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Distinct molecular profile and outcome of oligodendroglioma, *IDH*-mutant, 1p/19q-codeleted, and *TERT*p-wildtype: A grade 1 oligodendroglioma of young patients?

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Abstract

Background. Oligodendrogliomas, characterized by isocitrate dehydrogenase (*IDH)* mutations and 1p/19q codeletion, often exhibit telomerase reverse transcriptase promoter (*TERT*p) mutations, which have been linked to telomere maintenance (TM) and tumor proliferation. Although there are a few reports on a *TERT*p-wildtype subset of these tumors in adolescents and young adults, the frequency, molecular characteristics, and prognostic implications of *TERT*p-wildtype status in oligodendrogliomas remain elusive.

Methods. We retrospectively analyzed 166 *IDH*-mutant and 1p/19q-codeleted oligodendroglioma cases through comprehensive histopathological review and molecular analyses, including Sanger sequencing, DNA methylation profiling, and whole-exome sequencing (WES).

Results. A *TERT*p-wildtype status was observed in 20/166 cases (12.0%) and was significantly associated with noticeably young age (age range: 14–27, $P < .001$), CNS WHO grade 2 ($P = .003$), and the absence of additional DNA copy number variations (CNVs) beyond the pathognomonic 1p/19q codeletion ($P < .001$). Epigenetic profiling demonstrated *TERT*p-wildtype tumors shaped a distinct subgroup at the utmost periphery of *TERT*p-mutant oligodendrogliomas. Methylation analysis of the upstream and proximal *TERT*p regions revealed that, in line with the absence of genetic alterations, epigenetic regulation does not favor *TERT* overexpression in *TERT*p-wildtype oligodendrogliomas. WES showed no TM-related gene alterations in *TERT*p-wildtype cases. Cox regression analysis confirmed *TERT*p-wildtype status as an independent prognostic factor for more favorable progression-free survival (PFS) ($P = .009$).

Conclusions. In conclusion, "oligodendroglioma, *IDH*-mutant, 1p/19q-codeleted, and *TERT*p-wildtype" represent a distinct molecular subgroup associated with younger age and a better clinical course compared to CNS WHO grade 2 oligodendrogliomas.

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Key Points

- Telomerase reverse transcriptase promoter promoter (*TERT*p)-wildtype status is significantly associated with young age in oligodendroglioma.
- Molecular profiling identifies *TERT*p-wildtype oligodendrogliomas as a distinct subgroup.
- *TERT*p-wildtype status is an independent prognostic factor for more favorable PFS.

Importance of the Study

Oligodendrogliomas, *IDH*-mutant, 1p/19q-codeleted frequently harbor telomerase reverse transcriptase promoter (*TERT*p) mutations, critical in telomere maintenance and tumorigenesis. However, a subset that lacks *TERT*p mutations, particularly in adolescents and young adults, remains poorly understood, with limited insights into its frequency, molecular characteristics, and clinical implications. This study bridges this knowledge gap by providing a comprehensive analysis of *TERT*p-wildtype oligodendrogliomas, including a relevant cohort of young patients. Through comprehensive molecular profiling, our data identify *TERT*p-wildtype status as a distinct molecular subgroup linked to younger age and improved prognosis. Our findings reveal the absence of typical telomere maintenance alterations and a distinct epigenetic profile, positioning *TERT*p-wildtype oligodendrogliomas as a low-risk group. These insights have the potential to refine prognostic classifications and lead to more tailored management strategies for younger oligodendroglioma patients.

According to the fifth edition of the WHO Classification of the CNS Tumours,[1] the molecular entity-defining alterations in oligodendrogliomas include missense mutations in *IDH1* codon 132 or *IDH2* codon 172, combined with whole-arm deletions of 1p and 19q. Other nonessential diagnostic requirements, but frequent genetic hallmarks include capicua (*CIC*), far upstream element binding protein 1 (*FUBP1*), and telomerase reverse transcriptase promoter (*TERT*p) mutations, as well as the absence of alpha-thalassemia mental retardation X-linked (*ATRX*), and tumor protein 53 (*TP53)* alterations.[2]

Telomere maintenance (TM) is a key event in glioma development[3,4] and is believed to be usually achieved by 2 different pathways: (1) activation of *TERT* through promoter mutation that recruits de novo E26 transformation-specific transcription factor binding sites, which leads to transcriptional upregulation of *TERT* expression driving telomere stabilization, cellular immortalization, and proliferation,[3,5,6] or (2) a telomerase-independent mechanism known as alternative lengthening of telomeres, which relies on the homologous recombination of telomeric regions, resulting in heterogeneous length and sequence composition, and it is commonly reflected by *ATRX* mutation.[7,8] Unlike *IDH*-mutant astrocytomas,[9] the vast majority of *IDH*-mutant and 1p/19q-codeleted oligodendrogliomas are nonmutated for *ATRX*,[10] while over 90% of them harbor *TERT*p hotspot mutations.[11–16] A recent whole-tumor analysis[7] also showed *TERT*p mutation to be an early and clonal event in oligodendroglioma genesis, which remains stable during tumor progression and at recurrence.[17]

Although *TERT*p mutations are widely observed in oligodendroglioma, Lee et al.[18] described a case series of 3 *IDH*-mutant and 1p/19q-codeleted oligodendrogliomas who lacked *TERT*p mutation, typically found in adult cases. Reviewing data from major pediatric low-grade glioma genomics studies,[19–22] just a few additional cases of *IDH*-mutant and 1p/19q-codeleted oligodendrogliomas with confirmed *TERT*p-wildtype status have been reported.[19,20] In older studies and population-based registries, the *TERT*p mutation rate of histologically defined tumors is confounded by the inclusion of gliomas lacking *IDH* mutation and/or 1p/19q codeletion.[23]

The prognostic impact of *TERT*p mutations in diffuse gliomas is bivalent, depending on *IDH* status and tumor grade,[12] while its significance in conjunction with 1p/19q codeletion remains controversial and largely unknown.[15,24] Additionally, since *TERT*p mutations are almost universally found in *IDH*-mutant, 1p/19q-codeleted oligodendrogliomas,[15] our understanding of the molecular characteristics and clinical behavior of *TERT*p-wildtype cases is still extremely limited.[14,25]

To address this, we assessed the frequency, molecular characteristics, and the clinical behavior of *TERT*p-wildtype oligodendrogliomas through comprehensive histopathological, clinical, and molecular analyses, including Sanger sequencing, DNA methylation profiling, copy number variation (CNV) analysis, and whole-exome sequencing (WES).

Materials and Methods

Collection of Tissue Samples and Clinical Data

A total of 166 cases of oligodendroglioma, *IDH*-mutant, and 1p/19q-codeleted, were included in this study. The experimental design followed these inclusion criteria: (a) newly diagnosed oligodendrogliomas, *IDH*-mutant, and

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1p/19q-codeleted, between 2004 and 2024; (b) diagnosis confirmed by DNA methylation analysis; (c) molecular confirmation of whole-arm deletions of 1p and 19q; (d) availability of formalin-fixed, paraffin-embedded (FFPE) tissue samples; and (e) availability of clinical follow-up data.

The above-mentioned inclusion criteria have been applied to all cases present within the Biomaterial Bank Heidelberg (BMBH), which stores samples related to both internal cases and those from other national and international institutions that have sent samples over time for diagnostic purposes, additional molecular testing, or second opinion.

The collection and analysis of tissue samples and clinical data were conducted in accordance with the principles of the Declaration of Helsinki.

