors has been based on histopathological features and immunochemistry, but in 2016 the WHO (World Health Organization) took molecular characteristics into account in its reclassification of tumors, and in 2021 it underlined molecular markers after additional update classifications (4-6).

Gliomas have been found to have several genetic changes that maybe used as diagnostic, prognostic, predictive, and potentially therapeutic biomarkers. The most commonly reported genetic variations include mutations in *ATRX, BRAF, CDKN2A/B*, *IDH*, *NF1*, *RB1*, *TERT*, and *TP53* genes as well as *MGMT* promoter methylation status, copy number alterations such as 1p/19q codeletion, *EGFR* amplification and combined gain of chromosome 7 and loss of chromosome 10 (7+/10-), and rearrangements (7-9).

Subsequently, the advent of next-generation sequencing (NGS) technologies over the past ten years, has significantly influenced the field of molecular oncology in gliomas, demonstrating a significant variability by age, gender, and ethnicity (10,11).

Implementation and broad use of multigene panels on Formalin-Fixed, Paraffin-Embedded (FFPE) tissues enable the cost-effective study of many genes and detection of genetic abnormalities by massively parallel sequencing.

To date, a large number of investigations encompassing commonly affected and new candidate genes have been performed to more precisely categorize gliomas and identify distinct prognostic categories that may have treatment implications, with varied results being obtained (12,13). Therefore, the aim of the present study is to further elucidate and determine the prevalence of somatic mutations by implementing a broad 80-gene panel among 32 patients with gliomas, while reporting their clinicopathological features and outcomes. Subsequently, 14 of 32 tumor samples were examined using another multigene panel to evaluate the accuracy and clinical utility of the broad panel.

Materials and methods

This study initially included 48 patients diagnosed with high-grade gliomas and 48 available FFPE tissue samples selected in the clinical centers affiliated with Hellenic Cooperative Oncology Group (HeCOG). Sixteen out of these 48 tumor samples did not meet the quality control criteria for further analysis. Subsequently, thirty-two tumor paraffin blocks from 32 patients were examined. The median age of cancer diagnosis among the patients was 54.9 years (range: 25.2-77.8 years). Clinical data were obtained from the HeCOG data office. Written informed consent was obtained from all individuals prior to the use of their biological material for research purposes as specified in the Declaration of Helsinki. Tumor blocks were stored and centrally processed in the Laboratory of Molecular Oncology (MOL; Hellenic Foundation for Cancer Research/Aristotle University of Thessaloniki) for histology review, including confirmation of tumor tissue on the section; comparison to local typing; histologic grade; areas with necrosis; microvascular proliferation; assessment of tumor areas for macrodissection. The study was approved by the Bioethics Committee of the Aristotle University of Thessaloniki School of Medicine (AUTH #No 2/February 4, 2015) and by Cyprus National Bioethics Committee (ΕΕΒΚ/ΕΠ/2016/54, 22/4/2021).

Tissue processing, DNA extraction and NGS genotyping. Tumor dense areas were marked on H&Es and microdissected manually from 10 μm unstained FFPE sections prior to DNA extraction. FFPE samples from 48 patients diagnosed with gliomas were processed. DNA was isolated from FFPE tissue sections using the GeneRead DNA FFPE Kit (Qiagen GmbH, Hilden, Germany) in accordance with manufacturer's instructions. DNA was quantified with a fluorometric-based assay for FFPE tissue-derived DNA (Qubit flex fluorometer, Qubit dsDNA high sensitivity assay, ThermoFisher Scientific, Carlsbad, CA, USA). Sixteen samples that did not meet the minimum acceptable quality control (QC) criteria (a minimum of 10 ng of DNA and a minimum DNA concentration of 1 ng/μl) were excluded from the analysis. Therefore, 32 FFPE samples from 32 patients were included and analysed.

Tumor genotyping was performed in the Clinical Laboratory Improvement Amendments (CLIA)-certified laboratory, using a comprehensive, commercially available, pan-cancer tumor profile panel of 80 genes (NIPD Genetics, Nicosia, Cyprus, https://nipd.com/products/oncology/foresentia/), from which Single Nucleotide Variants (SNVs), Insertions and Deletions (InDels), Copy Number Alterations (CNAs) and Rearrangements were detected in accordance with manufacturer's instructions (Table SI). Targeted genomic loci were captured using an in-solution hybridization method (NIPD Genetics, Nicosia, Cyprus) (Supplementary materials and methods). Notably, 14 of 32 tumor samples were also genotyped on Ion Personal Genome Machine (Thermo Fisher/IonTorrent) at the MOL with a custom Ampliseq panel (IAD68363_167), targeting mutations in 55 genes, as previously described (14). Library construction with the MOL panel was performed with standard protocols, using 20 ng DNA per sample and the Ampliseq primers along with the Ampliseq Library Kit v.2.0 and Ion Xpress barcodes (Life Technologies, Carlsbad, CA). Resulting libraries, once normalized to 15 ng/ml, were clonally amplified on the One-Touch-2 instrument, enriched on the OneTouch ES station and sequenced on the Ion Personal Genome Machine sequencer. For data retrieval, base calling was performed on the Torrent Server with Torrent Suite v.4.4.2. Consequently, variants were annotated with Ion Reporter v.4 and accepted for analysis if they had P-value <0.0001; >100 amplicon reads; position coverage >100; variant coverage >40 for position coverage of 100; variant allele frequency (VAF) >5% and Indels without GC stretches. Finally, only tumor samples with mean depth >150 and at least 5 variants were considered eligible and included in the study (14). The study design is presented in Fig. 1.

Bioinformatics analysis. Sequencing data were de-multiplexed with bcl2fastq (v.2.16.0) and paired-end DNA

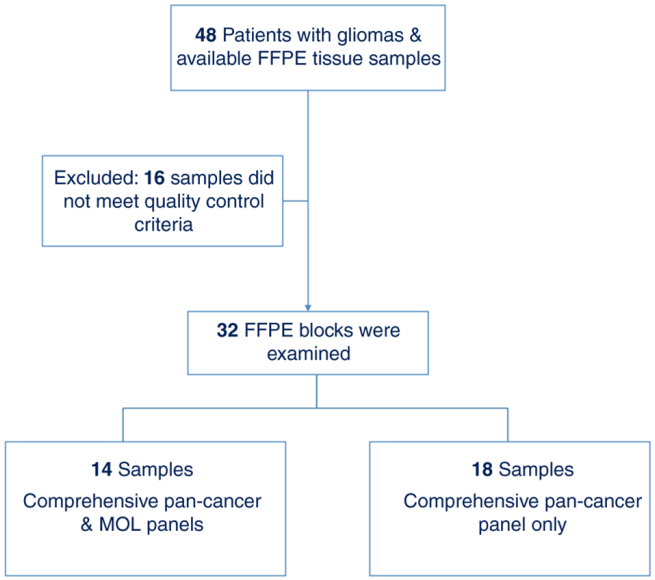

Figure 1. An illustration of the study's design, demonstrating the patients' enrolment and analysis of samples at each stage. FFPE, formalin-fixed, paraffin-embedded.

