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ANATOMICAL PATHOLOGY

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

Theoni Maragkou[1], Stefan Reinhard[1], Patric Jungo[1], Baptiste Pasquier[1], Maja Neuenschwander[1], Philippe Schucht[2], Erik Vassella[1], Ekkehard Hewer[1,3]

[1]*Institute of Tissue Medicine and Pathology, University of Bern, Bern, Switzerland;* [2]*Department of Neurosurgery, Inselspital, Bern University Hospital and University of Bern, Bern, Switzerland;* [3]*Institute of Pathology, Lausanne University Hospital and University of Lausanne, Lausanne, Switzerland*

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 pI61NK4a (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 a 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.[6] 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.[8–10]

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

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and peritoneal mesotheliomas.[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 *CDKN2A/B* HD for distinguishing malignant mesothelioma from mesothelial cell proliferation evoked by reactive changes.[15,16]

Pl61NK4a (p16), encoded by *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 *CDKN2A* gene in different types of human cancer, and is widely used for diagnostic purposes across different diagnostic fields in pathology.[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 *CDKN2A* HD, amongst other reasons likely due to variable constitutive expression in healthy and neoplastic tissues.[18] Whether the loss of p16 immunoreactivity correlates with *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 *CDKN2A/B* HD.[15,18] In the present study, we evaluate the utility of MTAP and p16 immunostainings as surrogate markers for *CDKN2A/B* 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.

MATERIAL AND METHODS

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 [ngTMA; n=301 (Cohort 2)].[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 Bern, Bern, 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.

Tissue microarray

NgTMA, which combines digital pathology and automated arraying for tissue processing steps, was constructed following the methodology of our department.[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 ngTMA paraffin block. The block was then sectioned at 4 µm and stained for the markers described below.

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 *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%). IDH1 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 [antibody type mouse/IgG1; clone 2G4; dilution 1:200; phosphate buffered saline (PBS) buffer for antigen retrieval (H1 30); Abnova], p16 [antibody type mouse; clone E6H4; dilution 1:5; EDTA buffer for antigen retrieval (H1 20); Ventana, USA], R132H-mutant IDH1 [antibody type mouse/IgG2a; clone H09; dilution 1:100; phosphate buffered saline (PBS) buffer for antigen retrieval (H2 30); Dianova, India], and ATRX [antibody type mouse/IgG2a; clone BSB-108; dilution 1:100; bovine serum albumin (BSA) 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, 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%).

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.[19,21]

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.[22] The *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 *CDKN2A/B* HD.[8,22] 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%).

Fluorescence *in situ* hybridisation

FISH for *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 µm 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 (GCL), Inselspital, Bern University Hospital and University of Bern, Bern, Switzerland, uses the Vysis LSI CDKN2A/CEP 9 dual colour probe set for detection of *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 *CDKN2A/B* was characterised by the absence of both p16

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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 Cohen'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) positivity was restricted in high grade gliomas. Epidemiological and basic clinical data as well as histological diagnoses and available molecular data of all cases of both cohorts are described in detail in Supplementary Table 1 (Appendix A).

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 stainability, 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 older 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

Astrocytomas	MTAP retention	MTAP loss
CNS WHO grade 2	36 cases	1 case
CNS WHO grade 3	24 cases	5 cases
CNS WHO grade 4	6 cases	3 cases

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

Oligodendrogliomas	MTAP retention	MTAP loss
CNS WHO grade 2	27 cases	1 case
CNS WHO grade 3	26 cases	5 cases

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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 oligodendroglioma-like morphology, which makes it also unlikely for these tumours to be classified in the group of oligodendrogliomas.

