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ORIGINAL ARTICLE

CDKN2A Loss Is Associated With Shortened Overall Survival in Lower-Grade (World Health Organization Grades II–III) Astrocytomas

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Abstract

Lower-grade (World Health Organization Grades II and III) gliomas vary widely in clinical behavior and are classified as astrocytic, oligodendroglial, or mixed. Anaplasia depends greatly on mitotic activity, with *CDKN2A* loss considered as the most common mechanism for cell cycle dysregulation. We investigated whether loss of the *CDKN2A* gene is associated with overall survival across pathologically and genetically defined glioma subtypes. After adjustment for *IDH* mutation, sex, and age, *CDKN2A* deletion was strongly associated with poorer overall survival in astrocytomas but not in oligodendrogliomas or oligoastrocytomas. Molecular classification of astrocytomas by *IDH* mutation, *TP53* mutation, and /or *ATRX* loss of expression revealed that *CDKN2A* loss in *IDH/TP53* mutated tumors was strongly associated with worse overall survival. *CDKN2A* loss in *IDH* mutated tumors with ATRX loss was only weakly associated with worse overall survival. These findings suggest that *CDKN2A* testing may provide further clinical aid in lower-grade glioma substratification beyond *IDH* mutation and 1p19q codeletion status, particularly in *IDH/TP53* mutated astrocytomas.

Key Words: Astrocytoma, *ATRX*, Biomarker, *CDKN2A*, *IDH*, Infiltrating glioma, Oligodendroglioma, p16, *TP53*.

INTRODUCTION

Infiltrating gliomas account for approximately 60% of primary malignant intracranial tumors (1) and have a devastating course given their widespread invasiveness, tendency toward biologic progression, and resistance to available adjuvant therapies. The World Health Organization (WHO) classifies infiltrating gliomas as follows: diffuse (WHO Grade II) astrocytoma (A-II), diffuse (WHO Grade II) oligodendroglioma (O-II), diffuse (WHO Grade II) oligoastrocytoma (OA-II), anaplastic (WHO Grade III) astrocytoma (A-III), anaplastic (WHO Grade III) oligodendroglioma (O-III), anaplastic (WHO Grade III) oligoastrocytoma (OA-III), and glioblastoma multiforme (WHO Grade IV) (2). Although diagnostic reproducibility among neuropathologists is high for glioblastoma multiforme, there is far lower concordance for cell type determination in other subtypes and in the distinction between WHO Grade II and WHO Grade III. The latter difficulty stems in part from different grading criteria for astrocytic and oligodendroglial tumors, vagaries in WHO grading criteria, and interobserver variability in the detection of mitotic figures and microvascular proliferation. In addition, although molecular criteria for more objectively distinguishing diffuse astrocytomas from oligodendrogliomas in adult patients have been published recently, it remains unclear whether previously devised grading criteria based solely on morphology can be appropriately applied to newly defined glioma subtypes based mostly on molecular definitions (3). As such, the development of clinically useful ''grading'' biomarkers for molecularly defined glioma subsets is sorely needed to improve diagnostic reproducibility.

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Most Grade II and Grade III gliomas, as well as so-called secondary glioblastoma multiforme derived from lower-grade gliomas, harbor mutations in the *IDH* gene, with *IDH1* being most common (4–7). Mutations in *TP53* and *ATRX* also occur frequently and are present in approximately 60% to 70% of tumors of astrocytic differentiation (2). In a recent study of lower-grade gliomas, *ATRX* mutation was closely associated with *TP53* mutation and was restricted to *IDH* mutated tumors (8). Consequences of *ATRX* mutation include activation of the alternative lengthening of telomeres pathway and telomerase-independent immortalization of tumor cells (9, 10). Conversely, approximately 70% of oligodendrogliomas harbor 1p19q codeletion and lack *TP53* and *ATRX* alterations (2, 8, 11–14). Approximately 80% of oligodendrogliomas have mutations in the *TERT* gene promoter, which reactivates telomerase activity (15). Mixed oligoastrocytomas remain poorly defined molecularly, with most showing classic molecular features of either astrocytoma or oligodendroglioma (16).

In addition to the alterations described previously, homozygous and hemizygous losses involving 9p21 have been observed at high frequency in infiltrating gliomas, with homozygous loss being most common (17–20). One of the consequences of 9p21 deletion is loss of the cyclin-dependent kinase inhibitor *CDKN2A* gene, which results in cellular proliferation and dysregulation of proapoptotic pathways (21). Cell cycle progression from G1 phase to S phase relies on complex formation between cyclin-dependent kinases (CDK4 or CDK6) and D-type cyclins, which subsequently leads to phosphorylation of retinoblastoma (RB1) protein, release of elongation factor (EF2) transcriptional factor, and activation of genes involved in G1-to-S transition (22, 23). Alterations in this pathway seem to be rare in Grade II gliomas but are frequent in higher-grade (Grades III and IV) tumors, suggesting a key role for the CDKN2A-CDK4-RB pathway in malignant progression (24–26). Because the main criterion for anaplastic designation in gliomas is either the presence of mitoses (astrocytomas) or high mitotic activity (oligodendroglial tumors), loss of the *CDKN2A* gene or p16 protein (*CDKN2A* product) seems to be an ideal candidate for distinguishing the molecular phenotypes of WHO Grade II and Grade III gliomas.

Several studies have reported worse prognosis for *CDKN2A* loss in gliomas (27–33). However, these studies were conducted before the realization that *IDH* mutation is an independent favorable prognostic factor in adult-type diffuse gliomas (6, 34, 35). Therefore, it is unclear whether *CDKN2A* loss remains a statistically significant prognostic factor after correcting for the effects of *IDH* mutation. Furthermore, prior studies have not explored the effects of *CDKN2A* loss on newly defined ''molecular'' Grade II to Grade III astrocytomas (i.e. *IDH* mutated, *TP53* mutated, and often with associated loss of ATRX protein expression) or ''molecular'' Grade II to Grade III oligodendrogliomas (i.e. *IDH* mutated and 1p19q codeleted). Our study addresses these questions by testing the hypothesis that *CDKN2A* loss determined by fluorescence in situ hybridization (FISH) and/or loss of p16 protein expression determined by immunohistochemistry (IHC) can enhance prognostication of overall survival in molecularly characterized lower-grade (WHO Grades II and III) adult-type diffuse gliomas. If so, these molecular biomarkers could potentially enhance future WHO grading criteria and improve interobserver concordance rates among pathologists.