sequencing reads were processed to remove adapter sequences and poor-quality reads. The remaining sequences were aligned to the human reference genome build (hg19) using the Burrows-Wheeler alignment algorithm (15). Duplicate read entries were removed and aligned reads files were converted to a binary (BAM) format (16). Variant calling was performed using a versatile somatic variant caller (17) and variant annotation was performed using the ENSEMBLE Variant Effect Predictor (VEP) (18). Subsequently, variants were filtered on the basis of variant mapping and read quality (amplicon coverage >100; and base coverage >50). Moreover, variants that fulfilled selection criteria for rarity (minor allele frequency <0.1% based on dbSNP, 5000 Exomes and ExAC) and were identified in coding regions and splice junctions were included in the study. The identified variants were classified and interpreted in accordance with ClinVar and COSMIC databases (19,20). Gene-level CNAs were detected using an in-house bioinformatics pipeline that implements a circular binary segmentation method (21). Rearrangement calling was performed by utilizing discordant pair and split-read alignments following local assembly, realignment, and an in-house filtering pipeline to refine the set of candidate events (22-24).

Statistical analysis. The objectives of the study were descriptive. Categorical variables were summarized using frequencies and percentages while continuous variables using median (min, max). Since Greece lacks a national Tumor Registry, we proportionally estimated the number of glioma patients to the country's approximate 10 million residents using information from glioblastoma (GBM) patients in Malta, a country that is geographically close to Greece. More specifically, a recent study reported that glioblastoma has an incidence rate of 4.5 cases per 100,000 population in Malta (25). Therefore, we assumed that there were 450 glioma patients in Greece and 48 patients would generate a confidence interval of maximum width +/- 14.07% for the percentage estimates. Associations between categorical and continuous variables were examined using the Kruskal-Wallis test whilst associations between categorical variables were examined using the chi-squared test. Overall survival was defined as the time elapsed from cancer diagnosis until death or last contact. The Kaplan-Meier method was used to estimate the survival rate since diagnosis. Univariable Cox regression was used to generate hazard ratios which were presented alongside 95% C.I. The long-rank test was used to examine differences in event-free probability between patient subgroups. Significance level was set to 0.05 for all significance tests. The data management and statistical analysis were mainly performed using the SAS software (version 9.4). The Python 3 and the R 4.2.0 programming languages were employed to build different types of charts for data visualization. The R packages readxl and GenVisR were used to produce the genetic variants' map.

Results

Patient characteristics. A total of 32 patients with high-grade gliomas were involved in the present study, of which 10 (31.3%) were female and 22 (68.7%) were male. The median age of cancer diagnosis was 54.9 years of age, with ages ranging from 25.2 to 77.8 years of age. Among the 31 patients who had a known type of surgery, biopsy was carried out on 16.1% (5/31) while the vast majority of patients underwent subtotal tumor excision (67.8%; 21/31). In our cohort, tumors were predominantly glioblastomas (81.2%; 26/32) whereas 12.5% (4/32) were grade 3 astrocytomas. Of 30 cancer patients with information on the tumor side, tumors were located in the right or in the left hemisphere with the same ratio (each 46.7%; 14/30) and two tumors in both hemispheres (6.6%; 2/30). All information is summarized in Table I.

Genomic landscape of somatic variants in gliomas. In total, 96 variants (SNVs and InDels) were identified in 30 of the 32 (93.75%) tumors in 34 genes, namely *APC*, *AR*, *ATM*, *ATRX*, *BARD1*, *BRCA1*, *CDKN2A*, *CHEK2*, *DICER1*, *EGFR*, *ERBB2*, *ERBB3*, *FANCA*, *FGFR1*, *FGFR2*, *IDH1*, *KEAP1*, *MLH1*, *MRE11*, *MSH2*, *NF1*, *NTRK1*, *NTRK3*, *RB1*, *PALB2*, *PIK3CA*, *POLD1*, *POLE*, *PTEN*, *RAD51D*, *STK11*, *TMPRSS2*, *TP53* and *ZNF276*. The number of identified variants per sample ranged from 1 to 14. Among the 30 tumors with variants, 6 (20%) had only one, 9 (30%) had two and 15 (50%) had three or more variants. Two tumor samples (6.25%; 2/32) demonstrated no variants in the targeted genomic regions. In total, the analysis revealed 38 pathogenic variants and 58 variants of unknown clinical significance (VUS) of which 5 were in-frame insertions/deletions. Among 38 pathogenic variants in 23 tumor samples in 13 genes, 19 were missense, 7 were frameshift, 6 were nonsense and 6 affected a conserved splice-site. *TP53* mutations were the most prevalent (10/38; 26.3%), followed by *PTEN* (9) and *IDH1* (4) (23.7 and 10.5% respectively). Four out of ten *TP53* pathogenic variants were located at mutational hotspot codons 248 and 273. Notably, all the *IDH1* mutations involved the p.(R132H) one. In *EGFR*, all pathogenic variants were located in the extracellular domain. The distribution of pathogenic variants per gene is shown in Fig. 2A.

Table I. Clinical and histopathological characteristics of patients diagnosed with gliomas.

Characteristic	Value
Age at diagnosis, years	
Median (minimum-maximum)	54.9 (25.2-77.8)
Sex	
Female	31.3% (10/32)
Male	68.7% (22/32)
Type of surgery	
Biopsy (<75% of the tumor)	16.1% (5/31)
Subtotal (75-99% of the tumor)	67.8% (21/31)
Total excision	16.1% (5/31)
Unknown	1
Histology	
Grade 3 astrocytoma	12.5% (4/32)
Glioblastoma	81.2% (26/32)
Other[a]	6.3% (2/32)
Hemisphere	
Bilateral	6.6% (2/30)
Left	46.7% (14/30)
Right	46.7% (14/30)
Unknown	2
Necrosis	
No	21.9% (7/32)
Yes	78.1% (25/32)
Hemorrhage	
No	56.3% (18/32)
Yes	43.7% (14/32)
Endothelial hyperplasia	
No	21.9% (7/32)
Yes	78.1% (25/32)

[a]Other: One glioblastoma tumor with oligodendroglioma elements and one anaplastic oligodendroglioma tumor. Values presented as Mean ± SD, Median (P25, P75), Median (min, max) or N (column %). Percentages were calculated with respect to the total number of patients with the known relevant information.

