RESEARCH ARTICLE

Editorial Process: Submission:10/14/0000 Acceptance:03/10/2023

Detection of IDH1 Mutation in cfDNA and Tissue of Adult Diffuse Glioma with Allele-Specific qPCR

Adil Husain[1,2], Sridhar Mishra[1], Mohammed Haris Siddiqui[3], Nuzhat Husain[1]*

Abstract

Background: The World Health Organization (WHO) classification of central nervous system (CNS) tumors necessitates testing of isocitrate dehydrogenase *(IDH) 1/2* gene mutation in patients with adult-type diffuse glioma (ADG) for better disease management. In clinical practice, the testing of *IDH1* is primarily achieved using immunohistochemistry (IHC) specific to IDH1-R132, which carries a sensitivity of 80% and specificity of 100%. However, in some cases, non-specific background staining or regional heterogeneity in the protein expression of *IDH1* may necessitate confirmatory genetic analysis. Robust and reliable assays are needed for IDH1/2 mutation testing. The aim of the current study was to detect *IDH1* mutation in cfDNA and tissue of adult-type diffuse glioma with allele-specific qPCR. **Materials and Methods:** In the current study, *IDH1-R132H* mutation was analyzed in tumor tissue with paired cell-free DNA (cfDNA) in patients with ADG (n = 45) using IHC and competitive allele-specific Taqman PCR (CAST-PCR). Genomic DNA was extracted from formalin-fixed paraffin-embedded (FFPE) tissue and matched serum for cfDNA using commercially available kits. CAST-PCR with IHC for the detection of *IDH1-R132H* mutation was also compared. **Results:** The *IDH1-R132H* mutation was detected in 46.67% (21/45) cases and 57.78% (26/45) cases using IHC and allele-specific CAST-PCR. In cfDNA of matched IDH1-mutant FFPE tissue DNA, *IDH1-R132H* mutation was detected in 11.54% (3/26) using CAST-PCR. The concordance rate for *IDH1-R132H*mutation between IHC and CAST-PCR was 80.77% (21/26). **Conclusion:** The CAST-PCR assay is more precise and sensitive for *IDH1-R132H*detection than traditional IHC, and *IDH1-R132H* mutation detection using cfDNA may add to the current methods of glioma genomic characterization.

Keywords: Adult- type Diffuse Glioma- *IDH1/2*- Cell-free DNA- CAST-PCR- Mutation diagnostics

Asian Pac J Cancer Prev, **24 (3)**, 961-968

Introduction

Adult-type diffuse gliomas (ADG) are characterized predominantly by astrocytic or oligodendroglial morphology (Rodriguez et al., 2016). Molecular parameters were included in the classification of central nervous system (CNS) tumors in the update 2016 CNS World Health Organization (WHO), which has broken the century-old rule of diagnosis dependent only on microscopy (Louis et al., 2016), and the last update in 2021 has further increased the diagnostic use of genetic alterations (Brat et al., 2020; Louis et al., 2021). Molecular markers in the CNS tumor have both diagnostic and prognostic importance. Various clinical decisions are being made based on the results of these biomarkers, whether for diagnosis, treatment, or prediction monitoring (Fan et al., 2020; P. Yang et al., 2016). The inclusion of isocitrate dehydrogenase (IDH) 1/2 as the "molecular signature" of gliomas and mutations in either *IDH1* or IDH2 as the primary prognostic factor and molecular diagnostic criterion for ADG in the 2016 WHO classification marked a significant departure from the previous morphology-alone classification (Louis et al., 2016; Parsons et al., 2008). *IDH1/2* mutation is a feature of 'oligodendroglioma, IDH-mutant, and 1p19q-codeleted' and 'astrocytoma IDH-mutant' while IDH-wildtype is a key diagnostic marker for 'Glioblastoma, IDH-wildtype' and 'Diffuse pediatric-type high-grade glioma, H3-wildtype, and IDH-wildtype' as recommended by the 2021 WHO classification of CNS (Brat et al., 2020; Louis et al., 2021).

Determining *IDH1* mutation is crucial for diagnosis and selecting an appropriate treatment strategy. Typically, the first step in treating a glioma is to perform the safest radical resection to provide enough tumor tissue for a reliable diagnosis. Regardless of tumor grade, any glioma expressing IDH-wildtype should be regarded as glioblastoma, IDH-wildtype (Louis et al., 2021) and treated with aggressive chemoradiotherapy according to the Stupp protocol (Stupp et al., 2005, 2014). The

*[1]Department of Pathology, Dr. Ram Manohar Lohia Institute of Medical Sciences, Lucknow, Uttar Pradesh, India. [2]Department of Biosciences, Integral University, Lucknow, Uttar Pradesh, India. [3]Department of Bioengineering, Integral University, Lucknow, Uttar Pradesh, India. *For Correspondence: drnuzhathusian@hotmail.com*

treatment of gliomas expressing mutated variations of IDH should be guided by the presentation of clinical and molecular features. For radically resected low-grade tumors exhibiting both the 1p/19q co-deletion and an IDH mutation, one might even consider omitting oncotherapy altogether and recommend watchful follow-up (Weller et al., 2017; Weller et al., 2021).

