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RESEARCH ARTICLE

Ki-67 and MCM6 labeling indices are correlated with overall survival in anaplastic oligodendroglioma, *IDH1*-mutant and 1p/19q-codeleted: a multicenter study from the French POLA network

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Keywords

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Abstract

Anaplastic oligodendroglioma (AO), *IDH*-mutant and 1p/19q codeleted (*IDH*mut+/1p19qcodelet), is a high-grade glioma with only limited prognostic markers. The primary objective of this study was to evaluate, by immunohistochemistry, the prognostic value of two proliferation markers, MCM6 and Ki-67, in a large series of *IDH*mut+/1p19qcodelet AO included in the POLA (“Prise en charge des Oligodendrogliomes Anaplasiques”) French national multicenter network. We additionally examined the transcriptome obtained from this series to understand the functional pathways dysregulated with the mRNA overexpression of these two markers. The labeling indices (LI) of MCM6 and Ki-67 were obtained via computer-assisted color image analyses on immunostained AO tissues of the cohort (n = 220). Furthermore, a subgroup of AO (n = 68/220) was used to perform transcriptomic analyses. A high LI of either MCM6 (≥50%) or Ki-67 (≥15%) correlated with shorter overall survival, both in univariate ($P = 0.013$ and $P = 0.004$, respectively) and multivariate analyses ($P = 0.027$; multivariate Cox model including age, mitotic index, MCM6 and Ki-67). MCM6 and Ki-67 LI also correlated with overall survival in an additional retrospective cohort of 30 grade II *IDH*mut+/1p19qcodelet oligodendrogliomas. The prognostic value of *MCM6* mRNA level was confirmed in The Cancer Genome Atlas (TCGA) *IDH*mut+/1p19qcodelet gliomas. The transcriptomic approach revealed that high transcriptional expressions of *MCM6* and *MKI67* were both linked positively with cell cycle progression, DNA replication, mitosis, pro-neural phenotype as well as neurogenesis, and negatively with microglial cell activation, immune response, positive regulation of myelination, oligodendrocyte development, beta-amyloid binding and postsynaptic specialization. In conclusion, the overexpression of MCM6 and/or Ki-67 is independently associated to shorter overall survival in *IDH*mut+/1p19qcodelet AO. These two easy-to-use and cost-effective markers could thus be used concurrently in routine pathology practice. Additionally, the transcriptomic analyses showed that AO with high proliferation index have down-regulated immune response and lower microglial cells activation, and bears pro-neural phenotype.

INTRODUCTION

According to the World Health Organization (WHO), *IDH*-mutant and 1p/19q-codeleted (*IDH*mut+/1p19qcodelet) anaplastic oligodendrogliomas (AOs) are high-grade gliomas, corresponding histologically to WHO grade III primary brain tumors (35). AO are rare, accounting for 0.5% of all primary brain tumors and one-third of oligodendroglial tumors (38). They are histologically defined as tumors of the oligodendroglial cells, with features of anaplasia, including increased cellularity and mitotic activity, microvascular proliferation and/or necrosis. Although Ki-67 labeling index (LI) is usually used to help differentiate low-grade from high-grade oligodendroglioma, no clear cut-off point has been established (12). By definition, and according to the 2016 update of the WHO Classification of Central Nervous System, AO carry both *IDH1/2* mutations and 1p/19q codeletion (10, 43). The standard of care for these tumors includes surgical resection in conjunction with adjuvant therapies like combined adjuvant radiochemotherapy with procarbazine, CCNU (lomustine), and vincristine (RT-PCV) to improve the overall survival (OS) (46).

The prognostic role of the WHO grading in *IDH*-mutant gliomas remains debatable, and this is particularly true in the case of oligodendrogliomas (43). In AO patients, young age, high Karnofsky index/clinical performance status, complete surgical resection and adjuvant therapy such as RT-PCV have been shown to associate with longer OS (45, 46). Histological features such as microvascular proliferation, mitotic index (MI) and necrosis have been found associated with three distinct prognostic subgroups of *IDH*-mut+/1p19qcodelet AO with other genomic alterations (19). These include mutations of *CIC* and *TCF12*, both linked to more aggressive AO (4, 22, 32), and allelic loss of 9p21.3, associated with shorter progression-free survival (PFS) and shorter OS in *IDH*-mut+/1p19qcodelet AO (2). Omics approaches using transcriptome, genome and methylome have also been used to identify potential prognostic factors. One such study has successfully classified oligodendroglial tumors into three molecular subgroups with distinct clinical behaviors (29). Machine learning algorithms aiming to identify copy number variations have also been applied recently to AO samples; such technique seems promising in helping with prognostic stratification (41). Despite of these advances, however, it is worth noting that all the above-mentioned markers of AO need expansive and time-consuming molecular analyses, and thus cannot be widely applied to all samples in all laboratories.

In contrast, immunohistochemical proliferation markers such as Ki-67, whose LI correlates strongly with prognosis of many tumor types, are simple, effective and economical means to assess tumor aggressivity. In the case of AO, past few evidence obtained using univariate—but not multivariate—analysis appeared to support the prognostic value of Ki-67 LI (40). However, as less than 50% of the AO tissues in this latter study (performed prior to the 2016 revision of the WHO classification) actually harbored *IDH* mutations and 1p/19q codeletion, its validity shall be questioned. In a more recent study, Zeng *et al* evaluated the prognostic

value of Ki-67 upon classifying gliomas into subgroups with either the presence or the absence of *IDH1/2*; however, data concerning 1p/19q values codeletion were lacking in the studied gliomas (49).

Several proliferation markers other than Ki-67 have also been studied in brain tumors. Notably, we previously reported that Minichromosome Maintenance Complex component 6 (MCM6) was overexpressed in meningioma, and was associated with higher histological grade and risk of recurrence (21). In adamantinomatous craniopharyngiomas, MCM6 correlated with a higher risk of long-term recurrence (48). In gliomas, especially in glioblastomas of the Chinese Cancer Genome Atlas (CCGA), *MCM6* mRNA overexpression was also reported to be correlated with poor overall survival (9).

Minichromosome Maintenance proteins (MCMs) play a key role in DNA synthesis and replication, forming a hexameric helicase complex around the DNA (31). All MCMs (MCM2-7) are detectable during the different phases of the cell cycle, including G1, S, G2 and M, but are absent in G0 (33). These proteins are also expressed earlier during G1, in comparison with Ki-67. In other solid tumors, like non-small cell lung carcinomas (47), hepatocellular carcinomas (34), endometrial carcinomas (26), low-grade chondrosarcomas (24) and mantle cell lymphomas (42), a high MCM6 LI correlated with a worse prognosis. To our knowledge since the revision of the 4th WHO classification of 2016, MCMs, and notably MCM6, have never been specifically studied in *IDH*mut+/1p19qcodelet AO.

The primary goal of this study was therefore to evaluate and compare, by immunohistochemistry, the prognostic value of MCM6 and Ki-67 in a large series of *IDH*mut+/1p19qcodelet AO obtained from the POLA (“Prise en charge des Oligodendrogliomes Anaplasiques”) French national multicenter network. We additionally examined the transcriptomes obtained from part of this series to understand the functional pathways dysregulated with the mRNA overexpression of these two markers.

MATERIAL AND METHODS

Population and clinicopathological data

Two hundred and thirty-one cases of *IDH*mut+/1p19qcodelet AO were retrieved from the French national multicenter POLA cohort. Clinical data, such as age, sex, extent of surgical removal, type of adjuvant treatment and OS are available in the database, as well as molecular data, including *IDH* mutation status and presence of 1p/19q codeletion. All cases included in this cohort have been centrally reviewed by the neuropathologists of the national board of French national POLA network and were classified according to the 2016 4th WHO classification update. The MI and Ki-67 LI were evaluated in whole tissue sections, as previously described (18). In 220 out of 231 patients, tissue microarray (TMA) blocks were designed with representative samples of tumors, in order to perform immunohistochemical analyses. One to three spots were available for each case.