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 oligodendrogliomas [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.[2,3] 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 oligodendrogliomas 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

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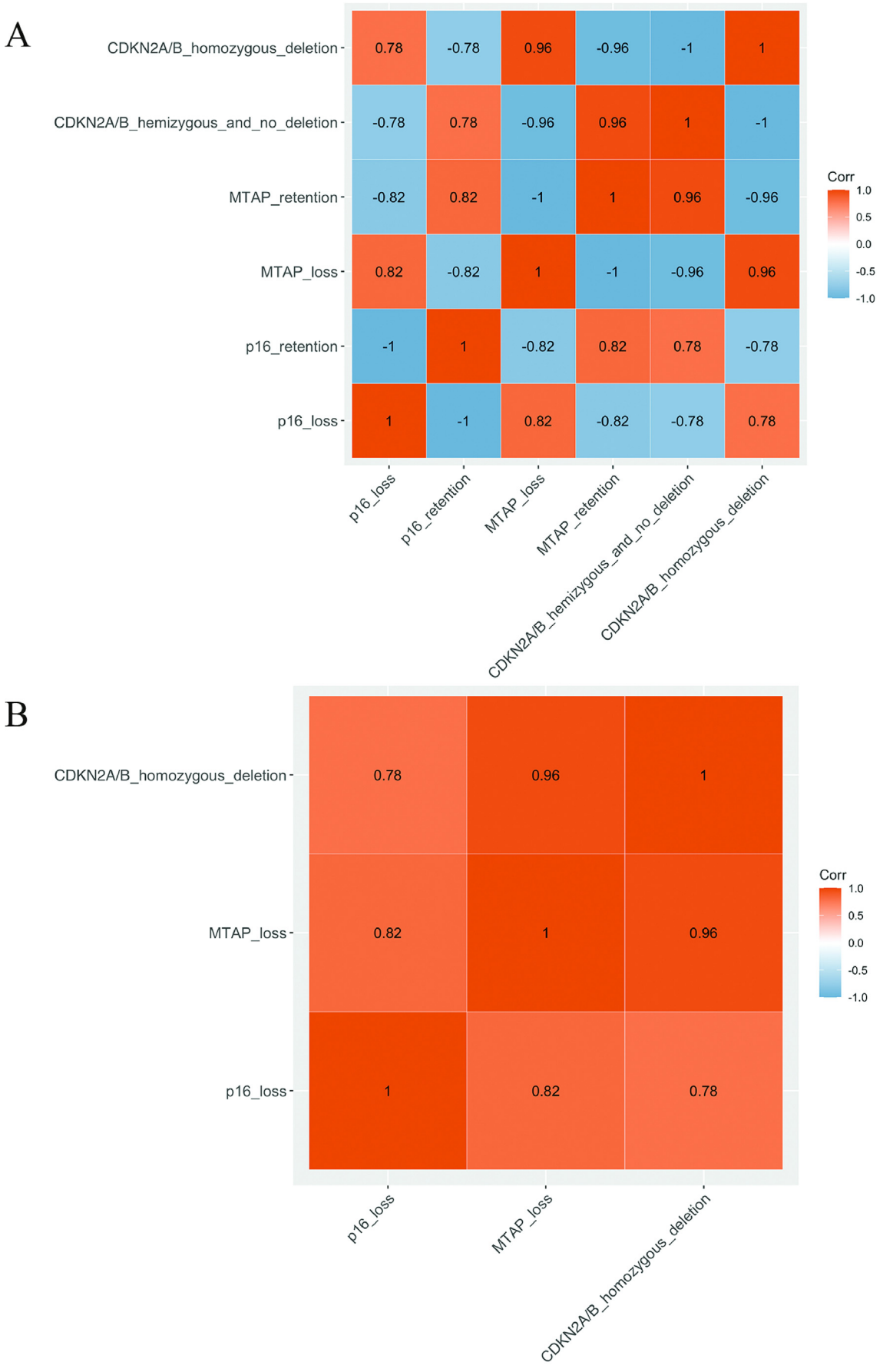

Fig. 1 Full correlation matrix of all variables (A) and correlation matrix only of MTAP loss, p16 loss and *CDKN2A/B* homozygous deletion (HD) variables (B). HD for *CDKN2A/B* derived from copy number variation plot displays an almost perfect correlation with immunohistochemical deficiency of MTAP (0.96), 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).

