LABORATORY INVESTIGATION

Prognostic significance of telomere maintenance mechanisms in pediatric high-grade gliomas

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Abstract Children with high-grade glioma, including diffuse intrinsic pontine glioma (DIPG), have a poor prognosis despite multimodal therapy. Identifying novel therapeutic targets is critical to improve their outcome. We evaluated prognostic roles of telomere maintenance mechanisms in children with HGG, including DIPG. A multi-institutional retrospective study was conducted involving 50 flash-frozen HGG (35 non-brainstem; 15 DIPG) tumors from 45 children (30 non-brainstem; 15 DIPG). Telomerase activity, expression of *hTERT* mRNA (encoding telomerase catalytic component) and TERC (telomerase RNA template) and alternative lengthening of telomeres (ALT) mechanism were assayed. Cox Proportional Hazard regression analyses assessed association of clinical and pathological variables, TERC and *hTERT* levels, telomerase activity, and ALT use with progression-free or overall survival (OS). High TERC and *hTERT* expression was detected in 13/28 non-brainstem HGG samples as compared to non-neoplastic controls. High TERC and *hTERT* expression was identified in 13/15 and 11/15 DIPG samples, respectively, compared to controls. Evidence of ALT was noted in 3/11 DIPG and 10/19 non-brainstem HGG specimens. ALT and telomerase use were identified in 4/19 non-brainstem HGG and 2/11 DIPG

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specimens. In multivariable analyses, increased TERC and *hTERT* levels were associated with worse OS in patients with non-brainstem HGG, after controlling for tumor grade or resection extent. Children with HGG and DIPG, have increased *hTERT* and TERC expression. In children with non-brainstem HGG, increased TERC and *hTERT* expression levels are associated with a worse OS, making telomerase a promising potential therapeutic target in pediatric HGG.

Keywords Pediatric · High-grade glioma · Telomerase

Introduction

The outcome for children with high-grade gliomas (HGG) remains poor despite multi-modal therapy with surgery, radiation, and conventional chemotherapy. Molecularly targeted agents may offer new therapeutic options for this devastating disease. One potential target is telomerase, a ribonucleoprotein complex that includes a catalytic subunit encoded by the *hTERT* gene and an RNA template (TERC) [1–3]. Telomerase elongates telomeric DNA by adding hexanucleotide repeats to compensate for progressive DNA loss with each cell division [1, 4].

Telomerase activity is detectable in 85–95 % of cancer types [5–7]. By contrast, normal somatic tissues, including brain, do not demonstrate telomerase activity [5, 7]. *hTERT* mRNA expression correlates tightly with telomerase activity and is generally restricted to tumor cells [8–10]. Although TERC RNA is present in most normal cells, TERC expression is upregulated during tumorigenesis [11, 12].

Telomerase activity is highly variable in adult anaplastic astrocytomas (10–100 %) and glioblastoma multiforme (GBM) (26–100 %) [13–17]. Higher telomerase activity levels are associated with shorter overall survival (OS) for adult GBM [16, 17]. A subset of the telomerase-negative tumors maintains telomere length by an alternative lengthening of telomeres (ALT) mechanism involving DNA homologous recombination [18, 19]. One study reported that 11 and 44 % of adult and pediatric GBM, respectively, demonstrated evidence of ALT [20]. Adults with ALT-positive GBM were reported to have longer survival times [21, 22]. ATRX and DAXX are members of a chromatin-remodeling complex that deposits histone H3.3 onto chromatin and telomeres [23–25]. It has recently been shown that mutations in *H3F3A*, encoding H3.3, and chromatin remodeling genes *ATRX* and *DAXX* drive pediatric glioma [26]. The presence of *H3F3A/ATRX/TP53* mutations was found to be strongly associated with ALT [26]. Three point mutations were identified which led to single amino acid substitutions at two sites in the H3.3 tail,

H3.3K27M and H3.3G34V/R [26–28]. Furthermore, 60–71 % of DIPGs harbored the H3.3K27M mutation, while none had H3.3G34R/V mutations [28]. H3.3K27M mutation defines a clinically and biologically distinct subgroup of DIPG and is associated with shorter survival [31].

