Evaluation of MTAP and p16 immunohistochemical deficiency as surrogate marker for CDKN2A/B homozygous deletion in gliomas

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Summary
Homozygous deletion (HD) of the CDKN2A/B locus has emerged as an unfavourable prognostic marker in diffuse gliomas, both IDH-mutant and IDH-wild-type. Testing for CDKN2A/B deletions can be performed by a variety of approaches, including copy number variation (CNV) analysis based on gene array analysis, next generation sequencing (NGS) or fluorescence in situ hybridisation (FISH), but questions remain regarding the accuracy of testing modalities. In this study, we assessed: (1) the utility of S-methyl-5'-thioadenosine phosphorylase (MTAP) and cellular tumour suppressor protein p16INK4a (p16) immunostainings as surrogate markers for CDKN2A/B HD in gliomas, and (2) the prognostic value of MTAP, across different histological tumour grades and IDH mutation status. One hundred consecutive cases of diffuse and circumscribed gliomas (Cohort 1) were collected, in order to correlate MTAP and p16 expression with the CDKN2A/B status in the CNV plot of each tumour. IDH1 R132H, ATRX and MTAP immunohistochemistry was performed on next generation tissue microarrays (ngTMAs) of 251 diffuse gliomas (Cohort 2) for implementing survival analysis. Complete loss of MTAP and p16 by immunohistochemistry was 100% and 90% sensitive as well as 97% and 89% specific for CDKN2A/B HD, respectively, as identified on CNV plot. Only two cases (2/100) with MTAP and p16 loss of expression did not demonstrate CDKN2A/B HD in CNV plot; however, FISH analysis confirmed the HD for CDKN2A/B. Moreover, MTAP deficiency was associated with shortened survival in IDH-mutant astrocytomas (n=75; median survival 61 vs 137 months; p<0.0001), IDH-mutant oligodendrogliomas (n=59; median survival 41 vs 147 months; p=0.0001) and IDH-wild-type gliomas (n=117; median survival 13 vs 16 months; p=0.011). In conclusion, MTAP immunostaining is an important complement for diagnostic work-up of gliomas, because of its excellent correlation with CDKN2A/B status, robustness, rapid turnaround time and low costs, and provides significant prognostic value in IDH-mutant astrocytomas and oligodendrogliomas, while p16 should be used cautiously.

Key words: Glioma; CNV plot; CDKN2A/B homozygous deletion; MTAP; p16.

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INTRODUCTION
Gliomas are the most common tumours of the central nervous system (CNS), representing a heterogeneous group of brain tumours; therefore, their grading is of considerable significance in the management of patients with brain tumours.1,2 Traditionally, histological tumour grading, based on mitotic activity, microvascular proliferation and/or necrosis, has been the mainstay of prognostication in tumours of the CNS, despite known limitations.3 However, the current World Health Organization (WHO) classification of tumours of the CNS (5th edition; 2021) incorporates molecular markers not only into the definition of certain tumour types, but also regarding grading, which enables a more accurate prediction of clinical behaviour.4–7 Notably, homozygous deletions (HD) of the CDKN2A/B locus, which confer an unfavourable prognosis among IDH-mutant astrocytomas, are recognised as a criterion of attribution of tumours into the newly defined group of IDH-mutant astrocytoma, CNS WHO grade 4.8 While the WHO classification of tumours of the CNS remains neutral regarding the choice of testing methodology for HD of the CDKN2A/B locus, most data available for analysis are based on copy number variation (CNV) plots derived from genome-wide DNA methylation analysis, as well as on fluorescence in situ hybridisation (FISH) analysis.9–10

The MTAP gene, encoding S-methyl-5'-thioadenosine phosphorylase (MTAP), is located adjacent to the CDKN2A locus on chromosome 9p21, and is frequently co-deleted with the latter in multiple human cancers. This gene encodes an enzyme which plays an important role in adenine and methionine salvage and may act as a tumour suppressor gene.11 The phenomenon of concomitant immunohistochemical deficiency of MTAP and CDKN2A/B HD has been discussed and proven in different studies examining pleural...
and peritoneal mesotheliomas.\textsuperscript{12-14} Hence, and due to its robust constitutive expression and the availability of suitable antibodies, loss of MTAP expression has been recognised as an accurate surrogate marker of \textit{CDKN2A/B} HD for distinguishing malignant mesothelioma from mesothelial cell proliferation evoked by reactive changes.\textsuperscript{15,16}

P61NK4a (p16), encoded by \textit{CDKN2A} on chromosome 9p21, has been acknowledged as a cellular tumour suppressor protein for the most part due to the frequency of genetic inactivation of \textit{CDKN2A} gene in different types of human cancer, and is widely used for diagnostic purposes across different diagnostic fields in pathology.\textsuperscript{17} Nevertheless, previous studies have found loss of its expression to be difficult to assess, and therefore characterised it as a suboptimal surrogate marker for \textit{CDKN2A} HD, amongst other reasons likely due to variable constitutive expression in healthy and neoplastic tissues.\textsuperscript{18} Whether the loss of p16 immunoreactivity correlates with \textit{CDKN2A} HD remains controversial.