In clinical practice, the testing of *IDH1*/*2* mutation is primarily based on immunohistochemistry (IHC) specific for *IDH1/2* protein expression, which is limited in terms of sensitivity, and cross-reactivity. In some cases, non-specific background staining or regional heterogeneity in *IDH1-R132H* protein expression may necessitate confirmatory genetic analysis. Assays such as competitive allele-specific TaqMan Polymerase chain reactions (CAST-PCR) are characterized by their high sensitivity and specificity to detect minimal amounts of mutated DNA in a sample containing large amounts of normal wildtype DNA (Barbano et al., 2015; Bolton et al., 2015). It can robustly detect mutant alleles at values as low as 0.1% in a wildtype background and has >99% concordance with other technologies, including technology based on digital PCR and Sanger sequencing (Yang et al., 2018).

In recent years, various approaches have been developed, including "liquid biopsies" (nucleic acid extracted from biological fluids such as plasma, urine, and cerebrospinal fluid (CSF)) for detecting the IDH mutation (Satomi et al., 2022); D2HG detection in body fluids; and advanced MRI imaging with specific D2HG detection by magnetic resonance spectroscopy (MRS) (Fujita et al., 2022; Mithraprabhu et al., 2021; Tuna et al., 2022). However, none of them is currently used in clinical practice. A non-invasive, rapid, sensitive, and cost-effective method for IDH mutation analysis is needed to enhance diagnosis and predict survival. Analysis of cell-free nucleic acids has entered clinical practice in tumors like lung and colon cancer (Kolenčík et al., 2020; Kwapisz, 2017). The current study evaluated the *IDH1-R132H* mutation in cfDNA and respective tissue of adult-type diffuse glioma using a CAST-PCR assay. Further, the sensitivity and effectiveness of the CAST-PCR assay were compared with IHC for *IDH1-R132H*.

Materials and Methods

Study samples

Our study included histologically confirmed ADG patients (n=45) according to the WHO 2021 CNS classification. When the study commenced, cases were diagnosed based on the WHO 2016 classification of CNS tumors (Louis et al., 2016). They have been reclassified as per WHO 2021 CNS classification, and the staining of *IDH1-IHC* was used for *IDH1* status in all cases (Louis et al., 2020; Louis et al., 2021). All IDH-mutated astrocytomas have been categorized as astrocytoma *IDH*-mutant. *IDH*-wildtype cases were further classified on the histological features, including proliferation, necrosis, and mitosis. The Institutional ethics committee cleared the study (IEC No.26/18), and all study participants gave informed consent and have therefore been performed under the ethical standards of the Declaration of Helsinki (World Medical Association, 2013)

Sample collection

Formalin-fixed paraffin-embedded (FFPE) tumor tissue blocks were obtained from the departmental tumor archive after histopathological diagnosis, and peripheral blood (3.0ml) was collected in silica gel vials (BD Vacutainer, UK) from post-op patients who underwent biopsy or with remnant tumor. Samples were collected within 22-65 days (Q1-Q3) after the procedure. The serum was separated by centrifugation at 4,000 rpm for 10 minutes and stored at −80°C until further processing. All the samples were processed within 02 hours of collection.

Immunohistochemistry (IHC) of Isocitrate Dehydrogenase 1 (IDH1)

Immunohistochemical analysis of IDH1 was performed using Anti-*IDH1* (R132H) (Dianova, USA Clone: H09-unconj) in a dilution of 1:50. All Immunohistochemical assays were performed on a VENTANA BenchMark XT automated staining instrument according to the manufacturer's instructions with onboard deparaffinization, retrieval, and staining (Ventana Medical Systems, Inc., Tucson, USA). An expert pathologist (NH) independently analyzed all immunohistochemically stained sections.

Staining interpretation of IDH1

The staining interpretation of IDH was as follows: Mutant: intense cell cytoplasm and nucleus of tumor cells, wildtype: weak diffuse staining of cytoplasm and staining of macrophages. Normal/residual glial and vascular endothelial cells were internal negative controls.