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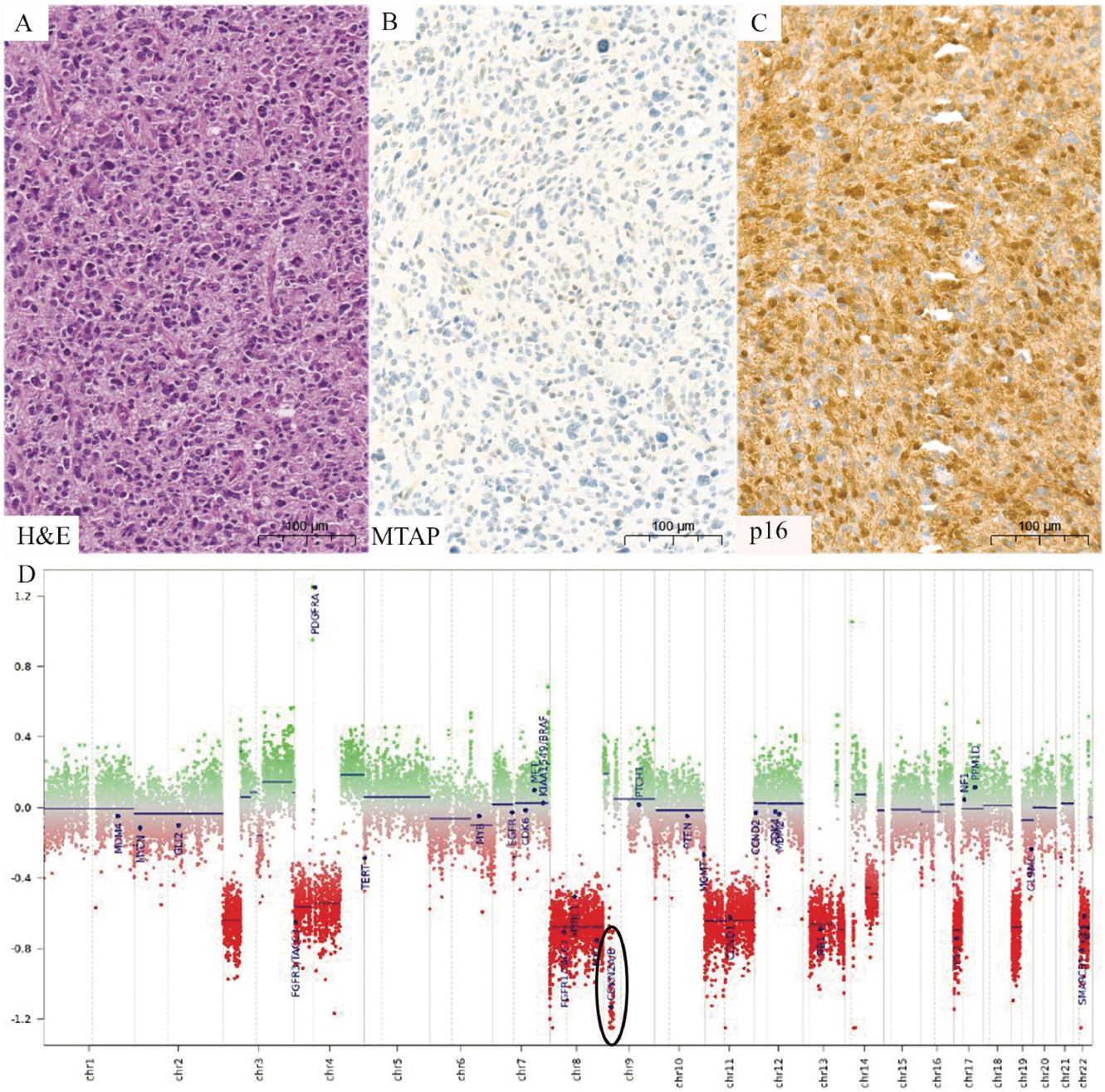