Lower availability of genome-wide DNA methylation analysis and/or FISH analysis, longer turnaround times, and higher expenses compared with immunohistochemistry, have induced great interest in a trustworthy immunohistochemical surrogate marker for \textit{CDKN2A} HD, amongst other reasons likely due to variable constitutive expression in healthy and neoplastic tissues.\textsuperscript{18} In the present study, we evaluate the utility of MTAP and p16 immunostainings as surrogate markers for \textit{CDKN2A} HD across various types of gliomas. Moreover, we assess the prognostic value of MTAP in IDH-mutant astrocytomas, IDH-mutant oligodendrogliomas and IDH-wild-type gliomas.

\section*{MATERIAL AND METHODS}

\subsection*{Sample identification}

All brain biopsies were collected from the archives of the Institute of Tissue Medicine and Pathology, University of Bern, Bern, Switzerland. For the different study parts, we analysed specimens of diffuse and circumscribed gliomas from our diagnostic practice \((n=100)\) (Cohort 1), as well as archival samples of diffuse gliomas included in previous next generation tissue microarray \((n\text{gTMA}=301)\).\textsuperscript{19} Cohort 1 was used for method validation purposes, while the purpose of Cohort 2 was to examine the biological potential of MTAP across different types of diffuse gliomas. From Cohort 2, 251 samples could be thoroughly analysed; eight samples did not have adequate tissue for further analysis, and from 42 samples no clinical follow-up data could be obtained. The patients included in Cohort 1 and Cohort 2 were operated upon between 1 January 2018 and 31 May 2022, as well as between 1 January 2006 and 31 December 2016 respectively, at the Department of Neurosurgery, Inselspital, Bern University Hospital and University of Lausanne, Switzerland. The inclusion criteria for Cohort 1 were brain biopsies of diffuse and circumscribed gliomas with adequate material, for which genome-wide DNA methylation analysis was already performed for diagnostic purposes, whereas for Cohort 2 the inclusion criteria were brain biopsies of diffuse gliomas with adequate material.

\subsection*{Tissue microarray}

\textit{NgTMA}, which combines digital pathology and automated arraying for tissue processing steps, was constructed following the methodology of our department.\textsuperscript{20} A single tissue core from the centre of each brain tumour biopsy was annotated on the haematoxylin and eosin (H&E) slide, and one punch with a diameter of 600 nm was included in the \textit{ngTMA} paraffin block. The block was then sectioned at 4 \textmu m and stained for the markers described below.

\subsection*{Immunohistochemistry}

MTAP and p16 immunohistochemistry was carried out in full sections of Cohort 1 cases, where not already available for diagnostic purposes, for correlating MTAP and p16 expression with the \textit{CDKN2A/B} status on the CNV plot of each tumour. The evaluation of MTAP immunohistochemistry as well as of the CNV plots in Cohort 1 was carried out by two board-certified neuropathologists (TM and EH), who were initially blinded to the CNV plots. The inter-rater reliability was excellent (100%). IDH R132H, ATRX and MTAP immunohistochemistry was performed on Cohort 2 for classifying the lesions into three groups [astrocytomas (A), oligodendrogliomas (O), and IDH-wild-type gliomas (IDHwtG)], in order to perform survival analysis in each group based on MTAP status. The classification of brain tumours into the above mentioned groups and MTAP assessment was carried out by a board certified neuropathologist (TM).

Immunostainings were performed on the Bond MAX immunostainer (Leica, Germany) using the following antibodies: MTAP \((\text{antibody type mouse/JgG1}; \text{clone 2G4}; \text{dilution 1:200}; \text{phosphate buffered saline (PBS) buffer})\) buffer for antigen retrieval (H1 30); Abn01, p16 \((\text{antibody type mouse}; \text{clone E6H4}; \text{dilution 1:5}; \text{EDTA buffer for antigen retrieval (H1 20); Ventana, USA})\), R132H-mutant IDH1 \((\text{antibody type mouse/JgG2a}; \text{clone H99}; \text{dilution 1:100}; \text{phosphate buffered saline (PBS) buffer})\) buffer for antigen retrieval (H2 30); Dianova, India, and ATRX \((\text{antibody type mouse/JgG2a}; \text{clone BSB-108}; \text{dilution 1:100}; \text{bovine serum albumin (BSA) buffer})\) buffer for antigen retrieval (H2 40 95); Bio SB, USA.