Histology and Immunohistochemistry

All cases underwent histological review according to the most recent WHO Classification of Tumours of the Central Nervous System (CNS5).[1] Histological features with established prognostic significance, including mitotic activity (counted in 10 high-power fields [HPFs] with a total area of 2.4 mm^2), as well as the presence of necrosis and microvascular proliferation, were assessed in all cases. Tumor paraffin blocks were subjected to immunohistochemical workup. Hematoxylin and eosin staining was performed according to standard protocols. The following antibodies for immunohistochemistry were used: GFAP (mouse monoclonal, clone GA5, dilution 1:2000, Cell Signaling), *IDH1*-R132H (mouse monoclonal, clone H09, Dianova), ATRX (mouse monoclonal, clone BSB-108, dilution 1:2000, Bio SB), Ki-67 (mouse monoclonal, clone MIB-1, dilution 1:100). For *ATRX* and Ki-67, the Ventana OptiView DAB IHC Detection Kit (Ventana Medical Systems) was used.

*TERT*p Sanger Sequencing

DNA was extracted from FFPE samples at the Department of Neuropathology, University Hospital Heidelberg, for all cases except 1, for which extracted tumor DNA was provided by the University Hospital Frankfurt. *TERT*p Sanger sequencing was performed on all oligodendrogliomas with unknown *TERT*p status at the Department of Neuropathology, University Hospital Heidelberg. Furthermore, to investigate whether *TERT*p status can be different during tumor progression, we analyzed all the recurrent and surgically treated *TERT*p-mutant cases with available both primary and recurrent tumor FFPE samples ($n = 6$).

DNA Methylation Profiling, DNA Methylation Analysis of *TERT*p and *HOXD12*, and Copy Number Analysis

Genome-wide DNA methylation data were obtained using the Infinium Methylation EPICv2 (935k) in 16 cases (9.6%), EPICv1 (850k) in 98 cases (59.0%), and the Infinium HumanMethylation450 (450k) BeadChip array in 52 cases (31.4%), following the manufacturer's instructions (Illumina), as previously described.[26] To integrate methylation data across different array versions, we processed raw signal intensities using the R package *minfi* for background correction and normalization. To minimize technical batch effects across Infinium 450k, EPICv1 (850k), and EPICv2 (935k) arrays, we applied the *swan* (Subset-quantile Within Array Normalization) method. Only probes common to all 3 platforms were included for downstream analysis. Additionally, beta values were normalized using functional normalization (*FunNorm*). These adjustments ensure robust comparability across datasets. The DNA methylation-based diagnostic validation of the study cases was performed using the Heidelberg Classifier (version 12.8, available at https://www.molecularneuropathology.org/mnp/). This classifier algorithm facilitates the rapid comparison of diagnostic cases with a reference cohort of more than 2800 cases and is based on the Random Forest algorithm, as previously published.[26] The output of the classifier is a tumor methylation class with an associated classification score that reflects the similarity to one of the predefined CNS tumor classes. Each class receives a score ranging from 0 to 1, with all class prediction scores summing to 1. A confident classification requires a methylation class score equal to or above the cutoff of 0.90.[26] Copy number profiles were generated from 450k and EPIC methylation arrays data using the conumee Bioconductor package version 1.12.0, as described before.[26,27] All computational analyses were conducted in R version 3.6.0 (R Development Core Team 2016, https://www.R-project.org). Copy number profiles were visually inspected to evaluate chromosomal gains and losses.[28,29] Alterations in key glioma-related genes—including *CCND1, CCND2, CDK4, CDK6, CDKN2A/B, EGFR, MDM4, MET, MYC, MYCN, NF1, NF2, PDGFRA, PPM1D, PTEN, RB1,* and *SMARCB1*—were assessed both through visual inspection and analysis using our proprietary algorithm. This algorithm outputs chromosomal status as balanced, gained, or lost, along with the affected region size (percentage) and the amplitude of the alterations in the listed genes. The specific genes and cutoff values have been previously published.[29] The *b*-values of 4 CpGs within upstream *TERT*p (cg11625005, cg07380026, cg17166338, cg26006951) and 1 CpG within proximal *TERT*p (cg10896616), covered by all arrays, were subsequently extracted. Recent studies have shown that the methylation status of 3 CpGs within the *HOXD12* gene (cg23130254, cg03964958, cg03371669) significantly correlates with *HOXD12* expression levels, age, and prognosis in oligodendrogliomas.[30] The beta value of cg23130254, covered by all arrays (450k, 850k, and EPICv2), was analyzed accordingly.

WES and Telomere Maintenance-Related Genes Mutational Analysis

Following *TERT*p Sanger sequencing, oligodendrogliomas with *TERT*p-wildtype status underwent further genetic analysis of tumor DNA by WES with SureSelect Human All Exon V8 (Agilent) on an Illumina NovaSeq platform (Illumina). The kit targets the most up-to-date protein-coding regions from RefSeq, GENCODE, and CCDS, including the *TERT*p region. WES data were analyzed for

brain tumors and TM-related genes, applying an in-silico panel listed in Supplementary Table 1. Genes of interest for TM were selected based on the TelNet Database,[31] a source provided by the German Cancer Research Center (DKFZ), which integrates information on TM-relevant genes and their specific cellular functions.

Statistical Analysis

Variables were summarized descriptively, namely median (range) for continuous variables and number (percentage) for categorical data. The association of clinicopathological parameters was analyzed through Mann–Whitney *U* test for continuous variables and Fisher's exact test for categorical variables. The prognostic value of clinically and pathologically relevant variables was assessed using univariate and multivariate Cox proportional hazards regression models (the latter applied to variables significant in univariate analysis) and expressed as hazard ratio (HR), adjusted HR (aHR), and 95% confidence interval (CI). The Kaplan–Meier (KM) method was used to estimate progression-free survival (PFS) probabilities, and differences were assessed using the log-rank test. PFS refers to the time from first diagnosis to the first documented disease progression, as evaluated in accordance with the Response Assessment in Neuro-Oncology criteria.[32] Further additional univariable and multivariable linear regression models were performed to investigate the association between *TERT*p status and *HOXD12* methylation. All the data were analyzed using GraphPad Prism 10.3.0 (GraphPad Software). All statistical tests were 2-sided, and $P < .05$ was considered significant.

Results

Demographic and Clinical Characteristics of the Whole Cohort

A total of 166 *IDH*-mutant and 1p/19q-codeleted oligodendrogliomas were included in the study, comprising 105 males (63.3%), with a median age at diagnosis of 39 years (range: 14–79). The cohort was distributed across the following age groups: 84 patients (50.6%) aged 14–39 years and 82 patients (49.4%) aged 40–79 years. The majority of tumors were located in the frontal lobe (105 patients, 63.3%). Gross-total resection (GTR) was achieved in 72 patients (43.4%). Adjuvant radiation therapy and/or chemotherapy regimens were administered to 89 patients (53.6%), while a "watch-and-wait" approach was applied in 77 patients (46.4%). Baseline clinical and pathological characteristics are provided in Table 1. A flowchart outlining the cohort and the experimental design is presented in Supplementary Figure 1.