Twenty-two out of 32 tumor samples (68.75%) showed evidence of structural variants (SVs) (Table SII). Overall, 33 SVs were identified of which the copy number deletions (53.2%; 17 out of 32 patients) were more frequent. Copy number amplifications involved 40.7% (13 out of 32 patients), followed by rearrangements (1 out of 32 patients; 3.1%) (Fig. S1A). Copy number deletions were identified in *CDKN2A* (46.9%; 15 out of 32 patients) and *PTEN* (9.4%; 3 out of 32 patients) whereas gene amplifications were identified most frequently in *EGFR* (31.3%; 10 out of 32 patients) followed by *MYC* (6.3%; 2 out of 32 patients), *FGFR3* and *KIT* (3.1%; 1 out of 32 patients for each gene). Interestingly, a *FGFR3-TACC3* rearrangement was also identified in one patient (1/32; 3.1%) concurrently with *FGFR3* amplification (Fig. S1B). And also interestingly, 8 out of 22 patients with copy number alterations had concurrent CNAs, predominately *CDKN2A* deletions with *EGFR* amplifications (Fig. S1C).

Finally, it was noteworthy to mention that 15 out of the 32 tumors had both SNVs/Indels and SVs. The distribution of genetic alterations encompassing SNVs, InDels, and SVs per gene and per tumor is demonstrated in Fig. 2B.

Comparison of mutational profiles derived from a comprehensive pan-cancer gene panel and EMO panel. To evaluate the newly implemented targeted sequencing panel, we compared the results from analysis of 14 tumor blocks genotyped with both the comprehensive pan-cancer and the MOL panels. Implementation of the comprehensive pan-cancer and the MOL panels resulted in the identification of 37 and 15 variants, respectively. Of those, 13 were common. Comprehensive pan-cancer panel identified 24 additional variants, 22 of which located in regions that were not targeted by the MOL panel. On the contrary, the MOL panel identified two additional variants. Of these, one was located in a non-targeted region by a comprehensive pan-cancer panel. Interestingly, six variants that were identified only by the comprehensive pan-cancer panel were pathogenic, whereas two variants identified only by the MOL panel were pathogenic.

Subsequently, 18 tumor blocks were genotyped with a comprehensive pan-cancer panel only. A total of 59 genetic variants were identified, of which 43 were located in regions targeted by the comprehensive pan-cancer panel only, while the remaining 16 variants were detected in regions by both panels. Notably, twenty-one of all detected variants were pathogenic.

Associations between clinicopathological features and mutations in IDH1, PTEN and TP53 genes

IDH1 mutational status. Patients with *IDH1* positive tumors were diagnosed with brain tumors at a statistically significant younger age than those with *IDH1* wild-type tumors (34.4 vs. 56.3 years, $P<0.05$). Moreover, *IDH1* mutational status was significantly associated with specific type of surgery (x^2 $P<0.05$). The majority (75%; 3/4) of patients with *IDH1* positive tumors carried out biopsy whereas 25% (1/4) underwent subtotal excision. In contrast, the majority (74.1%; 20/27) of patients with *IDH1* wild-type tumors underwent subtotal surgery, while 18.5% (5/27) performed total excision and 7.4% (2/27) carried out biopsy. Additionally, half of the *IDH1* positive tumors were glioblastomas, one was grade 3 astrocytomas and one was anaplastic oligodendroglioma. *IDH1* mutant tumors were located either in the right or left hemisphere with the same proportion (50% each). This was also the case with the *IDH1* wild-type tumors (46.2% each). All data are summarized in Table II.

PTEN mutational status. Tumors with *PTEN* mutations (SNVs and InDels) were identified in older patients when compared to *PTEN* wild-type tumors (59.6 vs. 52.1 years) although this finding was not statistically significant. The vast majority (77.8%; 7/9) of patients with *PTEN* mutant tumors underwent subtotal surgery, while all involved glioblastomas. No associations were observed between *PTEN* mutational status and the side of the tumor (Table II).