MATERIALS AND METHODS

Study Participants and Selection Criteria

Cases were selected from among participants of the San Francisco Bay Area Adult Glioma Study (AGS), which was conducted at the University of California San Francisco (UCSF), as previously described (36, 37). Briefly, the AGS enrolled patients who were newly diagnosed as having a histologically confirmed glioma at age 18 years or older between 1991 and 2012. All participants gave a written informed consent form, and the study was conducted under protocols approved by the UCSF Institutional Review Board.

The following criteria were used to select cases from the AGS for this analysis: Only cases classified as Grade II or Grade III astrocytoma, oligodendroglioma, or oligoastrocytoma

TABLE 1. Distribution of Histologic Subtype, Pathologic Diagnosis, Demographic Characteristics, and Clinical Characteristics of Patients With Glioma From the UCSF AGS

		Age (years)		
	n (%)	Mean	SE	Median
All cases	270 (100.0)	42.2	0.7	41.0
Histology				
Astrocytoma	113 (41.9)	42.4	1.3	41.0
Oligodendroglioma	104 (38.5)	44.5	1.0	44.5
Oligoastrocytoma	53 (19.6)	36.9	1.3	36.0
Pathology				
Diffuse astrocytoma (A-II)	49 (18.2)	41.4	1.9	40.0
Anaplastic astrocytoma (A-III)	64 (23.7)	43.2	1.8	41.5
Oligodendroglioma (O-II)	80 (29.6)	43.9	1.2	44.0
Anaplastic oligodendroglioma (O-III)	24 (8.9)	46.8	2.0	51.0
Oligoastrocytoma (OA-II)	42 (15.6)	37.7	1.6	36.0
Anaplastic oligoastrocytoma (OA-III)	11 (4.1)	34.1	2.4	32.0
Sex				
Male	147 (54.4)			
Female	123 (45.6)			
Surgery				
Biopsy	20 (7.4)			
Resection	250 (92.6)			
Adjunct therapy				
Chemotherapy				
No	107 (40.7)			
Yes	154 (58.6)			
Unknown	2 (0.8)			
Temozolomide	129 (47.8)			
Radiotherapy				
No	127 (47.0)			
Yes	143 (53.0)			
Outcome				
Alive	165 (61.1)			
Dead	105 (38.9)			

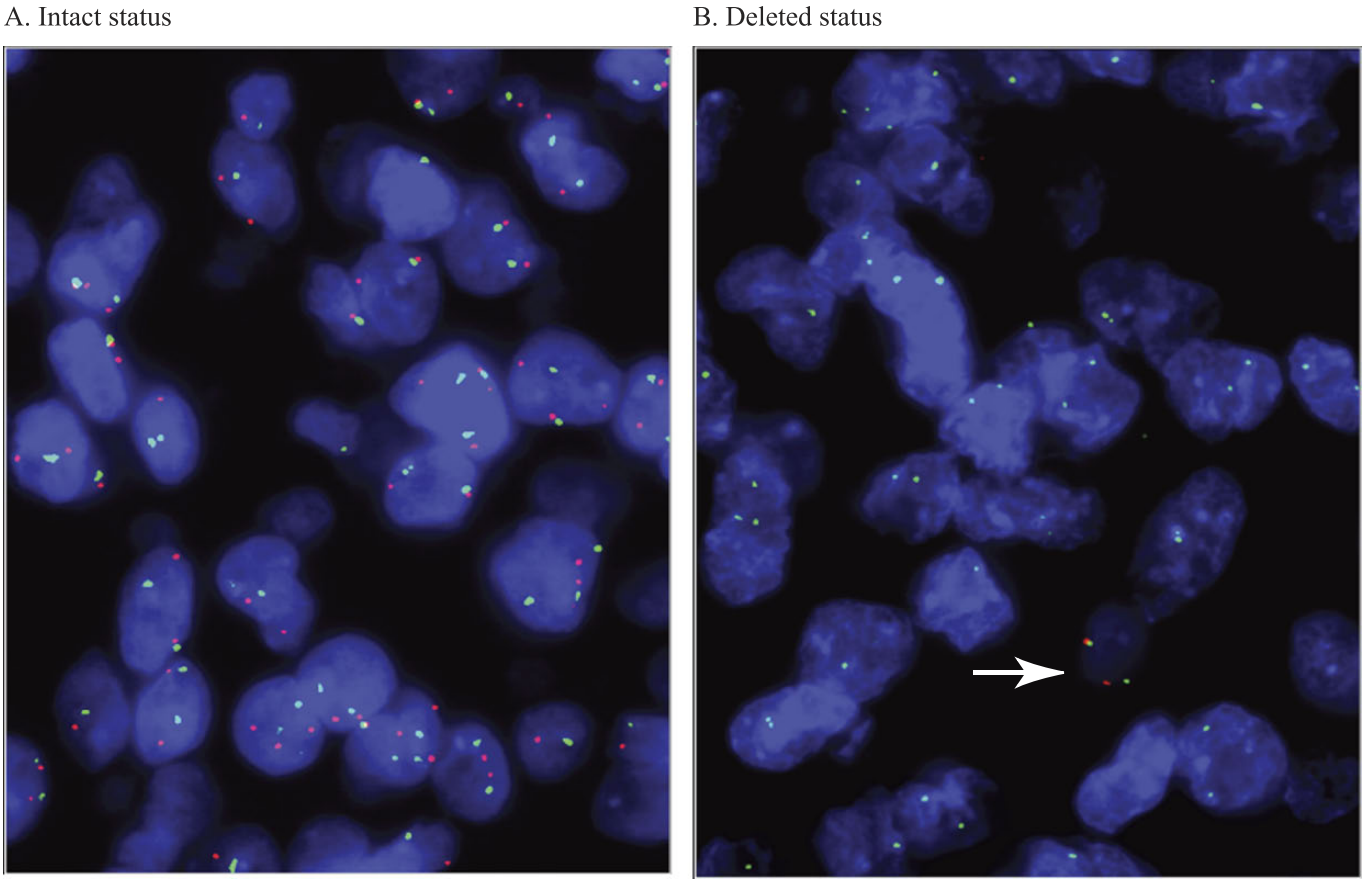

FIGURE 1. Results of FISH demonstrating intact **(A)** (CEP9-to-*CDKN2A* ratio near 1) and deleted **(B)** *CDKN2A* status. In the latter case, a nonneoplastic cell containing 2 green CEP9 signals and 2 red *CDKN2A* signals is seen (white arrow), whereas all tumor cells have only CEP9 signals.