Histological, Immunohistochemical, and Baseline Molecular Review

Of the 166 cases, 126 (75.9%) were classified as CNS WHO grade 2 tumors. The median mitotic activity was 1 mitosis per 10 HPFs (0.4 per mm^2). Necrosis and microvascular proliferation were observed in 11 cases (6.6%) and 40 cases (24.1%), respectively. Immunohistochemistry revealed *IDH1* R132H positivity in 138 cases (83.1%), with *ATRX* expression retained in 100% of cases. The median Ki-67 labeling index was 5% (range: 1%–30%). Molecular analysis from previously performed Sanger sequencing or NGS identified *IDH2* codon 172 mutations in 28 patients (16.9%). *CIC* mutation status was available for 72 patients (43.4%), with pathogenic somatic variants detected in 45 (62.5%). *FUBP1* molecular data were available for 73 patients (44.0%), of which 11 cases (15.1%) harbored a pathogenic variant.

Sequencing Analysis Identifies *TERT*p-Wildtype Status in the Vast Majority of Young Patients and WES Analysis Revealed Absence of Mutations in Telomere Maintenance-Related Genes of *TERT*p-Wildtype Cases

Sanger sequencing identified *TERT*p-wildtype status in 23 of 166 cases (13.8%). WES confirmed this status in 20 of these 23 cases (86.9%). In the remaining 3 cases (13.1%), WES detected canonical *TERT*p mutations (C228T in two cases and C250T in one case). Among the 20 confirmed *TERT*p-wildtype cases, 19 patients (95.0%) were ≤20 years old, and 1 patient (5.0%) was 27 years old (Figure 1A). No *TERT*p-wildtype case was detected in patients aged more than 30 years. Furthermore, *IDH2* codon 172 mutation was identified in 5/20 (25.0%) *TERT*-p wildtype cases versus 23/146 (15.8%) in the *TERT*p-mutant group (Figure 1B). Within *TERT*p-mutant cases, the C228T mutation (71.9%) was approximately 2 times, and half more frequent than the C250T (28.1%). Chi-square analyses showed that age ≤39 years ($P < .001$), CNS WHO grade 2 ($P = .003$), and a Ki-67 proliferation index less or equal than 5% (median value) were significantly associated with *TERT*p-wildtype status ($P = .001$). The age distribution across the study cohort illustrated in Figure 1A and 1B.

To explore the clonality of *TERT*p mutations, we selected two cases with markedly different Ki-67 expression levels. DNA was extracted from both areas using tissue punches, followed by *TERT*p Sanger sequencing, which revealed the presence of *TERT*p mutations in both regions (Supplementary Figure 2).

Similarly, to investigate whether *TERT*p mutations can be acquired during tumor progression, we analyzed recurrent *TERT*p-mutant cases with available primary tumor samples ($n = 6$; median PFS: 120 months [range: 32–234]). *TERT*p mutations were detected in all primary tumor samples of recurrent *TERT*p-mutant cases. Clinical and pathological features of the 6 analyzed patients are reported in Supplementary Table 2. In contrast, the single *TERT*p-wildtype case with tumor progression did not undergo surgical resection at recurrence, preventing the availability of recurrent tumor samples for similar analysis.

Comprehensive clinical, histopathological, and molecular data stratified by *TERT*p status are summarized in Table 1.

WES was performed on *TERT*p-wildtype cases identified through Sanger sequencing ($n = 23$) to assess TM-related

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Table 1. Baseline Characteristics of the Whole Cohort and According to *TERT*p Status

Variables	All patients (***n*** = 166)	***TERT***p-wildtype (***n*** = 20)	***TERT***p-mutant (n = 146)	***P***-value
Age Median (range)	39 (14–79)	17 (14–27)	41 (14–79)	**<.001**
Age range (years), *n* (%) 14–39 40–79	84 (50.6) 82 (49.4)	20 (100) 0 (0)	64 (43.8) 82 (56.2)	**<.001**
Gender, *n* (%) Male	105 (63.3)	12 (60.0)	93 (63.7)	.464
Site, *n* (%) Frontal	105 (63.3)	15 (75.0)	90 (61.6)	.181
CNS WHO grade, *n* (%) 2 3	126 (75.9) 40 (24.1)	20 (100) 0 (0)	106 (72.6) 40 (27.4)	**.003**
Mitosis, n/10 HPFs Median (range)	1 (0–19)	0 (0–1)	1 (0–19)	0.070
Necrosis, n (%) Yes	11 (6.6)	0 (0)	11 (7.5)	0.232
Microvascular proliferation, *n* (%) Yes	40 (24.1)	2 (10.0)	38 (26.0)	0.092
Ki-67 %, *n* (%) Median (range)	5 (1–30)	2 (1–6)	6 (1–30)	**0.001**
IDH mutation, *n* (%) *IDH1* codon 132 R132H R132G R132C *IDH2* codon 172 R172K R172M R172S R172W	138 (83.1) 133 (80.1) 4 (2.4) 1 (0.6) 28 (16.9) 20 (12.0) 6 (3.7) 1 (0.6) 1 (0.6)	15 (75.0) 15 (75.0) 0 (0) 0 (0) 5 (25.0) 4 (20.0) 0 (0) 1 (5.0) 0 (0)	123 (84.2) 118 (80.8) 4 (2.7) 1 (0.7) 23 (15.8) 16 (10.9) 6 (4.2) 0 (0) 1 (0.7)	0.229[a]
ATRX expression, *n* (%) Retained	166 (100)	20 (100)	146 (100)	-
*TERT*p mutation, *n* (%) C228T C250T Wildtype	105 (63.3) 41 (24.7) 24 (12.0)	- - 20 (100)	105 (71.9) 41 (28.1) -	-
CIC mutation, *n* (%) Yes Wildtype NA	45 (27.1) 27 (16.3) 94 (56.6)	12 (60.0) 8 (40.0) 0 (0)	33 (22.6) 19 (13.0) 94 (64.4)	.496
FUBP1 mutation, *n* (%) Yes Wildtype NA	11 (6.6) 62 (37.3) 93 (56.0)	1 (5.0) 19 (95.0) 0 (0)	10 (6.8) 43 (29.5) 93 (63.7)	.131
CDKN2A homozygous deletion, *n* (%) Yes	23 (13.9)	0 (0)	23 (15.8)	**.041**
DNA methylation-based score, *n* (%) ≥0.9 <0.9	140 (84.3) 26 (15.7)	6 (30.0) 14 (70.0)	134 (91.8) 12 (8.2)	**<.001**
MGMT, *n* (%) Methylated Unmethylated Undeterminable	156 (94.0) 2 (1.2) 8 (4.8)	13 (65.0) 0 (0) 7 (35.0)	143 (97.9) 2 (1.4) 1 (0.7)	-
CNV alterations, *n* (%) Isolated 1p/19q codeletion Additional CNV alterations	72 (43.4) 94 (56.6)	17 (85.0) 3 (15.0)	55 (37.7) 91 (62.3)	**<.001**
EOR, *n* (%) GTR STR Biopsy	72 (43.4) 69 (41.6) 25 (15.1)	7 (35.0) 12 (60.0) 1 (5.0)	65 (44.5) 57 (39.0) 24 (16.4)	.153

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Table 1. Continued

Variables	All patients (***n*** = 166)	***TERT***p-wildtype (***n*** = 20)	***TERT***p-mutant (n = 146)	***P***-value
Adjuvant therapies, *n* (%) Radiotherapy/Chemotherapy	89 (53.6)	3 (15.0)	86 (58.9)	-
Disease progression, *n* (%) Yes No	88 (53.0) 78 (47.0)	1 (5.0) 19 (95.0)	87 (59.6) 79 (40.4)	-
Follow-up time, (months) Median (25th—75th percentiles)	72 (32 – 116)	87 (22.75 – 115.75)	71 (33 – 116)	-

*TERT*p = *TERT* promoter, CNS = Central Nervous System, WHO = World Health Organization, HPFs = High-power fields, NA = not available, CNV = copy number variation, EOR = extent of resection, GTR = gross-total resection, STR = sub-total resection.
Statistically significant *P*-values are reported in bold.
[a]This analysis was performed considering *IDH1* codon R132 and *IDH2* codon R172 mutations as binary categorical variables.

gene mutations in *TERT*p-associated pathways. WES confirmed *TERT*p-wildtype status in 20 of these 23 cases, allowing a reassessment of *TERT*p status; however, no TM-related alterations were identified.