Little data exist regarding the role of telomere maintenance mechanisms and correlation to outcome in children with HGG or DIPG. To the best of our knowledge, this is the first report to describe telomerase expression and its prognostic significance in children with HGG, including DIPG.

Materials and methods

We conducted a retrospective study of children with Grade III (anaplastic astrocytomas) or IV gliomas (GBM), including DIPG, diagnosed between 1980 and 2011 who had flash-frozen tumor tissue available at Cincinnati Children's Hospital Medical Center (CCHMC; Cincinnati, OH, USA), Children's Memorial Hospital (Chicago, IL, USA), New York Medical Center (New York, NY, USA), Nationwide Children's Hospital (Columbus, OH, USA), Henry Ford Hospital (Detroit, MI), or the Brain Tumor Tissue Bank (Ontario, Canada). Institutional review board approval was obtained at CCHMC and at participating institutions (as applicable). Clinical information obtained included histopathology, extent of resection, primary tumor location, and time from diagnosis to progression/relapse, death, or last follow-up. Fifty HGG specimens from 45 patients were acquired, including 15 DIPGs and 35 non-brainstem HGGs. Four non-brainstem HGG patients provided multiple tumor samples at different times in their disease courses.

Tissue processing and analysis

All tumors were centrally reviewed for histological confirmation by an experienced neuropathologist (LM). Study controls included matched non-tumoral flash-frozen cortical brain specimens from autopsies of six DIPG patients on this study (median age 5.1 years; range 2.8–12.5 years), five flash-frozen non-tumoral cortical brain specimens from patients (age range 1.8–18 years) who had undergone epilepsy surgery, and commercial RNA derived from parietal cortex of a 26-year-old man (Agilent, Santa Clara, CA, USA), pooled pons from 20 people (age range 18–54 years; Clontech, Mountain View, CA, USA) and total brain from an 18-year-old man (Clontech, Mountain View, CA, USA). All tissue specimens were stored at −80 °C until processed for analysis.

Three measures of telomerase expression, (i) *hTERT* mRNA and (ii) TERC RNA levels by quantitative real-time polymerase chain reaction (qRT-PCR), and (iii) telomerase enzyme activity by telomeric repeat amplification protocol (TRAP) assay, were performed on tumor and control specimens. Although the TRAP assay provides a direct assessment of telomerase enzymatic activity, it may be subject to false negative results if telomerase is inactivated by time or heat during specimen processing. *hTERT* mRNA levels have been shown to correlate well with telomerase activity [8, 9, 29]. Telomeric restriction fragment (TRF) analysis by Southern blot was used to evaluate ALT activity [30]. Molecular assays were performed by investigators blinded to patients' outcome.

hTERT and TERC expression

Total RNA was extracted by RNAzol reagent (Molecular Research Center, Cincinnati, OH, USA), and 1 μg RNA was converted to cDNA using First Strand cDNA Synthesis kit (USB, Cleveland, OH, USA). *hTERT* mRNA expression analysis was performed by qRT-PCR using a primer/probe set that spans exon junctions 3–4 and therefore detects all gene splice forms (Hs00972650_m1; Applied Biosystems, Carlsbad, CA, USA). TERC RNA was performed similarly by qRT-PCR using commercially available primers and probes (Hs03297287_s1; Applied Biosystems, Carlsbad, CA, USA). mRNA levels of housekeeping genes glyceraldehyde phosphate dehydrogenase and TATA-box binding protein using commercially available primer/probe sets (Hs03929097_g1 and Hs00427621_m1, Applied Biosystems, Carlsbad, CA, USA) were used as normalizers. The mean *hTERT* and TERC levels from normal brain specimens were used as the reference level to which the tumor specimen levels were compared. qRT-PCR assays were performed for each sample at least twice in triplicate. Relative expression (expressed as relative quantification, RQ) levels of *hTERT* mRNA and TERC RNA levels were compared to the mean expression level from non-neoplastic control brain samples using the comparative Ct method [31]. RQ values >1 defined increased expression levels as compared to the mean values for control non-neoplastic specimens.