during the study's neuropathology review were included. In addition, only cases with sufficient available tissue for the planned assays were included. Because these subjects were also a subset of another study relating tumor markers to inherited single nucleotide polymorphism data, subjects were of European ancestry and had constitutive DNA available. Cases were prioritized with respect to available mutational status for isocitrate dehydrogenase (*IDH1* or *IDH2*) obtained by DNA sequencing, as previously described (37, 38). Tumor *TP53* mutation was also available for many of the subjects and assayed as described previously (39).

FISH Analysis

We assessed *CDKN2A* copy number alterations by FISH using commercially available probes (LSI CDKN2A [9p21] orange and CEP9 green spectra; Abbott Laboratories, North Chicago, IL). In brief, green and orange fluorescent signals were enumerated under an Olympus BX60 fluorescence microscope with appropriate filters (Olympus, Melville, NY). One hundred nonoverlapping nuclei were assessed for numbers of green and red signals, and cases were considered deleted regardless of whether the pattern suggested hemizygous or homozygous loss. An interpretation of deletion was made when the orange-to-green ratio was less than 0.8. Partial hybridization failure was ruled out by evaluating orange and green signals within vascular endothelial cells as internal control. Failed or weak hybridizations were repeated; uninterpretable cases were considered ''noninformative.'' Interpretable results were available for 253 cases (93.7%).

Fluorescence in situ hybridization images were captured using a black-and-white high-resolution COHU CCD camera, a Z-stack motor, and a CytoVision basic workstation (Applied Imaging, Santa Clara, CA) with sequential DAPI (1 level), fluorescein isothiocyanate (5 levels), and rhodamine (5 levels) filter settings. Resulting images were reconstituted with blue, green, and orange pseudocolors using CytoVision software.

1p19q deletion status was assayed according to published methods (40, 41). Tumors classified histologically as astrocytoma were not assayed for 1p19q deletion.

Immunohistochemistry

Immunohistochemistry for ATRX and p16 was performed at the Brain Tumor Research Center and Clinical Immunohistochemistry Laboratories, UCSF, using an automated staining processor (Ventana, Tucson, AZ). ATRX loss of expression was assessed using rabbit polyclonal antibody (HPA001906, 1:100; Sigma, St Louis, MO). Only cases with retained staining of endothelial cells (internal positive control) were considered interpretable. For p16 expression, Mtm Lab

kit (Ventana) and JC8 mouse antibody (1:100; Santa Cruz Biotechnology, Dallas, TX) were used, with most cases evaluated using the Mtm Lab kit. Quantitative analysis of staining pattern for 10 cases, using both antibodies, demonstrated a high correlation (r^2 = 0.8, p = 0.0004). Nuclear staining was required for a positive p16 result.

Image acquisition for p16 quantification was performed using an Olympus BX41 microscope (20× objective) and an Olympus DP72 digital camera. Areas of maximal staining were photographed to assess labeling index. JPEG images were imported into ImageJ (http://imagej.nih.gov/ij/), and ImmunoRatio plug-in (http://153.1.200.58/sites/default/files/software/immunoratio-plugin/index.html) was used to determine labeling index. Approximately 1,000 cells were required for an interpretable result.

Statistics and Survival Analysis

Comparisons among categorical variables were made using chi-square test and Fisher exact test. Cox proportional hazards regression was used to estimate overall survival. Models built based on histologic subtype (astrocytoma, oligodendroglioma, or oligoastrocytoma) or pathologic diagnosis (A-II, A-III, O-II, O-III, AO-II, or AO-III) were adjusted for sex, age, and *IDH* status, whereas models built based on molecular parameters (*IDH*, *TP53*, ATRX, or 1p19q status) were adjusted only for sex and age. Molecular subtypes of astrocytoma were defined based on the presence of *IDH/TP53* mutations (*IDH/TP53* group), combined *IDH/TP53* mutations with ATRX loss (*IDH/TP53/ATRX* group), or *IDH* mutation and ATRX loss (*IDH/ATRX* group). Molecular oligodendroglioma was defined as *IDH* mutated, 1p19q codeleted tumors. Logistic analysis was used to evaluate the association between *CDKN2A* loss by FISH and p16 labeling index by IHC, stratified by histologic subtype or pathologic diagnosis. Area under the curve was obtained from the receiver operating characteristic curve, which provides a graphic representation of the relationship between false-positive rate and true-positive rate (true-positive rate as defined by FISH). The optimal labeling index for p16 was extracted from the receiver operating characteristic table and used to dichotomize continuous p16 labeling index data into ''deleted'' and ''intact'' groups. Results with a value of $p < 0.05$ were deemed significant.

RESULTS

Patient Characteristics

The clinical and pathologic characteristics of the 270 study subjects are summarized in Table 1. The percentages of astrocytomas and oligodendrogliomas were similar (41.9% and 38.5%, respectively); tumors with mixed histology accounted for 19.6% of cases. The median follow-up time was 10.6 years (mean, 10.5 years; range, 0.2–22.3 years), with 105 deaths (38.9%) and 165 patients (61.1%) alive at last follow-up.

