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ARTICLE

Utility of methylthioadenosine phosphorylase immunohistochemical deficiency as a surrogate for *CDKN2A* homozygous deletion in the assessment of adult-type infiltrating astrocytoma

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Abstract

Homozygous deletion (HD) of *CDKN2A* is one of the most promising biomarkers for predicting poor prognosis of *IDH*-mutant diffuse gliomas. The Consortium to Inform Molecular and Practical Approaches to CNS Tumor Taxonomy (cIMPACT-NOW) recommendations propose that *IDH*-mutant lower-grade astrocytomas with *CDKN2A/B* HD be classified as grade IV tumors. Loss of methylthioadenosine phosphorylase (MTAP) immunohistochemistry staining has been proposed as a surrogate of *CDKN2A* HD in various tumors but its performance has not been fully investigated in diffuse glioma. This study determined whether MTAP immunoreactivity could serve as a proxy for *CDKN2A* HD in adult-type diffuse glioma, thereby contributing to stratifying patient outcome. MTAP immunohistochemistry staining using clone EPR6893 was scored in 178 diffuse glioma specimens consisting of 77 *IDH*-mutant astrocytomas, 13 *IDH*-mutant oligodendrogliomas, and 88 *IDH*-wildtype glioblastomas. The use of MTAP immunohistochemical deficiency to predict *CDKN2A* HD was good for *IDH*-mutant astrocytomas (sensitivity, 88%; specificity, 98%) and *IDH*-wildtype glioblastomas (sensitivity, 89%; specificity, 100%), but poor for *IDH*-mutant oligodendrogliomas (sensitivity, 67%; specificity, 57%). Both *CDKN2A* HD and MTAP immunohistochemical deficiency were significant adverse prognostic factors of overall survival for *IDH*-mutant astrocytoma ($P < 0.001$ each), but neither were prognostically significant for oligodendroglioma or *IDH*-wildtype glioblastoma. *IDH*-mutant lower-grade astrocytoma with *CDKN2A* HD and deficient MTAP immunoreactivity exhibited overlapping unfavorable outcome with *IDH*-mutant glioblastoma. MTAP immunostaining was easily interpreted in 61% of the cases tested, but scoring required greater care in the remaining cases. An alternative MTAP antibody clone (2G4) produced identical scoring results in all but 1 case, and a slightly larger proportion (66%) of cases were considered easy to interpret compared to using EPR6893. In summary, loss of MTAP immunoreactivity could serve as a reasonable predictive surrogate for *CDKN2A* HD in *IDH*-mutant astrocytomas and *IDH*-wildtype glioblastomas and could provide significant prognostic value for *IDH*-mutant astrocytoma, comparable to *CDKN2A* HD.

Introduction

Diffuse gliomas of adults comprise ~60–70% of primary malignant intracranial tumors in Japan [1]. These tumors are primarily graded according to histological findings, such as

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mitotic activity, microvascular proliferation, and/or necrosis [2–4]. In addition, the current World Health Organization (WHO) classification (WHO 2016) [5] incorporates genetics for defining histological types of gliomas, which enables a more accurate prediction of clinical behavior. Accordingly, adult-type diffuse glioma is subdivided into distinct categories based on isocitrate dehydrogenase (*IDH*) 1/2 mutation [6, 7] and 1p/19q codeletion [8]. Specifically, glioblastomas are categorized as *IDH*-wildtype (~90%) or *IDH*-mutant (~10%). Lower-grade diffuse gliomas are classified into three groups, *IDH*-mutant diffuse glioma without 1p/19q codeletion (the vast majority of diffuse astrocytic tumors), *IDH*-mutant diffuse glioma with 1p/19q codeletion (the vast majority of diffuse oligodendroglial tumors), and *IDH*-wildtype diffuse gliomas [9–11].

Homozygous deletion (HD) of the gene encoding cyclin-dependent kinase inhibitor 2 A (*CDKN2A*) is a recently reported biomarker for predicting poor prognoses of patients with *IDH*-mutant diffuse gliomas [12–17]. The Consortium to Inform Molecular and Practical Approaches to CNS Tumor Taxonomy (cIMPACT-NOW) update 5 recently recommended that *IDH*-mutant astrocytoma with *CDKN2A/B* HD be considered as a WHO grade IV tumor, even in the absence of microvascular proliferation or necrosis [18]. However, the prognostic value of *CDKN2A* HD in oligodendroglioma and *IDH*-wildtype glioma has not been established. Currently, *CDKN2A* HD is usually assessed using fluorescence in situ hybridization (FISH), array comparative genomic hybridization, or multiplex ligation-dependent probe amplification (MLPA), but newer techniques such as next generation sequencing and DNA methylation profiling microarray have also been used [19]. However, use of these molecular assays is limited due to their high costs, long turnaround time, and non-availability at all centers. This highlights the need to develop surrogate assays, such as immunohistochemistry-based approaches.

A historical candidate as a potential surrogate biomarker was the p16 protein, which is encoded by *CDKN2A* (9p21) and plays an important role in blocking G1 to S phase transition via CDK4 and CDK6 inhibition. However, whether the loss of p16 immunoreactivity correlates with *CDKN2A* HD is controversial [20–23]. More recently, the expression of methylthioadenosine phosphorylase (MTAP) has been extensively studied in various types of epithelial neoplasms [24–28] and malignant mesothelioma [29–32].

MTAP is essential for adenosine monophosphate and methionine salvage. As MTAP catalyzes the conversion of methylthioadenosine to adenine and methylthioribose-1-phosphate with the latter compound being further metabolized to methionine, it is expressed in virtually all cells [33, 34]. *MTAP* is located on 9p21 only 165 kb telomeric to *CDKN2A*, and in tumors, it is often simultaneously deleted with *CDKN2A* HD. *MTAP* HD results in the loss of MTAP immunohistochemical staining, which is accepted as a reliable surrogate marker of *CDKN2A* HD for differentiating malignant mesothelioma from reactive mesothelial cell proliferation (sensitivity: 74–93%, specificity: 96–100%) [35–37]. However, the predictive role of MTAP immunohistochemistry for *CDKN2A* HD has not been fully investigated in glioma. Therefore, we undertook the current study to determine (1) whether the loss of MTAP immunoreactivity could be used as a surrogate for *CDKN2A* HD in adult-type diffuse glioma and (2) whether MTAP staining had prognostic value for *IDH*-mutant astrocytoma. For comparison, we also evaluated in parallel p16 immunostaining.