Telomerase enzyme activity

Telomerase enzyme activity was assessed in tumor and control non-tumoral samples using the TRAPeze Telomerase Detection Kit (Millipore, Billerica, MA, USA) as previously described [32]. Recombinant RNasin ribonuclease inhibitor was added to the CHAPS lysis buffer before the extraction to a concentration of 200 units/ml lysis buffer. Testing was performed using 400 ng total protein per tumor/control specimen. Tumors were considered to have telomerase activity if a characteristic laddering pattern of telomere amplification products was detected by gel electrophoresis. Lysates without detectable enzymatic activity were reanalyzed at higher protein concentrations to detect low levels of telomerase activity. HeLa cell line (30 ng total protein) and CHAPS buffer were used as positive and negative controls, respectively.

ALT use

DNA was extracted from tumor specimens using the Gentra Puregene kit (Qiagen, Dusseldorf, Germany). Assessment of TRF lengths by Southern blot was performed on tumor specimens using the TeloTAGGG Telomere Length Assay Kit (Roche Diagnostics, Indianapolis, IN, USA). For all samples, 1 μg of total genomic DNA was digested and separated by gel electrophoresis. Samples were considered positive for ALT if they exhibited heterogeneous telomere lengths, including high molecular weight (>21 kb) telomeres. The osteosarcoma Saos-2 and histiocytic lymphoma U937 cell lines were used as positive and negative controls, respectively, for ALT.

H3F3A gene sequencing

Histone 3.3 gene *H3F3A* was partially sequenced as described by Wu et al. [30]. Briefly, a region of interest in *H3F3A* gene was sequenced by direct sequencing of PCR amplified products using the primers forward: 5′-GATTTT GGGTAGACGTAATCTTCA-3′, reverse: 5′-TTTCCTGT TACTCATCTTTTTGTT-3′ and a high fidelity DNA polymerase Optimase (Transgenomic, Omaha, NE, USA). Available matched non-neoplastic samples were also sequenced when available. The Sanger sequencing was performed on both strands using the above forward and reverse primers.

Statistical analyses

TERC expression levels were analyzed in base-2 log-transformed scale to provide hazard ratios (HR) in terms of doubling of qRT-PCR levels. *hTERT* is a low-copy-number transcript, and no amplification may be reported in qRT-PCR analysis with the maximum of 50 cycles. A constant of 1 was added to all *hTERT* expression RQ values to enable analysis in base2 log-transformed scale since the original data contained some values that were 0. TRAP and ALT variables were categorized as positive or negative, as described above. The following clinical and demographic variables were reviewed: age at diagnosis, pathological grade (WHO grade III vs. IV), tumor location (DIPG/brainstem vs.non-brainstem), specimen disease timing (initial vs. relapse), and extent of surgical resection (total vs. partial).

Table 1 Subject characteristics

	Results
Characteristic of subjects with non-brainstem HGG	
No. of patients	30
No. of tumor specimens	35
Median age (years)	13.1
Age range (years)	0.2–22.2
Male:female	18:12
Pathologic grade (III:IV)	8:22
Specimen timing (diagnosis:relapse)	21:11
Characteristic of subjects with DIPG	
No. of patients	15
No. of tumor specimens	15
Median age (years)	5.1
Age range (years)	2.7–12.5
Male:female	3:12
Pathologic grade (III:IV)	3:12
Specimen timing (diagnosis:autopsy)	1:14

HGG high-grade glioma, *DIPG* diffuse intrinsic pontine glioma

Associations of biological markers and clinical and demographic variables with progression-free survival (PFS) and OS were evaluated using Cox Proportional Hazard (PH) models and log-rank tests as appropriate. Due to sample size limitations, multivariable models were limited to two variables. Four patients had multiple tumor specimens ($n = 9$ specimens) from different time points, and the samples were analyzed for temporal changes in telomerase maintenance mechanisms descriptively. For the outcome analysis, assay results from the earliest specimen were used for patients who had multiple tumor samples available. PFS was defined as time from diagnosis to first disease progression or death and to last follow-up visit for patients without progression or death. OS was defined as time from diagnosis to death or last follow-up visit.