CDKN2A Loss Occurred More Frequently in Astrocytoma Than in Other Histologic Subtypes and Was Present in Relatively Similar Percentages in A-II and A-III

Examples of *CDKN2A* results by FISH are illustrated in Figure 1. The frequency of loss for each group is summarized

TABLE 2. Distribution of *CDKN2A*, *IDH*, ATRX, *TP53*, and 1p19q Status, by Pathologic Diagnosis, in Tumors of Patients With Glioma From the UCSF AGS

	All Cases	Diffuse Astrocytoma (A-II)	Anaplastic Astrocytoma (A-III)	Oligodendroglioma (O–II)	Anaplastic Oligodendroglioma (O-III)	Oligoastrocytoma (OA-II)	Anaplastic Oligoastrocytoma (OA-III)
CDKN2A							
Intact	159 (63)	26 (55)	30 (49)	57 (77)	16 (73)	25 (63)	5 (56)
Deleted	94 (37)	21 (45)	31 (51)	17 (23)	6 (27)	15 (38)	4 (44)
Total	253	47	61	74	22	40	9
IDH							
Wild type	65 (24)	10 (21)	26 (41)	9 (11)	8 (33)	8 (19)	4 (36)
Mutated	202 (76)	37 (79)	37 (59)	71 (89)	16 (67)	34 (81)	7 (64)
Total	267	47	63	80	24	42	11
ATRX							
Intact	156 (60)	22 (47)	24 (39)	68 (86)	20 (87)	18 (44)	4 (36)
Lost	106 (40)	25 (53)	37 (61)	11 (14)	3 (13)	23 (56)	7 (64)
Total	262	47	61	79	23	41	11
TP53							
Wild type	107 (63)	9 (31)	24 (48)	42 (89)	14 (82)	13 (65)	5 (63)
Mutated	64 (37)	20 (69)	26 (52)	5 (11)	3 (18)	7 (35)	3 (38)
Total	171	29	50	47	17	20	8
1p19q							
Intact	65 (42)	NA	NA	19 (25)	5 (23)	29 (74)	8 (89)
Codeleted	89 (58)			58 (75)	17 (77)	10 (26)	1 (11)
Total	154			77	22	39	9

Data are presented as n (%).
NA, 1p19q data not available for tumors classified histologically as astrocytoma.

TABLE 3. Association of *CDKN2A* Loss by FISH With Overall Survival Stratified by Histologic Subtype, Pathologic Diagnosis, and Molecular Subtype Using Cox Proportional Hazards Models in Patients With Glioma From the UCSF AGS

	CDKN2A Intact				*CDKN2A* Deleted				Unadjusted			Adjusted for Age and Sex		
	n	No. Deceased	Mean Overall Survival (years)	Median Overall Survival (years)	n	No. Deceased	Mean Overall Survival (years)	Median Overall Survival (years)	HR	95% CI	p Value	HR	95% CI	p Value
All cases*	159	56	10.3	11.8	94	42	8.8	7.2	1.7	1.1–2.5	0.01†	1.6	1.0–2.4	0.03
Astrocytoma*	56	23	8.7	9.5	52	31	5.5	4.4	2.6	1.5–4.6	0.0008†	2.0	1.1–3.5	0.02
Oligodendroglioma*	73	20	11.1	15.4	19	4	7.0		0.7	0.2–1.7	0.4	0.7	0.2–2.00	0.5
Oligoastrocytoma*	30	13	9.8	10.6	23	7	11.5	17.2	0.9	0.3–2.2	0.7	0.8	0.3–2.4	0.7
A-II*	26	9	10.2	12.8	21	9	6.6	6.4	3.9	1.4–11.2	0.01†	2.7	0.8–8.8	0.1
A-III*	30	14	5.0	6.9	31	22	4.7	3.3	2.1	1.1–4.2	0.03†	1.8	0.8–3.6	0.1
O-II*	57	14	9.4	11.8	17	2	7.6	NA	0.4	0.07–1.6	0.3	0.4	0.07–1.5	0.2
O-III*	16	6	11.2	15.4	6	2	1.9	NA	1.4	0.2–6.6	0.7	1.6	0.2–9.9	0.6
OA-II*	25	12	9.6	10.6	15	5	12.3	17.2	0.7	0.2–2.0	0.5	1.0	0.3–3.1	0.9
OA-III*	5	1	1.0	NA	4	2	3.4	3.7	2.8	0.3–60.0	0.4	0.2	0.0004–21.2	0.6
IDH/TP53 mutated, ATRX lost	21	10	10.2	12.9	21	14	6.4	4.8	3.3	1.4–8.8	0.008†	3.4	1.4–8.4	0.008†
IDH/TP53 mutated	28	12	10.6	12.8	22	15	6.3	4.8	4.3	1.8–10.1	0.001†	4.4	1.8–10.3	0.0008†
IDH mutated, ATRX lost	49	19	9.6	9.2	39	18	8.7	7.8	1.6	0.8–3.0	0.2	1.6	0.8–3.0	0.2
IDH mutated, 1p19q codeleted‡	56	16	9.2	10.6	16	0	NA	NA	NA	NA	NA	NA	NA	NA

*Hazard ratios for histologic groups and pathologic diagnosis were adjusted for *IDH* mutation status in addition to age and sex.
†Statistical significance at p = 0.05.
‡These tumors were also ATRX intact.
NA, median survival is not available because of lack of events.

in Table 2 and illustrated in Figure, Supplemental Digital Content 1, http://links.lww.com/NEN/A727. Astrocytomas demonstrated the highest frequency of *CDKN2A* loss (astrocytoma, 52 of 108 cases [55.3%]; oligodendroglioma, 23 of 96 cases [24.5%]; oligoastrocytoma, 19 of 49 cases [20.2%]; p = 0.006, chi-square test). No significant difference in the relative percentages of deleted cases in A-II versus A-III tumors was observed (A-II, 21 of 47 cases [44.7%]; A-III, 31 of 61 cases [50.8%]; p = 0.6, Fisher exact test).

The results for p16 expression by IHC are summarized in Table, Supplemental Digital Content 2, http://links.lww.com/NEN/A728. The p16 labeling index was highly variable across cases within a given histopathologic group.

CDKN2A Loss Was Associated With Poorer Survival in Astrocytomas But Not in Oligodendrogliomas or Oligoastrocytomas

As hypothesized, *CDKN2A* loss determined by FISH was associated with significantly worse overall survival in Grade II and Grade III gliomas after adjusting for age, sex, and *IDH* mutation (hazard ratio [HR], 1.6; 95% confidence interval [CI], 1.0–2.4; p = 0.03) (Table 3). This result was mostly related to a strong adverse effect of *CDKN2A* loss on patients with astrocytoma (HR, 2.0; 95% CI, 1.1–3.5) (Table 3; Figs. 2A–D). Results were similar for Grade II and Grade III astrocytomas (Table 3). There were nonsignificant inverse associations between *CDKN2A* loss and overall survival among patients with oligodendroglioma or oligoastrocytoma (Table 3). Thus, *CDKN2A* loss was associated with poor survival among patients with astrocytoma, but this effect was not observed among patients with oligodendroglioma or oligoastrocytoma.