Additionally, in order to evaluate the prognostic value of MCM6 and Ki-67 LI in low-grade tumors, 30 cases of grade II IDHmut+/1p19qcode1 oligodendrogliomas (IDHmut+/1p19qcode1 OII) were retrieved from the files of the Department of Neuropathology at Pitié-Salpêtrière Hospital (AP-HP, Paris; OncoNeuroTek database; Pitié-Salpêtrière) and the Department of Pathology at Nancy University Hospital (CHRU, Nancy; Centre de Ressources Biologiques, BB-0033-00035).

Ethics

Anonymity was strictly respected, according to the principles of the declaration of Helsinki and national ethical guidelines. Patients consent for clinical data collection and genetic analyses have been obtained prospectively, according to POLA network policies. The study was approved by the ethics committee of Hôpital Universitaire la Pitié-Salpêtrière.

Immunohistochemistry

Paraffin sections of 5 µm thickness were immersed in a 10mM sodium citrate buffer (pH 6) for 20 minutes at 97°C for dewaxing and antigen retrieval. The following primary antibodies were used: MCM6 (1/400; goat polyclonal, Santa Cruz Biotechnology, Heidelberg, Germany), Ki-67 (1/200; mouse monoclonal, MIB-1, Dako Cytomation, Glostrup, Denmark).

Immunohistochemistry was performed with Dako Autostainer Plus (Dako) and the Flex + Envision revelation system (Dako).

Ki-67 and MCM6 evaluation

Ki-67 and MCM6 labeling indices (LI) were defined as the percentage of cells with positive nuclear stain of the two individual markers, independently from the signal intensity. For each analyzed TMA spot, the field with the strongest immunostaining was selected by the observer, blinded to the clinical data and outcome of the respective patients. To limit inter-observer variability, cell counting was performed with a computerized color image analyzer (Olympus Cellsens Dimension, Olympus Medical System and Micro-Imaging Group, Hamburg, Germany). A x20 objective was used to take one microphotograph of the area of interest. A minimal object size of 50 pixels was required to count positive nuclei. Vessels and microcalcifications were excluded from the analysis. The percentage of positive and negative cells were automatically computed by the color image analyzer. A mean LI was calculated for each case. Additionally, in order to validate this computer-based LI, manual Ki-67 and MCM6 LI were evaluated in 50 randomly selected cases, by counting 1000 cells in TMA spots.

Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics for Windows, Version 23.0 (IBM Corp., Armonk, NY, USA).

Because the quantitative variables did not pass the Kolmogorov–Smirnov normality test, non-parametric tests

were used. Spearman correlation test was used to explore the correlation between Ki-67 LI, MCM6 LI and the MI. The concordance between image analysis and manual LI was evaluated with the intraclass correlation coefficient. The correlation between MCM6 and Ki-67 LI and WHO grade was evaluated with the Mann–Whitney Wilcoxon test. Overall survival (OS) analyses were performed with Kaplan–Meier estimation (log-rank test) and the Cox model using univariate and multivariate analyses. For Ki-67 and MCM6 LI, the optimal threshold was computed using the Cutoff Finder online tool (8), in order to separate patients in two groups. For Cox multivariate analyses, only variables which were significant on univariate analyses and WHO grade were integrated into the model. Survival analyses were performed in the POLA cohort of IDHmut+/1p19qcode1 AO, then in the IDHmut+/1p19qcode1 OII cohort. A *P*-value (*P*) of less than 0.05 was considered statistically significant.

Transcriptomic analyses

POLA cohort: Gene expression data from POLA (29) were downloaded from <http://gliovis.bioinfo.cnio.es/> under the “Kamoun” and “POLA Network” tags. From these publicly available data, we identified 68 samples satisfying the following phenotypic criteria: histology = oligodendroglioma, grade = III (anaplastic), co-deletion 1p-19q status = yes, IDH1 or IDH2 status = mutant. These data were normalized (lowess algorithm) against a median profile of all 68 samples and further underwent descriptive statistics, which helped define groups of samples before proceeding with downstream analyses. Hence, all samples were individually labeled MCM6- and MKI67-up or -down based on their relative MCM6 and MKI67 expression (Supplementary Figure S1).

K-means clustering, functional annotations, enrichments computations, differential expression statistics and renderings were achieved using protocols and tools as described before (20). Further hierarchical clustering was applied for each significant k-means cluster to evaluate the level of correlation and quickly delineate gene or sample outliers in each gene signature.

Differential expression *P*-values were computed on normalized data using a two-way t-test between both MCM6 and MKI67 up and down groups. These were adjusted to allow for false discovery rate (FDR) using the Benjamini–Hochberg procedure and FDR < 0.01 was considered to indicate statistical significance.

Linear correlations between MCM6 and MKI67 expression levels were performed with Pearson’s method, while Spearman’s rank correlation coefficients were used to explore the dependence between both MCM6 and MKI67 gene expressions with their respective MCM6 and Ki-67 immunohistochemical LI.

TCGA cohort: Additionally, in order to evaluate the results obtained on the POLA cohort, further analyses were run on a subset of The Cancer Genome Atlas Lower Grade Glioma cohort (TCGA-LGG). From the available RNA-Seq processed data (FPKM-UQ normalized counts), 98 samples corresponded to unique cases with the following criteria:

IDH1 and/or IDH2 mutated, presence of the 1p-19q codeletion, and clearly clinically labeled as grade III and/or Anaplastic OD (35 samples) or grade II OD (63 samples). The original 60483 gene Ensembl ID were re-annotated (using <https://biotools.fr>) and their expression values were aggregated to obtain a set of unique official gene symbols. Data were then filtered to remove genes with very low expression values across samples (FPKM-UQ < 10 in more than 9/10th of the samples), which left 16345 genes. Values were re-normalized according to library size and log₂ transformed. Two datasets were then created, one set containing the 35 grade III samples, with the aim to conduct validation analyses of the POLA high-grade OD transcriptomics, and another set with the entire selected TCGA cohort of 98 grade II + grade III samples for extending the results to lower grade OD. These two data sets finally underwent the same unsupervised workflow as described above (sample ordering according to *MCM6* mRNA expression levels, k-means clustering, functional annotations, enrichment analyses).

RESULTS

Clinicopathological data

The mean age of the patients of the POLA cohort was 49 years (range 19–80) (Table 1), with a male-to-female ratio of 1.22:1. A large majority of patients had surgery first, with 33% (72/220) of them showing macroscopic total resection, 31% (68/220) subtotal resection and 21% (46/220) partial resection. As adjuvant therapy, 32% (71/220) received RT-PCV therapy and 21% (46/220) combined radiotherapy with Temozolomide (Stupp Protocol). For 29% (64/220) of them, adjuvant radiotherapy was chosen. Fourteen patients did not receive any treatment (6%; 14/220). Follow-up data were available for 220 patients and were collected over a median follow-up of 40.9 (0.3–92) months. Disease-related death occurred in 9% (19/220) of patients. Using univariate analyses, age was the only variable significantly associated with survival ($P = 0.001$; HR: 1.055 [95%CI: 1.021–1.091]) (Table 2). A high MI (at least eight mitoses per 1.6 mm²) was significantly associated with a shorter OS ($P = 0.018$; HR: 2.587 [95%CI: 1.177–5.686]). No significant correlation between OS and microvascular proliferation ($P = 0.511$; HR: 1.493 [95%CI: 0.451–4.939]) or necrosis ($P = 0.192$; HR: 1.648 [95%CI: 0.778–3.492]) was found.