Materials and methods

Patients and tissue specimens

The study was approved by the institutional review board at the National Cancer Center, Tokyo, Japan (No. 2013-042). A total of 189 adult-type diffuse glioma specimens were obtained between 1997 and 2019 based on tissue availability. The status of *IDH*, 1p/19q, and *CDKN2A* was previously available for a subset of the tumors with the remaining tumors being newly tested as part of the current study. *IDH*-mutant gliomas that were previously determined to harbor *CDKN2A* HD at original diagnosis were preferentially selected for improved immunohistochemistry evaluation. All cases were diagnosed at the National Cancer Center Hospital (Tokyo, Japan), and their diagnoses were reviewed and confirmed according to the WHO 2016 classification [5], except for some *IDH*-mutant astrocytomas for which the loss of ATRX expression and strong diffuse p53 immunopositivity were considered equivalent to the absence of 1p/19q codeletion in accordance with the cIMPACT-NOW update 2 [38]. Among 189 tumors, 30 tumors were subjected to *BRAF* V600E testing by pyrosequencing, but none harbored the mutation. None of the cases in this study was screened for *FGFR* rearrangement. Of the 189 diffuse glioma specimens tested, 178 tumors were successfully scored using MTAP immunohistochemistry and *CDKN2A* MLPA or FISH, as detailed in the results section. Hence, these 178 tumors we used for subsequent analyses to determine the molecular/immunohistochemical correlation and prognostic implications.

The entire 178 tumor cohort was divided into three groups, the *IDH*-mutant astrocytoma group, the *IDH*-mutant oligodendroglioma group, and the *IDH*-wildtype group. The *IDH*-mutant astrocytoma group consisted of 77 tumors from 74 unique patients, including *IDH*-mutant gliomas that lacked 1p/19q codeletion as determined by MLPA or FISH and *IDH*-mutant gliomas that exhibited a loss of ATRX

nuclear expression and strong diffuse p53 immunopositivity. According to the WHO 2016 classification, the *IDH*-mutant astrocytoma group had 15 *IDH*-mutant diffuse astrocytomas (DA), 35 *IDH*-mutant anaplastic astrocytomas (AA), and 27 *IDH*-mutant glioblastomas. Two of the *IDH*-mutant glioblastomas and one of the AA were recurrences of DAs. The *IDH*-mutant oligodendroglioma group consisted of 13 tumors from 12 unique patients. Based on the WHO 2016 classification, five were oligodendrogliomas, *IDH*-mutant and 1p/19q-codeleted, and eight were anaplastic oligodendrogliomas, *IDH*-mutant and 1p/19q-codeleted. There was one pair of primary and recurrent anaplastic oligodendrogliomas from the same patient. Finally, the *IDH*-wildtype group consisted of 88 tumors from 88 unique patients. These included infiltrating gliomas without the *IDH* mutation as determined by either IDH1 (R132H) immunohistochemistry or sequencing. All these tumors corresponded to glioblastomas, *IDH*-wildtype, according to the WHO 2016 classification.

Molecular testing

Molecular testing was performed using either frozen or formalin-fixed paraffin-embedded (FFPE) specimens. DNA was extracted from the specimens using a DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA concentrations were determined using a Qubit DNA quantification system (Invitrogen, Carlsbad, CA, USA).

The *IDH1/2* mutation was analyzed using pyrosequencing as previously described [39]. 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 then underwent allele quantification analysis, which is an analysis mode available in the PyroMark Q24 software (Qiagen).

CDKN2A and 1p/19q testing was performed using MLPA and/or FISH. 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, and three probes for loci on *CDKN2A* and two probes for loci on *CDKN2B*. The data were analyzed using Coffalyser.Net (MRC-Holland) to determine the occurrence of 1p/19q codeletion and *CDKN2A* HD.

Dual-color *CDKN2A* or 1p/19q FISH studies were carried out using 4 µm-thick tissue specimens, either on whole tissue sections or tissue microarray (TMA). The probes used were Vysis LSI CDKN2A Spectrum Orange/CEP9 Spectrum Green probes (Abbott Molecular, Abbott park, IL, USA) and the 1p/19q probe set (Leica Biosystems, Tokyo, Japan). Images were captured using the Metafer Slide Scanning Platform (MetaSystems, Altlussheim, Germany) and a minimum of 100 non-overlapping tumor cells were examined. Tumors in which >10% of the cells showed loss of signal for both *CDKN2A* genes and at least one *CEP9* signal (0 Spectrum Orange/1–2 Spectrum Green) were considered HD [40]. Lymphocytes or endothelial cells with two *CDKN2A* and two *CEP9* signals served as internal controls. For 1p/19q codeletion, a minimum of 100 non-overlapping tumor cell nuclei with at least two green (1q or 19q) signals were evaluated. A 1p/19q codeletion was considered to be present when (1) >50% of tumor nuclei had only one orange (1p or 19q) signal or two orange and four green signals; and (2) the ratio of 1p/1q to 19q/19p was ≤0.8.

Although we used FISH and/or MLPA to obtain molecular data, these two methods have different capabilities to determine *CDKN2A* and *MTAP* copy number. The commercial FISH probe we used covered ~222 kb in the 9p21 region and hybridized to *MTAP*, *CDKN2A*, and *CDKN2B*, whereas the MLPA assay employed probes of smaller size (153, 190, and 253 bp) designed to hybridize to a specific region (~20 kb) within *CDKN2A* and did not hybridize to *MTAP*. Regardless of this difference, for the sake of brevity, we used the term *CDKN2A* HD for all cases in this paper unless otherwise specified. This choice was made based on the previous finding that in 564 diffuse gliomas, *MTAP* HD without *CDKN2A/B* HD is extremely rare (0.5%) [41], according to cBioPortal analysis [42, 43]. In addition, previous studies used a similar FISH assay to demonstrate the prognostic significance of *CDKN2A* HD in glioma [13, 16].

Immunohistochemistry

Immunohistochemistry staining was performed using either whole tissue sections or previously prepared TMAs with 2.0 mm cores. A 4 µm-thick section cut from the FFPE block was deparaffinized and the sections exposed to 3% hydrogen peroxide to block endogenous peroxidase activity. After heat-induced epitope retrieval in citrate buffer or Targeted Retrieval Solution, pH 9.0 (Dako), slides were incubated for 1 h at room temperature with primary antibodies, and subsequently labeled using the EnVision System (Dako, Glostrup, Denmark). Diaminobenzidine was used as the chromogen and hematoxylin as the counterstain.

For MTAP staining, we primarily used a rabbit monoclonal antibody EPR6893 (1:1000; Targeted Retrieval Solution, pH 9.0, Abcam, Cambridge, UK) that was one of the few reagents published on mesothelioma at the time this project was initiated [35]. However, because more recent publications on mesothelioma have favored different clones [36], we additionally used one of such newer clones 2G4 (1:200; Targeted Retrieval Solution, pH 9.0, Abnova,

Taiwan) and compared the performance between these two antibodies using the same 178 samples. For each antibody, the staining protocol was optimized using several different types of malignant tumors (e.g., mesothelioma) whose *CDKN2A* statuses were known. Normal cerebral tissue was diffusely positive using either protocol as expected. For p16 staining, we used mouse monoclonal antibody G175-405 (1:10; citrate buffer, BD Biosciences, San Jose, CA).