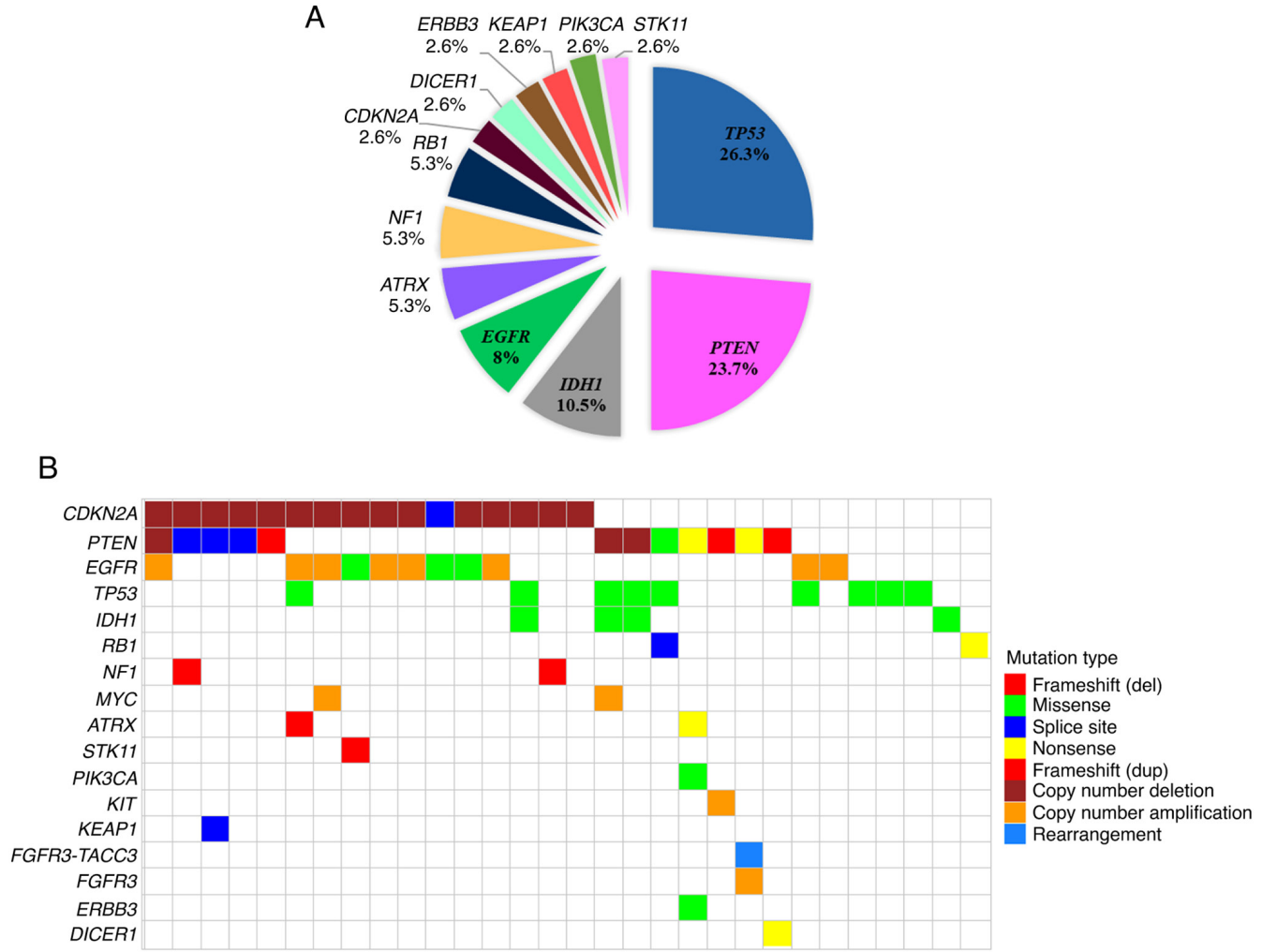

Figure 2. Mutational profiles of gliomas tumors. (A) Distribution of mutations (SNVs and InDels) among cancer genes. (B) Map of SNVs/InDels and SVs per gene and per type and per tumor. Del, deletion; Dup, duplication; InDels, insertions and deletions; SNVs, single nucleotide variants; SVs, structural variants.

TP53 mutational status. Tumors with *TP53* mutations were more frequently detected in younger patients, when compared to *TP53* wild-type tumors (42.6 vs. 58.7 years). One third of patients (3/9; 33.3%) with *TP53* positive tumors carried out biopsy. Most of*TP53* mutant tumors were glioblastomas (8/9; 88.9%). According to available information (n=30), five patients with *TP53* mutant tumors developed left-sided and four right-sided tumors, respectively (Table II).

Associations of the mutational status between IDH1, PTEN and TP53 genes. Wild-type *TP53* was present in *IDH1* wild-type tumors only (P<0.05). Similarly, wild-type *TP53* was more frequently identified in *PTEN* wild-type than *PTEN* mutated tumors. Additionally, *PTEN* mutations did not co-occur with *IDH1* mutations. All the data are summarized in Table III.

Patient outcomes. Herein, was assessed whether having a mutant tumor on a specific gene had an impact on patients' overall survival (OS). Notably, all the patients received standard of care treatment. The median follow-up of patients from diagnosis until death or last contact was 19.2 months and during this time period 29 deaths (90.6%; 29/32) occurred and three patients were alive (9.4%; 3/32). Our analysis showed that patients with *IDH1* mutant tumors had a statistically significant longer survival when compared to patients with *IDH1* wild-type tumors, although due to small size, a confidence interval was not available (Fig. 3).

Discussion

In the present study, we explored the genomic landscape of 32 tumor tissues from patients with high-grade gliomas by massively parallel sequencing using an 80 multigene panel. Subsequently, we examined the correlation between our results and patient outcomes as well as histopathological characteristics. Additionally, implementing two distinct panels on 14 common samples, we evaluated the molecular profiles of tumors.

Our findings showed that 96 genetic alterations (SNVS and InDels) were identified in the tumor samples. Of these, 38 were pathogenic and scattered among 13 distinct genes. *IDH1*, *PTEN*, and *TP53* pathogenic variants exhibited high incidence in mutational spectrum, with *TP53* being the most prevalent. These results were in line with those of other studies since the p53 pathway was deregulated in many cancers and these three genes, along with *EGFR*, were the most frequently altered genes in gliomas (26-29).

Table II. Associations between clinicopathological characteristics and the mutational status of several genes.

		IDH1 mutation			PTEN mutation			TP53 mutation		
Characteristic	Eligible	No	Yes	P-value	No	Yes	P-value	No	Yes	P-value
Age at diagnosis, years	32	56.3 (40.3,77.8)	34.4 (25.2,42.1)	0.003[a]	52.1 (25.2,77.8)	59.6 (40.3,69.2)	0.167	58.7 (40.3,77.8)	42.6 (25.2,59.1)	0.065
Sex	32			0.28			0.87			0.66
Female		10 (35.7)	0 (0.0)		7 (30.4)	3 (33.3)		7 (30.4)	3 (33.3)	
Male		18 (64.3)	4 (100.0)		16 (69.6)	6 (66.7)		16 (69.6)	6 (66.7)	
Surgery specify	31			0.02[a]			0.49			0.57
Biopsy (<75% of the tumor)		2 (7.4)	3 (75.0)		3 (13.6)	2 (22.2)		2 (8.7)	3 (33.3)	
Subtotal (75-99% of the tumor)		20 (74.1)	1 (25.0)		14 (63.6)	7 (77.8)		17 (73.9)	4 (44.4)	
Total excision		5 (18.5)	0 (0.0)		5 (22.7)	0 (0.0)		3 (13.0)	2 (22.2)	
Unknown								1 (4.3)	0 (0.0)	
Histology	32			0.15			0.45			0.99
Grade 3 astrocytoma		3 (10.7)	1 (25.0)		4 (17.4)	0 (0.0)		3 (13.0)	1 (11.1)	
Glioblastoma		24 (85.7)	2 (50.0)		17 (73.9)	9 (100.0)		18 (78.3)	8 (88.9)	
Other/Unknown		1 (3.6)	1 (25.0)		2 (8.7)	0 (0.0)		2 (8.7)	0 (0.0)	
Hemisphere	30			0.99			0.83			0.83
Bilateral		2 (7.7)	0 (0.0)		2 (9.1)	0 (0.0)		2 (9.5)	0 (0.0)	
Left		12 (46.2)	2 (50.0)		9 (40.9)	5 (62.5)		9 (42.9)	5 (55.6)	
Right		12 (46.2)	2 (50.0)		11 (50)	3 (37.5)		10 (47.6)	4 (44.4)	
Necrosis	32			0.20			0.36			0.46
No		5 (17.9)	2 (50.0)		6 (26.1)	1 (11.1)		6 (26.1)	1 (11.1)	
Yes		23 (82.1)	2 (50.0)		17 (73.9)	8 (88.9)		17 (73.9)	8 (88.9)	
Hemorrhage	32			0.61			0.40			0.99
No		15 (53.6)	3 (75.0)		14 (60.9)	4 (44.4)		13 (56.5)	5 (55.6)	
Yes		13 (46.4)	1 (25.0)		9 (39.1)	5 (55.6)		10 (43.5)	4 (44.4)	
Endothelial hyperplasia	32			0.20			0.36			0.46
No		5 (17.9)	2 (50.0)		6 (26.1)	1 (11.1)		6 (26.1)	1 (11.1)	
Yes		23 (82.1)	2 (50.0)		17 (73.9)	8 (88.9)		17 (73.9)	8 (88.9)	

[a]P<0.05. Percentages were calculated with respect to the total number of patients with the known relevant information

Table III. Associations of the mutational status between IDH1, PTEN and TP53.