In the grade II series, mean age was 44 years (range 18–81), with a male-to-female ratio of 1.21:1. Disease-related death occurred in 13% (4/30) of patients, with a median follow-up of 38.7 (2.4–79.9) months. When combining the two cohorts, WHO grade was not correlated to OS ($P = 0.642$).

MCM6 labeling index and correlation with survival

MCM6 staining was interpretable in 94% (206/220) of the cases with an average count of 1383 cells per case. Image

Table 1. Clinico-pathological characteristics and molecular data.

Variable	Results
Age (mean; min-max)	49; 19–80 years
Sex	Male-to-female ratio: 1.22:1 (121/99)
Surgery	Biopsy: 4.1% (9/220) Total resection: 32.7% (72/220) Subtotal resection 30.9% (68/220) Partial resection 20.9% (46/220) Missing data 11.4% (25/220)
Type of treatment	RT-PCV: 32.3% (71/220) Radiotherapy: 29.1% (64/220) PCV: 2.3% (5/220) Stupp protocol: 20.9% (46/220) Temozolomide: 4.1% (9/220) Other: 0.5% (1/220) No treatment: 6.4% (14/220) Missing data: 3.6% (8/220)
Survival	Progression: 30.7% (71/220) Death: 8.2% (19/220)
Molecular data	<i>TERT</i> promoter mutation: 98.3% (216/220) <i>CIC</i> loss: 61% (141/220)

RT-PCV = radiation therapy – procarbazine, CCNU (lomustine), and vincristine.

analysis-based LI was strongly correlated to manual counting (ICC: 0.864; 95%CI [0.772–0.920]). Mean MCM6 LI was 24% (range 0.1–87%; median 21.4; standard deviation 18.8) (Figure 1A,B). MCM6 LI was significantly correlated to MI ($\rho = 0.253$; $P < 0.0001$).

In the POLA cohort, log-rank survival analyses showed that patients with high MCM6 expression (upper than 50%) had significantly shorter overall survival (OS) ($P = 0.013$) (Figure 2A). Using univariate Cox model, MCM6 expression correlated inversely with OS ($P = 0.018$; HR: 3.28; 95%CI [1.22–8.83]) (Table 2). In the group of patients with grade II oligodendroglioma, a high MCM6 LI was also correlated to a shorter OS ($P = 0.001$) (Figure 3A). MCM6 LI was not significantly higher in grade III than in grade II oligodendrogliomas (23% vs. 18%; $P = 0.232$).

Ki-67 labeling index and correlation with survival

For Ki-67 LI, 91% (199/220) of the cases were interpretable. We found a high reproducibility between image analysis and manual counting (ICC: 0.900; 95%CI [0.827–0.943]). Ki-67 LI evaluated by image analysis was also strongly correlated to the Ki-67 LI previously obtained from whole slides ($\rho = 0.553$; $P < 0.0001$). The mean number of cells counted for each TMA was 1121. The mean Ki-67 LI was 6.3% (range 0.1–36.9; median 3.7; standard deviation 6.7) (Figure 1C,D). Ki-67 LI was significantly correlated to MI ($\rho = 0.275$; $P < 0.0001$) and MCM6 LI ($\rho = 0.449$; $P < 0.0001$).

The log-rank test and the univariate regression Cox model revealed that a Ki-67 LI equal to or greater than 15% was

Table 2. Univariate and multivariate Cox analyses for overall survival.

Variable	Cox univariate (OS)		Cox multivariate (OS) <i>Model 1</i>		Cox multivariate (OS) <i>Model 2</i>	
	HR [95%CI]	<i>P</i> -value	HR [95%CI]	<i>P</i> -value	HR [95%CI]	<i>P</i> -value
Age	1.055 [1.021–1.091]	0.001*	1.060 [1.020–1.103]	0.003*	1.051 [1.011–1.092]	0.012*
Mitotic index $\geq 8/1.6 \text{ mm}^2$	2.587 [1.177–5.686]	0.018*	1.439 [0.538–3.851]	0.469	1.588 [0.606–4.162]	0.347
MCM6 LI $\geq 50\%$	3.283 [1.221–8.826]	0.018*	2.896 [0.964–8.702]	0.058		
Ki-67 LI $\geq 15\%$	3.948 [1.442–10.41]	0.008*	2.713 [0.935–7.875]	0.066		
MCM6 LI $\geq 50\%$ and/or Ki-67 LI $\geq 15\%$	3.875 [1.603–9.370]	0.003*			2.872 [1.125–7.328]	0.027*

HR = hazard ratio; LI = labeling index; OS = overall survival.

*Statistically significant ($P < 0.05$).

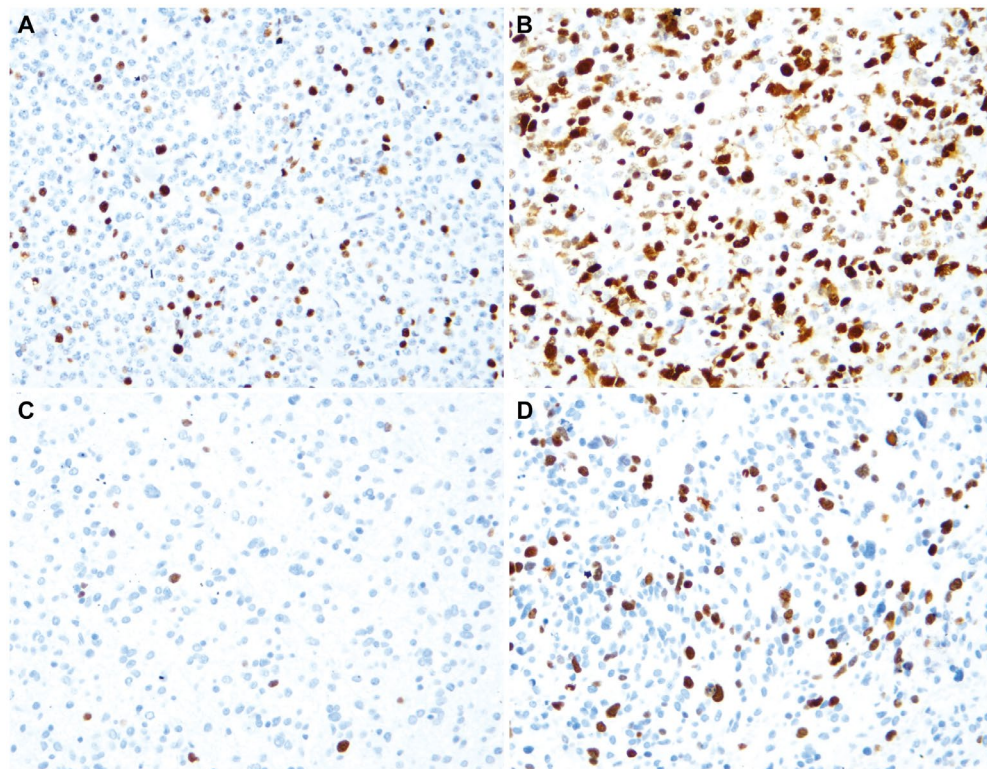


Figure 1. Immunolabeling for MCM6 and Ki-67 (immunohistochemistry, $\times 200$). A. Low MCM6 labeling index (LI). B. High MCM6 LI. C. Low Ki-67 LI. D. High Ki-67 LI (same case as B).

correlated with a shorter OS (log-rank: $P = 0.004$; Cox: $P = 0.008$; HR = 3.948; 95%CI [1.442–10.41]) (Figure 2B; Table 2). Similarly, in the group of patients with grade II oligodendroglioma, a high Ki-67 LI was correlated to shorter OS ($P = 0.027$; log-rank test) (Figure 3B). Ki-67 LI was significantly higher in grade III than in grade II tumors (6% vs. 3%; $P = 0.001$).