MTAP immunohistochemistry revealed cytoplasmic staining with or without nuclear labeling in normal cells, such as endothelial cells or lymphocytes. The loss of MTAP immunoreactivity in tumor cells was defined by the absence of cytoplasmic staining in the presence of reactivity of the internal positive controls. Tumors that exhibited cytoplasmic MTAP immunoreactivity but no nuclear staining were considered to retain MTAP staining [36]. We scored the staining only when the internal positive control was properly labeled, and any samples that failed to stain positive for endothelial cells/lymphocytes were considered uninterpretable. For all cases, the staining was independently interpreted by two pathologists (KS and AY) blinded to the molecular results. All scoring results were subsequently compared and any discrepancy or difficulty was discussed to reach consensus. In addition, for the cases evaluated using whole tissue sections, the level of difficulty in MTAP interpretation was graded as "low" or "high." The difficulty was considered low when the interpretation could accurately be performed even at low magnification (×10 objective). The rest of the cases definitely required higher magnification for scoring and were judged as high.

For p16 immunohistochemistry, nuclear and cytoplasmic stainings were evaluated separately. The p16 staining was often heterogeneous with a mosaic-like appearance, even if the staining was positive. Therefore, only complete loss of staining was considered p16-deficient with all other patterns of reactivity being considered as retained p16 expression. Interpretation of the p16 staining was performed by one pathologist (KS) blinded to the molecular results.

Statistical analysis

For statistical analysis, variables were compared using the Mann–Whitney U-test or Chi square test. To analyze performance, we evaluated the sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) for MTAP and p16 staining. Overall survival (OS) and progression free survival (PFS) after the first surgery were analyzed using the Kaplan–Meier method and compared using a log-rank test. $P < 0.05$ was considered statistically significant. All statistical analyses were performed using the Easy R (EZR) plug-in (Saitama Medical Center, Jichi Medical University, Saitama, Japan) [44], which is a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria).

Results

CDKN2A HD

CDKN2A HD was present in 44% (78/178) of all diffuse gliomas evaluated in our study. Specifically, it was present in 22% (17/77) of *IDH*-mutant astrocytomas, 46% (6/13) of *IDH*-mutant oligodendrogliomas, and 63% (55/88) of *IDH*-wildtype glioblastomas (Table 1). The frequency was significantly correlated with higher age at diagnosis ($P < 0.001$) and *IDH*-wildtype status ($P < 0.001$). Among *IDH*-mutant astrocytomas, 0% (0/15) of DA, 11% (4/35) of AA, and 48% (13/27) of *IDH*-mutant glioblastomas harbored *CDKN2A* HD. *CDKN2A* HD was significantly associated with WHO grade ($P < 0.001$; Table 1), but not related to patient age or sex or tumor location (Supplementary Table). Among the selected histological variables analyzed, microcystic change was significantly less common in tumors with *CDKN2A* HD than in those without *CDKN2A* HD ($P = 0.018$). Interestingly, two of the *IDH*-mutant DAs with intact *CDKN2A* recurred 7 and 13 years later as *IDH*-mutant glioblastoma with *CDKN2A* HD, suggesting a role for this deletion in tumor progression. The frequency of C*DKN2A* HD in *IDH*-mutant oligodendroglioma detected in our study was higher than that in previous studies [13, 15], likely because tumors with *CDKN2A* HD were preferentially selected by the study design.

MTAP immunohistochemistry (clone EPR6893)

Of 189 diffuse glioma samples initially tested, 107 tumors were included on TMAs. Excluding five tumors whose cores were unavailable, 80 of the 102 tumors (78%) were successfully scored. The remaining 22 tumors were difficult to score using TMAs due to high mixtures of reactive glial cells, relatively high background staining, or weak to no internal normal control positivity. The latter 22 tumors and 4 of the 5 tumors whose cores were lacking on the TMA, along with the remaining 82 tumors not included in the TMA, were evaluated using whole tissue sections. Of these 108 tumors evaluated, 100 tumors (100/108, 93%) were successfully scored, of which 2 cases were further excluded because *CDKN2A* data were unavailable. Overall, 178 tumors were successfully scored and formed the basis of the analyses.

Loss of MTAP immunoreactivity was observed in 40% (72/178) of all diffuse gliomas. The staining loss was uniformly seen in the evaluable tumor areas. Two representative gliomas with MTAP loss and *CDKN2A* deletion are

Table 1 Clinicopathological characteristics of all diffuse glioma cases in relation to *CDKN2A* homozygous deletion status.

	N (%)					
	All		*CDKN2A* HD		*CDKN2A* non-HD	
Number	178		78	(43.8)	100	(56.2)
Age at diagnosis						
Median (y)	49.0		61.0		41.5	
Range (y)	17–87		29–87		17–85	
Sex						
Male	99	(55.6)	47	(26.4)	52	(29.2)
Female	79	(44.4)	31	(17.4)	48	(27.0)
Group						
IDH-mutant astro[a]	77		17	(22.1)	60	(77.9)
WHO grade II		15 (19.5)		0 (0.0)		15 (19.5)
WHO grade III		35 (45.5)		4 (5.2)		31 (40.3)
WHO grade IV		27 (35.1)		13 (16.9)		14 (18.2)
IDH-mutant oligo[b]	13		6	(46.2)	7	(53.8)
WHO grade II		5 (38.5)		1 (7.7)		4 (30.8)
WHO grade III		8 (61.5)		5 (38.5)		3 (23.1)
IDH-wildtype	88		55	(62.5)	33	(37.5)

[a]*IDH*-mutant astro; *IDH*-mutant astrocytoma.

[b]*IDH*-mutant oligo; *IDH*-mutant oligodendroglioma.

illustrated in Fig. 1A–D. The loss was seen in 21% (16/77) of *IDH*-mutant astrocytomas, 54% (7/13) of *IDH*-mutant oligodendrogliomas, and 56% (49/88) of *IDH*-wildtype glioblastomas (Table 2). MTAP immunoreactivity was retained in the remaining cases, and one representative case with retained MTAP and intact *CDKN2A* copy number is illustrated in Fig. 1E, F. Among the *IDH*-mutant astrocytomas, 15/17 of the tumors with *CDKN2A* HD showed MTAP deficiency, while 59/60 of the tumors lacking *CDKN2A* HD retained MTAP expression (sensitivity = 88%, specificity = 98%, PPV = 94%, NPV = 97%; Table 2). MTAP expression was retained in two *IDH*-mutant glioblastomas with *CDKN2A* HD (Fig. 2). Although one MTAP-negative *IDH*-mutant AA showed no evidence of *CDKN2A* HD based on MLPA (Supplementary Fig. 1A, B), that tumor did show 9p21 HD based on FISH analysis (Supplementary Fig. 1C). For the *IDH*-wildtype group, 49/55 of the tumors with *CDKN2A* HD were deficient for MTAP, while all 33 tumors with no evidence of *CDKN2A* HD retained MTAP expression (sensitivity = 89%, specificity = 100%, PPV = 100%, NPV = 85%). The concordance between MTAP immunohistochemistry and *CDKN2A* HD for the *IDH*-mutant oligodendroglioma group was poorer than that for the other groups (sensitivity = 67%; specificity = 57%, PPV = 57%, NPV = 67%, Table 2).