Logistic analysis demonstrated a poor association between *CDKN2A* deletion by FISH and p16 expression by IHC (Table, Supplemental Digital Content 3, http://links.lww.com/NEN/A729). Furthermore, loss of p16 expression by IHC was weakly associated with poor overall survival in lower-grade (Grades II and III) gliomas (Table, Supplemental Digital Content 4, http://links.lww.com/NEN/A730).

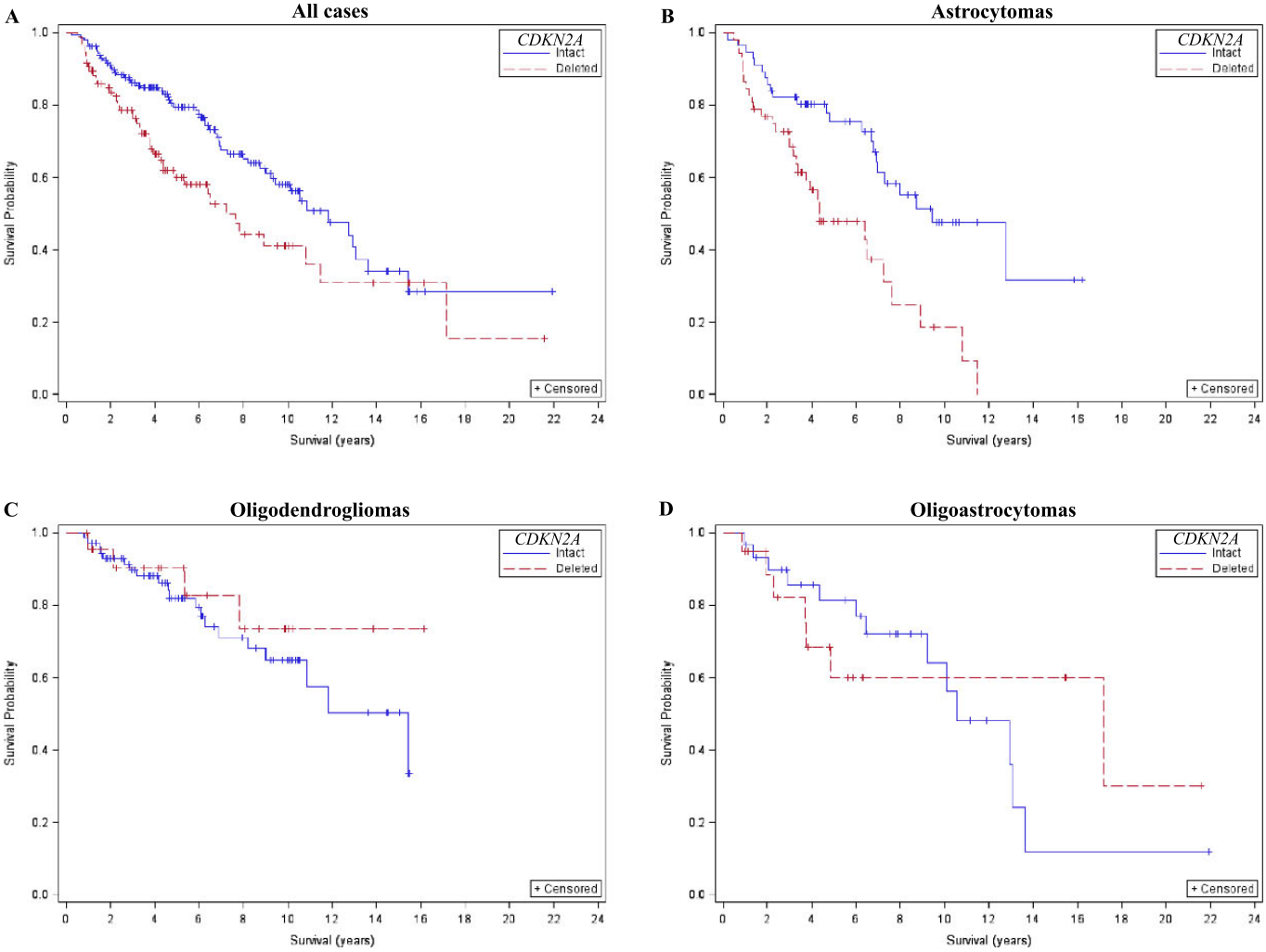

FIGURE 2. Effects of *CDKN2A* loss on overall survival in histologically classified lower-grade (WHO Grades II and III) gliomas. *CDKN2A* loss was associated with worse overall survival in lower-grade gliomas not stratified by histology **(A)** and in astrocytomas **(B)**, but not in oligodendrogliomas **(C)** or oligoastrocytomas **(D)**.

CDKN2A Loss Was Strongly Associated With Poor Survival in Certain Molecular Subsets of Astrocytoma

As an alternative to using histologic classification, we evaluated the effects of *CDKN2A* loss on the overall survival of certain molecular subsets of Grade II to Grade III astrocytomas, defined as *IDH* mutated tumors with both *TP53* mutation and ATRX loss (termed *IDH/TP53/ATRX* group), or combined with either *TP53* mutation (termed *IDH/TP53* group) or ATRX loss (termed *IDH/ATRX* group) (Table 2; Figure, Supplemental Digital Content 1, http://links.lww.com/NEN/A727). The co-occurrence of *IDH*, *TP53*, and ATRX alterations is provided in Table 4. Among 123 *IDH* mutated tumors with known *TP53* and ATRX status, 42 cases (34.1%) harbored both mutation in *TP53* and loss of ATRX expression; 21 cases (17.1%) harbored only ATRX loss; and 9 cases (7.3%) harbored only *TP53* mutation (Table 4). Fifty-one cases (41.5%) showed no alteration in either *TP53* or ATRX, of which 38 cases (74.5%) were 1p19q codeleted ''molecular oligodendroglioma.'' Overall, a strong association between occurrence of *TP53* mutation and ATRX loss was noted ($p < 0.0001$, Fisher exact test). Similarly, we found a strong association between the occurrence of *IDH* mutation and ATRX loss both in *IDH* mutated cases with 1p19q intact status ($p = 0.0004$, Fisher exact test) (Table 4) and in *IDH* mutated cases not evaluated for 1p19q because they were classic astrocytomas on histopathology ($p < 0.0001$, Fisher exact test) (Table 4); the latter was similar to observations made in *IDH/TP53* mutated cases. These data support a strong association between the occurrence of *IDH* mutation and *TP53* mutation and/or ATRX loss in Grade II to Grade III astrocytomas.