Multivariate survival analyses

The variables that were significant in univariate analyses (age, MI $\geq 8/1.6 \text{ m}^2$, MCM6 LI $\geq 50\%$, Ki-67 LI $\geq 15\%$) were included in the first multivariate Cox model. Only age remained significantly correlated to OS ($P = 0.003$), whereas

MCM6 and Ki-67 LI were close to significance ($P = 0.058$ and $P = 0.066$, respectively) (Table 2).

In order to evaluate if MCM6 could be an interesting marker to use in complement to Ki-67, we designed a second Cox multivariate model, with the variable “MCM6 LI $\geq 50\%$ and/or Ki-67 LI $\geq 15\%$ ”, that was strongly significant in univariate analysis (log-rank: $P = 0.001$; Cox: $P = 0.003$; HR: 3.875; 95%CI [1.603–9.370]). With this model, age and “MCM6 LI $\geq 50\%$ and/or Ki-67 LI $\geq 15\%$ ” were significantly correlated to OS ($P = 0.012$ and $P = 0.027$, respectively), but not MI ($P = 0.347$) (Figure 2C; Table 2).

Additionally, we evaluated the prognostic value of MCM6 and Ki-67 LI in all cases of IDHmut+/1p19qcodelet oligodendrogliomas, including grade II and III tumors. In a

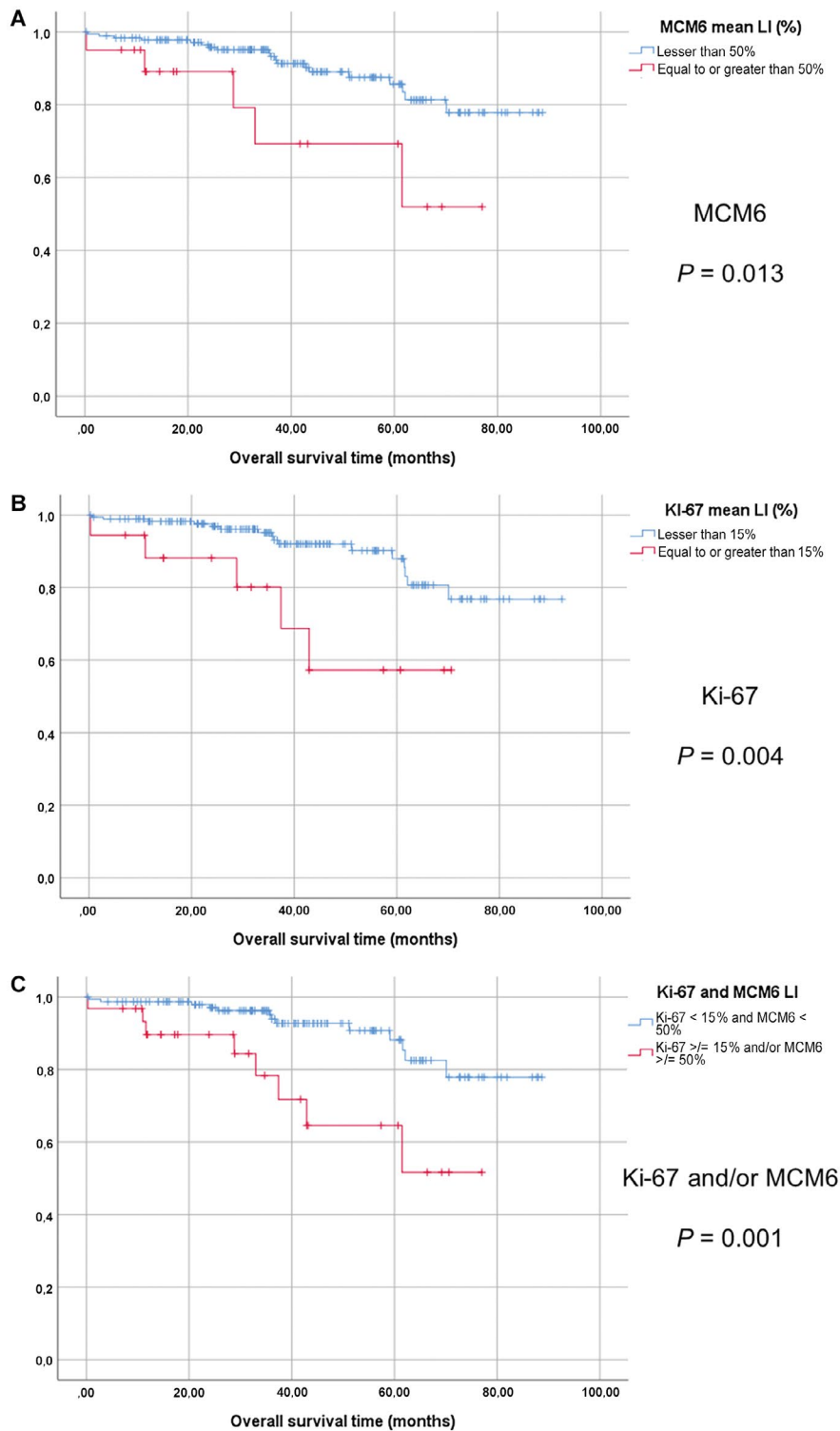


Figure 2. Survival analyses in the POLA cohort of anaplastic oligodendrogliomas. Kaplan-Meier curves with log-rank tests (overall survival). A. MCM6 labeling index, 50% threshold (LI). B. Ki-67 LI, 15% threshold. C. MCM6 LI \geq 50% and/or Ki-67 LI \geq 15% vs. MCM6 $<$ 50% and Ki-67 LI $<$ 15%.

multivariate model including age, MI, WHO grade and the two proliferation markers, we found that age ($P = 0.022$) and MCM6 LI \geq 50% and/or Ki-67 LI \geq 15% ($P = 0.001$;

HR: 4.148; 95%CI [1.735–9.920] were significantly associated with OS, but not WHO grade ($P = 0.108$) or MI ($P = 0.637$).