The level of difficulty in MTAP interpretation of specimens evaluated using whole tissue sections was low in 61% (60/98) of the cases and high in 39% (38/98) of the cases. Among 98 cases, 10 cases required discussion to reach consensus (10%, 10/98). As a result of internal positive control cells staining weakly, the MTAP scoring required close examination of more than one high-power field of tissue in 28% of the tumors (Fig. 3A, B). In 14% of tumors, the staining intensity within a slide was heterogeneous with the periphery of the tissue sections showing weak or no positivity in the internal normal controls. This was particularly notable for large resection specimens and was likely an artifact. Even in well-prepared samples, scoring required particular care in less-cellular, low-grade tumors to distinguish intermixed non-neoplastic glial cells from infiltrating tumor cells (Fig. 3C). Recognizing positive reactivity in smaller tumor cells with less cytoplasm was more challenging than in large pleomorphic cells with ample cytoplasm. Perinuclear halos were another challenge for interpretation and were present in 6% of tumors (Fig. 3D).

p16 immunohistochemistry

Loss of nuclear expression of p16 was observed in 57% (102/178) of all gliomas evaluated, and loss of cytoplasmic expression was seen in 51% (90/178) of glioma specimens. Loss of nuclear and cytoplasmic expression of p16 was observed in 42% (32/77) and 40% (31/77) of *IDH*-mutant astrocytomas, respectively, 77% (10/13) and 77% (10/13) of *IDH*-mutant oligodendroglioma, respectively, and 68% (60/88) and 56% (49/88) of *IDH*-wildtype glioblastomas, respectively (Table 2, Supplementary Fig. 2). Among the *IDH*-mutant astrocytomas, p16 nuclear and cytoplasmic immunohistochemistry showed sensitivities of 94% and 94%, respectively, and specificities of 73% and 75%,

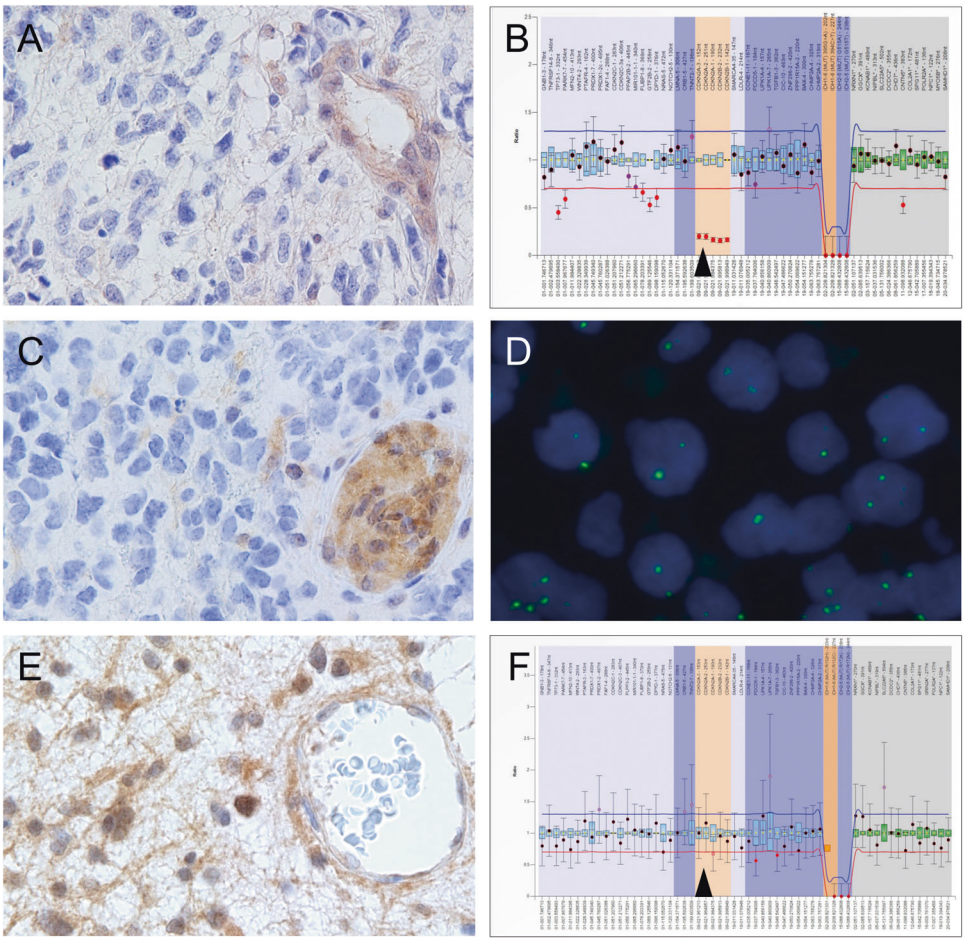

Fig. 1 MTAP immunohistochemistry and molecular tests for *CDKN2A*. **a** An *IDH*-mutant astrocytoma demonstrating the loss of cytoplasmic MTAP staining. Positively labeled endothelial cells serve as an internal control. **b** The same tumor from (**a**) demonstrating *CDKN2A* homozygous deletion (HD) according to MLPA. The arrow head indicates the region of *CDKN2A* probes. **c** An *IDH*-mutant astrocytoma showing loss of cytoplasmic MTAP staining. **d** The same tumor in (**c**) demonstrates *CDKN2A* HD by FISH. No orange *CDKN2A* signal was observed in the presence of two green *CEP9* signals. **e** An *IDH*-mutant astrocytoma retaining cytoplasmic MTAP staining. **f** Based on MLPA, this tumor harbored an intact copy number of *CDKN2A*. The arrow head indicates the region of *CDKN2A* probes.

respectively (Table 2). Similarly, for *IDH*-wildtype glioblastomas, p16 nuclear and cytoplasmic immunohistochemistry showed sensitivities of 91% and 78%, respectively, and specificities of 70% and 82%, respectively. The parameters of statistical relevance for *IDH*-mutant oligodendrogliomas were even poorer (Table 2). The combination of MTAP and p16 did not provide superior accuracy to MTAP staining alone for predicting *CDKN2A* HD in all gliomas ($n = 178$), *IDH*-mutant astrocytomas ($n = 77$), *IDH*-mutant oligodendrogliomas ($n = 13$), or *IDH*-wildtype glioblastomas ($n = 88$).