DNA extraction from formalin-fixed paraffin-embedded (FFPE) tissue and serum

FFPE tumor tissue was used to extract genomic DNA. The tumor area was defined on a Hematoxylin and Eosin (HE) stained slide and a corresponding section marked for DNA isolation. DNA was extracted using the QIAamp DNA FFPE Tissue Kit (Qiagen, Germany, Cat No. #56404). Serum cell-free DNA (cfDNA) was extracted using the ChargeSwitch® gDNA 1 mL Serum Kit (Invitrogen, USA) as per the manufacturer's instructions. The quality and quantity of the DNA were measured using Nanodrop (DeNoVix, USA, Model #DS-11).

IDH1 mutation detection by CAST-PCR

IDH1-R132H mutation was determined in 45 FFPE tissue DNA with paired cfDNA using CAST-PCR assay (TaqMan® Mutation Detection Assay). Each case was amplified using a reference assay (#Hs00001019_rf) and a mutation assay (#Hs00000981_mu) in a 20µl reaction, using a 10µl of genotyping master mix (#4371353), 1µl of primer and probe for reference and mutation assay, 1-9 µl of template DNA (up to 50ng) and volume were brought to 20µl by nuclease-free water. Real-time amplification was performed using the AriaMx Real-Time PCR system (Agilent Technologies, USA) as per the manufacturer's instructions. In each batch, NTC was run to check for cross-contamination (Figure 1i). ΔCT values were

calculated by CT (mutant allele assay)– CT (gene reference assay). A case was defined as mutated if the ΔCT values were ≤9.96 (TaqMan Mutation Detection Assays, #4467012, Applied Biosystems, USA).

Statistical analysis

The IBM SPSS v22 Statistical Package for Social Sciences (SPSS, IBM, USA) was used for the statistical analysis. Cohen's kappa statistics tested the agreement between IHC and CAST-PCR in FFPE and cfDNA. Reverse Kaplan-Meier was used to compute the median follow-up time. Kaplan-Meier and log-rank analyses were performed to assess survival concerning each parameter; differences were considered significant when $p \leq 0.05$.

Results

Patient's characteristics

Histologically confirmed oligodendroglioma (n=10), diffuse astrocytoma and (n=11) glioblastoma (n=24) were enrolled. As shown in Table 1, most patients (24/45) were ≤40 years old, with a male predominance (36/45). Most of the patients underwent GTE (36/45), the site of the tumor was frontal (19/45), temporal (05/45), parietal (02/45), and multiple (19/45), and all patients underwent a planned regimen. The median follow-up duration was 34 months (95%CI: 32-36). Among 45 patients, 28 (62.22%) died during the follow-up. The average OS was 22 months [95% CI: 18–26].

IDH1 mutation in formalin-fixed paraffin-embedded (FFPE) tissue

Out of 45 cases, IHC successfully detected *IDH1-R132H* mutation in 46.67% (21/45) of the cases. An *IDH1-R132H* mutation was determined in FFPE tissue DNA and cfDNA. The CAST-PCR assay successfully detected the *IDH1-R132H* mutation in 26/45 (57.78%) of FFPE DNA. Five cases showed mutations in the CAST-PCR test, but IHC showed they were all wildtype (Figure 1e-h). The rate of concordance for *IDH1-R132H* mutation between IHC and CAST-PCR was 80.77% (21/26) (p= 0.000; k= 0.780) (Table 2 and Supplementary Table 1).

IDH1 mutation in cell-free DNA (cfDNA)

In the CAST-PCR assay, 03/26 (11.54%) cfDNA samples were found as *IDH1-R132H* mutated (Figure 1a-d). Total cfDNA level of *IDH1*-wildtype (n =19) and mutant cases (n = 26) were 470.30±687.30ng/ml, and 175.00±206.90ng/ml respectively (p=0.04). Out of 26 mutated cases, *IDH1*-mutated cfDNA (n = 03) had mean cfDNA values of 189.70 (±235.80) ng/ml while wild type cfDNA (n = 23) have mean cfDNA value of 173.10 (±208.70) ng/ml (p = 0.89). There was slight agreement between the cfDNA and FFPE DNA for *IDH1* mutation detection (p =0.125; k = 0.099) (Tables 3, 4 and Supplementary Table 1).

Survival

The median follow-up duration was 34 months (95%CI 32-36). Among 45 patients, 28 (62.22%) died during the follow-up. The average OS was 22 months