Loss of MTAP, p16 and ATRX expression, respectively, was defined as a complete absence of staining in tumour cells with adequate internal positive control. Positivity for R132H-mutant IDH1 was defined as staining of any intensity attributable to tumour cells.

Unstained slides for a subset of brain tumours included in Cohort 1 were sent to the Institute of Pathology, Lausanne University Hospital and University of Lausanne, Switzerland, in order to perform immunohistochemistry for MTAP and p16 in an external laboratory, and thereby confirm our immunohistochemical results. Interlaboratory reproducibility was excellent (100%).

\subsection*{Molecular genetic analysis}

Assessment of loss of heterozygosity (LOH) at 1p and 19q chromosomal arms by microsatellite analysis and mutation analysis of isocitrate dehydrogenase one and two genes by Sanger sequencing were performed for diagnostic purposes in selected cases of Cohort 1 and 2, according to previously published protocols.\textsuperscript{19,21}

\subsection*{Copy number variations}

CNV plots were derived from genome-wide DNA methylation analysis on the Infinium MethylationEPIC array platform (Illumina, USA). For this purpose, we analysed the brain biopsies included in Cohort 1, and used the plots generated through the MolecularNeuropathology.org website hosted by the German Cancer Research Center. The generation of CNV plots and guidelines for their interpretation have been described by the developers.\textsuperscript{22} The \textit{CDKN2A/B} locus is highlighted by default in these plots. As in other studies suggested, we used a log2 value of −0.4 or lower as a criterion for a \textit{CDKN2A/B} HD.\textsuperscript{22} The genome-wide DNA methylation analysis was performed from tumour punches of Cohort 1 cases. For methylation analysis, we aimed at a tumour cell content of 70% or more in all cases included in Cohort 1. Retrospective examination of the punched areas on H&E staining revealed only two cases with a low tumour cell content (<70%).

\subsection*{Fluorescence in situ hybridisation}

FISH for \textit{CDKN2A/B} was performed on formalin fixed, paraffin embedded (FFPE) tissue sections for two cases included in Cohort 1, which showed a low tumour cell content (<70%), and for a subset of cases included in Cohort 2. In short, 4 \textmu m thick FFPE tissue sections were deparaffinised in xylene, rehydrated, treated with 2xsaline-sodium citrate (SSC), washed with 2xSSC, and then digested with a pepsin solution. Slides were co-denatured with the probes, allowed to hybridise overnight, washed according to the protocol of the manufacturer with a post-hybridisation buffer, counter-stained with DAPI, and cover slipped for analysis using a fluorescence microscope. The Clinical Genomics Lab (CGL), Inselspital, Bern University Hospital and University of Bern, Bern, Switzerland, uses the Vysis LSI \textit{CDKN2A/CEP} 9 dual colour probe set for detection of \textit{CDKN2A/B} deletion (Abbott, Japan). In order to preserve the diagnostic accuracy, 20 non-neoplastic cell nuclei were first evaluated by the molecular pathologists. For each tumour sample a total of 100 cells were examined. A HD for \textit{CDKN2A/B} was characterised by the absence of both p16
signals, while a hemizygous deletion was defined by the existence of only one p16 signal. A case where the total number of p16 signals was not greater than half of the total number of centromeric signals, was also classified as a hemizygous deletion.\(^{23}\)

**Clinical follow-up data**

Clinical follow-up data were collected from the database of the Department of Neurosurgery, Inselspital, Bern University Hospital and University of Bern, Bern, Switzerland. These data were used for performing survival analysis after classifying the brain tumours of Cohort 2 into groups and subgroups as described in detail in the section of results.

**Statistical analysis**

The variables MTAP loss, MTAP retention, p16 loss, p16 retention, no deletion of CDKN2A/B, hemizygous deletion of CDKN2A/B and HD of CDKN2A/B were correlated using a correlation matrix. Perfect correlation corresponds to 1. A Coher’s kappa test was carried out for inter-rater reliability and interlaboratory reproducibility. Agreement equal to 100% was considered excellent. The overall survival was analysed using the Kaplan–Meier method and compared using a log-rank test. A \(p\) value of <0.0001 was considered statistically significant. For Tables 1 and 2, a two-way ANOVA test and a Chi-square test were performed, respectively. A \(p\) value of <0.05 was considered statistically significant. All statistical analyses were performed using the R program (The R Foundation for Statistical Computing, Austria).