Survival analysis

Among the 178 tumors evaluated in the study, three pairs of *IDH*-mutant astrocytomas and one pair of *IDH*-mutant oligodendrogliomas were resected from the same patients at different times. Hence, the cohort was composed of 174 unique patients. The initially resected tumors from those four pairs were used for survival analysis with the subsequently resected specimens being excluded. All patients were treated with a combination of chemotherapy and radiotherapy in addition to surgical resection or biopsy. Survival data were available for all patients, including 74 patients with *IDH*-mutant astrocytoma, 12 patients with *IDH*-mutant oligodendroglioma, and 88 patients with *IDH*-wildtype glioblastomas. The median age of all 174 patients at diagnosis was 51.5 years (range, 17–87 y). The cohort included 97 men and 77 women. One teenage patient was included in the analysis as the tumor was of adult-type with *IDH* mutation. During the median follow-up of 17.2 months (range, 0.8–193.8 months), 118 patients died.

In the *IDH*-mutant astrocytoma group, both *CDKN2A* HD and MTAP immunohistochemical deficiency were significantly poor prognostic factors for OS ($P < 0.001$ each; Fig. 4A, B). In contrast, neither *CDKN2A* HD nor MTAP immunohistochemical deficiency stratified survival of the patients with *IDH*-mutant oligodendroglioma ($P = 0.799$ and $P = 0.671$, respectively; Fig. 4C, D). This was similar for patients with *IDH*-wildtype glioblastomas ($P = 0.158$ and $P = 0.484$, respectively; Fig. 4E, F).

For *IDH*-mutant astrocytomas, WHO grade was a significant prognostic factor for both OS ($P = 0.0237$; Fig. 5A) and PFS ($P = 0.0331$; Fig. 5D). However, when the analysis was limited to WHO grades II–III only, the survival differences did not reach statistical significance for either

Table 2 Correlation between *CDKN2A* homozygous deletion and MTAP or p16 immunohistochemistry.

	All ($n = 178$)		*IDH*-mutant astrocytoma ($n = 77$)		*IDH*-mutant oligodendroglioma ($n = 13$)		*IDH*-wildtype glioblastoma ($n = 88$)	
	CDKN2A HD	*CDKN2A* Non-HD	*CDKN2A* HD	*CDKN2A* Non-HD	*CDKN2A* HD	*CDKN2A* Non-HD	*CDKN2A* HD	*CDKN2A* Non-HD
MTAP_lost	68	4	15	1[c]	4	3	49	0
MTAP_retained	10	96	2	59	2	4	6	33
Sensitivity (%)	87.2		88.2		66.7		89.1	
Specificity (%)	96.0		98.3		57.1		100	
p16 nuc[a]_lost	70	32	16	16	4	6	50	10
p16 nuc_retained	8	68	1	44	2	1	5	23
Sensitivity (%)	89.7		94.1		66.7		90.9	
Specificity (%)	68.0		73.3		14.3		69.7	
p16 cyto[b]_lost	63	27	16	15	4	6	43	6
p16 cyto_retained	15	73	1	45	2	1	12	27
Sensitivity (%)	80.8		94.1		66.7		78.2	
Specificity (%)	73.0		75.0		14.3		81.8	

[a]p16 nuclear expression.

[b]p16 cytoplasmic expression.

[c]This tumor was anaplastic astrocytoma, *IDH*-mutant and harbored 9p21 homozygous deletion by FISH, although *CDKN2A* was intact by MLPA.

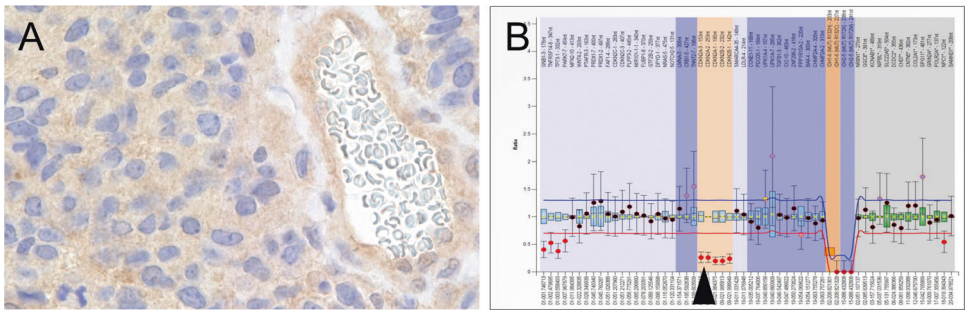

Fig. 2 Discrepancy between MTAP immunohistochemistry and *CDKN2A* copy number status. a An *IDH*-mutant glioblastoma exhibiting retained MTAP cytoplasmic immunoreactivity. **b** This tumor showed *CDKN2A* HD by MLPA. The arrow head indicates the region of *CDKN2A* probes.

OS ($P = 0.307$) or PFS ($P = 0.0551$), although grade II tended to do better than grade III (Fig. 5A, D). In this WHO grade II–III subgroup, both *CDKN2A* HD and MTAP loss remained significant prognostic factors for OS (*CDKN2A* HD: $P = 0.0124$; Fig. 5B; MTAP: $P < 0.01$; Fig. 5C) and PFS (*CDKN2A* HD: $P < 0.01$; Fig. 5E, MTAP: $P < 0.01$; Fig. 5F). Among *IDH*-mutant glioblastomas, MTAP deficiency was associated with a marginally significant shorter OS ($P = 0.0495$, Fig. 5C) and a trend toward shorter PFS ($P = 0.165$, Fig. 5F), while *CDKN2A* HD was associated with a trend toward both shorter OS ($P = 0.128$, Fig. 5B) and PFS ($P = 0.428$, Fig. 5E). Notably, *IDH*-mutant lower-grade astrocytoma with *CDKN2A* HD behaved similarly to *IDH*-mutant glioblastoma, with their differences not being significant for either OS ($P = 0.654$; Fig. 5B) or PFS ($P = 0.713$; Fig. 5E). Likewise, *IDH*-mutant lower-grade astrocytoma with deficient MTAP immunoreactivity showed overlapping unfavorable behavior with *IDH*-mutant glioblastoma, with their differences also not being significant for either OS ($P = 0.572$; Fig. 5C) or PFS ($P = 0.646$; Fig. 5F).

The results of p16 immunohistochemistry failed to provide significant prognostic stratification of any groups of patients with diffuse gliomas. The p16 immunohistochemistry results are shown in Supplementary Fig. 3.