Table 1. The Distribution of ADG Cases According to Clinical Findings

Characteristics		N (%)
Age	≤40 Years	24 (53.33)
	>40 Years	21 (46.67)
Median (Q1-Q3)	40.00 (32.25-54.00)	
Gender	Males	36 (80.00)
	Females	09 (20.00)
Karnofsky Performance Score	≤80	31 (68.89)
	>80	14 (31.11)
	Frontal	19 (42.22)
	Temporal	05 (11.11)
Tumor Site	Parietal	02 (4.44)
	Multiple	19 (42.22)
Extent of Excision	Gross Total Excision	36 (80.00)
	Partial Excision/Biopsy	09 (20.00)
WHO CNS 2021	Grade 2	13 (28.89)
Grade	Grade 3	07 (15.55)
	Grade 4	25 (55.55)
WHO CNS 2021	Astrocytoma IDH-mutant	11 (24.44)
Classification	Oligodendroglioma IDH-mutant 1p19q Deleted	10 (22.22)
	Glioblastoma IDH-wildtype	24 (53.33)
Adjuvant Radiotherapy	Radiotherapy	15 (33.33)
	Radiotherapy+Chemotherapy	30 (66.67)
Survival	Exitus	28 (62.22)
	Alive	17 (37.78)

Table 2. Agreement Test of *IDH1-R132H* between Immunohistochemistry and CAST-PCR Assay

		CAST-PCR Assay for IDH1		Total	p-Value	Kappa
		Mutant	Wildtype			
	Mutant	21	0	21		
IHC for IDH1	Wildtype	5	19	24	0.000	0.780
Total		26	19	45		

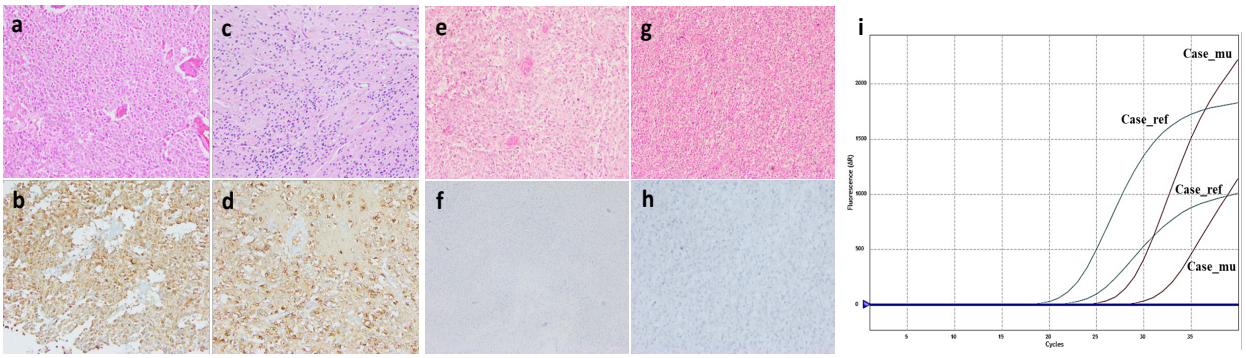

Figure 1. Immunohistochemical Analysis by Anti-IDH Antibody against Glioma tissue a,b) Oligodendroglioma, IDH-mutant, 1p19q co-deleted, 2021 WHO CNS Grade 3; c,d) Oligodendroglioma, IDH-mutant, 1p19q co-deleted, 2021 WHO CNS Grade 2; e,f) Glioblastoma, IDH-wildtype, 2021 WHO CNS Grade 4; g,h) Glioblastoma, IDH-wildtype 2021 WHO CNS Grade 4; i) Amplification plots of Competitive Allele-Specific TaqMan qPCR

[95% CI 18–26]. Patients in the lower age group (40 years) had better survival than patients in the higher age group (> 40years) in the Kaplan-Meier survival analysis (log-rank p = 0.010). Increased survival trends were observed with an increase in KPS score (log-rank p= 0.001), while survival decreased with an increase in WHO grade (log-rank p=0.001). *IDH1*-mutant patients had better survival than IDH1-wildtype patients (log-rank p=0.000). Gender (log-rank p= 0.332) and extent of excision (log-rank p=0.429) did not show a relationship to the patient's overall survival, as depicted in Table 5 & Figure 2.Patients with *IDH*-mutation in cfDNA showed OS survival of 25 months (95% CI; 11-39) and IDH-wildtype cfDNA showed OS of 22 months (95% CI; 18-26) (log-rank p=0.35).

Discussion

It has been found that *IDH1* gene mutations are common in diffuse gliomas (Parsons et al., 2008). Based on the mutation profiles of glioma subtypes and primary and recurrent tumors, *IDH1* mutations are considered one of the most significant genetic modifications in gliomagenesis (Johnson et al., 2014; Yan et al., 2009) that can aid in the diagnosis and prognosis. In addition, patients with known *IDH1* mutations may benefit from new *IDH1*-targeted chemotherapeutic regimens under development (Golub et al., 2019; Karpel-Massler et al.,

Table 3. Agreement Test of CAST-PCR Assay between FFPE DNA and cfDNA

		CAST-PCR Assay for *IDH1* in FFPE DNA		Total	p-Value	Kappa
		Mutant	Wildtype			
CAST-PCR Assay for *IDH1* in cfDNA	Mutant	3	0	3		
	Wildtype	23	19	42	0.125	0.099
Total		26	19	45		