In the *IDH/TP53/ATRX* group, *CDKN2A* loss was associated with worse overall survival after adjusting for age and sex (HR, 3.4; 95% CI, 1.4–8.4; $p = 0.008$) (Fig. 3A; Table 3). *CDKN2A* loss was strongly associated with poor survival in *IDH/TP53* mutated tumors (HR, 4.4; 95% CI, 1.8–10.3; $p = 0.0008$) (Fig. 3B; Table 3), but this association did not reach statistical significance in the *IDH/ATRX* group (HR, 1.6; 95%

TABLE 4. Distribution of *IDH/TP53* Mutation Status and ATRX Loss of Expression in Tumors of Patients From the UCSF AGS

***IDH* Mutated Cases With Both ATRX and *TP53* Data Available (n = 123)**			
ATRX	***TP53***	**n (%)**	**Fisher Exact Test p Value**
Intact	Wild type	51 (41.5)	<0.0001*
Intact	Mutated	9 (7.3)	
Lost	Wild type	21 (17.1)	
Lost	Mutated	42 (34.1)	
Total		123	
Cases With Both *IDH* and ATRX Data Available (n = 259)			
IDH	ATRX	n (%)	Fisher Exact Test p Value
Wild type	Intact	47 (18.1)	0.0017*
Wild type	Lost	14 (5.4)	
Mutated	Intact	108 (41.7)	
Mutated	Lost	90 (34.7)	
Total		259	
Cases With Both *IDH* and ATRX Data Available (Stratified by 1p19q Status)			
IDH	ATRX	n (%)	Fisher Exact Test p Value
1p19q codeleted (n = 87)			
Wild type	Intact	5 (5.7)	0.3
Wild type	Lost	1 (1.1)	
Mutated	Intact	78 (89.7)	
Mutated	Lost	3 (3.4)	
Total		87	
1p19q intact (n = 63)			
Wild type	Intact	16 (25.4)	0.0004*
Wild type	Lost	7 (11.1)	
Mutated	Intact	9 (14.3)	
Mutated	Lost	31 (49.2)	
Total		63	
1p19q not assayed (tumors histologically classified as astrocytoma) (n = 101)			
Wild type	Intact	26 (25.7)	<0.0001*
Wild type	Lost	6 (5.9)	
Mutated	Intact	17 (16.8)	
Mutated	Lost	52 (51.5)	
Total		101	

*Statistical significance at $p = 0.05$.