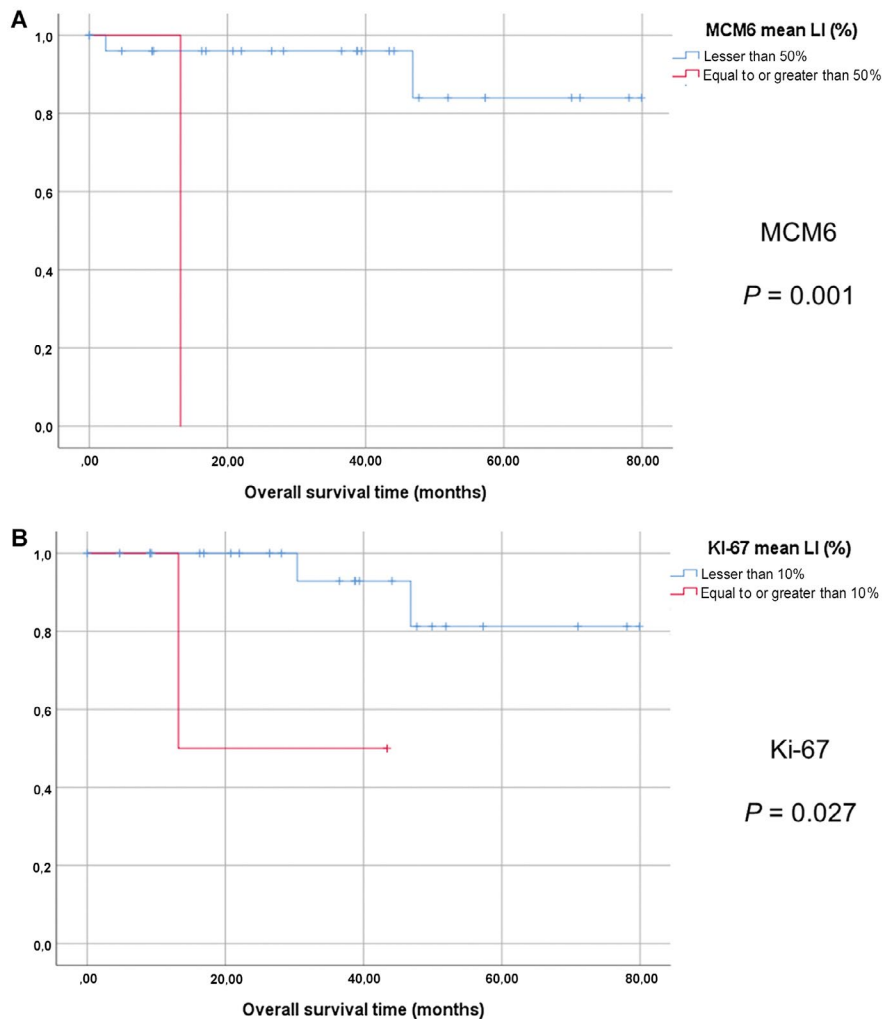


Figure 3. Survival analyses the cohort of grade II oligodendrogliomas. Kaplan-Meier curves with log-rank tests (overall survival). A. MCM6 labeling index, 50% threshold (LI). B. Ki-67 LI, 10% threshold.

Transcriptomics in the POLA cohort

For both *MCM6* and *MKI67*, we expected positive correlations between their LI and mRNA level in AO cases for which both transcriptomic data and LI were available. The Spearman tests indeed confirmed such correlations ($\rho = 0.43$, $P = 0.003$, $n = 46$; and $\rho = 0.63$, $P = 4.7 \times 10^{-5}$, $n = 38$, respectively) (Supplementary Figure S2). A highly positive correlation was also found between the mRNA level of *MCM6* and that of the *MKI67* in the AO transcriptomes of the POLA network ($n = 68$; Pearson’s test: 0.69; $P = 5.6 \times 10^{-11}$) (Supplementary Figure S3).

Molecular events associated with differential expressions of either *MCM6* or *MKI67* in these AO samples were subsequently analyzed ($n = 68$). This was achieved using the median expression levels for *MCM6* and *MKI67* as “up” and “down” subgroup delimiters (Supplementary Figure S1) in two separate analyses based on the relative expressions of *MCM6* and *MKI67*. When the median *MCM6* mRNA level was used to organize the 68 AO samples, 34 were classified

and labeled as *MCM6*-down (sample expression < median expression), and the remaining 34 as *MCM6*-up (expression > median). The same was applied for *MKI67*. In these two analyses, k-means clustering coupled with a distance computation based on the weighted correlation method, permitted the identification of clusters with distinct molecular functions differentiating the “up” from the “down” *MCM6* or *MKI67*-expresser AO (Figure 4). Remarkably, these *MCM6*- and *MKI67* gradient-driven clusterings resulted in individual clusters sharing high level of identity, as the clusters produced between the *MCM6* and *MKI67* series of experiments overlapped with each other (ranging from 85% to 99%, the most significant clusters presenting with the greatest identity) (Figure 4). Moreover, among the 10 clusters identified in each series (from c0 to c9), 2 clusters of upregulated genes (c3 and c7) and 3 clusters of downregulated genes (c1, c2 and c6) were highly conserved between the two series (98%, 99%, 97%, 97%, 96%, respectively; Figure 4). Further functional annotation of the up- and downregulated clusters revealed that tumors expressing higher levels of *MCM6* and *MKI67* were

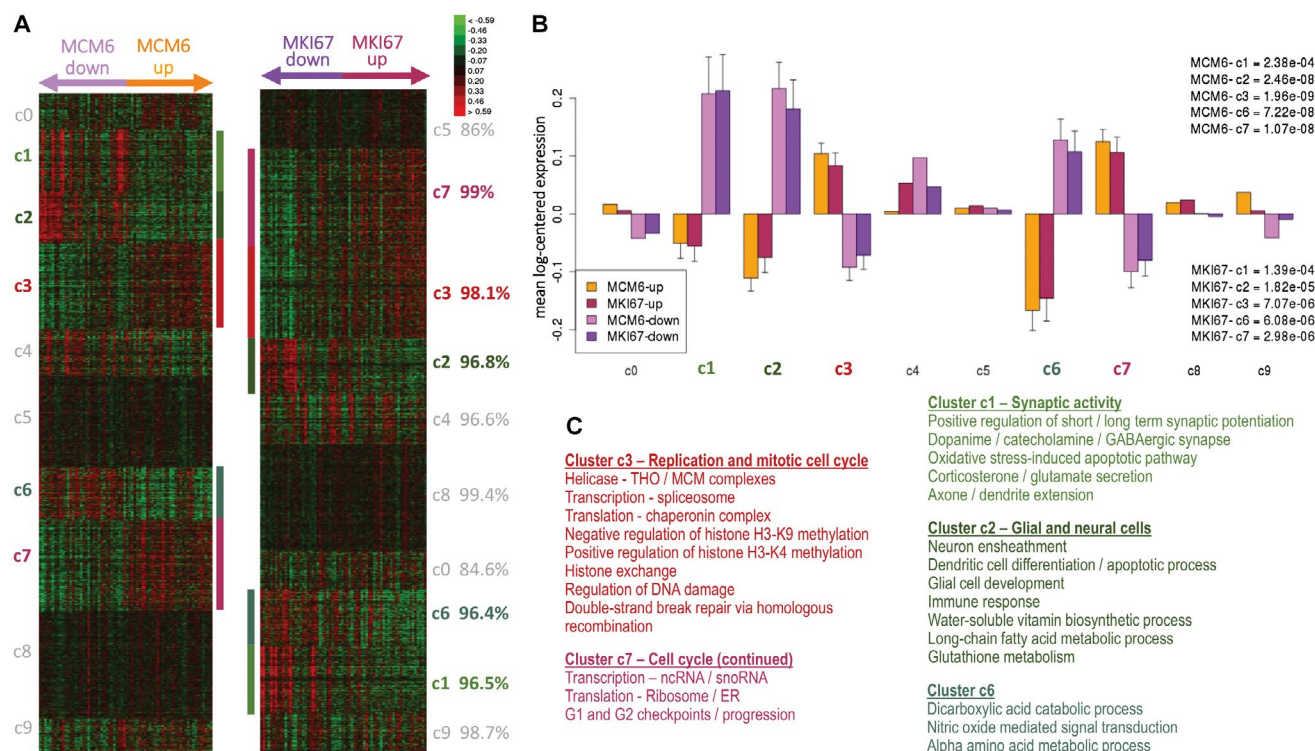


Figure 4. K-means clustering from MCM6 and MKI67 expression gradients (from lowest to highest) of 68 transcriptomes of anaplastic oligodendrogliomas from the POLA network. The two independent K-means clustering each delineated five significant groups of co-expressed genes ($P < 0.01$) showing highly correlated expression profiles. **A.** Heatmap of the ten identified k-means clusters in the two clustering results, and lowest identity (in %) found for each cluster (green, red and black indicate downregulated, upregulated, and median genes, respectively). **B.** Mean gene expression (overall with standard

error at the mean) for each cluster in MCM6 and MKI67 analyses where both upregulated samples for MCM6 or MKI67 are compared to their downregulated counterparts (AU – arbitrary units). Upregulated and downregulated samples are defined relatively to the median gene expression for either MCM6 or MKI67. **C.** Overview of the most relevant functional annotations for the significantly over- and under-expressed clusters (in red and green hues, respectively, all $p(FDR) \leq 0.01$).

also enriched with genes controlling replication and cell cycle (c3 and c7), but downregulated with genes associated with synaptic activity (c1), neuron ensheathment, glial cell development, immune and inflammatory response, vitamin biosynthetic process (c2), and alpha amino acid metabolic process (c6) ($FDR < 0.0001$) (Supplementary Table S1).