Table 4. Distribution of IDH Mutation in cfDNA and FFPE Tumor Tissue of ADG

Adult-type diffuse glioma	WHO Grade	IHC-IDH (MT/WT)	CAST-PCR-IDH (MT/WT)	Mean cfDNA (ng/mL)	IDH in cfDNA (MT/WT)
Astrocytoma IDH-mutant	Grade 2 (n=6)	6/0	6/0	280.80±361.80	0/6
	Grade 3 (n=4)	4/0	4/0	79.57±50.55	0/4
	Grade 4 (n=1)	1/0	1/0	66.70±0.00	0/1
Oligodendroglioma IDH-mutant 1p19q co-deleted	Grade 2 (n=7)	7/0	7/0	151.50±143.90	1/6
	Grade 3 (n=3)	3/0	3/0	293.20±203.20	1/2
Glioblastoma IDH-wildtype	Grade 4 (n=24)	0/24	5/19	394.80±626.90	1/23

Table 5. Predictors of Time to Overall Survival in Patients with Adult-Type Diffuse Glioma

Variables		N (%)	Overall Survival	
			Median (Months)	p-Value (Log-rank)
Age	≤44 Years	24 (53.33)	31	0.01
	>44 Years	21 (46.67)	12	
Gender	Male	36 (80.00)	22	0.332
	Female	09 (20.00	15	
Karnofsky Performance Score	70	15 (33.33)	10	0.001
	80	16 (35.56)	17	
	90	14 (31.11)	-	
Excision	GTE	36 (80.00)	22	0.429
	PE/Biopsy	09 (20.00)	14	
WHO CNS 2021 Type	Astrocytoma IDH-mutant	11 (24.44)	-	0.002
	Oligodendroglioma IDH-mutant 1p19q Deleted	10 (22.22)	24	
	Glioblastoma IDH-wildtype	24 (53.33)	12	
WHO CNS 2021 Grade	2	13 (28.89)	-	0.001
	3	07 (15.55)	31	
	4	25 (55.55)	13	
IHC-IDH	Mutant	21 (46.67)	-	0.000
	Wildtype	24 (53.33)	12	
CAST-PCR-IDH	Mutant	26 (57.78)	31	0.003
	Wildtype	19 (42.22)	12	

GTE, Gross Total Excision; PE, Partial Excision; RANO, Response Assessment in Neuro-Oncology; IDH1, Isocitrate Dehydrogenase- 1

2019; Mellinghoff et al., 2020).

A study by Matthias Preusser et al., (2011) showed the need for confirmatory genetic analysis in cases with non-specific background staining and/or regional heterogeneity of *IDH1-R132H* expression using the DIA H09 antibody (Preusser et al., 2011). Although Sanger sequencing is the "gold standard" for detecting mutations because of its low rate of false positives and high specificity, it has several drawbacks, including low sensitivity, long assay times, the necessity for high-quality tissue samples, and manual interpretation (Gao et al., 2016). Furthermore, next-generation sequencing (NGS), which is used to detect various mutations, has the drawback of taking too much time and being expensive to discover a single genetic variant. An alternative technique for mutation detection is a real-time CAST-PCR assay (Roma et al., 2013). Competitive allele-specific TaqMan PCR allows the selective amplification of minor alleles and blocks the amplification of non-mutant alleles.

In a significant subset of patients, immunohistochemical reactivity is not detected; it is likely but not certain that the tumor is *IDH*-wildtype. The study by Andrews and Prayson (2020) recommends PCR testing for all patients whose tumor is negative by IHC. However, this is not always performed, both because it is expensive and in some patient groups (e.g., elderly patients with tumors demonstrating necrosis), it is almost always negative (i.e., these are almost invariably glioblastoma). So, keeping this in mind, we decided to test the CAST PCR in *IDH*-negative tumors of all age groups.

In the current study, real-time PCR, combined with an MGB-blocking oligonucleotide to suppress the normal allele, was used to detect *IDH1-R132H* gene mutations in gliomas. The CAST-PCR identified 26/45 cases as *IDH1* mutated, and IHC detected 21/45 cases with an *IDH1* mutation. While both IHC and CAST-PCR assays confirmed 21 cases as *IDH1* mutants. A total of 05 cases were reported as mutated in CAST-PCR while were wild type in IHC. The reason leading to this inconsistency may be due to the sensitivity and specificity of antibodies (Preusser et al., 2011).

