ORIGINAL ARTICLE

Utility of real-time polymerase chain reaction for the assessment of *CDKN2A* homozygous deletion in adult-type *IDH*-mutant astrocytoma

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Abstract

The World Health Organization Classification of Tumors of the Central Nervous System 5th Edition (WHO CNS5) introduced a newly defined astrocytoma, *IDH*-mutant grade 4, for adult diffuse glioma classification. One of the diagnostic criteria is the presence of a *CDKN2A/B* homozygous deletion (HD). Here, we report a robust and cost-effective quantitative polymerase chain reaction (qPCR)-based test for assessing *CDKN2A* HD. A TaqMan copy number assay was performed using a probe located within *CDKN2A*. The linear correlation between the Ct values and relative *CDKN2A* copy number was confirmed using a serial mixture of DNA from normal blood and U87MG cells. The qPCR assay was performed in 109 *IDH*-mutant astrocytomas, including 14 tumors with *CDKN2A* HD, verified either by multiplex ligation-dependent probe amplification (MLPA) or CytoScan HD microarray platforms. Receiver operating characteristic curve analysis indicated that a cutoff value of 0.85 yielded optimal sensitivity (100%) and specificity (99.0%) for determining *CDKN2A* HD. The assay applies to DNA extracted from frozen or formalin-fixed paraffin-embedded tissue samples. Survival was significantly shorter in patients with than in those without *CDKN2A* HD, assessed by either MLPA/CytoScan or qPCR. Thus, our qPCR method is clinically applicable for astrocytoma grading and prognostication, compatible with the WHO CNS5.

Keywords Astrocytoma · *CDKN2A* · *IDH* · qPCR · WHO classification

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Introduction

Diffuse gliomas are the most common and aggressive primary brain tumors in adults. The revised 4th edition of the World Health Organization (WHO) Classification of Tumors of the Central Nervous System, published in 2016 (WHO2016CNS), molecularly defined diffuse gliomas based on the status of isocitrate dehydrogenase 1/2 (*IDH1/2*) mutations and 1p/19q co-deletion [1]. Although this was a significant step in the molecular classification of diffuse gliomas, new molecular markers that predict prognosis and may define malignancy grades have emerged. One of these is the cyclin-dependent kinase inhibitor 2A/2B (*CDKN2A/B*) [2, 3]. Multiple studies have identified homozygous deletion of *CDKN2A/B* as a marker of poor prognosis in patients with *IDH*-mutant diffuse gliomas without 1p/19q co-deletion [4–6]. Based on these findings, the Consortium to Inform Molecular and Practical Approaches to CNS Tumor Taxonomy

update 5 recommended that *IDH*-mutant astrocytoma with *CDKN2A/B* homozygous deletion (HD) be considered a WHO grade 4 tumor, even in the absence of necrosis or microvascular proliferation [2]. In the new WHO Classification, published in 2021 (5th Edition, WHO CNS5), adult-type *IDH*-mutant and 1p/19q non-codeleted diffuse gliomas are classified as a single tumor type, astrocytoma, *IDH*-mutant, and tumors with *CDKN2A/B* HD are classified as CNS WHO grade 4 [3]. Therefore, assessment of *CDKN2A* expression is mandatory for precise grading and prognostication.

Currently, *CDKN2A* HD is assessed using various methods, including fluorescence in situ hybridization, multiplex ligation-dependent probe amplification (MLPA), or more recently, next-generation sequencing or DNA methylation microarrays [2]. However, the use of these molecular assays is often limited by their high cost, long turnaround time, and requirement for dedicated equipment.

Quantitative real-time PCR (qPCR) is a method used to quantify the amount of target DNA by detecting amplified PCR products in real time during PCR cycles using fluorescent or intercalating dyes. The approach has been used to assess copy number variation (CNV) in many types of cancer cells [7, 8]. qPCR is a simple, inexpensive, and robust method that is readily applicable in clinical settings to allow accurate diagnosis and WHO grading of diffuse gliomas.