A, *IDH1* mutation

Mutation	No	Yes	P-value
TP53 mutation			0.25
No	22 (78.6)	2 (50.0)	
Yes	6 (21.4)	2 (50.0)	
PTEN mutation			0.55
No	20 (71.4)	4 (100.0)	
Yes	8 (28.6)	0 (0.0)	

B, *PTEN* mutation

Mutation	No	Yes	P-value
TP53 mutation			0.18
No	15 (65.2)	8 (88.9)	
Yes	8 (34.8)	1 (11.1)	
IDH1 mutation			0.54
No	20 (87.0)	9 (100.0)	
Yes	3 (13.0)	0 (0.0)	

C, *TP53* mutation

Mutation	No	Yes	P-value
PTEN mutation			0.18
No	15 (65.2)	8 (88.9)	
Yes	8 (34.8)	1 (11.1)	
IDH1 mutation			0.017[a]
No	23 (100.0)	6 (66.7)	
Yes	0 (0.0)	3 (33.3)	

[a]P<0.05.

In the present study, the most frequently detected mutations at codons 248 and 273 of the *TP53* gene were also found in *TP53* positive tumors, but at a decreased frequency compared to other series (30,31). Additionally, among *IDH1* mutated tumors, the p.(R132H) variant was solely found. Given that more than 90% of all *IDH1* mutants have the present variant, this finding is consistent with other reports (32). Genetic alterations were also detected in genes that were involved in cell cycle regulation, DNA damage response pathways, the MAPK/PI3K pathway, the receptor tyrosine kinase pathway and telomere maintenance including *ATRX*, *CDKN2A*, *NF1* and*RB1*. These genes could also serve as therapeutic targets for the design of a more individualized treatment protocol (33).

Furthermore, the use of the comprehensive pan-cancer panel allowed for the detection of structural variations. The identification of *CDKN2A* deletions and *EGFR* amplifications is consistent with previous research since both genetic changes are frequent in glioblastomas and have previously been linked to a poor prognosis (34). Additionally, an *FGFR-TACC3* rearrangement was identified; these genomic events are present in approximately 2-3% of glioblastomas. Clinical studies and case reports have provided some preliminary evidence that suggests that this result could serve as an actionable therapeutic target in advanced solid tumors. Erdafitinib, a pan-FGFR tyrosine kinase inhibitor, produced one stable disease and two partial responses in three glioblastoma patients with *FGFR-TACC3* positive tumors in two phase I studies (35,36).

We also assessed the extended panel's clinical usefulness and accuracy. We presented a comparison of the results from the tumor analysis using both the comprehensive pan-cancer panel and the MOL panel. We observed that there was a high concordance (86.7%; 13/15) between the two panels since 13 variants were common. It's noteworthy that a significant portion (22/24; 91.6%) of additional variants that were identified by the comprehensive pan-cancer panel occurred in regions that were not targeted by the MOL panel, emphasizing the necessity of implementing a broader panel with additional targeted regions.

In this study, a mutation in exon 14 of the *EGFR* gene was found in the tumor of a glioblastoma patient, using the comprehensive pan-cancer panel; this exon was not included in the MOL panel. Although numerous prior studies involving EGFR tyrosine kinase inhibitors have failed to demonstrate anti-tumor efficacy in tumors with *EGFR* mutations, there is still continuing research using anti EGFR-immunotherapy approaches, such as antibody-based strategies and vaccines (37,38).

Subsequently, variant calling parameters; such as relatively low variant allele frequency (VAF; ≤5%) and/or low base coverage (≤50%), explained why despite the fact that two genetic alterations although were targeted by the MOL panel, they were not detected and reported.

Moreover, a mutation in the *LZTR1* gene's Kelch domain was only detected by the MOL panel due to the absence of the *LZTR1* gene from the extended panel. This could be considered a limitation of the comprehensive pan-cancer panel, because it has been demonstrated that *LZTR1* mutations facilitate glioma sphere development and self-renewal, these mutations could be considered as a potential therapeutic target for glioma (39,40). Additionally, the *IDH1* p.(R132H) mutation was identified by the MOL panel but not by the comprehensive pan-cancer panel, although it was targeted by the latter, and sufficient quality control parameters were reached, ruling out the likelihood of decreased assay sensitivity. This disagreement may be explained by the fact that both assays used DNA that was isolated from two separate sets of FFPE sections from the same FFPE block, demonstrating intra-tumor heterogeneity (41,42). Finally, our findings showed that SVs could be identified using NGS data if the suitable NGS platform and multigene panel were implemented. This is essential for the comprehensive molecular characterization of tumor profiles.

Therefore, the implementation of a pan-cancer panel enables the identification of a larger number of mutations as well as rare genomic events, thus providing more therapeutic choices for patients with gliomas. Consequently, these findings may help with glioma patient diagnosis, prognosis, eligibility