Epigenetic Profiling Reveals Distinct Clustering of *TERT*p-Wildtype and *TERT*p-Mutant Oligodendrogliomas

A focused *t*-distributed stochastic neighbor embedding (*t*-SNE) analysis was performed to assess the clustering patterns of oligodendrogliomas with different *TERT*p mutation statuses from the study cohort in relation to a reference cohort of 340 glial tumors from the Heidelberg Brain Tumour Classifier reference set (version v12.8).[26] The reference cohort included the following *IDH*-wildtype and *IDH*-mutant diffuse gliomas: glioblastoma, *IDH*-wildtype (*n* = 50); astrocytoma, *IDH*-mutant, lower grade (*n* = 50); astrocytoma, *IDH*-mutant, high grade (*n* = 50); diffuse hemispheric glioma, H3 G34-mutant (*n* = 30); diffuse midline glioma, H3 K27-altered (*n* = 30); diffuse leptomeningeal glioneuronal tumor (n = 40); diffuse pediatric-type high-grade glioma, H3 wildtype and *IDH* wildtype (*n* = 40); dysembryoplastic neuroepithelial tumor (*n* = 30); and oligosarcoma, *IDH*-mutant (*n* = 20).

The *t*-SNE analysis revealed that while *TERT*p-wildtype oligodendrogliomas exhibited the highest epigenetic resemblance to their mutant counterparts, they clustered together at the periphery of the *TERT*p-mutant group, representing an "epigenetic" extreme pole of oligodendrogliomas (Figure 2A). Further stratification by *CIC* mutation status, CNS WHO grade, and age at diagnosis revealed no significant distinctions between these subgroups or overlapping with the clusters based on *TERT*p status (Supplementary Figure 3A–C). These findings suggest that the distinct epigenetic profiles within oligodendroglioma are primarily associated with *TERT*p status among the assessed parameters (Figure 2B).

Analysis of CNVs derived from the DNA methylation data showed whole-arm deletions of 1p and 19q in 166/166 (100%) of cases. These concurrent entity-defining alterations were alone in 72 (43.4%) cases, whereas additional single or multiple CNV alterations were observed in 94 (56.6%) cases. *CDKN2A/B* homozygous deletion was observed in 23/166 (15.8%) cases, all of them belonging to the *TERT*p-mutant subgroup. Interestingly, an association was found between the presence of isolated 1p/19q codeletion, with no additional CNV alteration, and *TERT*p-wildtype oligodendrogliomas ($P < .001$), as shown in Figure 1B. CNV plots (Figure 3) confirmed a trend of higher CNV alterations from *TERT*p-wildtype oligodendrogliomas in teenagers to their *TERT*p-mutant adult counterparts, as well as from CNS WHO 2 to grade 3 tumors ($P = .024$).

Methylation Analysis of Upstream and Proximal *TERT*p Regions

By analyzing data extracted from the available methylation arrays, we aimed to investigate whether the epigenetic regulation of *TERT*p in *TERT*p-wildtype oligodendrogliomas might be, contrary to the genetic alteration, in favor of *TERT* overexpression compared to their mutant counterparts. For this purpose, we compared the average of the extracted beta values for 5 CpGs within *TERT*p, covered by various arrays, between *TERT*p-wildtype and *TERT*p-mutant subgroups using 2-tailed unpaired *t*-tests. We observed a considerable DNA methylation heterogeneity between *TERT* upstream and proximal promoter regions. In line with prior reports,[33–35] showing that upstream hypermethylation and proximal hypomethylation of *TERT*p are associated with *TERT* overexpression, in our study the upstream and proximal promoter regions were remarkably hypermethylated and hypomethylated, respectively (0.70 vs. 0.09 methylation level; *P*-value <.0001). The analysis showed a lower level of methylation across 4 CpG sites within the upstream promoter region in *TERT*p-wildtype cases compared to *TERT*p-mutant (0.59 vs. 0.65 methylation level; $P = .015$), whereas there was no difference in the methylation level of the single CpG within the *TERT* proximal promoter region (Supplementary Table 3). Thus, the epigenetic regulation of *TERT*p in oligodendroglioma favors *TERT* expression less in *TERT*p-wildtype cases compared to their mutant counterparts.

Methylation Analysis of the *HOXD12* Gene

To investigate whether methylation levels at *cg23130254* in the *HOXD12* gene differ according to *TERT*p status, we first

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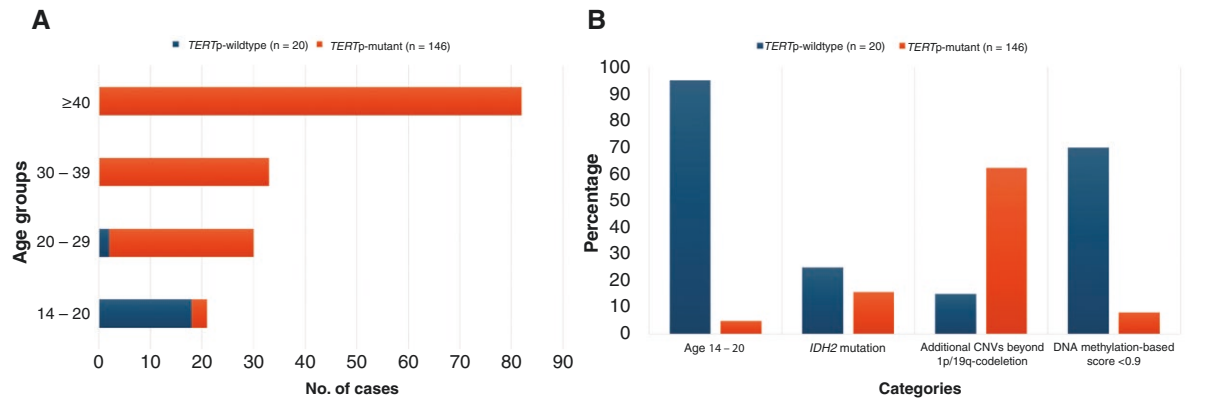

Figure 1. Characterization of *TERT* promoter (*TERT*p) mutation in the study cohort. (A) Age distribution of *TERT*p-wildtype cases across decades. (B) Bar chart comparing the distribution of age (≤20 years), *IDH2* mutation, copy number variations and DNA methylation score between *TERT*p-wildtype and *TERT*p-mutant groups.

performed a univariable linear regression of *TERT*p status on *HOXD12*, which revealed significantly lower methylation levels in *TERT*p-wildtype compared to *TERT*p-mutant (β = –0.111, 95% CI: –0.180 to –0.043). However, given that both age and CNS WHO grade were known to be independently associated with *HOXD12* gene methylation,[30] as also confirmed in our sample (Supplementary Table 4) and were found to be associated with *TERT*p status (Table 1), we subsequently conducted a multivariable linear regression including these 2 covariates. In this adjusted model, the association between *TERT*p status and *HOXD12* was no longer statistically significant (β = –0.033, 95% CI: –0.101 to 0.035), suggesting that the methylation difference may not be independently attributable to *TERT*p status, but may rather reflect underlying differences in age distribution or tumor biology between the groups.