In this study, we developed a qPCR assay to detect *CDKN2A* HD, validated it in a series of adult *IDH*-mutant astrocytoma samples and compared its efficacy with that of other conventional methods such as DNA microarray or MLPA.

Materials and methods

Patient and tissue specimens

This study was approved by the Institutional Review Board of Juntendo University Hospital, Tokyo, Japan (No. 2010–014) and the National Cancer Center, Tokyo, Japan (No. 2013–042).

A total of 109 adult-type diffuse glioma specimens, obtained from 104 patients between 1997 and 2020, were used, based on tissue availability. All cases were evaluated at Juntendo University Hospital (Tokyo, Japan) or the National Cancer Center Hospital (Tokyo, Japan) and molecularly diagnosed according to the WHO2016 classification. Among them, 14 samples had been resected from recurrent tumors. The status of *IDH*, 1p/19q, and *CDKN2A* had been previously assessed for a subset of tumors [9] with the remaining tumors were newly tested in the current study. The 109 diffuse glioma specimens were *IDH*-mutant astrocytomas,

consisting of 51 *IDH*-mutant diffuse astrocytomas, 45 *IDH*-mutant anaplastic astrocytomas, and 13 *IDH*-mutant glioblastomas. None of the tumors had a 1p/19q co-deletion.

Molecular testing

Molecular testing was performed using frozen or formalin-fixed paraffin-embedded (FFPE) specimens. For frozen samples, DNA was extracted using a DNeasy Blood & Tissue Kit or QIAamp Fast DNA Tissue Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. For FFPE samples, DNA was extracted from three 10-μm paraffin sections per specimen, using the QIAamp DNA FFPE Tissue Kit (Qiagen). DNA concentrations were determined using a Quantus FLUOROMETER (Promega Corp., Madison, WI, USA) or a Qubit DNA quantification system (Invitrogen, Carlsbad, CA, USA).

The *IDH1/2* mutation was analyzed by pyrosequencing, as previously described [10]. Briefly, an 86-bp fragment of *IDH1* containing the targeted codon 132 and an 85-bp fragment of *IDH2* containing the targeted codon 172 were amplified. The amplified fragments were then subjected to allele quantification analysis, using the appropriate analysis mode in PyroMark Q24 software (Qiagen). BT142 glioma cells bearing the *IDH1* R132H mutation [11] were obtained from the American Type Culture Collection (Manassas, VA, USA) and used as the control for the *IDH* mutation. Testing for 1p/19q co-deletion was performed using MLPA. The SALSA MLPA P088 Oligodendroglioma 1p-19q probemix (MRC-Holland, Amsterdam, The Netherlands) was used according to the manufacturer's instructions. The kit contained 19 probes for loci on 1p and three probes for loci on 1q, two probes for loci on 19p, and 11 probes for loci on 19q. The data were analyzed using Coffalyser.Net (MRC-Holland) to determine the occurrence of 1p/19q co-deletion. *CDKN2A* testing was performed using MLPA or a CytoScan HD microarray platform (Affymetrix, Santa Clara, CA, USA), as previously described [12]. The SALSA MLPA P088 probemix described above contained three probes for *CDKN2A* and two probes for *CDKN2B* loci. The CytoScan HD microarray platform contains 2.67 million probes, including 1.9 million copy number probes and 0.75 million SNP probes. Array analysis and interpretation were performed using Chromosome Analysis Suite (ChAS 4.0.0.385 [r 28959]) software (Affymetrix). Array data were analyzed using the Chromosome Analysis Suite software package.

Quantitative PCR for *CDKN2A*

CDKN2A HD was validated by TaqMan copy number assay using the probe Hs02738179_cn (Applied Biosystems, Waltham, MA, USA) [13], according to the manufacturer's recommendations. This probe (Chr.9:21,974,451