Additionally, we explored differential expression statistics (two-sided t-test) between MCM6-up and MCM6-down, and between MKI67-up and MKI67-down samples. Four significant gene lists were produced (all $FDR < 0.01$), one list for over- and one for under-expressed genes in MCM6-up samples (871 and 461 genes; fold changes $> 4/3$ and $< 3/4$, respectively), and likewise for MKI67-up samples (352 and 237 genes, respectively) (Supplementary Table S2). MCM6 overexpressed samples presented with a significant enrichment in genes involved in cell cycle functions (Table 3), including most notably DNA replication, mitotic centrosome separation and mitotic chromosome condensation and genes involved in internal ribosome entry site (IRES) dependent translational initiation. Tumors harboring lower MCM6 expression showed significant upregulation of pathways involved in glial differentiation and immune response, such

as microglial cell activation, myelination, oligodendrocyte development and oligodendrocyte differentiation. Similarly, tumors with higher expression of MKI67 (Table 4) were enriched in genes involved with cell cycle functions such as DNA strand elongation in DNA replication during cell cycle, pre-replicative complex assembly, mitotic chromosome condensation and MCM complex. Tumors with lower MKI67 expression were significantly enriched in genes involved in beta-amyloid binding, synaptic vesicle, axon terminus, trans-synaptic signaling, neuronal cell body and cell projection.

When focusing on genes associated with a pro-neural signature, such as SOX2, SOX4, SOX11, OLIG1, OLIG2, INSM1, FERMT1, DCX, CTTNBP2, ATOH8 and ASCL1, we found a significant overexpression of these genes in tumors showing higher levels of MCM6 and MKI67 ($FDR < 0.01$) (Supplementary Figure S4A). Surprisingly, a number of genes involved in immunological responses were significantly downregulated in these tumors (CX3CRI, TLR4, SYK, FAS, HSPA2, CEBPB, TRIM59, ITGB5, PLP1, C3AR1, PREX1, MND, GAB2, VAMP3; $FDR < 0.01$) (Supplementary Figure S4B). Genes controlling the production of molecular mediators involved in inflammatory response were more significantly

Table 3. Top 12 Gene Ontology functional annotations associated with the expression of MCM6 (sorted by fold enrichment).

GO biological process	Fold enrichment	FDR
<i>Enrichment in tumors showing MCM6 higher expression</i>		
Cell cycle DNA replication initiation (GO:1902292)	22.1	<0.0001
DNA strand elongation involved in cell cycle DNA replication (GO:1902296)	21.7	0.0001
Ndc80 complex (GO:31262)	21.7	0.0001
Pre-replicative complex assembly (GO:36388)	21.3	<0.0001
Condensed nuclear chromosome kinetochore (GO:778)	15.2	<0.0001
Mitotic centrosome separation (GO:7100)	15.2	0.001
Mitotic chromosome condensation (GO:7076)	14.8	<0.0001
IRES-dependent translational initiation (GO:2192)	14.6	0.0001
Aster (GO:5818)	13.8	0.0001
Exodeoxyribonuclease activity (GO:4529)	12.6	0.003
Mitotic prophase (GO:88)	12.1	<0.0001
Regulation of transcription involved in G1/S transition (GO:83)	11.9	<0.0001
<i>Enrichment in tumors showing MCM6 lower expression</i>		
Myelin sheath adaxonal region (GO:35749)	11.2	0.009
Negative regulation of inclusion body assembly (GO:90084)	8.7	0.02
Sodium ion export from cell (GO:36376)	8.7	0.02
Positive regulation of organic acid transport (GO:32892)	8.2	0.02
Microglial cell activation (GO:1774)	8.4	<0.0001
Macrophage activation involved in immune response (GO:2281)	8.2	0.009
Central nervous system myelination (GO:22010)	7.6	0.002
Positive regulation of myelination (GO:31643)	6.5	0.02
Main axon (GO:44304)	4.6	<0.0001
Proteoglycan binding (GO:43394)	4.5	0.01
Oligodendrocyte development (GO:14003)	4.2	0.005
Positive regulation of glial cell differentiation (GO:45687)	4.0	0.02

FDR = false discovery rate; GO = gene ontology.

downregulated with *MCM6*-up than *MKI67*-up (*EPHX2*, *APOD*, *VAMP3*, *SYK*, *ALOX5*, *DUSP10*, *SNX6*, *ADORA3*, *ALOX5AP*, *TNF*, *LYN*, *BTK*, *FCER1G*, *IL17RA* and the pro-inflammatory mediator *IL17D*; FDR < 0.01) (Supplementary Table S2).

Other gene signatures were also found to overexpress with *MCM6* and *MKI67* high transcription, such as epigenetic markers (*DNMT1A*, *EZH2*, *TYMS*, *DHFR*; FDR < 0.01) and cell cycle progression key protagonists (all *MCMs*, *PCNA*, *CCNBI*, *CCND1*, *CDK1*, *CDK4*; FDR < 0.01), whereas myelination and glial cell differentiation genes were under-expressed with *MCM6* (FDR < 0.01) but less clearly with *MKI67* high levels, which is in accordance with the functional annotations reported above (Supplementary Figures S4B,C,E,F). Strongly upregulated KEGG pathways were all associated with an *MCM6* or *MKI67* overexpression (ribosome, RNA transport, splicing and degradation, DNA replication and repair, P53 signaling; *q*-value < 0.05). Both *MCM6*-up and *MKI67*-up sets of significantly disturbed pathways were nearly identical, with cell cycle controlling genes showing the same overexpression patterns (Supplementary Figure S5).

Confirmation analyses based on TCGA data

To validate the observations made on the POLA cohort, we ran a validation analysis with 98 similar cases identified in TCGA (The Cancer Genome Atlas) database. These were all identified as IDH1/IDH2 codeleted OD cases and included

35 fully annotated grade III and 63 grade II samples. Our survival analyses of the 98 TCGA cases showed that similar to that observed in our own grade II/III cohort, high levels of *MCM6* mRNA (equal to or greater than the median expression; arbitrary unit) indeed correlated to a shorter OS ($P = 0.027$) (Supplementary Figure S6A). However, the mRNA level of *MKI67* in the TCGA samples did not correlate with OS ($P = 0.398$) (Supplementary Figure S6B). WHO grade did not correlate to survival ($P = 0.617$) (Supplementary Figure S6C). Only age was independently correlated to survival (Cox multivariate analysis, $P = 0.007$). *MCM6* and *MKI67* mRNA levels were strongly correlated ($\rho = 0.623$; $P < 0.0001$). No significant correlation existed between the WHO grade and the mRNA level of either *MCM6* or *MKI67* ($P > 0.50$).

We also compared the transcriptomic signatures obtained with anaplastic POLA samples with the 35 grade III independent cases retrieved from TCGA (Supplementary Figure S7). We did so by conducting the analyses with the same unsupervised protocols on the TCGA data set as were applied to the POLA study. As sample grouping according to *MCM6* and *MKI67* median expression levels led to the same configuration, results for both comparisons were fused as one. Similar molecular signatures were indeed uncovered in TCGA samples, each signature presenting with an enrichment with its POLA counterpart ranging from 3.3 to 7.6 times higher than expected (all $P < 1 \times 10^{-100}$, Fisher's exact test). These showed a downregulation of inflammation, glial differentiation,

Table 4. Top 12 Gene Ontology functional annotations associated with the expression of MKI67 (sorted by fold enrichment).