Our results showed that the IDH1 mutation detection rate in gliomas was significantly different (p=0.000) between IHC and CAST-PCR (κ=0.780). A study by Agarwal et al., (2013) compared the performance of IHC and DNA sequencing for IDH1 mutation and found a concordance rate of 88% (44/50) (Agarwal et al., 2013). Similarly, in our study, the concordance rate between CAST-PCR and IHC in detecting IDH1 mutation was 80.77%. Moreover, the CAST-PCR assay was more sensitive than IHC in identifying the IDH1 mutation. Further, cfDNA and FFPE DNA show concordant results only in 11.54% of cases; this low concordance may be due to a lack of tumor-derived DNA or a low copy number of mutated DNA. All cases with cfDNA-positive for IDH1 on CAST-PCR were <55 years, including two cases of oligodendroglioma, IDH-mutant, 1p19q co-deleted, CNS WHO grade 2 and 3, respectively, and one case of Glioblastoma, IDH-wildtype CNS WHO grade 4 by IHC analysis.

We have included only the *IDH1-R132H* mutation in both IHC and CAST-PCR, and other mutations of *IDH1* &

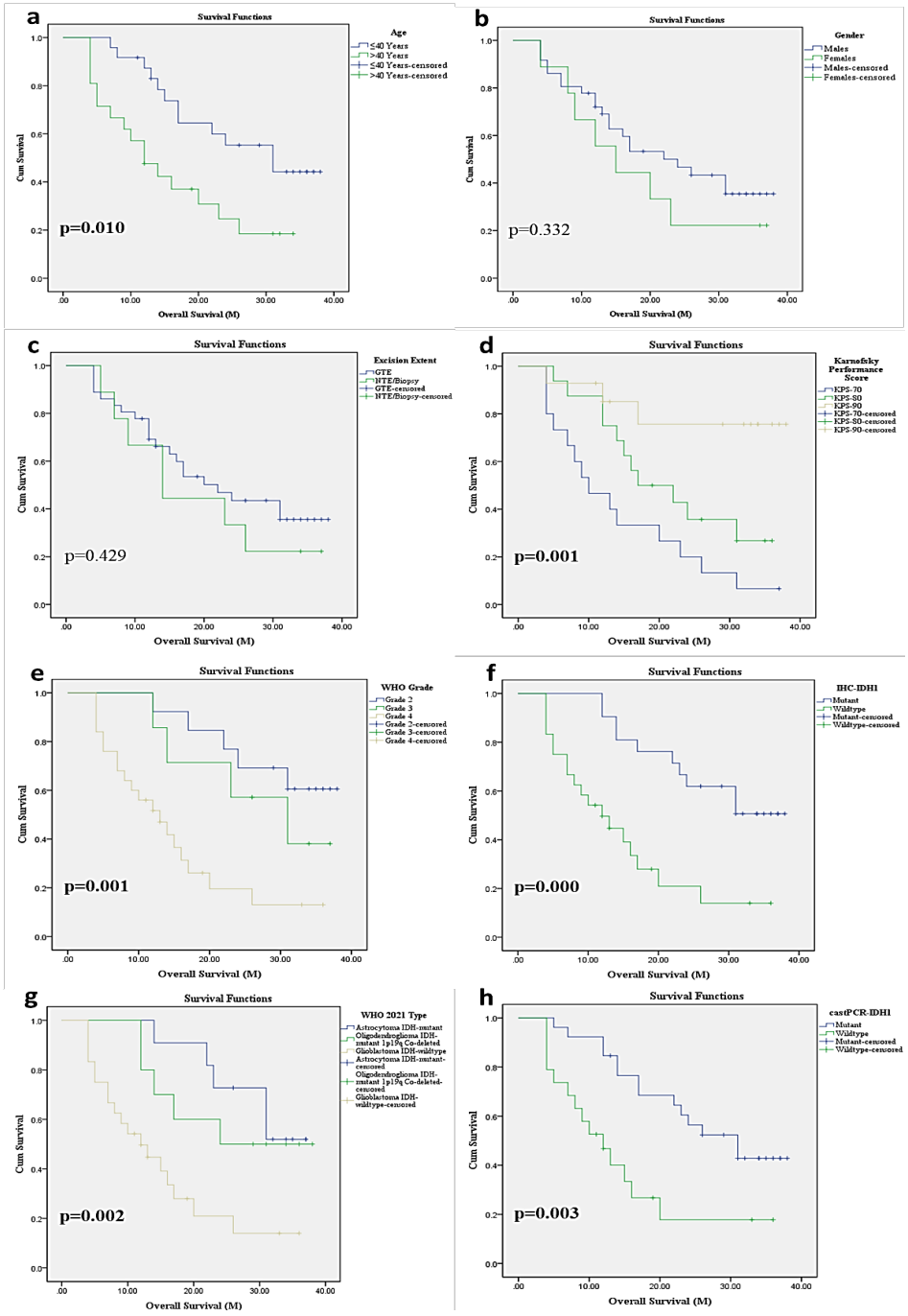

Figure 2. Kaplan-Meier Curves. Overall survival rate (OS) for patients with adult-type diffuse glioma. a) OS according to age. b) OS according to gender. c) OS according to excision extent. d) OS according to KPS. e) OS according to WHO 2021 grade. f) OS according to IHC-IDH1 expression. g) OS according to WHO 2021 adult-type diffuse glioma. h) OS according to CAST-PCR-IDH1 expression. The log-rank test calculated p-values.