on GRCh38, https://www.thermofisher.com/) was located within intron 1 of *CDKN2A* (NM_000077), between exon 1α (ENSE00001833804) and exon 2 (ENSE00003475459). Pooled blood-derived DNA from healthy donors was used as a control to assess normal copy number status. DNA from the glioblastoma cell line U87MG (obtained from the American Type Culture Collection), known to have *CDKN2A* HD [14], was used as the positive control for homozygous deletion. RNase P (4,403,326; Applied Biosystems) on chromosome 14 was used as the reference gene. Numerical alterations of chromosome 14 is known to be rare in gliomas, and therefore RNase P was considered suitable as the reference gene. Briefly, all assays were run in triplicate on an ABI7500 Real-time PCR or QuantStudio 6 Flex Real-Time PCR System in a 20-μl reaction volume containing 10 μl of TaqPath ProAmp Master Mix ($\times$10) (A30668, Applied Biosystems), 1 μl of TaqMan Copy Number Assay, 1 μl of TaqMan Copy Number Reference Assay, and 5 ng of genomic tumor DNA, under the following PCR conditions: an initial activation step at 95 °C for 10 min, followed by 40 cycles each consisting of 95 °C for 15 s and 60 °C for 1 min. The $2^{-\triangle\triangle Ct}$ method was used to determine the gene copy number status. CNVs were analyzed using ABI7500 Softwarev2.3 (Applied Biosystems) or QuantStudio™ Real-Time PCR Software v1.6.1, and Copy Caller Software v.2.1 (Applied Biosystems).

Statistical analysis

For statistical analysis, variables were compared using the Mann–Whitney *U* test or the chi-square test. Paired sample comparison and correlation analysis between fresh-frozen and FFPE samples were performed using linear regression analysis. Receiver operating characteristic (ROC) curve analysis was performed to compare predictive validity. Sensitivity and specificity were calculated at all possible cutoff points to determine the optimal cutoff value. Overall survival (OS) after the first surgery was analyzed using the Kaplan–Meier method and compared using a log-rank test. Statistical significance was set at $p < 0.05$. All statistical analyses were performed using GraphPad Prism 8.4.3 (GraphPad, La Jolla, CA, USA).

Results

CDKN2A HD

CDKN2A HD was detected in 13% (14/109) of all *IDH*-mutant astrocytomas evaluated in the present study either by MLPA or CytoScan HD microarray (Table 1, Supplementary Table S1a, S1b). *CDKN2B* HD was solely detected in cases with *CDKN2A* HD. According to WHO2016CNS, 6% (3/51)

Table 1 Clinicopathological characteristics of all IDH-mut 1p/19q non-codel diffuse astrocytoma cases according to *CDKN2A* homozygous deletion status

	N (%)		
	All	CDKN2A HD	CDKN2A non-HD
Number	109	14 (13%)	95 (87%)
Age at diagnosis			
Median (y)	39	38	39
Range (y)	21–82	28–62	21–82
Sex			
Male	62	10	52
Female	47	4	43
Group			
WHO grade II	51	3 (6%)	48 (94%)
WHO grade III	45	9 (20%)	36 (80%)
WHO grade IV	13	2 (15%)	11 (85%)

HD homozygous deletion, *WHO* World Health Organization

of grade 2, 20% (9/45) of grade 3, and 15% (2/13) of *IDH*-mutant glioblastoma (grade 4) harbored *CDKN2A* HD, all of which are now re-classified as astrocytoma, *IDH*-mutant, and as grade 4 tumors according to the WHO CNS5. The presence of *CDKN2A* HD was not associated with patient age, sex, or WHO grade as defined by the WHO2016CNS (Table 1).

Quantitative PCR

Patient tumor samples inevitably contain non-neoplastic cell components to variable degrees, which may mask homozygous deletions in tumor cells. To determine the sensitivity of a qPCR-based detection of *CDKN2A* HD, DNA from the U87MG cell line, which is known to harbor *CDKN2A* HD, was serially diluted with peripheral blood lymphocyte (PBL) DNA from a healthy donor. The amount of PBL and U87MG cell line DNA was adjusted so that the Ct values for RNase P were the same for both samples. The *CDKN2A* copy number was analyzed by qPCR using a serial mixture of DNA derived from normal PBL and from U87MG cells in the ratios of 0, 20, 40, 60, 80, and 90% to demonstrate whether the assay can quantitatively measure the *CDKN2A* gene dosage in a mixture of HD and wild-type samples. When the Ct values and the ratio of U87MG DNA to normal DNA were plotted, the Ct values correlated linearly ($R^2 = 0.983$) with the relative amounts of U87MG DNA in the mixtures (Fig. 1a).