**RESULTS**

**Epidemiology and clinical presentation**

The age of patients at the time of diagnosis ranged for Cohort 1 from 1 to 88 years (median age 47.5), with 44 females and 56 males, and for Cohort 2 ranged from 6 to 83 years (median age 52), with 102 females and 149 males. All patients presented with typical brain tumour symptoms depending on tumour location, such as headache, nausea, vomiting, and/or visual disturbances. The high grade gliomas typically showed contrast enhancement following gadolinium injection and oedema in magnetic resonance imaging (MRI), while the low grade gliomas displayed ill-defined margins on T2-weighted (hyperintense) as well as T1-weighted (hypointense) without contrast enhancement. Intraoperative 5-aminolevulinic acid (5-ALA) was used for performing survival analysis after classifying the brain tumours of Cohort 2 into groups and subgroups as described in detail in the section of results.

General neuropathology and classification of brain tumours

The neuropathological diagnostic criteria of neoplastic lesions consistent with glial tumours were seen in all brain biopsies of Cohorts 1 and 2. The astrocytic tumours were histomorphologically characterised by hypercellular brain tissue diffusely infiltrated by elongated or irregular hyperchromatic nuclei and eosinophilic cytoplasm. On the contrary, oligodendrogliomas presented with rounded nuclei, frequently with perivascular halos, calcifications and fine, branching blood vessels. The glial origin of the tumour cells was confirmed with the immunohistochemical marker glial acidic fibrillary protein (GFAP). Tumours with nuclear atypia and no mitoses were assigned into CNS WHO grade 2, tumours with nuclear atypia and increased mitotic activity were assigned into CNS WHO grade 3, whereas microvascular proliferation and/or necrosis defined CNS WHO grade 4 tumours.\(^{24}\)

MTAP immunostaining showed no heterogeneity in the tumour cells of all examined cases (Cohort 1 and 2), such as partial staining, apart from a slightly weak staining of tumour cells, but still unequivocal cytoplasmic reactivity, in a very small number of cases, which probably correlates well with the CDKN2A/B hemizygous deletion confirmed by the CNV plot. On the other hand, p16 showed heterogeneous immunostaining in a small number of cases, while in some others positively stained reactive astrocytes were observed among negative tumour cells.

The brain tumours of Cohort 1 were epigenetically classified, additionally to histopathological classification, using the genome-wide DNA methylation data (Supplementary Table 1, Appendix A). For the brain tumours of Cohort 2, we used the nomenclature and concept of integrative diagnosis of the WHO classification of tumours of the CNS (5th edition; 2021), considering all available histomorphological, immunohistochemical and molecular data (Supplementary Table 1, Appendix A), in order to classify them, as mentioned above, into the three following distinct groups.\(^{25}\)

Astrocytomas (A) were defined by the identification of IDH mutation by immunohistochemistry or mutation analysis, and either loss of ATRX or absence of 1p/19q-codeletion. Oligodendrogliomas (O) were defined by the presence of IDH mutation by immunohistochemistry or mutation analysis, and of 1p/19q-codeletion. IDH-wild-type gliomas (IDHwtG) were defined by the loss of IDH mutation by immunohistochemistry or mutation analysis, and preservation of ATRX. All astrocytomas (A) were negative for 1p/19q-codeletion as determined by LOH testing, while the presence of LOH at 1p and 19q chromosomal arms, a feature defining oligodendrogliomas, was confirmed in all brain tumours classified as oligodendrogliomas (O). IDH1/2 mutation and ATRX loss with unclear status of 1p/19q-codeletion were defined as an exclusion criterion; however, no such case was detected. The IDH-wild-type gliomas (IDHwtG) included diffuse high grade gliomas, mostly glioblastomas and, according to old terminology, some diffuse and anaplastic astrocytomas, IDH-wild-type, which nowadays correspond to molecular glioblastomas. In the same group, 79 of 117 patients were younger than 55 years, a fact that by itself rules out an IDH mutation. The remaining 38 patients were younger than 55 years, making

**Table 1** Group of IDH-mutant astrocytomas classified according to initial central nervous system World Health Organization grade and MTAP status

<table>
<thead>
<tr>
<th>Astrocytomas</th>
<th>MTAP retention</th>
<th>MTAP loss</th>
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<tbody>
<tr>
<td>CNS WHO grade 2</td>
<td>36 cases 1 case</td>
<td></td>
</tr>
<tr>
<td>CNS WHO grade 3</td>
<td>24 cases 5 cases</td>
<td></td>
</tr>
<tr>
<td>CNS WHO grade 4</td>
<td>6 cases 3 cases</td>
<td></td>
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</table>

**Table 2** Group of IDH-mutant and 1p/19q-codeleted oligodendrogliomas classified according to initial central nervous system World Health Organization grade and MTAP status