Fig. 2 A case of glioblastoma, IDH-wild-type, CNS WHO grade 4, showing opposing results between MTAP and p16 immunohistochemistry and homozygous deletion (HD) for *CDKN2A/B* on copy number variation (CNV) plot. Histopathology reveals a hypercellular glial tumour with pleomorphic nuclei (A), with deficiency of MTAP in tumour cells by adequate internal positive control (B), while expression of p16 is retained in tumour cells (C). The CNV plot clearly reveals a HD, consistent with loss of MTAP, as the *CDKN2A/B* locus (in circle) is observed far below the −0.4 value (D).

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

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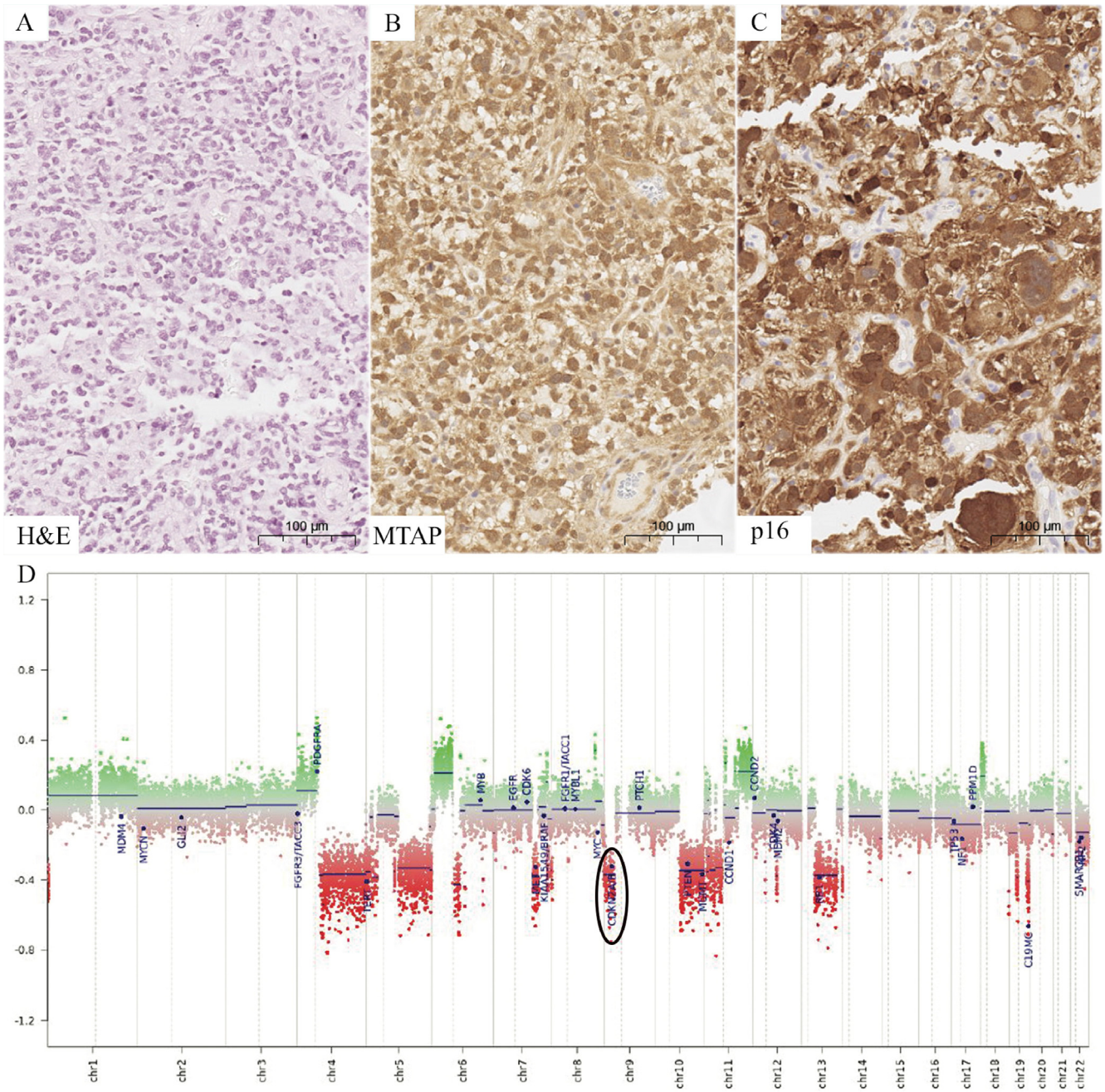