To compare the effect of preservation methods on copy number assessment by qPCR, we assessed frozen and matched FFPE samples from 12 randomly selected patients (Fig. 1b). For these samples, the copy numbers measured by qPCR showed a high concordance between DNA from
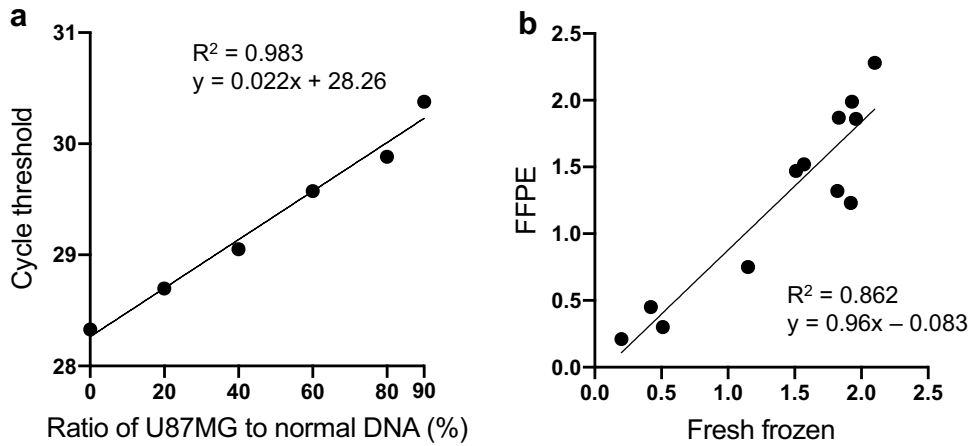
Fig. 1 Copy number comparisons. **a** The cycle threshold (Ct) values of *CDKN2A* quantitative real-time polymerase chain reaction (qPCR) for a serial mixture of U87MG DNA with normal DNA. The Ct values correlated linearly with the relative amount of U87MG DNA. **b** Comparison of copy numbers assessed by qPCR in the matched fro- zen tissues and formalin-fixed paraffin-embedded (FFPE) samples from 12 randomly selected patients. The copy numbers showed high concordance between DNA from frozen tissues and FFPE samples ($R^2 = 0.862$)

frozen tissues and FFPE samples ($R^2 = 0.862$, Fig. 1b). These data indicated that DNA from either frozen or FFPE tissue samples could be analyzed in the same manner.

Based on these findings, we measured the *CDKN2A* copy number of 109 diffuse gliomas using qPCR (Fig. 2a). The mean *CDKN2A* copy number from the tumors with