<table>
<thead>
<tr>
<th>Oligodendrogliomas</th>
<th>MTAP retention</th>
<th>MTAP loss</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNS WHO grade 2</td>
<td>27 cases 1 case</td>
<td></td>
</tr>
<tr>
<td>CNS WHO grade 3</td>
<td>26 cases 5 cases</td>
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testing for IDH1/2 mutation, which cannot be assessed by immunohistochemistry, mandatory. In 18 of 38 cases an absence of IDH1/2 mutation was confirmed by mutation analysis. In the remaining 20 of 38 cases, in which IDH1/2 mutation analysis was not performed in the frame of diagnostics, preservation of ATRX expression was found, which makes it unlikely for these tumours to be classified in the group of astrocytomas. Ultimately, nine of 20 cases with preserved ATRX showed no 1p/19q-codeletion, and 11 of 20 cases with preserved ATRX showed no oligodendrogliona-like morphology, which makes it also unlikely for these tumours to be classified in the group of oligodendroglomias.

**Immunohistochemical expression of MTAP and p16 in relation to CDKN2A/B status**

The correlation of MTAP and p16 status, defined as MTAP loss, MTAP retention, p16 loss, p16 retention, based on immunohistochemistry with the CDKN2A/B status, defined as HD, hemizygous deletion, no deletion, derived from CNV plots of Cohort 1 is depicted in Fig. 1. Regarding MTAP expression, 67 of 100 gliomas showed MTAP retention, and either a hemizygous deletion or no deletion on CNV plot. The remaining 33 gliomas displayed MTAP loss, with 31 of 33 gliomas presenting with a HD on CNV plot, and two of 33 gliomas with no deletion. These two discrepant cases, both of which were epigenetically classified as glioblastomas, IDH-wild-type, subtype mesenchymal, were later tested through FISH analysis for CDKN2A/B HD, which confirmed the HD. Concerning p16 expression, 65 of 100 gliomas exhibited p16 retention, with 62 of 65 gliomas presenting with either a hemizygous deletion or no deletion on CNV plot, and three of 65 gliomas with a HD on CNV plot. The remaining 35 of 100 gliomas displayed p16 loss, with 28 of 35 gliomas showing a HD on CNV plot, while seven of 35 gliomas demonstrated no deletion. Complete loss of MTAP and p16 expression by immunohistochemistry was 100% and 90% sensitive as well as 97% and 89% specific for CDKN2A/B HD, respectively, as identified on CNV plot derived from genome-wide DNA methylation analysis. The two discrepant cases, which were confirmed through FISH analysis for HD, are not included in these percentages. Hence, after confirming the HD in these two discrepant cases by FISH, we found that immunohistochemical deficiency of MTAP shows an excellent correlation with CDKN2A/B HD and brings the sensitivity and specificity up to 100%. In eight of 100 gliomas we observed opposing results between MTAP and p16 expression. These samples included two glioblastomas, IDH-wild-type, subtype RTK I; one glioblastoma, IDH-wild-type, subtype mesenchymal; two glioblastomas, IDH-wild-type, epigenetically not further classified; one diffuse leptomeningeal glioneuronal tumour; one astrocytoma, IDH-mutant; and one diffuse hemispheric glioma, H3 G34-mutant. False negative staining in entities such as pilocytic astrocytoma was not determined in any case. In Fig. 2–4 we present three cases: one case with opposing results between MTAP and p16 immunohistochemistry and HD for CDKN2A/B on CNV plot; one case with retained expression of MTAP and p16 and no CDKN2A/B deletion on CNV plot; and one case with immunohistochemical deficiency for MTAP and p16 and HD for CDKN2A/B on CNV plot.

**Confirmation of CDKN2A/B HD through FISH**

As mentioned previously, a HD for CDKN2A/B was not detected on the CNV plot, provided by genome-wide DNA methylation analysis, in two discrepant cases of Cohort 1, whereas both MTAP and p16 markers demonstrated a complete deficiency in tumour cells. Both cases had a histological and molecular diagnosis of glioblastoma, IDH-wild-type, CNS WHO grade 4; however, both tumour profiles based on CNV plots were flat, indicative of a low number of tumour cells. Therefore, a FISH analysis was carried out, as previously described in the methodology, to confirm the CDKN2A/B status. Ultimately, the FISH analysis verified the presence of CDKN2A/B HD in both cases, a result compatible with the initial immunohistochemical analysis. This result confirms the excellent correlation of MTAP expression with CDKN2A/B status and brings the sensitivity and specificity up to 100%. A retrospective examination of the punched area of these two cases on H&E staining revealed a low tumour cell content (<70%), indicating that MTAP is a more sensitive method for detecting CDKN2A/B HD. In Fig. 5 we present one of the two discrepant cases. Additionally, we assessed CDKN2A/B status by FISH in a subset of cases included in Cohort 2, where we also found 100% concordance between HD for CDKN2A/B and loss of MTAP immunohistochemical expression (data not shown).