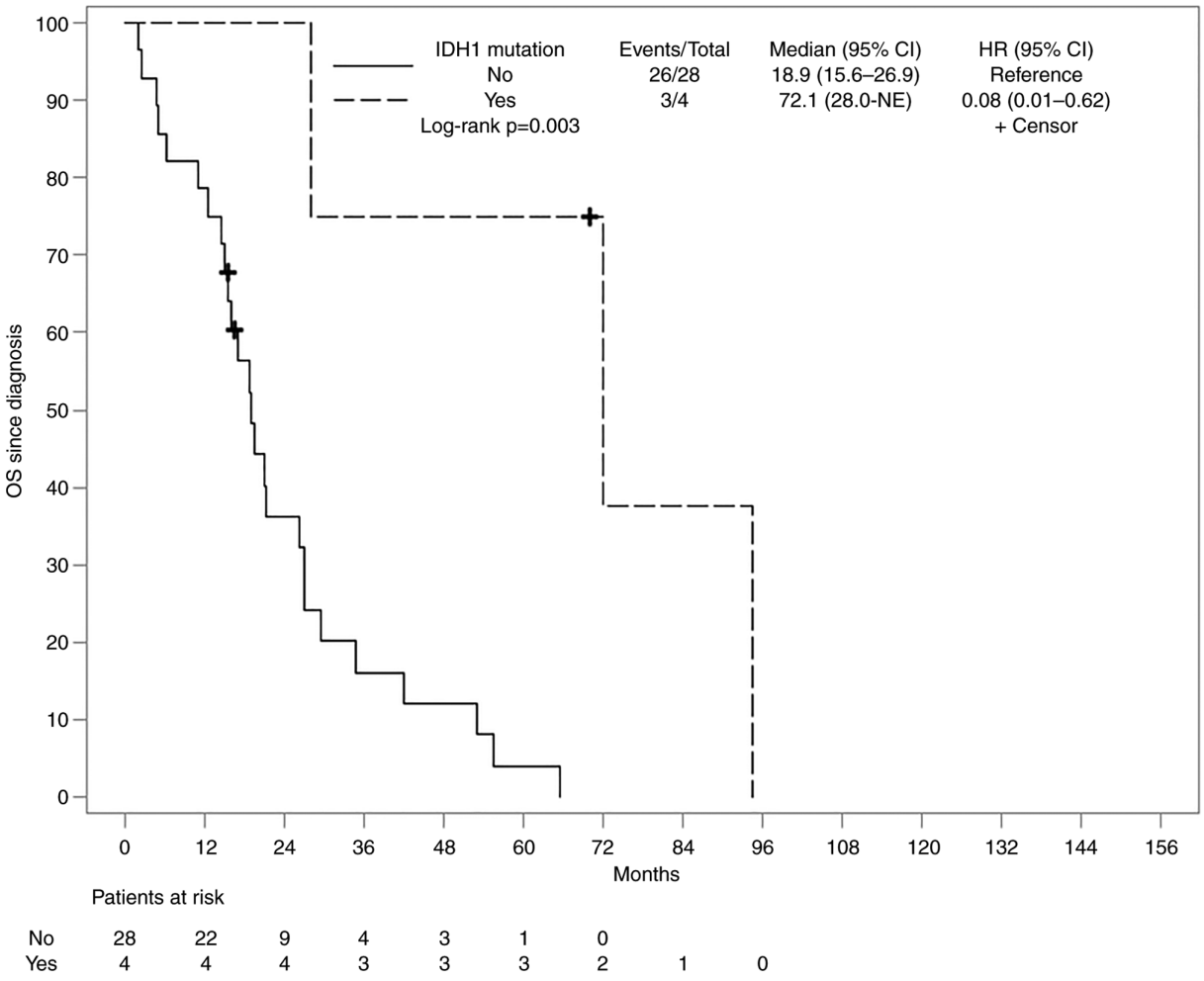

Figure 3. Kaplan-Meier Curve with respect to overall survival based on the mutational status of *IDH1* gene in the entire cohort. IDH, isocitrate dehydrogenase; OS, overall survival; HR, hazard ratio.

for clinical trial enrollment, and treatment as previously described (12,28,43).

The application of broad panel sequencing also provided insightful information about clinicopathological traits and patient outcomes. We found that *IDH1* positive tumors were more frequently detected in younger patients, when compared to *IDH1* wild-type tumors. Patients with *IDH1* mutated tumors were characterized by better prognosis compared to patients with *IDH1* wild-type tumors which is consistent with previous studies (44-46). Patients with *IDH1* mutant tumors mainly underwent biopsies rather than tumor resections; that were more frequently diffused and cannot be completely removed.

Moreover, we observed that patients with *TP53* positive tumors were more likely to be diagnosed with cancer at a younger age than patients with wild-type *TP53* tumors, as expected (47). Interestingly, *PTEN* mutations were mutually exclusive with *IDH1* mutations and wild-type *TP53* tumors were presented in *IDH1* wild-type tumors only (14,26,48). However, these data should be regarded with caution due to the small examined number of tumors.

All patients in our study have received standard-of-care and have long term follow up that reflects actual survival. This, gives our study a benefit in identifying prognostic molecular biomarkers for glioma patients.

These observations are consistent with other reports and established findings as mentioned above.

Furthermore, both platforms verified the tumor genotyping results for the common samples. This study also demonstrates the importance of collaboration between many institutions and clinics in the acquisition of tumor blocks, along with comprehensive clinical information, histology reports, and patient outcomes. On the other hand, there are certain restrictions with the present study.

First of all, the small sample size restricts the statistical power of our research, necessitating careful interpretation of the results. Second, tumors were chosen based on the quantity and quality of available tissue, thereby bringing selection bias into the study.

Overall, utilizing FFPE samples in clinical and research contexts, the use of a broader sequencing panel enables the simultaneous identification of several genetic alterations in a wide range of genes across gliomas, improving tumor molecular characterization and classification. The application of the new technology improves clinical outcomes by facilitating accurate molecular diagnosis as well as the identification of known and candidate prognostic biomarkers that could serve as possible therapeutic targets for the personalized treatment of glioma patients.

Acknowledgements

Not applicable.

Funding

This work was supported by an internal HeCOG research grant (grant no. HE R_17/15), by NIPD Genetics Limited and by a HeSMO Grant.

Availability of data and materials

We have submitted our data to the 'Figshare' Repository. They are available from Romanidou, Ourania (2022): Accession no. figshare. Dataset. Version 1 25.10.22, 13:15 first online date https://doi.org/10.6084/m9.figshare.21394077.v1 and the link https://doi.org/10.6084/m9.figshare.21394077.