IDH2 were not analyzed. However, these mutation types become secondary due to low frequency because their testing costs may burden the patients. Sanger sequencing is considered the gold standard for *IDH1* & IDH2 mutation detection; however, we could not validate our results using the sequencing method.

Finally, the CAST-PCR technique for detecting glioma *IDH1* gene mutations has high sensitivity, good reproducibility, ease of use, and accurate results. It offers a more precise method for detecting mutations in the *IDH1* gene in formalin-fixed paraffin-embedded tissue samples but lacks sensitivity using an alternative source, cfDNA, in the case of tissue scarcity. Despite the small sample size, evidence suggests that CAST-PCR assays outperform IHC. More sensitive techniques may be required to detect IDH mutations in cfDNA.

Author Contribution Statement

Conceptualization, Funding acquisition, Project administration, Resources, Software, Supervision, Validation, Writing - original draft, Writing - review & editing: Nuzhat Husain; Data curation, Investigation, Methodology, Writing - original draft, Writing - review

& editing: Adil Husain; Methodology, Writing - review & editing: Sridhar Mishra; Resources, Software, Supervision, Writing - review & editing: Mohammed Haris Siddiqui.

Acknowledgements

Funding statement

The authors would like to acknowledge the University Grant Commission, New Delhi, Government of India, for the JRF-NET Fellowship to Adil Husain (Fellowship No. NOV-2017-343185) and Dr. Ram Manohar Lohia Institute of Medical Sciences, Lucknow, for an Intramural Research Grant to Prof. Nuzhat Husain (26/18).

Ethics Approval

Institutional Ethics Committee of Dr. Ram Manohar Lohia Institute of Medical Sciences (RMLIMS), Lucknow (IEC-26/18) and Integral University, Lucknow (Manuscript Communication Number-IU/R&D/2022-MCN0001618).

Conflict of Interest

None.

References

Agarwal S, Sharma MC, Jha P, et al (2013). Comparative study of *IDH1* mutations in gliomas by immunohistochemistry and DNA sequencing. *Neurooncology*, **15**, 718-26.

Andrews C, Prayson RA (2020). IDH mutations in older patients with diffuse astrocytic gliomas. *Ann Diagn Pathol*, **49**, 151653.

Barbano R, Pasculli B, Coco M, et al (2015). Competitive allele-specific TaqMan PCR (Cast-PCR) is a sensitive, specific and fast method for BRAF V600 mutation detection in Melanoma patients. *Sci Rep*, **5**, 1-11.

Bolton L, Reiman A, Lucas K, Timms J, Cree IA (2015). KRAS Mutation Analysis by PCR: A Comparison of Two Methods. *PLoS One*, **10**, e0115672.

Brat DJ, Aldape K, Colman H, et al (2020). cIMPACT-NOW Update 5: Recommended Grading Criteria and Terminologies for IDH-mutant Astrocytomas. *Acta Neuropathol*, **139**, 603-8.

Fan Z, Liu Y, Li S, et al (2020). Association of tumor growth rates with molecular biomarker status: a longitudinal study of high-grade glioma. *Aging*, **12**, 7908-26.

Fujita Y, Nunez-Rubiano L, Dono A, et al (2022). *IDH1* p. R132H ctDNA and D-2-hydroxyglutarate as CSF biomarkers in patients with IDH-mutant gliomas. *J Neurooncol*, **159**, 261-70.

Gao J, Wu H, Wang L, et al (2016). Validation of targeted next-generation sequencing for RAS mutation detection in FFPE colorectal cancer tissues: comparison with Sanger sequencing and ARMS-Scorpion real-time PCR. *BMJ Open*, **6**, e009532.

Golub D, Iyengar N, Dogra S, et al (2019). Mutant Isocitrate Dehydrogenase Inhibitors as Targeted Cancer Therapeutics. *Front Oncol*, **9**, 417.

Johnson BE, Mazor T, Hong C, et al (2014). Mutational Analysis Reveals the Origin and Therapy-Driven Evolution of Recurrent Glioma. *Science*, **343**, 189-93.