Fig. 3 A case of astrocytoma, IDH-mutant, CNS WHO grade 4, displaying retained expression of MTAP and p16 and no *CDKN2A/B* deletion on copy number variation (CNV) plot. Histopathology presents a diffuse astrocytic glioma (A), with retained expression of MTAP (B) as well as of p16 in tumour cells (C). 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%.

histopathological classification of brain tumours, which often has an immediate impact on tumour grading.[25] 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.[18] 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 and 1p/19q-codeleted oligodendrogliomas,

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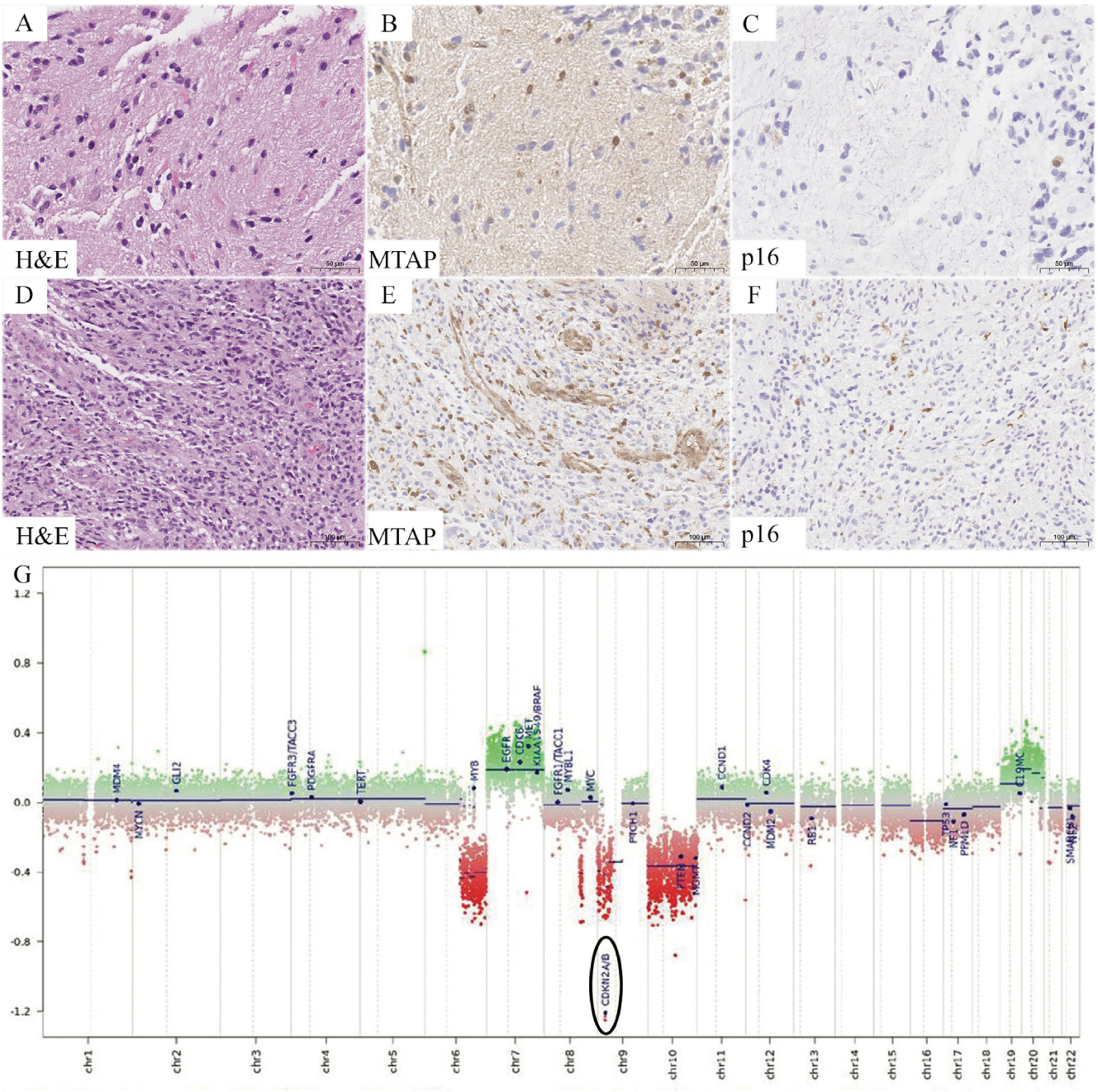