PFS Analysis Indicated a Favorable Impact on PFS of *TERT*p-Wildtype Status in Oligodendroglioma

Follow-up data were available for all patients included in the cohort ($n = 166$). Median follow-up was 72.00 months across the whole cohort (25th and 75th percentiles: 32.00 and 116.00, respectively); 87.00 months in *TERT*p-wildtype patients (25th and 75th percentiles: 22.75 and 115.75, respectively); 71.00 months in *TERT*p-mutant patients (25th and 75th percentiles: 33.00 and 116.00, respectively). Tumor progression was observed in 88 (51.2%) cases, of which 87 (98.8%) in the *TERT*p-mutant group. Interestingly, within the *TERT*p-wildtype patient group, recurrence was exclusively observed in $n = 1$ patients over 20 years of age (27 years). The median PFS was 41.00 months (25th and 75th percentiles: 22.00 and 74.25, respectively) considering the whole cohort; 40.00 months in *TERT*p-mutant subgroup (25th and 75th percentiles: 22.75 and 71.00, respectively), and the PFS of the single *TERT*p-wildtype case with disease progression was 17 months.

*TERT*p-wildtype status ($P = .003$ [HR: 0.050 (95% CI: 0.007–0.362)]), age >39 years ($P = .049$ [HR: 1.531 (95% CI: 0.998–2.349)]), CNS WHO grade 3 ($P < .001$ [HR: 2.657 (95% CI: 1.701–4.151)]), mitotic count >1/10HPFs ($P = .001$ [HR: 2.242 (95% CI: 1.462–3.438)]), microvascular proliferation ($P = .012$ [HR: 1.785 (95% CI: 1.134–2.807)]), Ki-67 expression >5% ($P = .006$ [HR: 1.986 (95% CI: 1.218–3.238)]), and the presence of additional CNV alterations beyond 1p/19q codeletion ($P = .001$ [HR: 2.091 (95% CI: 1.327–3.294)]) emerged as statistically significant variables for PFS at univariate analysis, as shown in Table 2. The observed significant prognostic impact of *TERT*p-wildtype status on PFS was further confirmed by Cox regression multivariate analysis ($P = .009$ [HR: 0.068 (95% CI: 0.009–0.512)]). Full correlations between clinical, pathological, molecular variables and PFS at Cox regression models are detailed in Table 2.

KM analysis (Figure 4A–D), conducted to explore the prognostic significance of *TERT*p-wildtype status throughout the whole cohort as well as within distinct patient subgroups, revealed a statistically significant favorable impact on PFS for *TERT*p-wildtype status versus *TERT*p-mutant across the whole cohort ($P < .001$ [HR 0.053 (0.030–0.091)]) as well as within patients ≤39 years ($P < .001$ [HR: 0.052 (95% CI: 0.026–0.103)]), within CNS WHO grade 2 tumors ($P < .001$ [HR: 0.066 (95% CI: 0.035–0.126)]) and according to different extents of resection ($P < .001$).

The methylation status of a previously identified prognostically relevant CpG site within *HOXD12* did not correlate with PFS in a Cox proportional hazards regression analysis, either across the entire cohort ($P = 0.063$ [HR 1.228 (0.989–1.525)]), within the younger cohort (≤39 years; $P = .053$ [HR 2.048 (1.024–4.097)]), or within the subgroup of CNS WHO grade 2 patients ($P = .345$ [HR 1.298 (0.755–2.233)]).

Discussion

Herein, we described a peculiar subset of *IDH*-mutant and 1p/19q-codeleted oligodendroglioma patients lacking *TERT*p mutation, showing considerably favorable PFS and distinct molecular characteristics.

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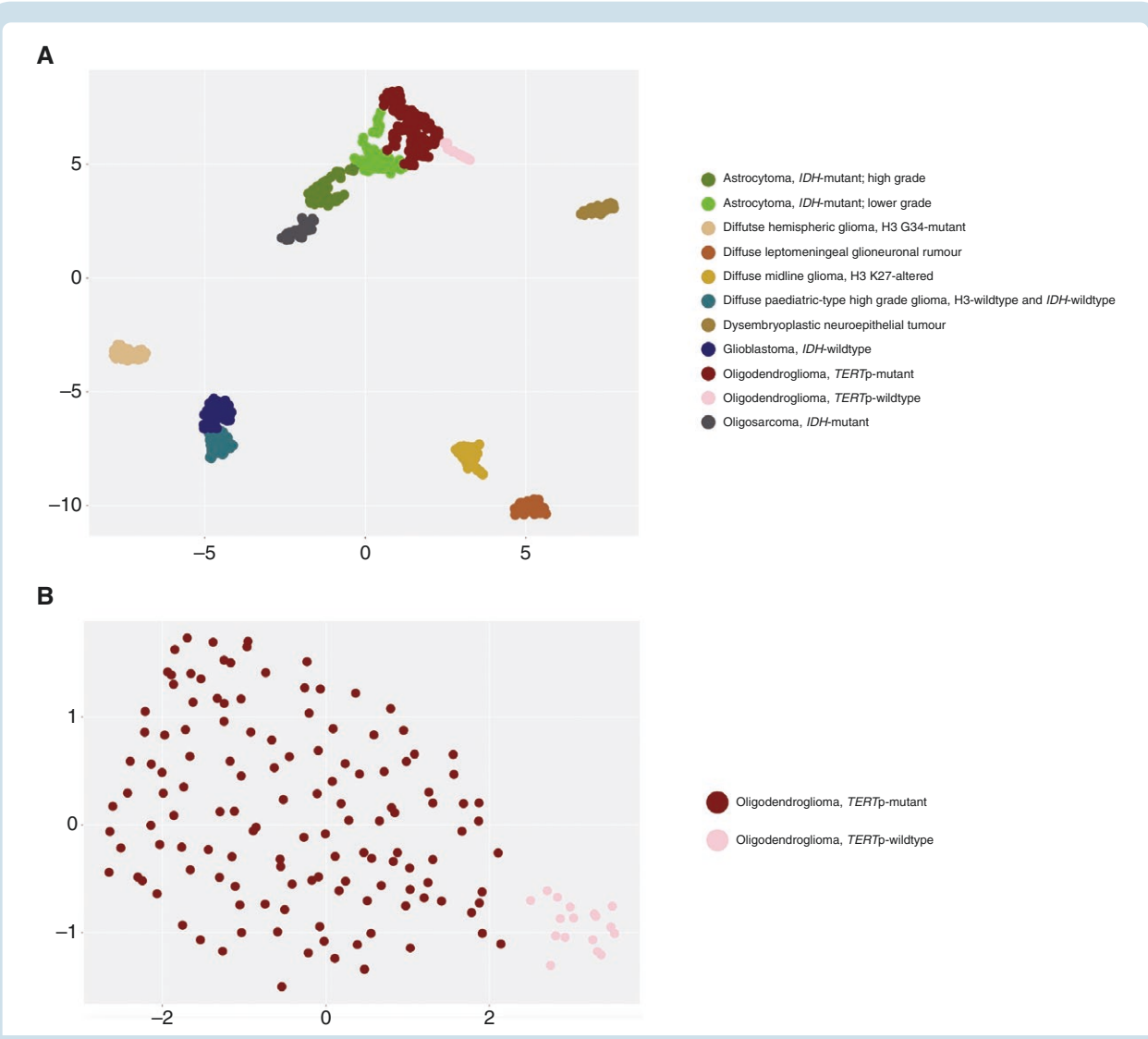

Figure 2. Unsupervised, nonlinear *t*-distributed stochastic neighbor embedding (*t*-SNE) of DNA methylation profiles from 450 glial tumor samples, being included from reference set of Heidelberg´s brain tumor classifier revealed that while *TERT*p-wildtype oligodendrogliomas showed the highest epigenetic resemblance to their mutant counterparts, they clustered together at the periphery of the *TERT*p-mutant group, representing an "epigenetic" extreme pole of oligodendrogliomas (A). Despite the epigenetic proximity between *TERT*p-wildtype and *TERT*p-mutant oligodendrogliomas, no overlap was observed in further t-SNE analyses considering the entire oligodendroglioma set (B).