Karpel-Massler G, Nguyen TTT, Shang E, Siegelin MD (2019). Novel *IDH1*-Targeted Glioma Therapies. *CNS Drugs*, **33**, 1155-66.

Kolenčík D, Shishido SN, Pitule P, et al (2020). Liquid Biopsy in Colorectal Carcinoma: Clinical Applications and Challenges. *Cancers*, **12**, 1376.

Kwapisz D (2017). The first liquid biopsy test approved. Is it a new era of mutation testing for non-small cell lung cancer?. *Ann Transl Med*, **5**, 46.

Louis DN, Perry A, Reifenberger G, et al (2016). The 2016 World Health Organization Classification of Tumors of the Central Nervous System: a summary. *Acta Neuropathol*, **131**, 803-20.

Louis DN, Wesseling P, Aldape K, et al (2020). cIMPACT-NOW update 6: new entity and diagnostic principle recommendations of the cIMPACT-Utrecht meeting on future CNS tumor classification and grading. *Brain Pathol*, **30**, 844-56.

Louis DN, Perry A, Wesseling P, et al (2021). The 2021 WHO Classification of Tumors of the Central Nervous System: a summary. *Neurooncology*, **23**,1231-51.

Mellinghoff IK, Ellingson BM, Touat M, et al (2020). Ivosidenib in Isocitrate Dehydrogenase 1–Mutated Advanced Glioma. *J Clin Oncol*, **38**, 3398-06.

Mithraprabhu S, Chen M, Savvidou I, Reale A, Spencer A (2021). Liquid biopsy: an evolving paradigm for the biological characterisation of plasma cell disorders. *Leukemia*, **35**, 2771-83.

Parsons DW, Jones S, Zhang X, et al (2008). An Integrated Genomic Analysis of Human Glioblastoma Multiforme. *Science*, **321**, 1807-12.

Preusser M, Wöhrer A, Stary S, et al (2011). Value and Limitations of Immunohistochemistry and Gene Sequencing for Detection of the *IDH1-R132H* Mutation in Diffuse Glioma Biopsy Specimens. *J Neuropath Exp Neur*, **70**, 715-23.

Rodriguez FJ, Vizcaino MA, Lin MT (2016). Recent Advances on the Molecular Pathology of Glial Neoplasms in Children and Adults. *J Mol Diagn*, **18**, 620-34.

Roma C, Esposito C, Rachiglio AM, et al (2013). Detection of EGFR Mutations by TaqMan Mutation Detection Assays Powered by Competitive Allele-Specific TaqMan PCR Technology. *BioMed Res Int*, **2013**, 385087.

Satomi K, Yoshida A, Matsushita Y, et al (2022). Clinical application of a highly sensitive digital PCR assay to detect a small fraction of IDH1 R132H-mutant alleles in diffuse gliomas. *Brain Tumor Pathol*, **39**, 210-7.

Stupp R, Weller M, Belanger K, et al (2005). Radiotherapy plus Concomitant and Adjuvant Temozolomide for Glioblastoma. *N Engl J Med*, **352**, 987-996

Stupp R, Brada M, van den Bent MJ, Tonn JC, Pentheroudakis G (2014). High-grade glioma: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann Oncol*, **25**, 93-101.

Tuna G, Dal-Bekar NE, Akay A, et al (2022). Minimally Invasive Detection of *IDH1* Mutation With Cell-Free Circulating Tumor DNA and D-2-Hydroxyglutarate, D/L-2-Hydroxyglutarate Ratio in Gliomas. *J Neuropath Exp Neur*, **81**, 502-10.

Weller M, van den Bent M, Tonn JC, et al (2017). European Association for Neuro-Oncology (EANO) guideline on the diagnosis and treatment of adult astrocytic and oligodendroglial gliomas. *Lancet Oncol*, **18**, 315-29.

Weller M, van den Bent M, Preusser M, et al (2021) EANO guidelines on the diagnosis and treatment of diffuse gliomas of adulthood. *Nat Rev Clin Oncol*, **18**, 170-86.

World Medical Association (2013). World Medical Association Declaration of Helsinki: Ethical Principles for Medical Research Involving Human Subjects. *JAMA*, **310**, 2191-4.

Yan H, Parsons DW, Jin G, et al (2009). *IDH1* and IDH2 Mutations in Gliomas. *N Engl J Med*, **360**, 765-73.

Yang P, Cai J, Yan W, et al (2016). Classification based on mutations of TERT promoter and IDH characterizes subtypes in grade II/III gliomas. *Neurooncology*, **18**, 1099-08.
Yang Y, Meng Y, Zhang H, et al (2018). Detection of EGFR and BRAF mutations by competitive allele-specific TaqMan polymerase chain reaction in lung adenocarcinoma. *Oncol Lett*, **15**, 3295-04.