Authors' contributions

OR, PA, VK, GF and PCP conceptualized the study. KK, AA, CLo, CLe, AP and FF analyzed the data. KT, AE, KP and VK performed the experiments. OR, MI, EK, GK, GR, IX, GF and PCP acquired the data. KT, AP and FF validated the reproducibility of results. KT and KP designed the methodology of next generation sequencing. AA, ChrL and AP software development. AE, AA and ChaL curated the data. MI, EK and GK supervised the study. PCP was project administrator and acquired funding. OR, PA, AE and GF wrote the original draft. PA, KK, KT, AA, CLo, CLe, MI, EK, GK, KP, AP, GR, IX, FF, VK and PCP wrote or revised critically the manuscript. KP and AE confirm the authenticity of all the raw data. All authors read and approved the final manuscript and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The study was approved by the Bioethics Committee of the Aristotle University of Thessaloniki School of Medicine (approval no. 2/February 4, 2015) and by Cyprus National Bioethics Committee (approval no. ΕΕΒΚ/ΕΠ/2016/54; 22/4/2021).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Schwartzbaum JA, Fisher JL, Aldape KD and Wrensch M: Epidemiology and molecular pathology of glioma. Nat Clin Pract Neurol 2: 494-503, 1, 516, 2006.
2. Stupp R, Brada M, van den Bent MJ, Tonn JC and Pentheroudakis G; ESMO Guidelines Working Group: High-grade glioma: ESMO clinical practice guidelines for diagnosis, treatment and follow-up. Ann Oncol 25 (Suppl 3): iii93-iii101, 2014.
3. Ostrom QT, Cioffi G, Gittleman H, Patil N, Waite K, Kruchko C and Barnholtz-Sloan JS: CBTRUS statistical report: Primary brain and other central nervous system tumors diagnosed in the United States in 2012-2016. Neuro Oncol 21 (Suppl 5): v1-v100, 2019.
4. Wesseling P and Capper D: WHO 2016 classification of gliomas. Neuropathol Appl Neurobiol 44: 139-150, 2018.
5. Louis DN, Perry A, Reifenberger G, von Deimling A, Figarella-Branger D, Cavenee WK, Ohgaki H, Wiestler OD, Kleihues P and Ellison DW: The 2016 World Health Organization classification of tumors of the central nervous system: A summary. Acta Neuropathol 131: 803-820, 2016.
6. Śledzińska P, Bebyn MG, Furtak J, Kowalewski J and Lewandowska MA: Prognostic and predictive biomarkers in gliomas. Int J Mol Sci 22: 10373, 2021.
7. Weller M and Reifenberger G: Beyond the World Health Organization classification of central nervous system tumors 2016: What are the new developments for gliomas from a clinician' perspective? Curr Opin Neurol 33: 701-706, 2020.
8. Kristensen BW, Priesterbach-Ackley LP, Petersen JK and Wesseling P: Molecular pathology of tumors of the central nervous system. Ann Oncol 30: 1265-1278, 2019.
9. Reinhardt A, Stichel D, Schrimpf D, Sahm F, Korshunov A, Reuss DE, Koelsche C, Huang K, Wefers AK, Hovestadt V, *et al*: Anaplastic astrocytoma with piloid features, a novel molecular class of IDH wildtype glioma with recurrent MAPK pathway, CDKN2A/B and ATRX alterations. Acta Neuropathol 136: 273-291, 2018.
10. Ostrom QT, Kinnersley B, Wrensch MR, Eckel-Passow JE, Armstrong G, Rice T, Chen Y, Wiencke JK, McCoy LS, Hansen HM, *et al*: Sex-specific glioma genome-wide association study identifies new risk locus at 3p21.31 in females, and finds sex-differences in risk at 8q24.21. Sci Rep 8: 7352, 2018.
11. Ostrom QT, Cote DJ, Ascha M, Kruchko C and Barnholtz-Sloan JS: Adult glioma incidence and survival by race or ethnicity in the united states from 2000 to 2014. JAMA Oncol 4: 1254-1262, 2018.
12. Zacher A, Kaulich K, Stepanow S, Wolter M, Köhrer K, Felsberg J, Malzkorn B and Reifenberger G: Molecular diagnostics of gliomas using next generation sequencing of a glioma-tailored gene panel. Brain Pathol 27: 146-159, 2017.
13. Kim HS, Kwon MJ, Song JH, Kim ES, Kim HY and Min KW: Clinical implications of TERT promoter mutation on IDH mutation and MGMT promoter methylation in diffuse gliomas. Pathol Res Pract 214: 881-888, 2018.
14. Razis E, Kotoula V, Koliou GA, Papadopoulou K, Vrettou E, Giannoulatou E, Tikas I, Labropoulos SV, Rigakos G, Papaemmanoyil S, *et al*: Is there an independent role of TERT and NF1 in high grade gliomas? Transl Oncol 13: 346-354, 2020.
15. Li H: Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM. arXiv 3: 13033997, 2013.
16. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G and Durbin R; 1000 Genome Project Data Processing Subgroup: The sequence alignment/map format and SAMtools. Bioinformatics 25: 2078-2079, 2009.
17. Li MM, Datto M, Duncavage EJ, Kulkarni S, Lindeman NI, Roy S, Tsimberidou AM, Vnencak-Jones CL, Wolff DJ, Younes A and Nikiforova MN: Standards and guidelines for the interpretation and reporting of sequence variants in cancer: A joint consensus recommendation of the association for molecular pathology, American society of clinical oncology, and college of American pathologists. J Mol Diagn 19: 4-23, 2017.
18. McLaren W, Gil L, Hunt SE, Riat HS, Ritchie GR, Thormann A, Flicek P and Cunningham F: The ensembl variant effect predictor. Genome Biol 17: 122, 2016.
19. Landrum MJ, Lee JM, Benson M, Brown GR, Chao C, Chitipiralla S, Gu B, Hart J, Hoffman D, Jang W, *et al*: ClinVar: Improving access to variant interpretations and supporting evidence. Nucleic Acids Res 46 (D1): D1062-D1067, 2018.
20. Tate JG, Bamford S, Jubb HC, Sondka Z, Beare DM, Bindal N, Boutselakis H, Cole CG, Creatore C, Dawson E, *et al*: COSMIC: The catalogue of somatic mutations in cancer. Nucleic Acids Res 47 (D1): D941-D947, 2019.
21. Seshan VE and Olshen AB: DNAcopy: A package for analyzing DNA copy data. Bioconductor Vignette, 1-7, 2014.
22. Chen X, Schulz-Trieglaff O, Shaw R, Barnes B, Schlesinger F, Källberg M, Cox AJ, Kruglyak S and Saunders CT: Manta: Rapid detection of structural variants and indels for germline and cancer sequencing applications. Bioinformatics 32: 1220-1222, 2016.