Comparison of *hTERT* and TERC expression values between DIPG and non-brainstem HGG specimens and between relapsed and newly-diagnosed non-brainstem HGG specimens was performed using the Kruskal–Wallis test. Results with *p* values <0.05 were reported as statistically significant without any correction for multiplicity.

Results

Patient characteristics

Fifty tumor samples from 45 patients were included. Table 1 summarizes the clinical characteristics of 35 non-brainstem HGG tumors from 30 patients and of 15 DIPG tumors from 15 patients. Two non-brainstem samples were secondary HGG that developed after low-grade astrocytoma or medulloblastoma.

hTERT and TERC expression

Table 2 summarizes clinical and demographic information and results from telomere maintenance assays, including *hTERT* and TERC expression analysis. Among 15 DIPG tumor specimens, 86.7 and 73.3 % had increased TERC RNA and *hTERT* mRNA expression, respectively, compared to the mean normal controls. Of 28 non-brainstem HGG specimens analyzed, 46.4 % had increased TERC RNA and *hTERT* mRNA expression levels compared to normal controls. Median TERC values were significantly (3.4-fold) higher in children with DIPG compared to those with non-brainstem HGG (Kruskal–Willis, $p = 0.001$) (Table 3). Median *hTERT* values trended towards being significantly higher (12-fold) in the DIPG cohort compared to the non-brainstem HGG cohort (Kruskal–Willis, $p = 0.056$). When *hTERT* and TERC values were compared between the non-brainstem HGG specimens from diagnosis ($n = 22$) versus relapse ($n = 6$), no statistically significant differences were noted.

Telomerase activity by TRAP

Thirty-eight tumor (26 non-brainstem HGG; 12 DIPG) and five non-neoplastic matched brain specimens were analyzed for telomerase activity by TRAP (Supplemental Fig. 1; Table 2). Twenty-five percent (3/12) of DIPG specimens and 42 % (11/26) of non-brainstem HGG samples had telomerase activity, compared to none of the non-neoplastic control specimens. 91 % (11/12) of TRAP-positive non-brainstem HGG samples were Grade IV tumors. The lower level of telomerase-positive DIPG specimens may be secondary to technical issues related to time to processing of autopsy specimens and resulting inactivation of any telomerase enzyme present in tumor cells, especially in light of the lower correlation of TRAP results with *hTERT* mRNA levels in specimens obtained by autopsy versus surgery (Table 4).

ALT use

Telomere length of 30 (19 non-brainstem HGG; 11 DIPG) specimens was analyzed by Southern blot (Supplemental Fig. 2; Table 2). 27 % (3/11) of DIPG and 53 % (10/19) non-brainstem HGG specimens showed evidence of ALT use. Interestingly, 21 % (4/19) of non-brainstem HGG and 18 % (2/11) of DIPG specimens used both ALT and telomerase. Specifically, 6/13 (46 %) ALT-positive (non-brainstem and DIPG) tumors also showed evidence of

Table 2 Summary of clinical characteristics and telomere maintenance assay results