Comparison with an alternative MTAP antibody (clone 2G4)

We performed MTAP staining using clone 2G4 on the same 178 samples and compared the performance with that of EPR6893. The scoring results were concordant in all but one sample (177/178, 99%). The discrepant case was an *IDH*-mutant glioblastoma with *CDKN2A* HD, in which MTAP immunohistochemistry was scored as retained using clone 2G4 but as lost using clone EPR6893. Hence, among

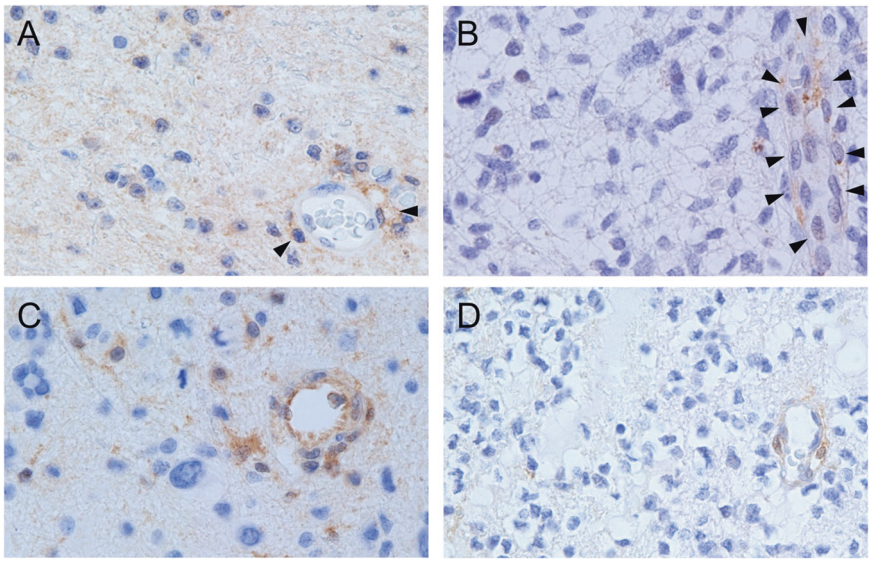

Fig. 3 Challenges in interpretation of MTAP immunohistochemistry. a An *IDH*-mutant astrocytoma showing positive MTAP staining in fine cytoplasmic processes with weakly positive internal normal controls (arrow head). **b** Loss of MTAP staining in an *IDH*-mutant astrocytoma showing weak positivity for the internal normal controls (arrow head). **c** An *IDH*-mutant astrocytoma showing loss of MTAP staining with intermixed non-neoplastic reactive astrocytes retaining MTAP positivity. **d** An *IDH*-mutant oligodendroglioma, with perinuclear halos that made MTAP interpretation challenging. The case was interpreted as MTAP deficient, although the tumor lacked *CDKN2A* HD.

the *IDH*-mutant astrocytomas, the performance parameters of clone 2G4 were slightly lower (sensitivity = 82%, specificity = 98%, PPV = 93%, NPV = 95%) than those of EPR6893 (sensitivity = 88%, specificity = 98%, PPV = 94%, NPV = 97%).

Regarding the level of interpretational difficulty based on whole tissue sections only, it was considered as low in 66% (65/98) of cases using 2G4 and in 61% of cases (60/98) using EPR6893. Specifically, 2G4 enabled easier interpretation in 34% (13/38) of specimens that were hard to interpret using EPR6893, but the interpretation became harder with 2G4 in 13% (8/60) of samples that were easy to interpret using EPR6893. The specific challenges associated with 2G4 were similar to those associated with EPR6893, but overall, 2G4 provided a stronger staining and fewer cases associated with weak internal positive control cells and heterogeneous staining intensity than did EPR6893. Distinguishing staining of tumor cells from that of reactive glia and interpretation of cells with perinuclear halo were equally challenging for both 2G4 and EPR6893.

Discussion

Discovery of the *IDH* mutation has revolutionized the diagnosis and management of diffuse glioma in adults as the mutation portends significantly better prognosis and response to therapy [45, 46]. However, risk stratification of patients with *IDH*-mutant infiltrating astrocytomas remains an issue, with the assessment of lower-grade tumors being particular problematic [10, 11, 14, 45, 47–51]. *CDKN2A* HD has been reported as one of the most promising prognostic biomarkers in *IDH*-mutant lower-grade astrocytomas [13], *IDH*-mutant astrocytic tumors of all grades [14], and *IDH*-mutant astrocytic and oligodendroglial tumors [15]. Our current data are in line with this emerging view. We revealed a significant correlation between *CDKN2A* HD and OS in the *IDH*-mutant astrocytoma group. Moreover, when we focused the analysis on WHO grades II–III *IDH*-mutant astrocytoma, *CDKN2A* HD remained a significant adverse prognostic factor, while histological grade failed to significantly stratify prognosis. Furthermore, *IDH*-mutant lower-grade astrocytoma with *CDKN2A* HD exhibited overlapping survival with *IDH*-mutant glioblastoma in our cohort, which, albeit based on a small number, supports the cIMPACT-NOW update 5 recommendation that *CDKN2A* HD in *IDH*-mutant astrocytoma should be given a grade IV designation, even in the absence of necrosis or microvascular proliferation [18]. In contrast to a previous report [17], our study failed to demonstrate a significant survival difference among patients with *IDH*-mutant glioblastoma in relation to *CDKN2A* HD. This may have been due to the smaller size of the cohort evaluated.

In our current study, we evaluated the utility of MTAP immunohistochemistry as a proxy for *CDKN2A* HD in the assessment of diffuse glioma. We showed that the loss of MTAP staining provided a high concordance with *CDKN2A*