Fig. 4 A case of glioblastoma, IDH-wild-type, CNS WHO grade 4, with an additional area of infiltration zone demonstrating immunohistochemical deficiency for MTAP and p16 and homozygous deletion (HD) for *CDKN2A/B* on copy number variation (CNV) plot. Histopathology shows an infiltration zone with evidence of normal/reactive cells and some pleomorphic tumour cells spread in between (A) as well as a diffuse infiltrative glial tumour (D), which are both negative for MTAP (B,E) and p16 (C,F) by adequate internal positive control, consisting of endothelial cells lining the vessels and some intratumoural inflammatory infiltrates. The CNV plot indicates a HD, in concordance with immunohistochemistry, as the *CDKN2A/B* locus (in circle) is placed much below the −0.4 value (G).

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.[18,26] 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

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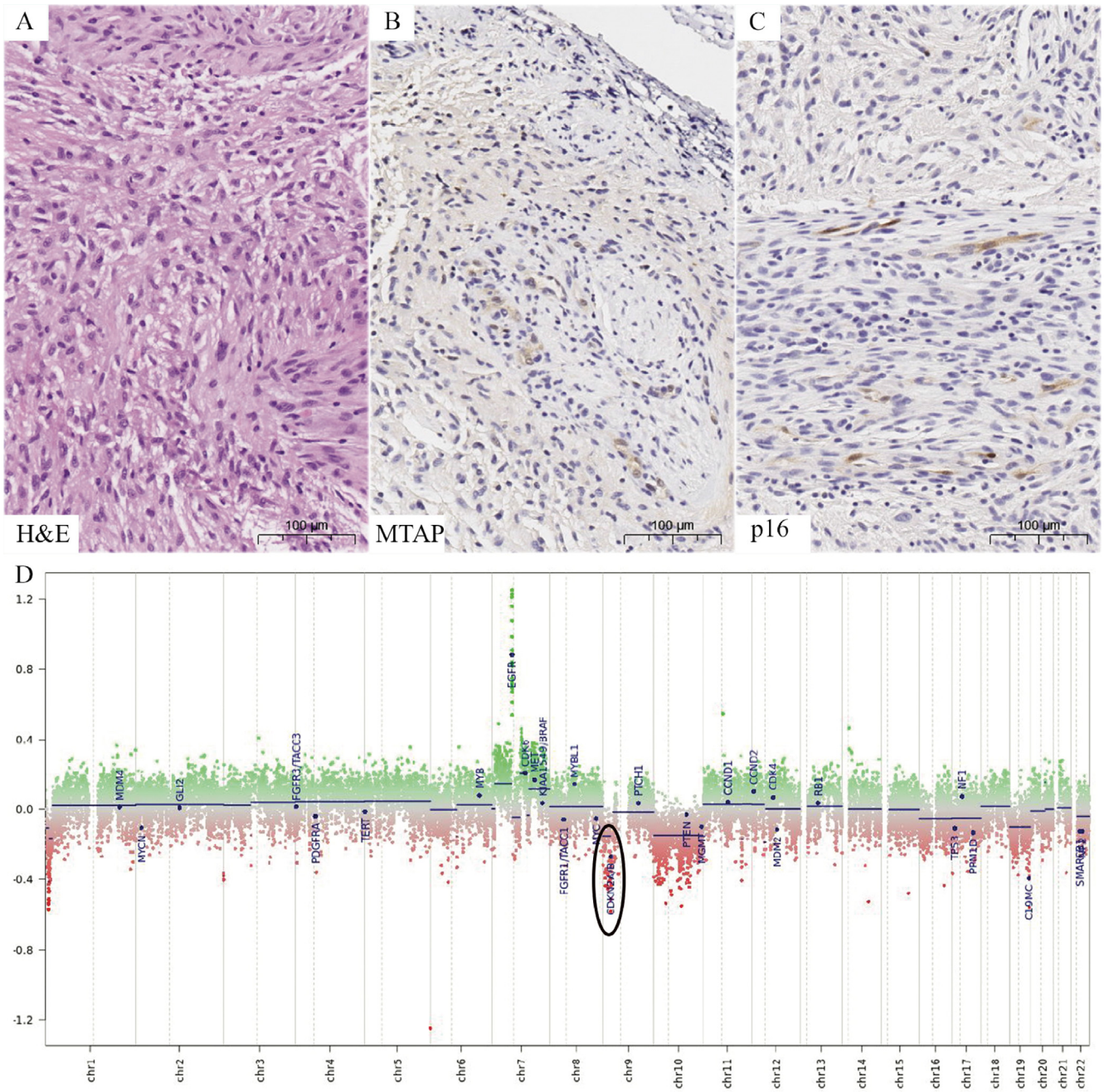

Fig. 5 A case of glioblastoma, IDH-wild-type, CNS WHO grade 4, showing loss of MTAP and p16 expression, and no homozygous deletion (HD) for *CDKN2A/B* on copy number variation (CNV) plot. Histopathology demonstrates a glial tumour with numerous elongated nuclei (A), and deficiency of MTAP (B) and p16 (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%.

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]