Fig. 2 Copy number variation analysis in patient specimens. **a** The *CDKN2A* copy numbers measured by quantitative real- time polymerase chain reaction (qPCR) in all 109 diffuse gliomas. **b** A receiver operating characteristic (ROC) curve anal- ysis of copy number assessed by qPCR and determined by either multiplex ligation-dependent probe amplification (MLPA) or CytoScan
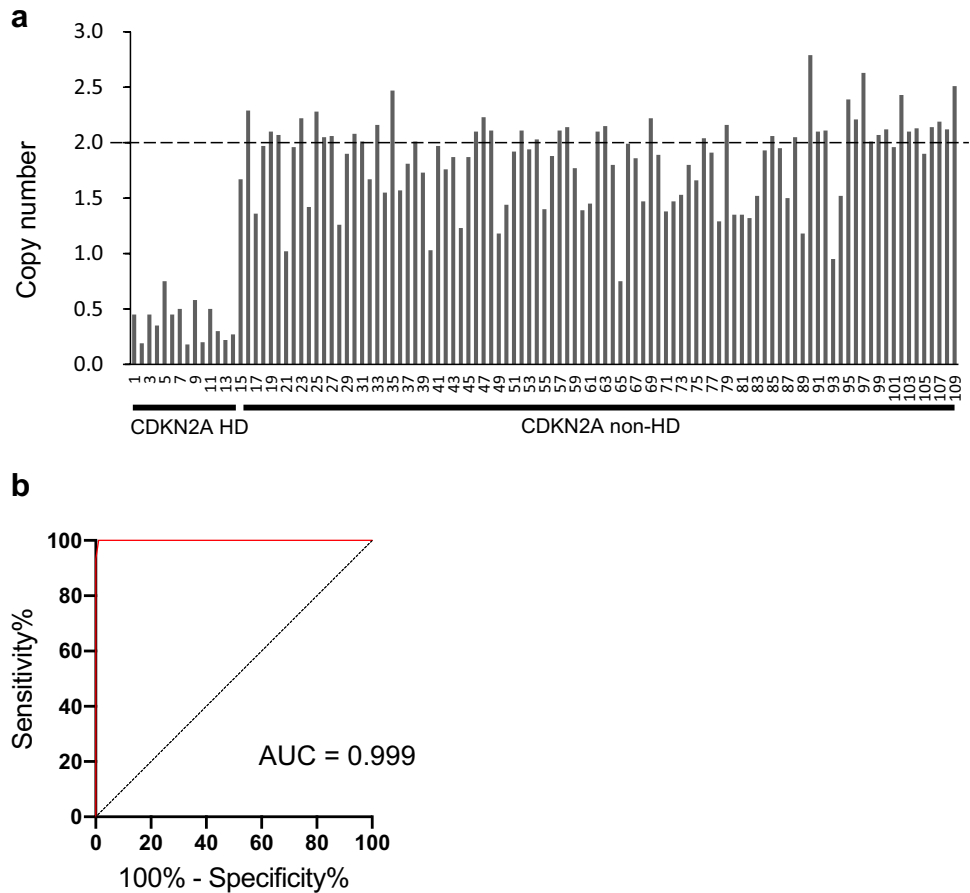
CDKN2A HD validated by MLPA or CytoScan (CDKN2A HD group) and those similarly validated as *CDKN2A* non-HD (CDKN2A non-HD group) were 0.39 (SD 0.17, range 0.18–0.75) and 1.85 (SD 0.39, range 0.75–2.79), respectively.

ROC curve analysis was used to evaluate the diagnostic potential of *CDKN2A* HD and to test its sensitivity and specificity (Fig. 2b). The copy number calculated by qPCR showed high diagnostic potential (AUC = 0.999; $p < 0.0001$). ROC analysis indicated that a cutoff value of 0.85 resulted in the optimal sensitivity (100%) and specificity (99.0%). Using this cutoff, all *CDKN2A* HD determined by MLPA or CytoScan were judged as HD by qPCR, except that one case judged as non-HD by CytoScan showed a *CDKN2A* copy number of 0.75 by qPCR.

Variant allele frequency of IDH1 R132H and *CDKN2A* copy number

The impact of tumor cell content on the assessment of *CDKN2A* copy number by qPCR was determined. The variant allele frequencies (VAF) of *IDH1* R132H measured by pyrosequencing were used as a surrogate for tumor cell content, considering that the *IDH1* R132H mutation is a founder mutation [15], implying its presence in all tumor cells. We first assessed whether the VAF of *IDH1* R132H accurately reflected the ratio of tumor DNA to normal DNA. Pyrosequencing to quantify the VAF of *IDH1* R132H [10] was performed on the DNA from BT142 glioma cells (known to harbor a homozygous *IDH1* R132H mutation) serially diluted with PBL-DNA from a healthy donor to achieve mutant DNA ratios of 0, 1, 5, 10, 20, 30, 40, or 50%, in triplicate. The *IDH1* R132H mutant allele was detected as distinct peaks in the pyrogram of samples containing 5% or more mutant DNA (Fig. 3a). The mean VAF determined in triplicate experiments showed a strong linear correlation with the expected ratio ($R^2 = 0.987$, $p < 0.001$) (Fig. 3b). The results suggested that the VAF of *IDH1* R132H could be used to estimate tumor cell content even in a mixture of normal and tumor cells.