A major finding of this study relates to the high frequency (95.0%) of *TERT*p-wildtype status in tumors arising in young patients (≤20 years). The reported frequency of *TERT*p mutation ranges from 70%[36] to 96%,[14,15] most likely due to revisions in the definition of oligodendroglioma, shifting from a histological entity to one defined by *IDH* mutation and 1p/19q codeletion as essential molecular diagnostic criteria. The most recent studies consistently report a *TERT*p mutation rate exceeding 90% in *IDH*-mutant and 1p/19q-codeleted cases,[16,25] regardless of patient age. To the best of our knowledge, this study presents the largest cohort of *TERT*p-wildtype cases in *IDH*-mutant and 1p/19q-codeleted oligodendroglioma, expanding on previous findings ($n = 3$) that highlighted a strong link between *TERT*p-wildtype status and the teenage years.[18]

A key finding of our study is the prognostic impact of *TERT*p status in oligodendroglioma patients. *TERT*p-wildtype patients showed significantly better PFS in both KM and multivariate regression analyses, stratified by age, CNS WHO grade, and extent of resection. While *IDH* mutations, combined with 1p/19q codeletions, are well known to define oligodendroglioma as a glial tumor with significantly better clinical outcomes compared to both *IDH*-wildtype glioblastoma and *IDH*-mutant astrocytoma,[37] our findings indicate that *TERT*p status adds an additional layer of prognostic stratification in oligodendrogliomas, allowing to identify a subgroup of tumors with a relevantly better outcome than CNS WHO grade 2 *TERT*p-mutant oligodendrogliomas. Our findings expand the concept of molecular grading in adult-type diffuse gliomas and, for the first time in oligodendrogliomas, suggest the relevance

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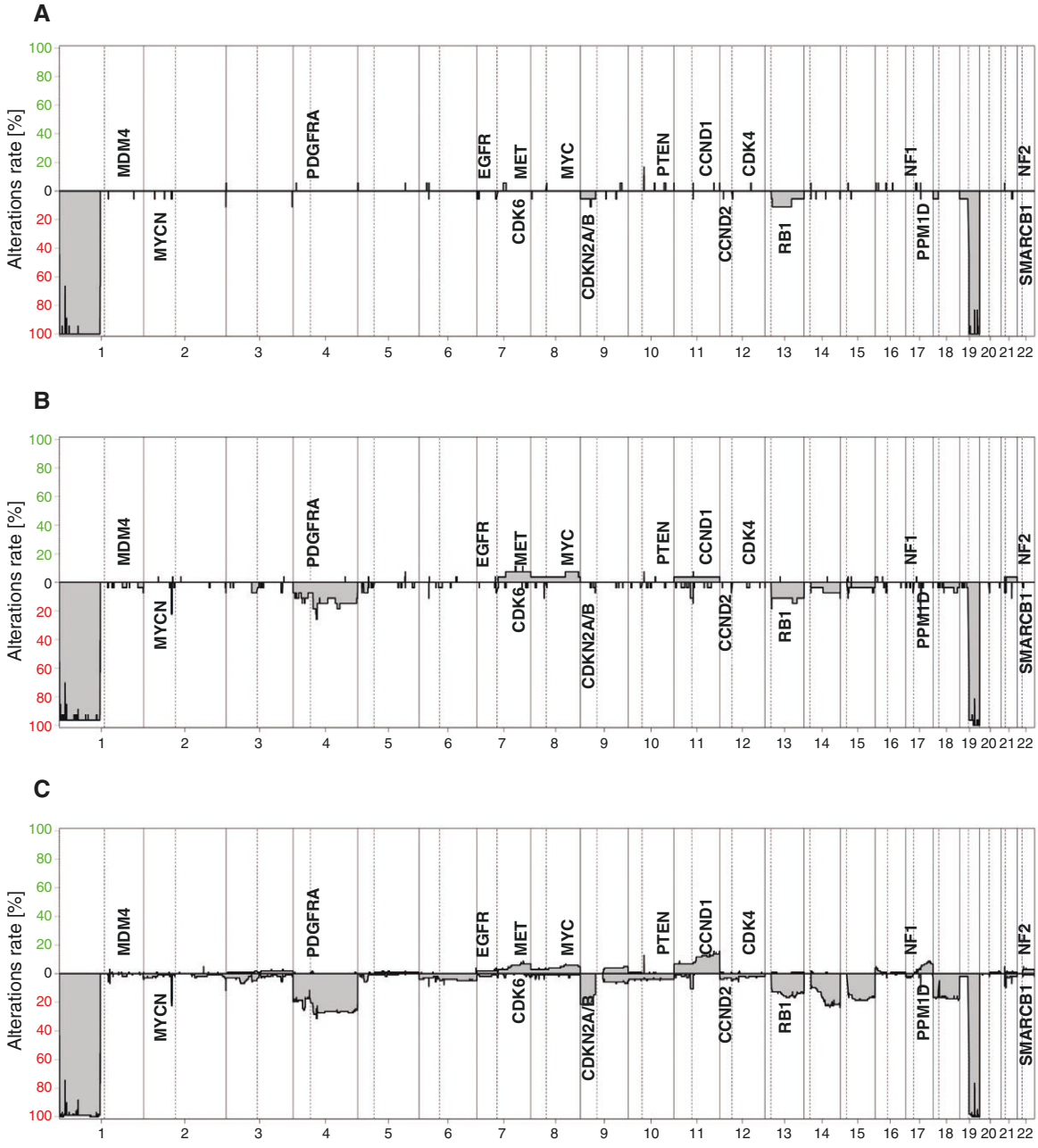

Figure 3. Cumulative copy number variations (CNV) plots of *TERT*p-wildtype cases (A), *TERT*p-mutant cases in young patients (≤30 years) (B), and *TERT*p-mutant cases in older patients (C). CNV profiles show a trend of increasing CNVs from *TERT*p-wildtype oligodendrogliomas in teenagers (A) to more alterations in the *TERT*p-mutant cases in adults (C). Annotations of the clinically relevant glioma-related gene loci have been added with descriptive purposes.

of a molecular marker to identify a lower-risk subgroup of patients. Therefore, "oligodendroglioma, IDH-mutant, 1p/19q-codeleted, and *TERT*p-wildtype" could represent a CNS WHO grade 1 oligodendroglioma associated with young age, as well as *CDKN2A/B* deletion has recently drawn attention as a potential marker able to identify the malignant counterpart of the oligodendroglioma spectrum.[38] However, the clinical significance of *CDKN2A/B* alterations in oligodendroglioma still remains debated.[39]The prognostic impact of *TERT*p-wildtype status represents a compelling novel finding, as the extremely limited number of *TERT*p-wildtype cases in prior oligodendroglioma cohorts[14,24,25] has made it impracticable to statistically evaluate the impact of *TERT*p status on PFS. In a recent study[40] aiming at analyzing long-term outcomes in patients with CNS WHO grade 2 oligodendroglioma, the median PFS was 6.8 years, and the 5-year PFS rate was 60%. In our cohort, *TERT*p-wildtype patients showed a 5-year PFS rate of

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Table 2: Univariate and multivariate Cox regression analysis for PFS.