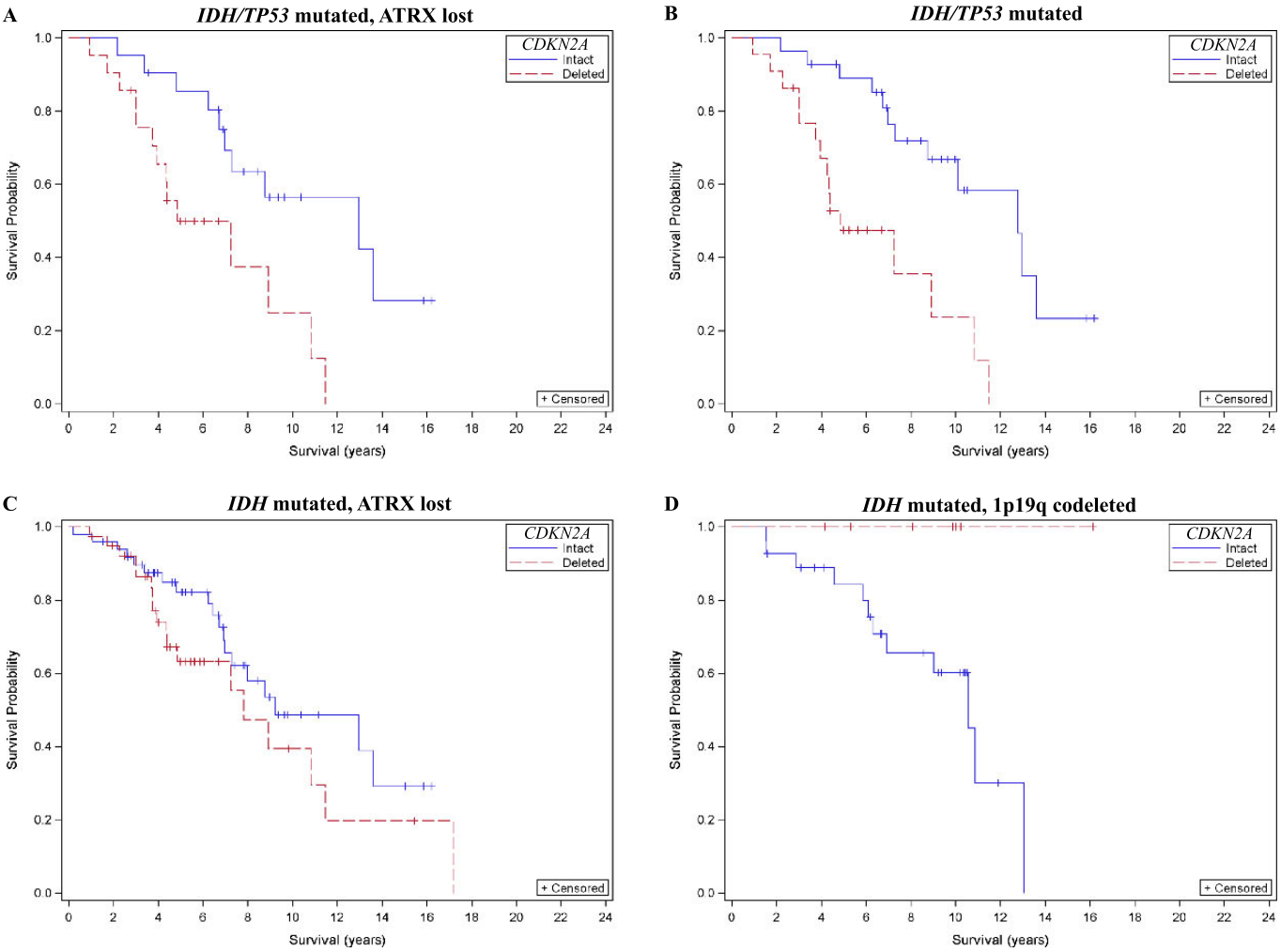

FIGURE 3. Effects of *CDKN2A* loss on overall survival in subsets of molecularly defined astrocytomas and oligodendrogliomas. *CDKN2A* loss by FISH was associated with worse overall survival in *IDH/TP53* mutated tumors with ATRX loss (*IDH/TP53/ATRX* group) **(A)** and *IDH/TP53* mutated tumors (*IDH/TP53* group) **(B)** but not in *IDH* mutated tumors with ATRX loss (*IDH/ATRX* group) **(C)**. **(D)** Patients with molecularly defined oligodendrogliomas in the *CDKN2A* deleted group seemed to have improved survival; however, the statistical significance of the association between *CDKN2A* loss and overall survival could not be accurately assessed because none of the subjects in the *CDKN2A* deleted group died during the follow-up period.

CI, 0.8–3.0; p = 0.2) (Fig. 3C; Table 3). The 2 groups overlapped partially, such that of the 115 *IDH* mutated cases with known status for *CDKN2A*, *TP53*, and ATRX, 42 cases (36.5%) showed ATRX loss and *TP53* mutation, 7 cases (6.1%) showed intact ATRX and mutated *TP53*, and 20 cases (17.4%) showed ATRX loss without *TP53* mutation (Fig. 4). The *IDH/TP53* and *IDH/ATRX* groups contained a similar proportion of Grade II and Grade III tumors. Furthermore, subjects in each group were of nearly identical (mean ± SE) age at the time of diagnosis (*IDH/TP53*, 36.5 ± 1.3 years; *IDH/ATRX*, 36.4 ± 1.2 years). Thus, in both histologically and molecularly defined astrocytoma (in particular, in astrocytomas defined by the presence of *IDH* and *TP53* mutations), *CDKN2A* loss was strongly associated with poor survival after adjusting for age and sex.

With respect to molecularly defined oligodendrogliomas, the effects of *CDKN2A* loss on *IDH* mutated, 1p19q codeleted tumors were examined (Fig. 3D). This group consisted of 16 patients with no deaths (Table 3); therefore, a comparison using Cox proportional hazards regression would not be reliable, and estimates of the association between *CDKN2A* loss and survival in molecular oligodendroglioma could not be determined.

DISCUSSION

This study demonstrates an independent adverse effect of *CDKN2A* loss on the overall survival of patients with lower-grade (WHO Grades II and III) astrocytomas after accounting for *IDH* mutation, age, and sex. Tumors for 270 patients were classified and graded according to WHO criteria by expert neuropathologists, and molecular data for *CDKN2A*, *IDH*, *TP53*, ATRX, and 1p19q were included. Although most patients (61.1%) were alive at censoring, survival analysis yielded unequivocal results with good concordance between histologic and molecular analyses, in particular for the molecular subset of astrocytoma defined by