23. Cameron DL, Schröder J, Penington JS, Do H, Molania R, Dobrovic A, Speed TP and Papenfuss AT: GRIDSS: Sensitive and specific genomic rearrangement detection using positional de Bruijn graph assembly. Genome Res 27: 2050-2060, 2017.
24. Layer RM, Chiang C, Quinlan AR and Hall IM: LUMPY: A probabilistic framework for structural variant discovery. Genome Biol 15: R84, 2014.
25. Grech N, Dalli T, Mizzi S, Meilak L, Calleja N and Zrinzo A: Rising incidence of glioblastoma multiforme in a well-defined population. Cureus 12: e8195, 2020.
26. Nie Q, Hsiao MC, Chandok H, Rowe S, Prego M, Meyers B, Omerza G, Hesse A, Uvalic J, Soucy M, *et al*: Molecular profiling of CNS tumors for the treatment and management of disease. J Clin Neurosci 71: 311-315, 2020.
27. Ballester LY, Fuller GN, Powell SZ, Sulman EP, Patel KP, Luthra R and Routbort MJ: Retrospective analysis of molecular and immunohistochemical characterization of 381 primary brain tumors. J Neuropathol Exp Neurol 76: 179-188, 2017.
28. Zeng C, Wang J, Li M, Wang H, Lou F, Cao S and Lu C: Comprehensive molecular characterization of Chinese patients with glioma by extensive next-generation sequencing panel analysis. Cancer Manag Res 13: 3573-3588, 2021.
29. Zheng S, Alfaro-Munoz K, Wei W, Wang X, Wang F, Eterovic AK, Shaw KRM, Meric-Bernstam F, Fuller GN, Chen K, *et al*: Prospective clinical sequencing of adult glioma. Mol Cancer Ther 18: 991-1000, 2019.
30. Gillet E, Alentorn A, Doukouré B, Mundwiller E, van Thuijl HF, Reijneveld JC, Medina JA, Liou A, Marie Y, Mokhtari K, *et al*: TP53 and p53 statuses and their clinical impact in diffuse low grade gliomas. J Neurooncol 118: 131-139, 2014.
31. Pessôa IA, Amorim CK, Ferreira WAS, Sagica F, Brito JR, Othman M, Meyer B, Liehr T and de Oliveira EHC: Detection and correlation of single and concomitant TP53, PTEN, and CDKN2A alterations in gliomas. Int J Mol Sci 20: 2658, 2019.
32. Parsons DW, Jones S, Zhang X, Lin JC, Leary RJ, Angenendt P, Mankoo P, Carter H, Siu IM, Gallia GL, *et al*: An integrated genomic analysis of human glioblastoma multiforme. Science 321: 1807-1812, 2008.
33. Park C, Kim TM, Bae JM, Yun H, Kim JW, Choi SH, Lee ST, Lee JH, Park SH and Park CK: Clinical and genomic characteristics of adult diffuse midline glioma. Cancer Res Treat 53: 389-398, 2021.
34. Reis GF, Pekmezci M, Hansen HM, Rice T, Marshall RE, Molinaro AM, Phillips JJ, Vogel H, Wiencke JK, Wrensch MR, *et al*: CDKN2A loss is associated with shortened overall survival in lower-grade (World Health Organization grades II-III) astrocytomas. J Neuropathol Exp Neurol 74: 442-452, 2015.
35. Loriot Y, Schuler MH, Iyer G, Witt O, Doi T, Qin S, Tabernero J, Reardon DA, Massard C, Palmer D, *et al*: Tumor agnostic efficacy and safety of erdafitinib in patients (pts) with advanced solid tumors with prespecified fibroblast growth factor receptor alterations (FGFRalt) in RAGNAR: Interim analysis (IA) results. J Clin Oncol 40 (16 Suppl): S3007, 2022.
36. Costa R, Carneiro BA, Taxter T, Tavora FA, Kalyan A, Pai SA, Chae YK and Giles FJ: FGFR3-TACC3 fusion in solid tumors: Mini review. Oncotarget 7: 55924-55938, 2016.
37. Yang K, Wu Z, Zhang H, Zhang N, Wu W, Wang Z, Dai Z, Zhang X, Zhang L, Peng Y, *et al*: Glioma targeted therapy: Insight into future of molecular approaches. Mol Cancer 21: 39, 2022.
38. Higa N, Akahane T, Hamada T, Yonezawa H, Uchida H, Makino R, Watanabe S, Takajo T, Yokoyama S, Kirishima M, *et al*: Detection of EGFR mutation distribution and transcriptional variants in IDH-wildtype high-grade gliomas using a next-generation sequencing oncopanel. Res Sq, pp1-11, 2021.
39. Wang Y, Zhang J, Zhang P, Zhao Z, Huang Q, Yun D, Chen J, Chen H, Wang C and Lu D: LZTR1 inactivation promotes MAPK/ERK pathway activation in glioblastoma by stabilizing oncoprotein RIT1. bioRxiv, 2020.
40. Frattini V, Trifonov V, Chan JM, Castano A, Lia M, Abate F, Keir ST, Ji AX, Zoppoli P, Niola F, *et al*: The integrated landscape of driver genomic alterations in glioblastoma. Nat Genet 45: 1141-1149, 2013.
41. Comba A, Faisal SM, Varela ML, Hollon T, Al-Holou WN, Umemura Y, Nunez FJ, Motsch S, Castro MG and Lowenstein PR: Uncovering spatiotemporal heterogeneity of high-grade gliomas: From disease biology to therapeutic implications. Front Oncol 11: 703764, 2021.
42. Sottoriva A, Spiteri I, Piccirillo SG, Touloumis A, Collins VP, Marioni JC, Curtis C, Watts C and Tavaré S: Intratumor heterogeneity in human glioblastoma reflects cancer evolutionary dynamics. Proc Natl Acad Sci USA 110: 4009-4014, 2013.
43. Tirrò E, Massimino M, Broggi G, Romano C, Minasi S, Gianno F, Antonelli M, Motta G, Certo F, Altieri R, *et al*: A custom DNA-based NGS panel for the molecular characterization of patients with diffuse gliomas: Diagnostic and therapeutic applications. Front Oncol 12: 861078, 2022.
44. Zou P, Xu H, Chen P, Yan Q, Zhao L, Zhao P and Gu A: IDH1/IDH2 mutations define the prognosis and molecular profiles of patients with gliomas: A meta-analysis. PLoS One 8: e68782, 2013.
45. Yan H, Parsons DW, Jin G, McLendon R, Rasheed BA, Yuan W, Kos I, Batinic-Haberle I, Jones S, Riggins GJ, *et al*: IDH1 and IDH2 mutations in gliomas. N Engl J Med 360: 765-773, 2009.
46. Sun H, Yin L, Li S, Han S, Song G, Liu N and Yan C: Prognostic significance of IDH mutation in adult low-grade gliomas: A meta-analysis. J Neurooncol 113: 277-284, 2013.
47. Park Y, Park J, Ahn JW, Sim JM, Kang SJ, Kim S, Hwang SJ, Han SH, Sung KS and Lim J: Transcriptomic landscape of lower grade glioma based on age-related non-silent somatic mutations. Curr Oncol 28: 2281-2295, 2021.
48. Ichimura K, Pearson DM, Kocialkowski S, Bäcklund LM, Chan R, Jones DT and Collins VP: IDH1 mutations are present in the majority of common adult gliomas but rare in primary glioblastomas. Neuro Oncol 11: 341-347, 2009.