GO biological process	Fold enrichment	FDR
<i>Enrichment in tumors showing MKI67 higher expression</i>		
Condensin complex (GO:796)	50.2	<0.0001
Replication fork protection complex (GO:31298)	42.1	<0.0001
DNA strand elongation involved in cell cycle DNA replication (GO:1902296)	40.1	<0.0001
Ndc80 complex (GO:31262)	40.1	<0.0001
Pre-replicative complex assembly (GO:36388)	35.1	<0.0001
Leading strand elongation (GO:6272)	35.1	<0.0001
Condensed nuclear chromosome kinetochore (GO:778)	31.6	<0.0001
MCM complex (GO:42555)	28.1	0.0001
Mitotic centrosome separation (GO:7100)	28.1	0.0001
Mitotic chromosome condensation (GO:7076)	27.5	<0.0001
Kinetochore microtubule (GO:5828)	25.5	0.0001
Aster (GO:5818)	25.5	0.0001
<i>Enrichment in tumors showing MKI67 lower expression</i>		
Regulation of inclusion body assembly (GO:90083)	12.3	0.03
Beta-amyloid binding (GO:1540)	10.5	0.005
Neurotrophin TRK receptor signaling pathway (GO:48011)	7.9	0.04
Import into cell (GO:98657)	6.6	0.01
Sodium ion transmembrane transport (GO:35725)	4.7	0.03
Synaptic vesicle (GO:8021)	4.1	0.007
Postsynaptic specialization (GO:99572)	3.7	0.001
Axon part (GO:33267)	3.7	<0.0001
Excitatory synapse (GO:60076)	3.7	0.001
Myelin sheath (GO:43209)	3.4	0.01
Dendrite (GO:30425)	2.6	0.001
Trans-synaptic signaling (GO:99537)	2.5	0.001

FDR = false discovery rate; GO = gene ontology.

myelin sheath and synaptic activity in tumors that overexpressed *MCM6* and/or *MKI67*, and an upregulation of mitotic cell cycle and DNA replication (all FDR < 0.01).

We further asked if these results extended to grade II cases and applied the same experimental protocol on TCGA combined grades II/III cohort (n = 98; Supplementary Figure S8). Clusters of genes involved in mitotic cell cycle/replication, myelin sheath/glia cell differentiation and axoneme/oxacid metabolic process were indeed strongly differential between *MCM6*-down and *MCM6*-up groups (all FDR < 0.01), and presented with high identities with their POLA counterparts (73.6%, 60.6% and 48.2%, respectively). However, we did not find well-defined signatures such as those reported for grade III alone of both POLA and TCGA cohorts for the other clusters. The immune response/inflammation signature, in particular, was lost when including grade II samples to the mixture.

DISCUSSION

The aim of our study was to evaluate the prognostic value of *MCM6* and *Ki-67* LI in AOs. Previously, other members of *MCM* family, such as *MCM2*, *MCM3* and *MCM7*, have indeed been studied in gliomas, and their overexpression was associated with a poor overall prognosis; however, their applicability in clinical practice seems to be unclear (14, 16, 27). The prognostic value of *MCM6* has been confirmed in various

types of brain tumors like meningiomas, adamantinomatous craniopharyngiomas and gliomas (9, 21, 48). In the present study, we specifically focused on AOs with the presence of defined mutations of *IDH1/2* mutations and 1p/19q codeletion to avoid confusing factors due to molecular alterations. Indeed, *IDH1/2* mutations and 1p/19q codeletion are known to correlate with better outcome, due notably to a better response to adjuvant chemotherapy. Only few prognostic markers have been reported in the specific group of *IDHmut+/1p19qcodelet* AO. In this work, PFS was not assessed, because a consensual definition of progression in glioma is still lacking (1, 7, 28).

In this study, we found a good correlation between the mean LIs of *MCM6* and *Ki-67*. *MCM6* LI was always higher than *Ki-67* LI in a given tumor (Figure 1). This is consistent with data of the literature, and is due to the fact that *MCMs* are expressed earlier in the cell cycle (15, 30). Furthermore, we have identified a cutoff point at 50% *MCM6* LI as the optimal criteria for survival prediction: at greater than a LI of 50%, *MCM6* overexpression correlated with a shorter overall survival. Based on the results we obtained earlier in meningioma, in which high *MCM6* LI were found correlated with survival (21), this marker may even be applicable to other brain tumor types. Consequently, this high labeling index of *MCM6* at 50% threshold can be considered as an easy-to-use tool for routine practice of the pathology laboratories, in complement to *Ki-67*.

We also found that high mean LIs of *Ki-67* were associated with poor outcome, with a threshold of 15%. This

result is consistent with previous studies using the EORTC (European Organization for Research and Treatment of Cancer) Brain Tumor Group (40).

Given the results reported above, it shall be noted that although Ki-67 LI had good inter-observer agreement in previous studies (11, 39), there is no consensual method for counting Ki-67 LI in AO. Usually, most of the neuropathologists select areas showing the highest proliferation. Here, in order to have a more reproducible counting method for both Ki-67 and MCM6, we adapted computerized color image analyses based on previous results for Ki-67 cell scoring (6, 36). Consequently, a wide area was analyzed for each TMA spot. However, as we were not sure whether the selected spots corresponded to areas with the highest cell proliferation (despite the fact that individual spots were made with histologically representative samples of the tumors), we determined in our series the correlation between the Ki-67 LI obtained from TMA and the one obtained from whole slides; we found that these values correlated significantly ($\rho = 0.553$; $P < 0.0001$; data not shown), confirming the adequacy of our prepared TMA spots.

Multivariate analyses showed that MCM6 may be an interesting marker to use in association to Ki-67. Indeed, tumors harboring MCM6 LI $\geq 50\%$ and/or Ki-67 LI $\geq 15\%$ were significantly correlated to shorter survival, both in univariate and multivariate analyses. High MCM6 and Ki-67 LI were also correlated to shorter survival in grade II tumors, while in our study WHO grade was not significantly correlated to survival. However, these results are limited by a relatively short median follow-up time and a low number of OS events, thus the observed survival differences were based on few patients. Another limitation is that we used a statistical method that specifically seeks for the best cutoff in a given data set, which may not be applicable in another cohort. Further studies are needed in order to confirm the validity of these thresholds.

Analyses of the transcriptome data of the POLA grade III cohort showed that *MCM6* and/or *MKI67* overexpressing tumors share near identical whole transcriptional profiles, in spite of a correlation between *MCM6* and *MKI67* mRNA levels not being perfect. These tumors upregulated genes linked to DNA replication during the cell cycle (including all *MCMs*), DNA strand elongation, pre-replicative complex assembly, mitotic centrosome condensation and separation or regulation of helicase activity. This is consistent with the highly proliferative activity of these aggressive cancers. In addition, the *MCM6*-up and *MKI67*-up tumors of the POLA AO cohort overexpressed pro-neuronal differentiation genes such as *SOX4*, *SOX11*, *INSM1* and *ASCL1*. These findings are consistent with the pro-neuronal signature of the AO previously highlighted by Bielle *et al*. These authors identified a subgroup of AO overexpressing neuronal intermediate progenitor genes, associated with immunohistochemical similarities to embryonic subventricular zone, expression of *INSM1* and no expression of *SOX9* (5).