Variables	Univariate			Multivariate		
	HR	95% CI	*p*-value	HR	95% CI	*p*-value
*TERT*p Mutant (reference) Wildtype	1 0.050	0.007-0.362	**0.003**	1 0.068	0.009-0.512	**0.009**
Age ≤39 y/o (reference) >39 y/o	1 1.531	0.998-2.349	**0.049**	1 1.043	0.674-1.614	0.849
Gender Male (reference) Female	1 1.173	0.754-1.826	0.479	_		_
Site Frontal (reference) Other	1 0.908	0.590-1.399	0.663	_		_
CNS WHO grade 2 (reference) 3	1 2.657	1.701-4.151	**<0.001**	1 2.368	0.929-6.036	0.071
Mitosis[a] ≤1/10 HPFs (reference) >1/10 HPFs	1 2.242	1.462-3.438	**<0.001**	1 1.025	0.476-2.210	0.949
Necrosis Absent (reference) Present	1 1.857	0.919-3.756	0.085	_		_
Microvascular proliferation Absent (reference) Present	1 1.785	1.134-2.807	**0.012**	1 0.831	0.377-1.833	0.646
Ki-67[a] ≤5% (reference) >5%	1 1.986	1.218-3.238	**0.006**	1 0.887	0.494-1.591	0.687
CNV alterations Isolated 1p/19q codeletion (reference) Additional CNV alterations	1 2.091	1.327-3.294	**0.001**	1 1.381	0.851-2.240	0.191
CDKN2A/B homozygous deletion Absent (reference) Present	1 1.616	0.922-2.834	0.094	_		_
IDH mutations *IDH1* R132 (reference) *IDH2* R172	1 0.902	0.509-1.601	0.725	_		_
CIC mutation[b] Absent (reference) Present	1 0.912	0.473-1.757	0.782	_		_
FUBP1 mutation[c] Absent (reference) Present	1 0.508	0.248-1.043	0.065	_		_
HOXD12 methylation[e] Absent (reference) Present	1 1.228	0.989-1.525	0.063	_		_
EOR Biopsy/STR (reference) GTR	1 0.664	0.426-1.035	0.071	_		_
Adjuvant treatment Yes (reference) No	1 1.442	0.935-2.225	0.098	_		_

*TERT*p = *TERT* promoter, PFS = progression-free survival, CNS = Central Nervous System, WHO = World Health Organization, HPFs = High-power fields, CNV = Copy Number Variation, EOR = extent of resection, GTR = gross-total resection, STR = sub-total resection.
[a]Considered as categorical binary variables, according to the median value.
[b]Calculated on 72 cases (based on data availability).
[c]Calculated on 73 cases (based on data availability). [d]Calculated on 158 cases (based on data availability).
[e]Based on beta values of *HOXD12* gene CpG site cg23130254. Multivariate analysis was performed on variables that reached significance on univariate analysis. Statistically significant *p*-values are reported in bold.

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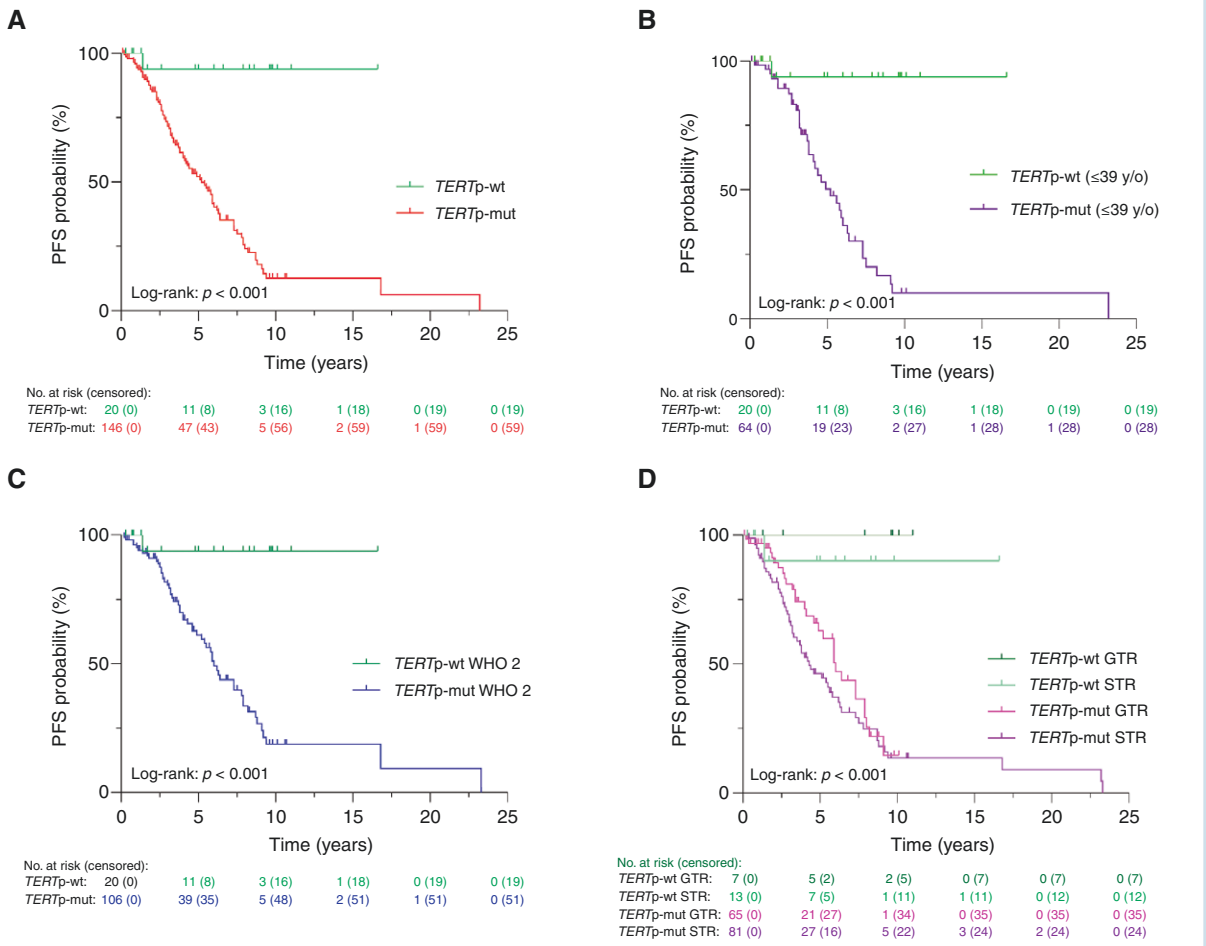

Figure 4. Kaplan–Meier curves by log-rank test. *TERT*p-wildtype status was significantly associated with better progression-free survival (PFS) in the overall cohort (A), and this association remained significant in specific patient subgroups such as in patients aged ≤39 years (B), in CNS WHO grade 2 tumors (C) and by subgrouping patients based on different extent of resection (D).