ID	Gender	Age (years)	Tumor	Pathology	Specimen timing	Resection	Telomerase activity by TRAP	ALT	TERT (RQ)	TERC (RQ)	Time to death or last follow-up (years)	Deceased
1	M	8.8	DIPG	GBM	A	N/A	–	+	5.38	8.56	0.84	Y
2	F	12.1	DIPG	AA	D	Partial	N/A	N/A	52.29	1.46	2.26	Y
3	F	22.2	HGG	GBM	D	Partial	–	+	0	0.31	1.53	N
4	F	12.0	DIPG	AA	A	N/A	–	+	0.50	5.03	1.21	Y
5	F	10.2	HGG	AA	D	Partial	–	+	0.11	0.23	11.19	N
6	F	17.9	HGG	GBM	R	Partial	N/A	N/A	0.49	1.58	3.47	Y
7	M	17.9	HGG	AA	D	Partial	+	N/A	80.21	1.40	1.18	Y
8	F	2.5	HGG	AA	D	Total	–	N/A	0	4.031	5.36	N
9[#]	M	17.9	HGG	AA	D	Partial	–	+	0	0.84	2.98	Y
10[#]	M	17.9	HGG	GBM	R	Partial	+	+	4.058	0.32	2.98	Y
11	M	5.9	HGG	GBM	D	Partial	N/A	N/A	0.44	1.70	2	N
12	M	13.8	HGG	AA	D	Total	N/A	N/A	0.36	1.18	0.27	N
13	M	12.1	HGG	GBM	D	Total	N/A	N/A	0	0.43	3.23	N
14	M	3.7	HGG	AA	D	Partial	–	N/A	0.14	0.21	0.26	N
15[*]	M	12.1	HGG	GBM	R	Total	N/A	N/A	57.91	5.29	3.23	N
16[*]	M	12.1	HGG	GBM	R	Total	N/A	N/A	71.41	1.73	3.23	N
17[^]	F	0.02	HGG	GBM	R	Partial	–	N/A	N/A	N/A	21.38	N
21	F	5.1	DIPG	GBM	A	N/A	–	N/A	3.58	5.22	1.16	Y
22	F	3.2	DIPG	AA	A	N/A	N/A	N/A	5.44	26.016	0.56	Y
23	M	5.4	DIPG	GBM	A	N/A	+	–	6.37	2.47	0.68	Y
24	F	12.5	DIPG	GBM	A	N/A	+	–	4.83	6.40	0.81	Y
25	F	12.1	DIPG	GBM	A	N/A	N/A	N/A	0.80	2.38	0.78	Y
26	M	2.8	DIPG	GBM	A	N/A	–	–	1.68	2.45	2.64	Y
27	F	3.4	DIPG	GBM	A	N/A	–	–	0.12	0.53	0.42	Y
36	F	4.7	DIPG	GBM	A	N/A	–	–	9.027	2.65	1.58	Y
37	F	4.8	DIPG	GBM	–	–	3.92	5.82	1.25	Y		
45	F	9.6	DIPG	GBM	–	+	15.22	5.92	0.64	Y	21.38	N
46	F	4.2	DIPG	GBM	–	–	0.14	0.84	0.81	Y	1.15	Y
47	F	4.6	DIPG	GBM	+	–	7.12	2.85	1.95	Y	0.98	Y
28	F	6.9	HGG	GBM	R	Total	+	–	5.31	1.056	3.58	Y
29	F	20	HGG	GBM	R	Partial	–	+	0.33	0.18	0.83	N
30	M	6	HGG	GBM	D	Partial	–	N/A	8.45	19.36	0.08	Y
31	F	15.8	HGG	AA	D	Partial	–	–	0.30	1.44	2.25	N
32	F	13.8	HGG	GBM	D	Total	+	–	499.71	0.20	1	Y
33	M	15.2	HGG	GBM	D	Partial	+	–	2.99	3.21	1.42	Y
34	F	7.4	HGG	AA	D	Partial	–	+	0	3.13	1.58	Y
35	M	15.2	HGG	GBM	D	Total	+	–	15.46	0.15	3	N
38	M	17.8	HGG	AA	R	Partial	–	+	0.087	0.62	1.5	N
39[+]	M	19.2	HGG	GBM	D	Total	–	+	1.18	0.42	4.58	N
40[+]	M	19.2	HGG	GBM	R	Total	–	–	0.30	0.20	4.58	N
41	M	16.6	HGG	GBM	D	Partial	–	N/A	0.30	0.41	0.65	Y
42	M	8.4	HGG	GBM	D	Partial	–	–	0.23	0.76	0.8	Y
43	F	11.6	HGG	GBM	D	Partial	–	+	1.83	8.20	0.77	Y
44	M	12.4	HGG	GBM	D	Partial	+	–	19.80	0.70	1.87	Y
48	M	15	HGG	GBM	D	Total	+	–	6.65	0.84	0.91	Y
49	M	12	HGG	GBM	R	Total	–	+	4.33	0.90	2.24	Y
50	M	20	HGG	GBM	D	Total	–	–	0.37	1.12	0.45	Y