On the contrary, in AO cases showing an overall lower expression of *MCM6* and/or *MKI67*, transcriptomic analyses revealed enrichments of genes involved in myelin sheath production, myelination or oligodendroglial differentiation. These results suggest that both *MCM6*-down and

MKI67-down tumors lack a pro-neuronal signature, and unlike their *MCM6*-*MKI67*-up counterparts, are well-differentiated oligodendroglial-like tumors.

In the *MCM6*-down subgroup, we also found genes like *CX3CR1*, *TLR4* and *SYK*, involved in immune response and microglial cells activation. Such downregulations in immune responsive genes have been observed in other types of glioma. For example, in murine models, loss of *CX3CR1* expression promoted gliomagenesis in glioblastoma with pro-neuronal signature through larger inflammatory monocytes accumulation (17). Lower levels of *TLR4*, a member of the toll-like receptors involved in macrophagic immune response, have been implicated in the immune evasion of glioblastoma stem cells (3). The immune evasion in high-grade gliomas is well documented. The non-neoplastic cells of the glioma microenvironment, such as fibroblasts, endothelial cells or microglial cells, produce survival and growth factors, helping tumors cells to grow and infiltrate the brain parenchyma (23). The stimulatory role of spleen tyrosine kinase receptors (*SYK*) in gliomagenesis, brain invasion, and recruitment of immune cells has recently been studied in glioblastoma stem cells by Moncayo *et al* using *SYK*-inhibitors, but their involvement has never been specifically studied in AO (37). In another study, *SYK* appeared as a tumor suppressor gene in breast carcinomas (13). First-generation *SYK*-inhibitors have been successfully tested in some hematologic malignancies, as B-acute lymphoid leukemia and chronic lymphocytic lymphoma (25, 44). The assessment of their efficiency in high-grade gliomas needs further investigations.

Finally, the predictive prognostic power of *MCM6* and/or *MKI67* was tested using a TCGA cohort of *IDH1*-*IDH2*-codel glioma composing of both grade II ($n = 68$) and III ($n = 35$) tumors. We found that high *MCM6* expression indeed correlated with OS, but not *MKI67*.

CONCLUSION

In this study, we assessed the prognostic value of MCM6 and Ki-67 LI in *IDH*-mutant and 1p/19q codeleted AOs of the POLA cohort. Our multivariate analyses showed that the overexpression of MCM6 and/or Ki-67 was independently correlated to shorter survival. The prognostic value of MCM6 was confirmed in TCGA grade II–III *IDH*-mutant and 1p/19q codeleted gliomas. These two easy-to-use and cost-effective markers could thus be used concurrently in routine pathology practice. Potentially, these markers could also be integrated into therapeutic as well as clinicoradiological monitoring strategies to improve disease outcomes. We also would like to underline the important new insight gained from the transcriptomic analyses of the AO, which is that AO with high proliferation have downregulated immune response and lower microglial cells activation.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web site:

Figure S1. *MCM6* and *MKI67* mRNA level distributions into two subgroups of higher (“-up”) and lower (“-down”) expressions. Transcriptomic samples (n = 68) are separated at the median expression for each gene and ordered according to their expression gradient for either *MCM6* or *MKI67*.

Figure S2. Correlations between immunohistochemistry (IHC) and mRNA level for Ki-67 (*MKI67*) and *MCM6*.

Figure S3. Correlation between mRNA expression levels of *MKI67* and *MCM6*.

Figure S4. Log₂-normalized gene expression levels for the upregulated pro-neural (A), epigenetic (B), and cell cycle (C) signatures, as well as the downregulated immune response (D), myelination (E) and glial cells differentiation (F) signatures for both *MCM6* and *MKI67* “-down” and “-up” samples (transcriptomics).

Figure S5. Significantly dysregulated KEGG pathways with an overall upregulation (Table as obtained with Gage) and map

of the cell cycle (GraphViz rendered with Pathview) in both *MCM6* (A) and *MKI67* (B) upregulated samples. Significantly (FDR < 0.01) down- and upregulated genes are colored from green to red, with respect to their fold change of expression (log2).

Figure S6. Survival analyses in grades II/III IDH-mutant/1p19q codeleted oligodendrogliomas selected from TCGA database. Kaplan–Meier curves with log-rank tests (overall survival). A. *MCM6* mRNA level, median threshold (arbitrary units). B. *MKI67* mRNA level, median threshold (arbitrary units). C. WHO 2016 grade.

Figure S7. K-means clustering based on *MCM6* and/or *MKI67* mRNA expression gradient (from lowest to highest) of the 35 transcriptomes of anaplastic IDH-mutant/1p19q codeleted oligodendrogliomas selected from TCGA bank. The K-means clustering delineated five significant clusters of co-expressed genes ($P < 0.05$) with highly correlated molecular functions. A. Heatmap of the 10 k-means clusters organized based on the extent of *MCM6* and *MKI67* expression (green, red and black indicate downregulated, upregulated and median genes, respectively). B. Overview of the most relevant functional annotations for the significantly over- and under-expressed clusters (in red and green hues, respectively, all $P(FDR) \leq 0.01$). Each of these clusters was compared to the clusters found of the POLA cohort, and their identities calculated based on the corresponding POLA clusters. C. Mean gene expression (overall with standard error at the mean) for each cluster where both upregulated samples for *MCM6* and/or *MKI67* are compared to their downregulated counterparts (AU—arbitrary units). Upregulated and downregulated samples are defined relatively to *MCM6* median gene expression.

Figure S8. K-means clustering based on *MCM6* and/or *MKI67* mRNA expression gradient (from lowest to highest) of the 98 transcriptomes of grades II and III IDH-mutant/1p19q codeleted oligodendrogliomas selected from TCGA bank. The K-means clustering delineated five significant clusters of co-expressed genes ($P < 0.05$) with highly correlated molecular functions. A. Heatmap of the ten k-means clusters organized based on the extent of *MCM6* and *MKI67* expression (green, red and black indicate downregulated, upregulated and median genes, respectively). B. Overview of the most relevant functional annotations for the significantly over- and under-expressed clusters (in red and green hues, respectively, all $P(FDR) \leq 0.01$). Each of these clusters was compared to the clusters found of the POLA cohort, and their identities calculated based on the corresponding POLA clusters. C. Mean gene expression (overall with standard error at the mean) for each cluster where both upregulated samples for *MCM6* and/or *MKI67* are compared to their downregulated counterparts (AU—arbitrary units). Upregulated and downregulated samples are defined relatively to *MCM6* median gene expression.

Table S1. K-means clustering from *MCM6* and *MKI67* expression gradients of 68 transcriptomes of anaplastic oligodendrogliomas from the POLA network. Functional annotations of

the genes present in the five significant groups of co-expressed genes ($P < 0.01$), sorted by gene ontology enrichment (FDR, false discovery rate; GO, gene ontology).

Table S2. Differential gene expression statistics (t-test) between *MCM6*-up and *MCM6*-down, and between *MKI67*-up and *MKI67*-down samples, sorted by *P*-value (BH, Benjamini–Hochberg; FDR, false discovery rate; GO, gene ontology; SD, standard deviation).

Table S3. K-means clustering from *MCM6* (or *MKI67*) expression gradients of 35 transcriptomes of grade III oligodendrogliomas from the TCGA bank. Functional annotations of the genes present in the five significant groups of co-expressed genes ($P < 0.01$), sorted by gene ontology enrichment (FDR, false discovery rate; GO, gene ontology).

Table S4. K-means clustering from *MCM6* expression gradients of 98 transcriptomes of grade II and grade III oligodendrogliomas from the TCGA bank. Functional annotations of the genes present in the six significant groups of co-expressed genes ($P < 0.01$), sorted by gene ontology enrichment (FDR, false discovery rate; GO, gene ontology).

APPENDIX

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