**Survival analysis of the three groups in relation to MTAP status**

After classifying the brain tumours into the above mentioned groups, each group was subsequently divided into two subgroups in relation to MTAP status, defined as MTAP retention (MTAP+) and MTAP loss (MTAP−), to assess the prognostic value of MTAP in each group. In all groups, the gliomas displaying MTAP deficiency were associated with shortened survival in comparison to the gliomas with a retained MTAP expression, IDH-mutant astrocytomas [n=75; median survival 61 vs 137 months; p=0.000094 (p<0.0001)], IDH-mutant and 1p/19q-codeleted oligodendroglomias [n=59; median survival 41 vs 147 months; p=0.000017 (p<0.0001)] and IDH-wild-type gliomas [n=117; median survival 13 vs 16 months; p=0.011 (p<0.0001)] (Fig. 6).

**When should we look for a CDKN2A/B HD in IDH-mutant gliomas?**

As we continue to better characterise and prognosticate gliomas, greater attention should be paid to the emerging molecular alterations as they are being detected. Traditional histological grading criteria do not necessarily ensure prognostic power when IDH gene status is taken into consideration, and particular molecular markers are more influential and therefore have been integrated into the grading system. In an attempt to define the prognostic effect of CDKN2A/B HD in low grade IDH-mutant gliomas, and to examine whether finding a CDKN2A/B HD in low grade IDH-mutant gliomas can be often expected, the IDH-mutant astrocytomas and IDH-mutant and 1p/19q-co-deleted oligodendroglomias of Cohort 2 were assigned a CNS WHO grade (Tables 1 and 2), based on histological criteria, such as mitotic rate, microvascular proliferation and/or necrosis. After performing an analysis of variance for Tables 1 and 2, no statistical
Fig. 1  Full correlation matrix of all variables (A) and correlation matrix only of MTAP loss, p16 loss and CDKN2A/B homozygous deletion (HD) variables (B). HD for CDKN2A/B derived from copy number variation plot displays an almost perfect correlation with immunohistochemical deficiency of MTAP (0.96), due to the two discrepant cases which were later confirmed by FISH for CDKN2A/B HD, while p16 scoring is in general less satisfactory (0.78).
significance was observed between the groups (p>0.05). However, this could be explained by the relatively low number of low grade IDH-mutated gliomas that were included in the analysis.

Furthermore, we re-examined the histology of astrocytoma CNS WHO grade 2 and oligodendroglioma CNS WHO grade 2 (Tables 1 and 2), which both revealed MTAP loss, to look for particular features. The astrocytoma displayed extensive microcystic changes in tumour stroma, while no mitoses, microvascular proliferation, necrosis, or KM enhancement were reported. The oligodendroglioma showed pronounced cellularity with isolated mitoses in a proliferative nodule, however no increased mitotic activity, microvascular proliferation, necrosis, or KM enhancement were determined.

**DISCUSSION**

In recent years, DNA methylation profiling has become an important tool for tumour classification and identification of new molecular subclasses in neuropathology. The current WHO classification of tumour of the CNS (5th edition 2021) has introduced the concept of an integrated diagnosis, incorporating important molecular information into the
Histopathological classification of brain tumours, which often has an immediate impact on tumour grading. Such important molecular markers are highlighted by default in CNV plots obtained by genome-wide DNA methylation analysis, using the German Cancer Research Center classifier. However, Infinium MethylationEPIC array is not available worldwide, and is associated with higher costs and longer turnaround time compared to immunohistochemistry. Moreover, the possibility of analysing a tissue area with a low number of tumour cells, leading to inadequate information provided by the CNV plot, is not low. Thus, detecting immunohistochemical surrogate markers for important molecular alterations can be of great help.

This study is of particular interest because data investigating potential surrogate markers for CDKN2A/B status in gliomas are limited. To the best of our knowledge, this is the second study to focus on the investigation of MTAP and p16 immunohistochemical expression in relation to CDKN2A/B status, and to present data of survival analysis taking into account the MTAP status in a large cohort of both IDH-mutant and IDH-wild-type gliomas.