HGG non-brainstem high-grade glioma, *DIPG* diffuse intrinsic pontine glioma, *GBM* glioblastoma multiforme, *AA* anaplastic astrocytoma, *RQ* relative quantification, specimen timing, *A* autopsy, *D* diagnosis, *R* relapse

#, ^, *, + Serial specimens from same patient

Table 3 *hTERT* and TERC expression by tumor location

Variable	Tumor	n	RQ			
			Mean	SD	Median	Range
hTERT	DIPG	15	7.76	12.95	4.83	0.12–52.3
	Non-brainstem HGG	28	23.3	94.6	0.4	0–499
TERC	DIPG	15	5.24	6.19	2.85	0.53–26.01
	Non-brainstem HGG	28	1.97	3.79	0.84	0.15–19.34

HGG high-grade glioma, *DIPG* diffuse intrinsic pontine glioma, *RQ* relative quantification, *SD* standard deviation

Table 4 Multivariable Cox Proportional Hazards model results for non-brainstem HGG assessing association with OS

Variable	HR	*p* value
Extent of resection	2.1	0.18
hTERT	1.3	0.021
Extent of resection	1.6	0.41
TRAP	2.2	0.14
Extent of resection	1.4	0.55
TERC	1.4	0.079
Extent of resection	1.0	1.0
ALT use	0.5	0.3
Tumor grade	2.6	0.21
hTERT	1.2	0.11
Tumor grade	2.9	0.17
TRAP	1.4	0.49
Tumor grade	5.0	0.042
TERC	1.6	0.016
Tumor grade	3.7	0.21
ALT use	0.6	0.46

HGG high-grade glioma, *OS* overall survival

telomerase use. Finally, 3/19 (16 %) non-brainstem HGG and 2/11 (18 %) DIPG tumors exhibited no telomere maintenance mechanism (i.e. demonstrated no telomerase activity on TRAP, no evidence of ALT on TRF analysis and had *hTERT* mRNA RQ ≤1).

H3F3A gene sequencing

Partial sequencing of *H3F3A* gene, which encodes histone 3 variant H3.3, was performed on 12 DIPG and 23 non-brainstem HGG specimens. Six (50 %) DIPG specimens demonstrated a K27M mutation, but none had G34V/G34R mutations. Three (13 %) K27M mutations, one (4.5 %) G34V mutation and one (4.5 %) G34R mutation were detected in non-brainstem HGG specimens. Patients with K27M mutations tended to be younger (7.4–11.6 years).

Both tumor specimens with G34R/G34V mutations used ALT to maintain telomere length and were from young adult patients (20–22 years). When considering the DIPG specimens only, 3/6 of tumors with K27M mutations used ALT and 0/5 wild-type H3.3 tumors used ALT.

Association between telomere maintenance mechanism and patient outcome

Overall median survival times for patients with DIPG and non-brainstem HGG were consistent with published literature. For the DIPG cohort, median PFS and OS were 0.63 years (range 0.13–1.25 years) and 0.84 years (0.42–2.64 years), respectively. For the non-brainstem HGG cohort, median PFS and OS were 0.83 years (0.08–3.67 years) and 2.25 years (0.08–11.19 years), respectively. Kaplan–Meier plots display OS distributions for non-brainstem cohort stratified by *hTERT* level or TERC level and extent of resection or tumor grade (Fig. 1a–d). Note that conversion of *hTERT* and *TERC* into dichotomous variables by splitting them into high and low levels at their median value in the HGG cohort for the Kaplan–Meier analysis lead to reduction in power and thus loss of statistical significance seen with the Cox analysis where these markers were treated as continuous variables. For the DIPG cohort, telomerase activity, ALT use, *hTERT* and TERC values were not associated with either PFS or OS in neither single- nor multivariable models (Supplementary Fig. 3).

In the non-brainstem HGG cohort, extent of resection (gross-total vs. subtotal), age at diagnosis, and tumor grade (III vs. IV) were considered using single-variable models. Higher tumor grade was associated with a worse PFS that approached statistical significance (log-rank; $p = 0.072$) in the single-variable models; no other variable demonstrated statistically significant association with PFS.