The *IDH1* R132H VAF and *CDKN2A* copy number in the *CDKN2A* HD group were compared (Fig. 3c). Only newly diagnosed tumors sampled during the initial surgery were included in this analysis (see below). The *CDKN2A*

Fig. 3 Pyrosequencing results. **a** Pyrograms of pyrosequencing for the *IDH1* R132H mutation using the variable mixtures of DNA from BT142 cells, which harbored homozygous *IDH1* R132H, and normal peripheral blood leukocytes (PBL). **b** Comparison between the ratio of the serial mixture of BT142 cells to PBL DNA and the mean variant allele frequencies (VAF) of the triplicate experiments of *IDH1* pyrosequencing shows strong linear correlation ($R^2 = 0.987$). **c** Comparison between the *IDH1* R132H VAF with the *CDKN2A* copy number in the newly diagnosed tumors sampled during the initial surgery in the *CDKN2A* HD group showed an inverse correlation ($R.^2 = 0.78$)
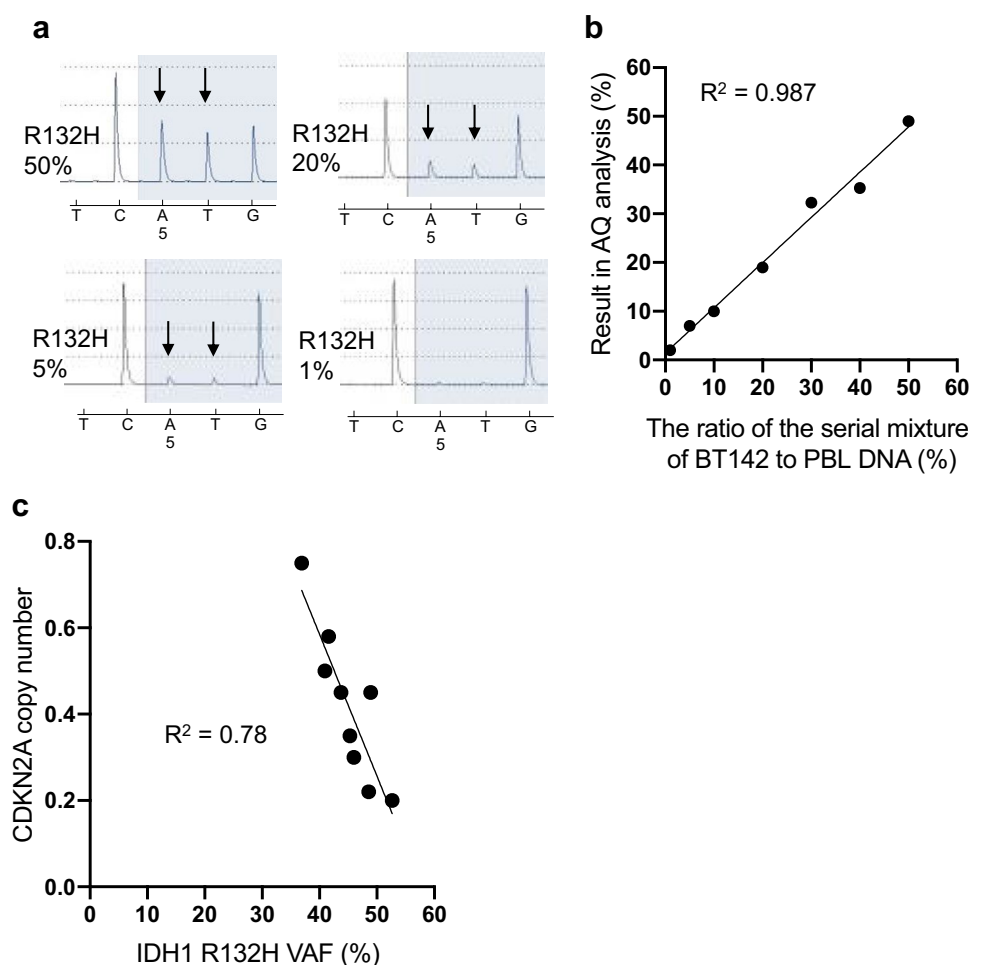
copy number in the HD cases was inversely correlated with *IDH1* R132H VAF ($R^2 = 0.78$), suggesting that the measured values of *CDKN2A* copy number in the *CDKN2A* HD cases reflected the number of co-existing normal cells in the tissue. However, the *CDKN2A* copy number and *IDH1* R132H VAF were not necessarily concordant in recurrent tumors. A diffuse astrocytoma grade 2, resected in the initial operation in a patient aged 17 years, harbored *IDH1* R132H. However, no copy number change of *CDKN2A* was observed. When the tumor recurred 13 years later in this patient, the recurrent tumor had developed *CDKN2A* HD while retaining *IDH1* R132H. The *CDKN2A* copy number (0.50) and *IDH1* R132H VAF (24.6%) were discordant in the recurrent tumor. A similar discordance was observed in a tumor resected at a second recurrence in this patient 6 months later.