Our results demonstrate that immunohistochemical loss of expression of MTAP correlates outstandingly with HD for CDKN2A/B, while p16 should be used cautiously. Furthermore, we show that the IDH-mutant astrocytomas as well as the IDH-mutant 1p/19q-codeleted oligodendrogliomas,

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Fig. 3 A case of astrocytoma, IDH-mutant, CNS WHO grade 4, displaying retained expression of MTAP and p16 and no CDKN2A/B deletion on copy number variation (CNV) plot. Histopathology presents a diffuse astrocytic glioma (A), with retained expression of MTAP (B) as well as of p16 in tumour cells (C). In compatibility with the immunohistochemical results, the CNV plot indicates a hemizygous deletion, as there is focal shifting of the blue line accompanied by multiple red dots including the CDKN2A/B locus (in circle) and are located slightly above the value of ~0.4 (D). Retrospective examination of the case revealed a tumour cell content of >70%.
displaying MTAP deficiency, were associated with statistically significant shortened survival in comparison to the gliomas with a retained MTAP expression. The analysis of variance performed in Tables 1 and 2 showed no statistical significance between the groups, which could be due to the low number of cases that were included in the analysis. Nevertheless, whether it is worth looking in low grade IDH-mutant gliomas for a CDKN2A/B HD, we conclude that it is a rare phenomenon to detect HD in CNS WHO grade 2 tumours than in CNS WHO grade 3 or 4 tumours, which more often show a HD. However, IDH-mutant gliomas CNS WHO grade 2 must be analysed for CDKN2A/B HD, when MTAP loss is determined. Furthermore, we believe that looking for a CDKN2A/B HD is particularly helpful when there is doubt whether classifying an astrocytoma as a CNS WHO grade 3 or 4, or whether classifying an oligodendroglioma as a CNS WHO grade 2 or 3. The choice of testing methodology for HD of the CDKN2A/B locus is another important question. While FISH and array technology have been the gold standard over the past years, challenges arise when enough tumour material is not available or the tumour cell content of the biopsy is too low, which could lead to a false result. For this matter, we examined the immunohistochemical expression of MTAP and p16 on the borders of a glioblastoma biopsy with HD for CDKN2A/B on CNV plot, where the
tumour cells displayed a clear deficiency of MTAP and p16 on the infiltration zone by adequate internal positive control (Fig. 4 A–C). This indicates that immunohistochemistry can be much more reliable, in brain biopsies dealing with low tumour cell content or an infiltration zone area, than FISH or genome-wide DNA methylation analyses. MTAP staining showed no heterogeneity in the tumour cells of all examined cases, such as partial stainability, which is another advantage attributed to this study. This may represent a tumour specific phenomenon, as heterogeneous MTAP immunostaining has been reported in malignant pleural mesotheliomas and meningiomas, while the same antibody clone was used as in our study.15,27

Multiple studies published over the past few years have delivered convincing evidence of relatively high specificity of MTAP immunohistochemistry for identifying CDKN2A/B HD.9,15,16,18,27,28 Chapel et al. reported that MTAP loss by immunohistochemistry was 78% sensitive and 96% specific for CDKN2A/B HD, and that MTAP is a trustworthy surrogate marker for CDKN2A/B FISH in the diagnosis of malignant mesothelioma.15

Fig. 5 A case of glioblastoma, IDH-wild-type, CNS WHO grade 4, showing loss of MTAP and p16 expression, and no homozygous deletion (HD) for CDKN2A/B on copy number variation (CNV) plot. Histopathology demonstrates a glial tumour with numerous elongated nuclei (A), and deficiency of MTAP (B) and p16 (C) in tumour cells by adequate internal positive control. The CNV plot reveals a hemizygous deletion for CDKN2A/B (in circle) as the locus here is also observed above the –0.4 value (D); however, the flat tumour profile is suspicious considering the histopathological tumour classification as glioblastoma. Hence, FISH analysis was additionally carried out and confirmed the presence of HD. Retrospective examination of the case revealed a tumour cell content of <70%.
Fig. 6  Overall survival of all groups and subgroups of diffuse gliomas included in Cohort 2 (A) and only of astrocytoma and oligodendroglioma groups and subgroups (B) through a Kaplan–Meier analysis and log rank tests. Astrocytomas with MTAP loss (A, MTAP−) and oligodendrogliomas with MTAP loss (O, MTAP−) are associated with statistically significant shortened survival compared to astrocytomas with MTAP retention (A, MTAP+) and oligodendrogliomas with MTAP retention (O, MTAP+). IDH-wild-type gliomas with deficiency of MTAP (IDHwtG, MTAP−) also display a shorter survival than IDH-wild-type gliomas with retention of MTAP (IDHwtG, MTAP+) but without statistical significance.
In addition, Satomi et al. examined whether MTAP deficiency could serve as a replacement for CDKN2A/B HD, detected by FISH or multiplex ligation-dependent probe amplification (MLPA), in adult-type diffuse gliomas, and the results showed 88% sensitivity and 98% specificity for IDH-mutant astrocytomas, 89% sensitivity and 100% specificity for IDH-wild-type glioblastomas, as well as 67% sensitivity and 57% specificity for IDH-mutant oligodendrogliomas. In the same study, it was presented that CDKN2A/B HD and MTAP deficiency was associated with statistically significant shortened overall survival in IDH-mutant astrocytomas, but none of these were prognostically significant for IDH-wild-type glioblastomas or IDH-mutant oligodendrogliomas. In none of them were prognostically significant for IDH-mutant astrocytomas, 89% sensitivity and 100% specificity for IDH-WT glioblastomas, as well as 67% sensitivity and 57% specificity for IDH-mutant oligodendrogliomas. In our study we present evidence that loss of MTAP was 100% sensitive and specific for CDKN2A/B HD, constituting an excellent surrogate marker, which could replace FISH and array technology. Furthermore, our overall survival analysis demonstrated that MTAP immunohistochemical deficiency was a significant adverse prognostic factor not only for group 1 IDH-mutant astrocytomas (p=0.000094; p<0.0001), but also for group 2 IDH-mutant and 1p/19q-codeleted oligodendrogliomas (p=0.000017; p<0.0001). Our group of oligodendrogliomas included 59 brain biopsies, whereas only 13 were investigated by Satomi et al. Moreover, in order to validate MTAP immunostaining as a trustful method for detecting CDKN2A/B HD, we compared expression of MTAP with two different techniques, CNV plot based on genome-wide DNA methylation analysis (Cohort 1) and FISH analysis (subset of Cohort 2 cases), whereas Satomi et al. used one technique, either FISH or MLPA. Regarding performance of p16 immunostaining as a method for detecting CDKN2A/B HD in gliomas, our sensitivity and specificity percentages (90% and 89%, respectively) are somewhat similar to the ones reported by Satomi et al. and we agree that combination of MTAP and p16 does not yield higher accuracy or additive benefit to MTAP immunostaining alone.