Among patients with non-brainstem HGG, 24 experienced progression and 18 had died at the time of analysis; therefore, our multivariable models could only accommodate two variables simultaneously. Given the well-established role of resection status and tumor grade in determining outcome, we chose to fit two-variable models that included either resection status or tumor grade and one telomere maintenance variable. Age at diagnosis was also considered but was not associated with PFS or OS in our cohort. In a Cox model incorporating *hTERT* and extent of resection, increased *hTERT* expression level was associated with worse OS ($p = 0.021$) in children with non-brainstem HGG. Similarly, when modeled simultaneously with tumor grade, increased TERC expression level was associated with worse OS ($p = 0.016$). Our data also contained some evidence for an association between higher TERC expression and worse OS based on a Cox model also
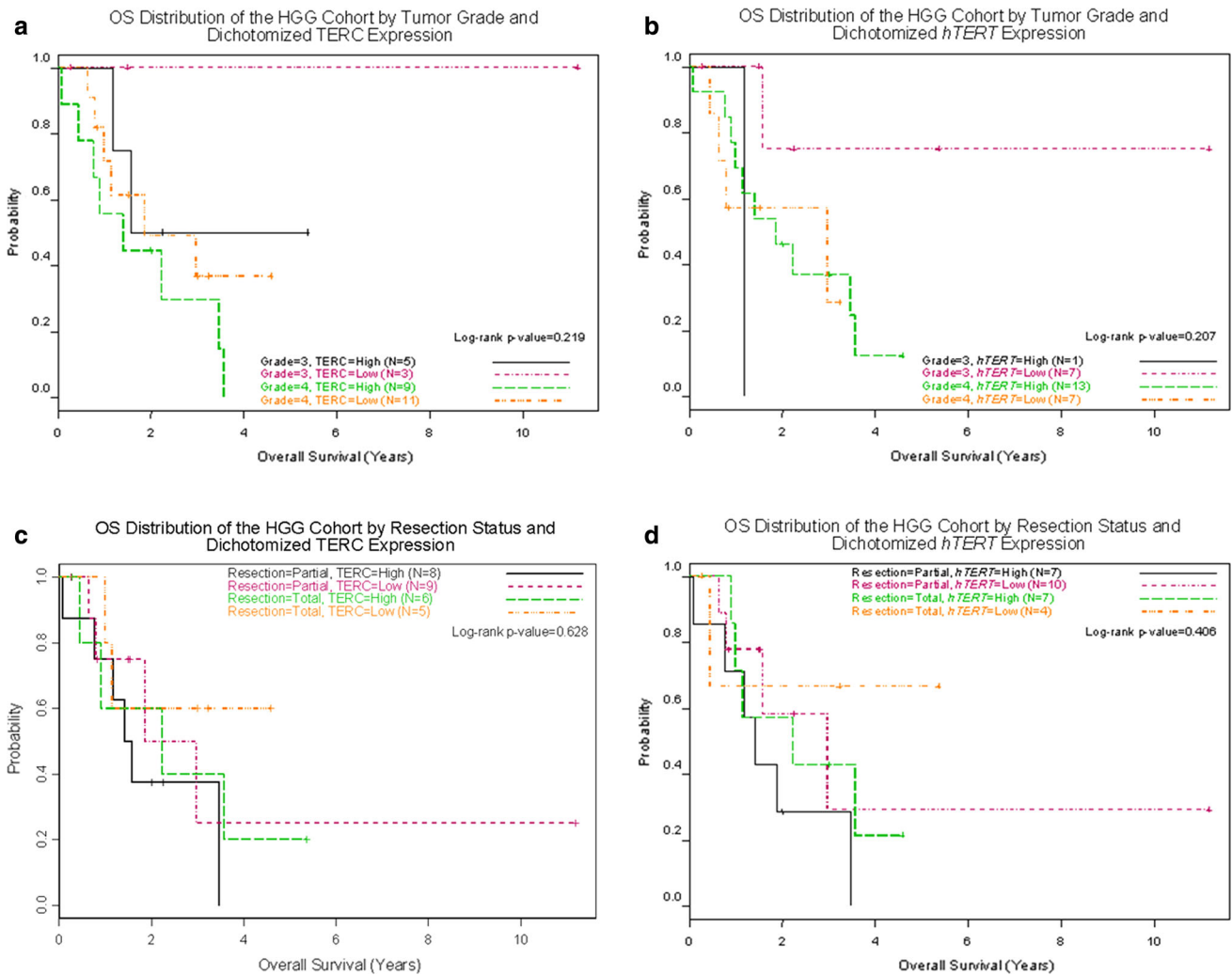
Fig. 1 a–d Overall survival distributions by Kaplan–Meier analysis of non-brainstem HGG cohort stratified hTERT expression and by resection status or tumor grade. hTERT and TERC expression were dichotomized into high (>median value) and low (<median value) groups for these plots only. The Cox model results are based on the continuous version of this variable