Survival analysis

Among the 109 tumors evaluated in this study, samples from patients with recurrent tumors were excluded. Survival data from initial surgery were available for 90 patients. The median age of the 90 patients at diagnosis was 39 years (range, 21–82 years). The study cohort included 49 men and 41 women. During a median follow-up period of 43 months (range, 4–177 months), 32 patients died. The median OS for the *CDKN2A* HD and *CDKN2A* non-HD groups was 30 and 126 months, respectively. *CDKN2A* HD, assessed by either MLPA/CytoScan or qPCR, was a significantly poor prognostic factor for OS ($p < 0.0001$; Fig. 4).
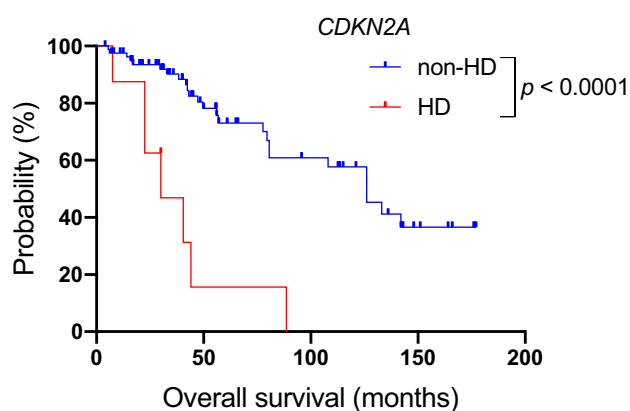

Fig. 4 A Kaplan–Meier curve of the 90 patients for whom survival data were available according to the *CDKN2A* status assessed by quantitative real-time polymerase chain reaction (qPCR). The patients with *CDKN2A* homozygous deletion (HD) showed significantly shorter overall survival ($p < 0.0001$)

Discussion

In this study, we showed that *CDKN2A* HD can be readily detected using our qPCR assay. We proposed an optimal cutoff value of 0.85 copies (normal = 2), which provided sensitivity and specificity comparable to that of MLPA or CytoScan.

CDKN2A HD, as determined by qPCR, predicted shorter OS in patients with IDH-mutant astrocytomas, as suggested by multiple recent studies. In clinical research, *CDKN2A/B* status has mostly been analyzed using fluorescent in situ hybridization (FISH) [16] or microarray [4, 17, 18]. FISH is widely used to detect copy number abnormalities; however, due to the presence of overlapping or partially sectioned nuclei, the assessment of HD is sometimes challenging. Microarray is a robust method for determining the genome-wide copy number status; however, it is costly and requires specialized equipment, making it impractical as a clinical test. MLPA is a robust and cost-effective method for detecting copy number abnormalities, and the kit used in our study is designed to evaluate some, but not all, *IDH* mutations as well as *CDKN2A/B* HD simultaneously, although it is rather labor-intensive and requires a capillary sequencer equipped with costly analytical software. qPCR requires real-time PCR equipment, which is now widely available and is highly time-efficient, allowing same-day production of results. We also showed that this method is applicable to both frozen specimens and FFPE samples. Thus, qPCR may serve as a method of choice for detecting *CDKN2A* HD to diagnose and determine malignancy grade in a manner fully compatible with the WHO CNS5 classification in diffuse gliomas.