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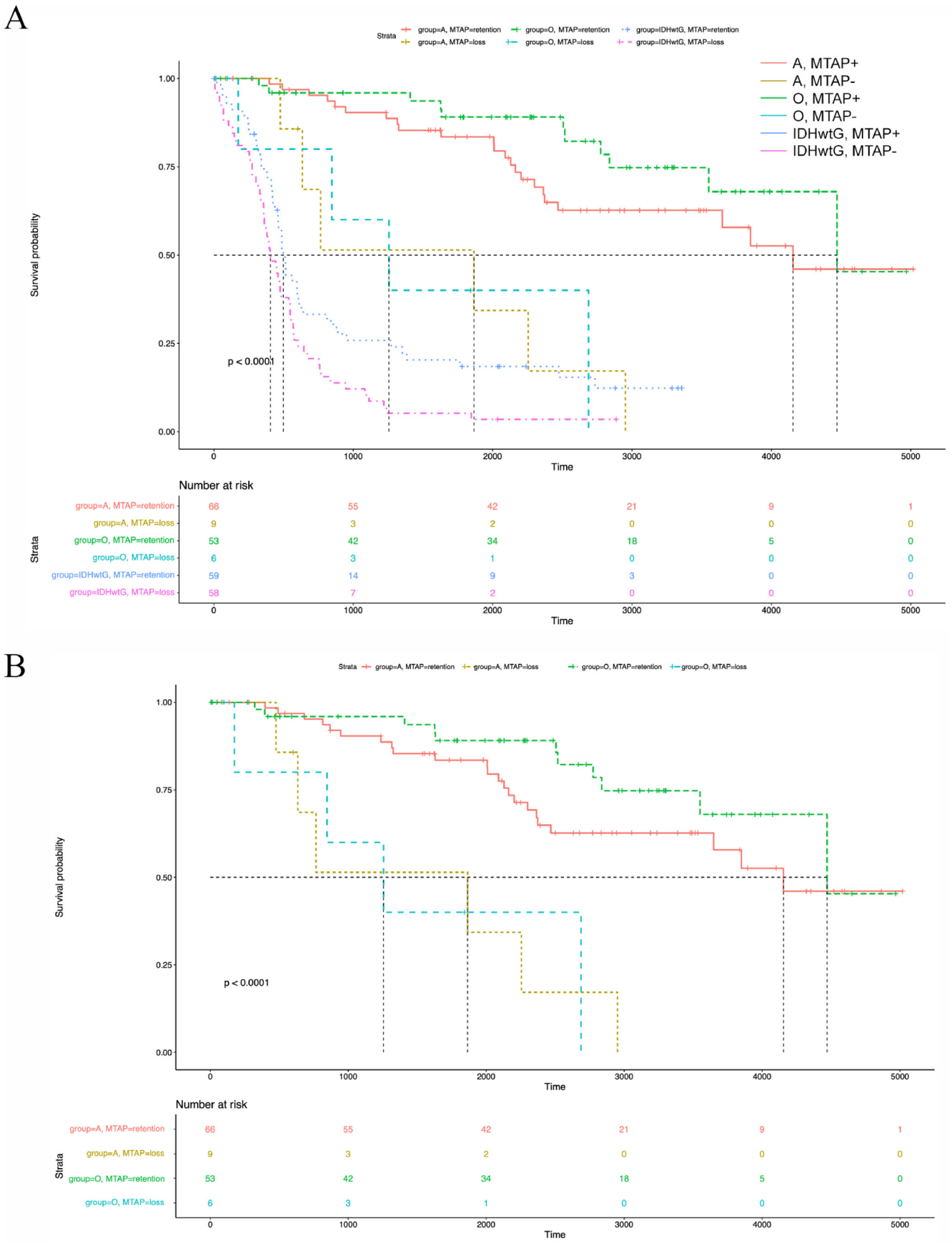

Fig. 6 Overall survival of all groups and subgroups of diffuse gliomas included in Cohort 2 (A) and only of astrocytoma and oligodendroglioma groups and subgroups (B) through a Kaplan–Meier analysis and log rank tests. Astrocytomas with MTAP loss (A, MTAP−) and oligodendrogliomas with MTAP loss (O, MTAP−) 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.

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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 them were prognostically significant for IDH-wild-type glioblastomas or IDH-mutant oligodendrogliomas.[18] 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.[27] Interestingly, MTAP loss was significantly associated with meningiomas displaying a high mitotic activity [four or more mitosis 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.[29] 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.[30] 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.

Ethics approval statement: This study was performed with permission of the Ethics Commission of the Canton of Bern (KEK 2014-200 and 2017-1189).

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

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pathol.2023.01.005.

Address for correspondence: Dr Theoni Maragkou, Institute of Tissue Medicine and Pathology, University of Bern, Hochschulstrasse 6, 3012, Bern, Switzerland. E-mail: theoni.maragkou@unibe.ch

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