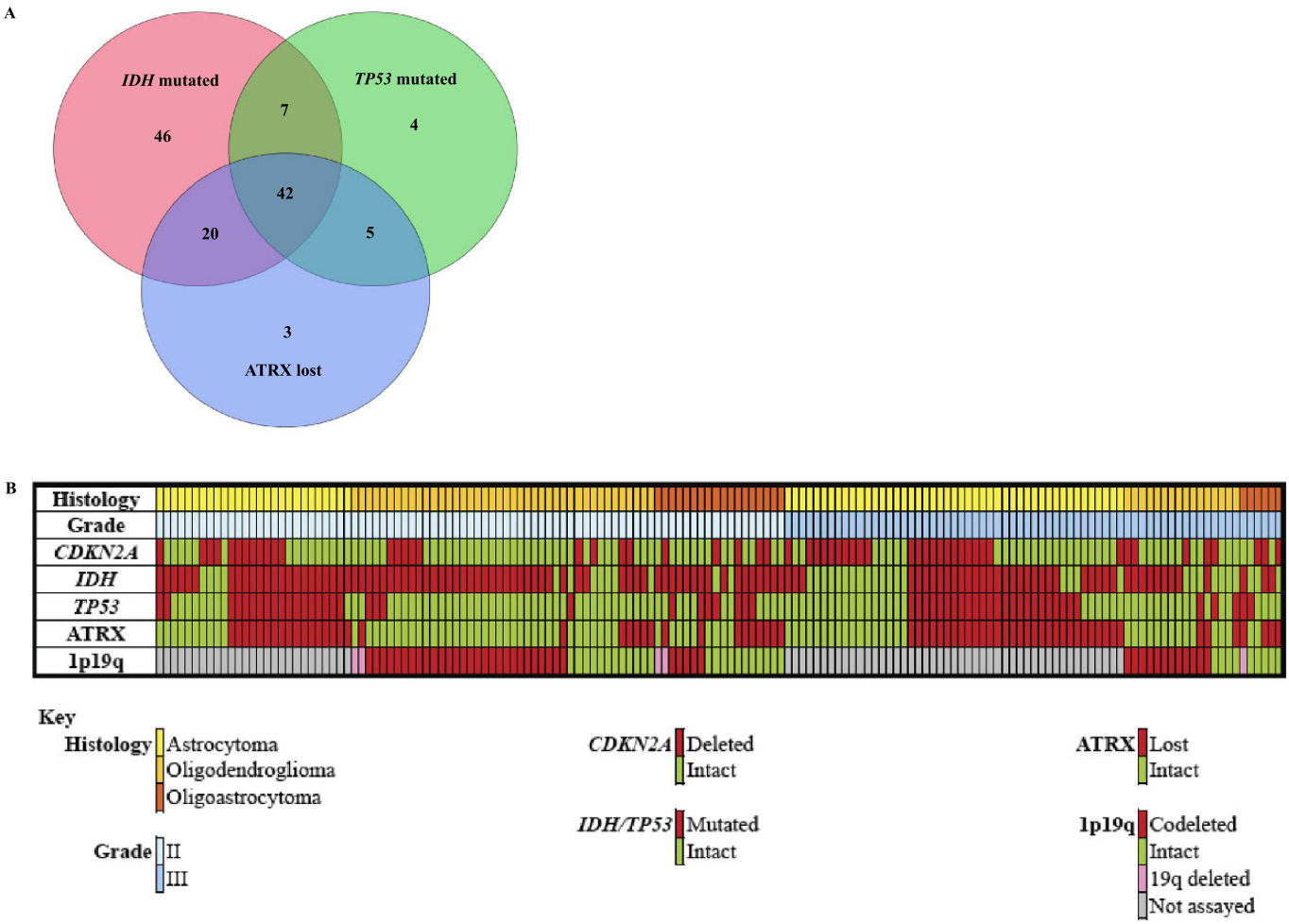

FIGURE 4. Overlap between *IDH/TP53* and *IDH/ATRX* groups. **(A)** The astrocytoma groups defined by *IDH/TP53* mutation (*IDH/TP53* group) or *IDH* mutated tumors with ATRX loss (*IDH/ATRX* group) showed partial overlap such that, among 115 *IDH* mutated cases with known status for *CDKN2A*, *IDH*, *TP53*, and ATRX, 42 cases (36.5%) showed ATRX loss and *TP53* mutation, 7 cases (6.1%) had mutated *TP53* and intact ATRX, and 20 cases (17.4%) had intact ATRX and wild-type *TP53*. **(B)** OncoPrint of molecular parameters grouped by histology and grade.

IDH/TP53 mutation. Unfortunately, a conclusion for molecular oligodendrogliomas could not be reached because of the absence of deaths in the *CDKN2A* deleted group. This analysis will require a follow-up interval longer than 10.9 years (median for the molecular oligodendroglioma subgroup included here).

In previous studies, loss of *CDKN2A* was associated with worse survival in both astrocytomas and oligodendrogliomas (27–33). This study shows that *CDKN2A* loss is associated with worse survival in astrocytomas, but this effect was not observed in oligodendrogliomas. The disagreement between the findings here and those of prior studies may stem from the powerful effect of *IDH* mutation on survival, which was not considered previously because the role of *IDH* mutation in gliomas was not yet known. However, we cannot exclude other factors, such as limited statistical power, in the oligodendroglioma subgroup because there were few deaths among these patients in our cohort.

Most cases of *CDKN2A* loss in infiltrating gliomas are homozygous deletions in the 9p21 region (17–20). Such genomic alterations would be predicted to result in loss of p16 protein expression. Surprisingly, the expression of p16 by IHC correlated poorly with *CDKN2A* deletion by FISH. Several factors could explain this discrepancy. For example, although hemizygous losses are relatively straightforward to detect by FISH, the loss of p16 expression might be minimal and might result in no significant detectable change in p16 expression by IHC. Indeed, in relating our findings to those of The Cancer Genome Atlas lower-grade glioma data set (publically available at http://www.cbioportal.org/), a significant decrease in *CDKN2A* messenger RNA expression was observed only for homozygous deletion of the *CDKN2A* gene but not for hemizygous deletion (Figure, Supplemental Digital Content 5, http://links.lww.com/NEN/A731). Alternatively, *CDKN2A* gene expression may be lost through promoter hypermethylation (42, 43) or point mutations (26), which are seen in a small subset of cases but are undetectable by FISH. If both copies of *CDKN2A* were inactivated by either mechanism, p16 expression by IHC would be expected to decrease, whereas *CDKN2A* status by FISH would be ''intact.''

Regardless of the reasons for the discrepancy, it should be emphasized that the strong association between *CDKN2A* loss by FISH and poor survival in astrocytoma, as opposed to the weak association between p16 loss of expression by IHC and poor survival in astrocytoma, argues strongly in favor of using FISH to evaluate the status of *CDKN2A* when prognosticating astrocytomas.

The analysis of molecular subsets of astrocytomas revealed a strong association between *CDKN2A* loss and poor survival in patients harboring *IDH/TP53* mutations, but a weaker effect was observed in the subset of molecular astrocytomas defined by *IDH* mutation and ATRX loss of expression. The concordance between ATRX loss and *TP53* mutation in our study (76%) was similar to that reported in previous studies on *ATRX* (range, 71%–78%) (8, 10, 44). Although a small fraction of cases with missense mutations (~10%) may be missed by IHC, most mutations in *ATRX* result in loss of protein expression (9, 44). Prior data strongly suggested that biologic differences may exist between the *IDH/TP53* group and the *IDH/ATRX* group (9, 44–46). For example, other pathways of gliomagenesis, including telomerase-dependent mechanisms (47, 48), could predominate in *IDH/TP53* mutated tumors with intact *ATRX*. Alternatively, mutations in other genes involved in the alternative lengthening of telomeres pathway could be present. Future studies focusing on these questions should help clarify this issue.

Whether the combination of *IDH* mutation and *TP53* mutation or the combination of *IDH* mutation and *ATRX* loss of expression should be considered the ''gold standard'' for defining molecular astrocytomas in the clinical setting remains unresolved. The strong association between *CDKN2A* loss and poor survival in *IDH/TP53* astrocytomas reported here relied on the determination of *CDKN2A* status by FISH and *TP53* mutation by sequencing. The poor correlation between *CDKN2A* status by FISH and p16 loss by IHC shows that the assay of choice can play a crucial role. Because molecular information on these markers will be an integral component of standard integrated diagnoses in clinical reports, as recently proposed by the WHO (3), our findings suggest that comparative studies using both sequencing and IHC methods should be conducted to better define the standard of care and to avoid confusion.

In conclusion, our study demonstrates that *CDKN2A* loss is highly prognostic in *IDH/TP53* mutated astrocytomas. Testing for *CDKN2A* loss could be a potentially useful ''grading'' biomarker for this adult glioma subtype.

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