Accurate assessment of *CDKN2A* HD largely depends on the tumor cell content of tissue. Wild-type *CDKN2A* signals from a large number of infiltrating stromal cells in the tumor tissue (> 50%) would mask HD in the tumor cells, which could result in false non-HD judgment. We showed that the variant allele frequency of *IDH1* R132H may serve as a reliable surrogate marker for estimating tumor cell content in tissues. Considering that the cutoff copy number for judging HD was 0.85 in our study, our assay theoretically allows a stromal cell presence up to approximately 40% stromal cell presence. In other words, more than 60% of tumor cell content (> 30% *IDH1* R132H VAF) is required to judge HD accurately. This would be a limitation universally applicable to any method to quantitatively assess copy number of *CDKN2A*. When tumor cell contents are estimated below this cutoff, microdissection of tissue specimens, to enrich tumor cells, may be considered.

We observed notable discrepancies between *CDKN2A* copy number and *IDH1* R132H VAF in recurrent tumors.

One patient developed *CDKN2A* HD at recurrence, but HD was not observed in the tumor resected during the initial operation. While *CDKN2A* HD was clearly observed in the recurrent tumor, the *IDH1* R132H VAF was below the level at which HD may be accurately determined (see above). It has been shown that *CDKN2A* deletions may be acquired at recurrence in *IDH*-mutated gliomas and that this is involved in their malignant progression [19]. It has also been reported that acquisition of a *CDKN2A* deletion exclusively occurs in post-radiation *IDH*-mutated gliomas [20]. This is consistent with the fact that the patient received radiation therapy (54 Gy) after the initial surgery. A relative decrease in the mutant *IDH1* VAF at recurrence has also been documented [21]. Therefore, tumor cell content estimation using *IDH1* R132H VAF should be used with caution, particularly in recurrent tumors.

CDKN2A and *CDKN2B* are tandemly located on 9p21, within a 41.5-kb region. Because of their proximity, these two genes are almost always simultaneously involved in HD of this region, with a few exceptions in which *CDKN2A* alone, but not *CDKN2B* alone, may be homozygously deleted [14, 22]. In addition, inactivating point mutations may occasionally be found, although rarely, in the coding region of *CDKN2A* (https://cancer.sanger.ac.uk/cosmic/gene/analysis?ln=CDKN2A) but rarely in *CDKN2B* (https://cancer.sanger.ac.uk/cosmic/gene/analysis?ln=CDKN2B) [23]. Although both CDKN2A/p16INK4A and CDKN2B/INK4B function as inhibitors of CDK4/CDK6 kinases by competing with cyclin D1 for their binding, *CDKN2A* is considered the primary target of HD at 9p21, that is, it is a *bona fide* tumor suppressor gene [23]. Thus, we targeted our qPCR probe to intron 1 of *CDKN2A*, considering that *CDKN2A*, even alone, is always involved in HD. In our cases for which *CDKN2A/B* HD was evaluated by MLPA or CytoScan HD, we confirmed that *CDKN2B* HD was solely detected in cases with *CDKN2A* HD. No cases showed HD only at *CDKN2B*.

In conclusion, the WHO CNS5 introduced molecularly defined astrocytoma, IDH-mutant, grade 4, which was a ground-breaking change in the structure of the adult diffuse glioma classification. The tumor types and malignancy grades of adult gliomas are primarily determined by molecular profiling. Although the new classification enabled objective malignancy grading, *CDKN2A* testing has been made mandatory in routine clinical practice. Our simple and robust qPCR test to detect *CDKN2A* HD may help clinicians in accurate diagnosis and grading of astrocytoma, *IDH*-mutant grade 4, in a manner fully compatible with the WHO CNS5.

Data Availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare that they have no conflicts of interest related to this work.

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