A very recent study by Sasaki et al. investigated the same question of MTAP being a trustworthy proxy for CDKN2A/B HD in meningiomas CNS WHO grade 2 and 3, showing that MTAP loss was in perfect harmony with CDKN2A/B HD, and reported 100% sensitivity and specificity. Interestingly, MTAP loss was significantly associated with meningiomas displaying a high mitotic activity [four or more mitoses in 10 high-power fields (HPF)] and an increased Ki-67 labeling index. Similarly, in diffuse gliomas we expect to find MTAP deficiency, hence CDKN2A/B HD, mostly in high grade gliomas and rarely in low grade gliomas.

In recent years and, most importantly now, with the new WHO classification of tumour of the CNS (5th edition 2021), there is an emerging relevance of CDKN2A/B HD in IDH-mutant gliomas. The issue of prognostic significance of CDKN2A/B has been often addressed in studies. In one of them they concluded that HD for CDKN2A/B is an important prognostic factor for survival outcomes of IDH-mutant glioma patients across multiple histological CNS WHO grades. Despite that, greater understanding of how detecting this deletion can help in the stratification of management for these tumours to improve clinical course is still needed. For this matter, adding pre and postoperative imaging analyses during the histological and molecular analyses of diffuse IDH-mutant gliomas, in means of correlating histology with MRI and clinical findings, seems to be of great significance. Therefore, further multi-centric studies are required to determine to which extent imaging and clinical data can prove to be of help in the diagnostic work-up of gliomas.

The results should be interpreted with full knowledge of the retrospective design of the study, which excluded some brain tumours from the overall survival analysis due to lack of clinical follow-up data and/or tumour material (Cohort 2). Furthermore, MTAP immunohistochemistry was performed for all cases included in Cohort 2, while data regarding CDKN2A/B status were available only in a subset of Cohort 2 cases through FISH analysis. Nevertheless, throughout the study there was no evidence of poor performance of MTAP immunohistochemistry in both IDH-mutant and IDH-wild-type tumours. Moreover, we recognise the relatively low number of low grade IDH-mutated gliomas included in the study.

In conclusion, MTAP immunostaining is an important complement for diagnostic work-up of gliomas, because of its excellent correlation with CDKN2A/B status in IDH-mutant astrocytomas, IDH-mutant oligodendrogliomas and IDH-wild-type gliomas, robustness, rapid turnaround time and low costs, which could replace FISH and array technology. On the contrary, p16 should be used cautiously, but might be considered as a marker when MTAP is not available. Discovering CDKN2A/B HD through MTAP immunohistochemistry seems to be a more reliable method than the CNV analysis derived from genome-wide DNA methylation data when tumour cell content is low. MTAP immunohistochemical deficiency represents a significant adverse prognostic factor not only in IDH-mutant astrocytomas, but also in IDH-mutant and 1p/19q-codeleted oligodendrogliomas.

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Data availability statement: The data that support the findings of this study are available from the corresponding author, upon reasonable request.

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APPENDIX A. SUPPLEMENTARY DATA

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