93.7% versus 52.1% of *TERT*p-mutant. Taking into account patients with CNS WHO grade 2 only ($n = 126$), *TERT*p-wildtype patients showed a 5-year PFS rate of 93.7% versus 61.1% of *TERT*p-mutant. Notably, *TERT*p-wildtype cases undergoing STR showed a better prognosis compared to *TERT*p-mutant cases with surgical GTR. Moreover, it would have been interesting to confirm the significantly superior outcome of *TERT*p-wildtype oligodendrogliomas also in terms of overall survival (OS), and the lack of this endpoint could represent a limitation of our study. However, median OS in oligodendrogliomas is a too-prolonged measure to be investigated under acceptable conditions, as even shown by the most valued clinical trials.[41]

Our findings hold clinical relevance in light of recent results from the INDIGO study,[41] a phase 3 trial of vorasidenib, an *IDH1*/*IDH2* inhibitor, which improved PFS and delayed intervention in patients with CNS WHO grade 2 *IDH*-mutant gliomas. The study focused on "low-risk" patients in the earliest phase of *IDH*-mutant glioma, typically managed with a "watch and wait" approach. Gatto et al.[42] identified an "intermediate risk" subgroup within this population that could benefit most from vorasidenib patients with stable, slow-growing tumors who do not need immediate adjuvant therapy.

Our findings on the role of *TERT*p status in refining risk stratification in CNS WHO grade 2 oligodendrogliomas may provide a more reproducible approach within the low-risk subgroup, helping identify the best candidates for "watch and wait" versus "vorasidenib treatment."

We further explored whether the *TERT*p-wildtype subgroup represents "early" or "distinct" oligodendrogliomas, as the timeline of driver genetic events in gliomagenesis remains debated.[43–45] To explore this, we collected samples from regions with markedly different Ki-67 expression within the same paraffin block and performed *TERT*p sequencing on both high and low Ki-67 areas. *TERT*p was found to be equally mutant in both areas, suggesting that *TERT*p status in oligodendrogliomas is independent of the sampled region. However, investigations on a larger number of cases are mandatory to give consistency to the trend we observed on just 2 cases. To further explore whether *TERT*p mutation might be acquired over time during tumorigenesis, we analyzed 6 *TERT*p-mutant recurrent cases with available primary tumor samples. *TERT*p

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sequencing showed the mutation was present in both primary and recurrent samples in all 6 cases. These findings align with a recent study that investigated *TERT*p mutation clonality through whole-tumor analysis, showing that *TERT*p mutation, along with *IDH* mutation,[45] is among the earliest events in glioma development and evolution.[7] The considerably long PFS of the 6 investigated cases supports even more the stability of *TERT*p mutation.

In terms of the epigenetic profile, *TERT*p-wildtype oligodendrogliomas closely resembled their mutant counterparts, but shaped a distinct cluster at the periphery of *TERT*p-mutant oligodendrogliomas. While *IDH*-mutant and 1p/19q-codeleted oligodendroglioma achieved the highest score in Heidelberg's brain tumor classifier v12.8, the vast majority of *TERT*p-wildtype cases had scores below the 0.9 cutoff, indicating a slightly different epigenetic profile, which was further confirmed by *t*-SNE analysis. CNV analysis revealed a trend of increased alterations from *TERT*p-wildtype oligodendrogliomas in teenagers to their *TERT*p-mutant adult counterparts.

To investigate whether *TERT* overexpression in *TERT*p-wildtype oligodendrogliomas might be epigenetically regulated, we analyzed the methylation status of CpGs within *TERT*p, including key upstream (cg11625005) and proximal promoter sites (cg10896616). Previous studies have shown that upstream hypermethylation and proximal hypomethylation of *TERT*p lead to *TERT* overexpression in various cancer cell lines and tissues.[33–35] Based on this, our findings suggest that *TERT* overexpression due to *TERT*p mutation is not epigenetically compensated in *TERT*p-wildtype oligodendrogliomas, potentially further explaining the favorable clinical outcomes in these patients. However, further validation at the protein expression level may be needed since the impact of allele-specific DNA methylation within the *TERT*p on gene transcription still remains controversial.[46]

Given that genetic and epigenetic regulations do not favor *TERT* overexpression in *TERT*p-wildtype oligodendrogliomas as they do in their mutant counterparts, we performed WES analysis to explore alternative mechanisms of replicative senescence evasion. However, *TERT*p-wildtype cases did not reveal any meaningful mutations in TM-related genes. Although TM has been considered a key event in glioma development,[4] it may not be mandatory in *IDH*-mutant and 1p/19q-codeleted oligodendrogliomas in young patients.

*TERT*p mutation analysis is virtually limited to DNA-based methods, sequencing, and pyrosequencing in particular. Due to the high cytosine-guanosine content in the *TERT*p sequence, both the analysis through Sanger and NGS-based methods can be challenging.[23,47] The testing through Sanger sequencing was conducted by using genomic DNA and a specific pair of primers to cover the whole promoter region and identify all mutation variants. To further assess *TERT*p status and minimize the error margin of the analysis, cases without sequence variations on Sanger sequencing ($n = 23$) were tested with WES, and the wildtype status was confirmed in in 20/23 (86.9%) cases, supporting the importance of a multi-method approach to explore *TERT*p status. Unfortunately, no reliable immunohistochemical marker can be used in routine practice as an indirect indication of the presence or absence of *TERT*p mutations.[48]

We further investigated *HOXD12* methylation status, recently shown to correlate significantly with age and prognosis in oligodendrogliomas.[30] Our findings showed higher levels of *HOXD12* methylation in *TERT*p-mutant patients compared to *TERT*p-wildtype. However, multivariable linear regression analysis suggested that the association between *TERT*p status and *HOXD12* methylation, observed by univariable analysis, may be confounded by age and WHO grade. Notably, 2 other CpG sites previously reported to have prognostic significance were not consistently included across the various arrays used in the present study (450k, 850k, and EPICv2.0), and were therefore not analyzed.

In summary, *TERT*p-wildtype oligodendrogliomas represent a distinct subgroup of *IDH*-mutant, 1p/19q-codeleted gliomas, characterized by unique genetic and epigenetic features, as well as distinct age distribution and clinical course. Incorporating this finding into risk stratification may improve the standard of care for these patients.

Supplementary material

Supplementary material is available online at *Neuro-Oncology* (https://academic.oup.com/neuro-oncology).

Keywords

adolescents and young adults | methylation | oligodendroglioma | progression-free survival |TERT promoter

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Conflict of interest statement

R.R. is a fellow of the AI Health Innovation Cluster Clinician Scientist Program. A.v.D. and F.S. are co-founders of Heidelberg Epignostix GmbH. The other authors declare no conflict of interest.

Author Contributions

F.S. and L.B. conceived, designed, and supervised the study. S.O.A., A.A.R., and A.M. curated the formal analysis. F.N., R.R., A.v.D., D.E.R., R.B., P.S., H.B., A.K.S., F.H., and F.K.A. offered pathological review. F.N., R.R., D.S., D.F., K.G., L.F., and S.P. curated molecular analysis. T.S., G.F., J.R.H., D.A.K., P.S., T.M., T.J., O.W.S., C.H.M., S.M.P., W.W., and S.M.K. provided clinical data. S.S., L.S., K.K., M.M., S.Z., C.S.N., R.C., J.S., B.G., N.E., and M.R.

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provided tissue specimens and offered pathological review. F.N. and R.R. wrote the manuscript and all authors reviewed and approved the manuscript for publication.

Ethics Statement

Collection and analysis of tissue samples and clinical data were performed in accordance with the principles of the Declaration of Helsinki.

Data Availability

Data will be made available upon reasonable request.

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