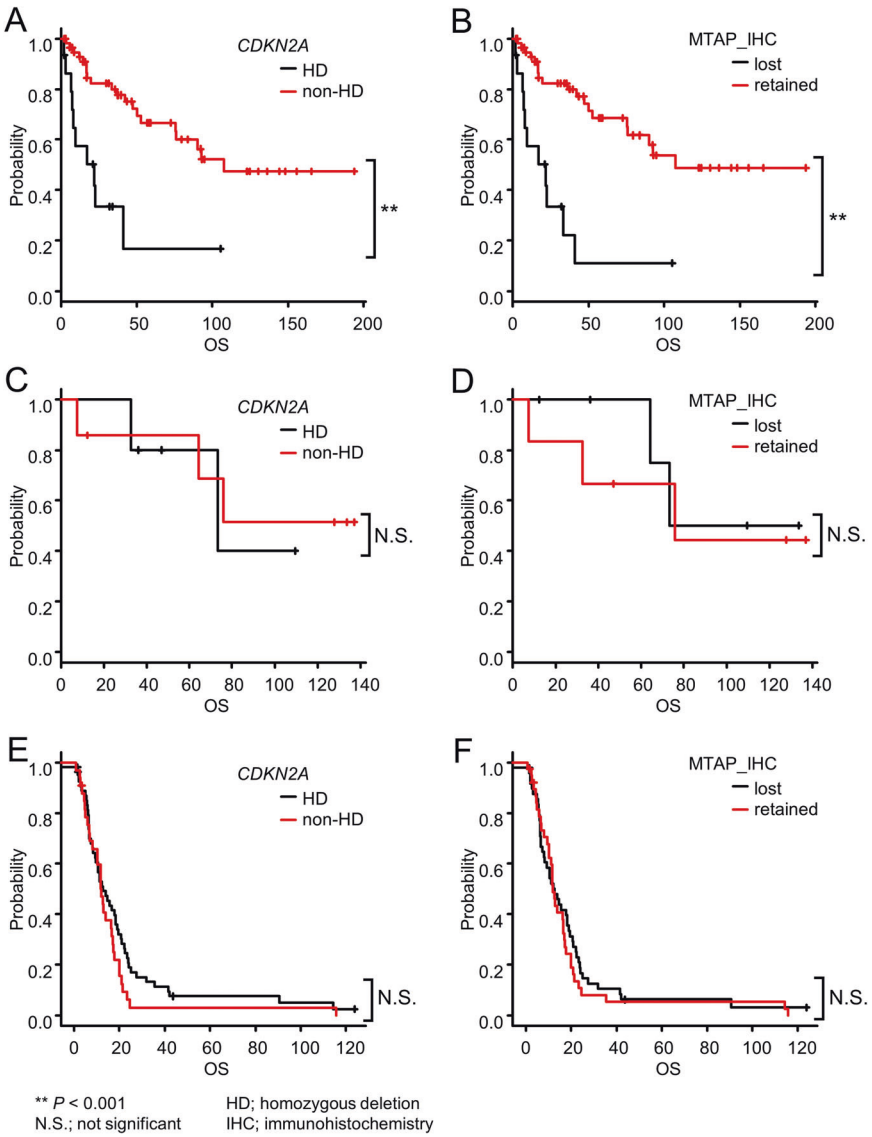

Fig. 4 Kaplan–Meier analysis and log-rank tests regarding overall survival (OS) of patients with *IDH*-mutant astrocytoma, *IDH*-mutant oligodendroglioma, and *IDH*-wildtype glioblastoma stratified by *CDKN2A* HD and MTAP staining status. a Among patients with *IDH*-mutant astrocytomas, shorter OS correlated with *CDKN2A* HD. **b** Shorter OS also correlated with MTAP immunohistochemical deficiency in patients with *IDH*-mutant astrocytomas. In contrast, OS was not significantly stratified by *CDKN2A* HD (**c**) or MTAP immunohistochemical deficiency (**d**) in *IDH*-mutant oligodendrogliomas. OS was also not significantly stratified by *CDKN2A* HD (**e**) or MTAP immunohistochemical deficiency (**f**) in *IDH*-wildtype glioblastomas. Survival time is in months.

HD among *IDH*-mutant astrocytomas (sensitivity = 88%, specificity = 98%) and *IDH*-wildtype glioblastomas (sensitivity = 89%, specificity = 100%). In addition, MTAP immunohistochemistry enabled a comparable degree of prognostic stratification as that of *CDKN2A* HD for the entire *IDH*-mutant astrocytoma and *IDH*-mutant lower-grade astrocytoma subgroup. Similar to *CDKN2A* HD, loss of MTAP staining in *IDH*-mutant lower-grade astrocytoma indicated an unfavorable survival, overlapping with that of *IDH*-mutant glioblastoma. These results suggest that MTAP immunohistochemistry may serve as a useful surrogate for *CDKN2A* molecular testing of infiltrating astrocytoma.

Our study also revealed several caveats in the use of MTAP immunohistochemistry in the assessment of diffuse glioma. First, there were a few cases of discordance between MTAP staining and *CDKN2A* HD. MTAP staining loss had overall lower sensitivity than specificity, which is consistent with published data that *CDKN2A* HD occurs more commonly than *MTAP* HD, with a small subset harboring only *CDKN2A* HD. It has been reported that in 230 diffuse gliomas with *CDKN2A* HD, *MTAP* copy number is retained in 10% of the specimens, while *MTAP* deletion without *CDKN2A* HD is rare (0.5%) [41], according to analysis using the cBioPortal database [42, 43]. In our current study, two *IDH*-mutant glioblastomas and six *IDH*-wildtype glioblastomas retained MTAP staining, despite *CDKN2A* HD. These cases may have harbored only *CDKN2A* HD. An alternative explanation for two of these cases is subclonal *CDKN2A* deletion, as the *CDKN2A* status was evaluated via MLPA on fresh frozen tissues whose origin may not match the exact site of samples used for IHC. However, in the remaining six cases, *CDKN2A* status was obtained via FISH on the very tumor sites showing retained MTAP expression, which makes it unlikely that

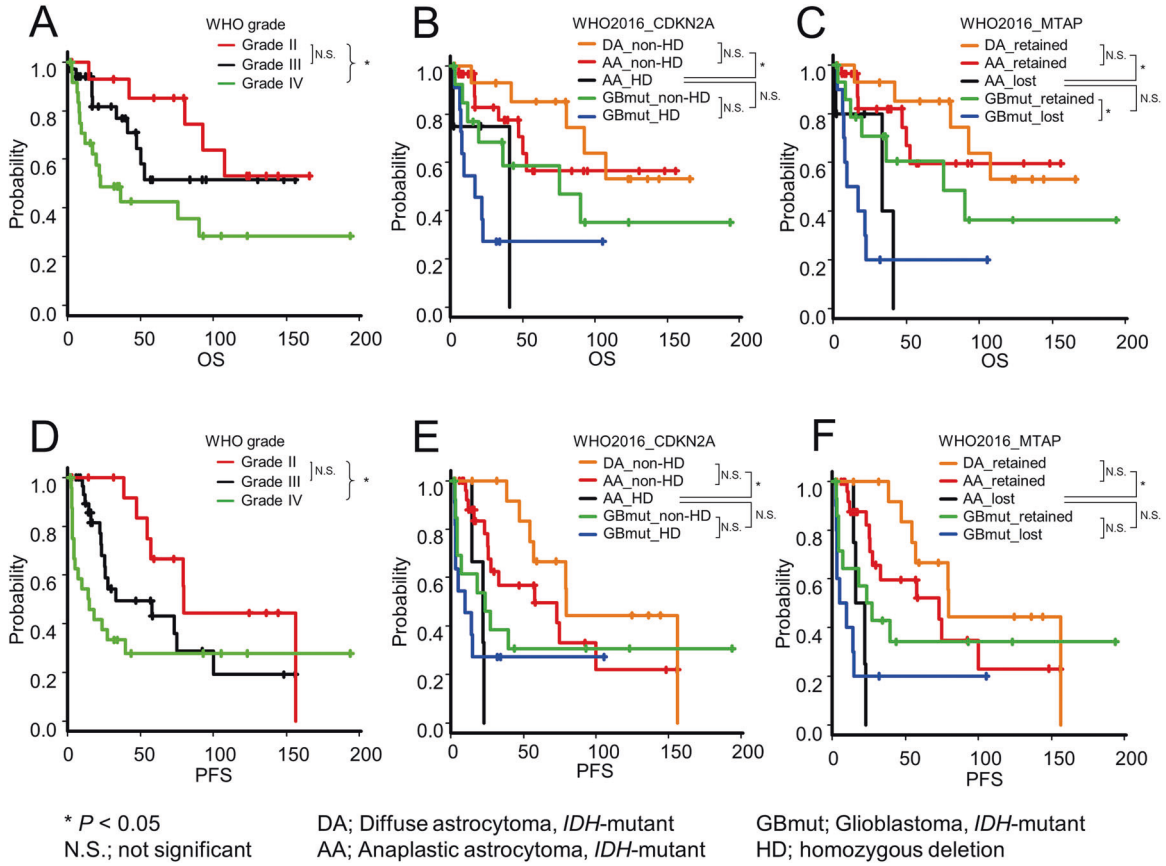

Fig. 5 In-depth survival analysis of the *IDH*-mutant astrocytoma cohort. Kaplan–Meier analysis and log-rank tests of overall survival (OS) (**a**) and progression free survival (PFS) (**d**) among patients with *IDH*-mutant astrocytoma in relation to WHO grade. OS (**b**) and PFS (**e**) were significantly stratified by *CDKN2A* HD in lower-grade (grades II–III) *IDH*-mutant astrocytoma. OS (**c**) and PFS (**f**) were significantly stratified by loss of MTAP immunoreactivity in lower-grade *IDH*-mutant astrocytoma (grades II–III). Note that neither OS nor PFS was significantly different between *IDH*-mutant glioblastoma and *IDH*-mutant lower-grade astrocytoma with *CDKN2A* HD (**b, e**) or loss of MTAP immunoreactivity (**c**, **f**). Survival time is in months.

discordance was caused by subclonal genetic changes. Conversely, the only *IDH*-mutant AA that was MTAP deficient, despite intact *CDKN2A* (determined by MLPA), may have harbored only *MTAP* HD as that case showed a loss of 9p21 signals by FISH. Overall, our data suggest that *CDKN2A* molecular testing of astrocytoma may be omitted when MTAP staining is lost, but there may be a small chance of discordance when MTAP expression is retained. Whether such a minor discordance should always be resolved using confirmatory *CDKN2A* molecular testing may be a subject of debate as MTAP staining itself provided a comparable degree of prognostic stratification to *CDKN2A* HD in *IDH*-mutant astrocytoma. Although *MTAP* HD is often perceived as a passenger genetic variant to *CDKN2A* HD, recent data suggest that MTAP dysfunction itself may actively contribute to gliomagenesis via epigenetic alteration [52]. The clinical impact of discordant *CDKN2A*/*MTAP* status in glioma is currently unclear, in part due to the relatively small number of events. Future validation using a larger cohort of *IDH*-mutant astrocytoma would be necessary to firmly establish the role of MTAP staining in diagnostic algorithms.

Second, the interpretation of MTAP immunohistochemistry was not always straightforward. The results were equivocal in a fifth of the samples initially tested using TMA, requiring re-testing using whole tissue sections. Even then, the staining remained uninterpretable in 7% of the specimens. Overall, MTAP immunohistochemistry was considered easy to interpret (i.e., low difficulty) in only 61% of the specimens successfully scored, with the remaining specimens presenting variable degrees of difficulty. Specific challenges included weak staining of internal positive controls, artifactual heterogeneous labeling, perinuclear halos, tenuous tumor cytoplasm in low-grade tumors, and differentiating tumor cells from admixed non-neoplastic elements. This could be partly alleviated using an alternative clone such as 2G4, which is becoming more favored than EPR6893 in the field of malignant mesothelioma [36], because it provided a stronger staining with a larger proportion (66%) of cases becoming easy to

interpret, while maintaining a virtually comparable power to predict *CDKN2A* HD. Nonetheless, it had some similar challenges to EPR6893, and, in a small number of samples, the scoring was considered more difficult than when using EPR6893. Although sample size, tumor cell fraction, and tissue quality dictate success rates for any biomarker testing, we believe that these variables affected MTAP immunohistochemistry more profoundly than did many other immunohistochemical antibodies that are routinely used in practice, including IDH1 (R132H) immunohistochemistry. Future studies are required to determine whether different combinations of staining platforms and/or antibody clones may resolve these issues, and interobserver variability studies would also be necessary before translating MTAP staining into practice.

The third caveat concerns the poor performance of MTAP immunohistochemistry in *IDH*-mutant oligodendrogliomas. This likely was primarily related to the fact that the staining was less reliable for scoring oligodendroglioma than for scoring astrocytoma. Oligodendroglioma nuclei are surrounded by an artifactual perinuclear halo with only a small amount of cytoplasm [53]. Therefore, cytoplasmic MTAP staining may be scarcely visible and dismissed, even if MTAP is expressed. The practical impact of this limitation is currently uncertain as the prognostic implication of *CDKN2A* HD in oligodendroglioma is controversial [13, 15, 54] and *CDKN2A* molecular assays have not been recommended in this setting.

We also found that p16 staining was of limited use in predicting *CDKN2A* HD. Consistent with previous reports [20, 21, 23], loss of p16 staining had low specificity. Consequently, neither nuclear nor cytoplasmic staining was found to be prognostic for diffuse glioma. Low specificity may be caused by *CDKN2A* being inactivated by other mechanisms or due to the antibody having low epitope affinity [55].

A minor, yet novel histological observation was that microcystic change inversely correlated with the presence of *CDKN2A* HD in *IDH*-mutant astrocytoma. Microcystic change has been reported as a favorable prognostic factor in glioblastoma [56] and is often observed in slowly growing gliomas [5]. The absence of microcystic change in tumors with *CDKN2A* HD might be a histological correlate of more aggressiveness.

In conclusion, we showed that the loss of MTAP immunoreactivity could serve as a reasonable surrogate for predicting *CDKN2A* HD in *IDH*-mutant astrocytomas and *IDH*-wildtype glioblastomas. MTAP immunohistochemical deficiency may provide a significant prognostic value for *IDH*-mutant astrocytoma, comparable to *CDKN2A* HD. Despite several caveats, MTAP immunohistochemistry may be applicable to diagnostic practices of brain tumors, as a rapid, economical, and accessible tool.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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