accounting for extent of resection ($p = 0.079$). No such associations with PFS were noted. Neither ALT use nor telomerase activity was associated with PFS or OS in single-variable or multiple-variable models for the non-brainstem HGG cohort.

Discussion

This study is the first to describe telomerase expression and its prognostic significance in children with HGG, including DIPG. We report that children with HGG, particularly DIPG, have increased hTERT and TERC levels compared to normal controls. More importantly, increased hTERT mRNA and TERC RNA expression are associated with worse OS in children with non-brainstem HGG. These findings are similar to reports in other pediatric cancers, including neuroblastoma, ependymoma and Wilms tumor [33–35]. Neither telomerase activity nor ALT use was associated with PFS or OS in our non-brainstem HGG cohort, which may be due to lack of power to detect differences in these categorical variables. Similarly, no statistically significant association between patient outcome and telomere maintenance variables, including expression levels of the telomerase components, telomerase activity and ALT use, were identified in the DIPG cohort, which may have been because of the smaller sample size and short survival times typical of children with DIPG.

In the current study, 46 % of HGG specimens (56 % of non-brainstem HGG; 25 % of DIPG tumors) used ALT to maintain telomere lengths, which builds on a prior study that included pediatric GBM tumors [20]. The prevalence and pattern of somatic H3F3A mutations in our cohort are generally consistent with prior studies [26, 28].

Specifically, G34V/G34R mutations were not found in our DIPG cases. Our finding of K27M and G34V mutations in 13 and 9 %, respectively, of non-brainstem HGG specimens is consistent with prior publications [27]. Our K27M mutation prevalence in DIPG of 50 % (95 % CI 21.1–78.9 %) is comparable to the reported prevalence [27, 28]. Although previous studies have identified the connection between ATRX/DAXX mutations, G34R/G34V mutations and ALT use, they have not fully evaluated any potential link between K27M mutations and ALT use. Our findings that all 3 DIPG patients with ALT use also had K27M mutations, suggests that the acquisition of a K27M mutation may be necessary but not sufficient for the induction of ALT use in DIPG. Further investigation of this relationship between K27M mutation and ALT use in a larger DIPG cohort is needed.

A novel observation in this study is that 21 % of non-brainstem HGG and 18 % of DIPG specimens showed both telomerase expression and ALT use. It is unclear whether these tumors are composed of separate cell populations (e.g. neural stem cell and tumor bulk populations) that utilize different telomere maintenance mechanisms or one cell population that uses both mechanisms. Further studies are required to fully understand this finding and its potential impact on response to therapies, especially in light of a prior publication that has suggested that the use of ALT in human glioma stem cells confers radiation resistance [36]. Finally, 16 % of non-brainstem HGG and 18 % of DIPG samples demonstrated no telomere maintenance mechanism (i.e. no telomerase activity, increased *hTERT* mRNA levels or ALT use), compared to 40 % of adult GBM [37].

Normal somatic cells without telomerase activity have been shown to constitutively express TERC but not *hTERT*. However, TERC is known to be upregulated during tumorigenesis [12, 38]. Our study analyzed several serial tumor samples from patients at different disease time points for evidence of known telomere maintenance mechanisms. Our data from a small subset of patients suggest that telomerase activity and *hTERT* mRNA levels, but not TERC levels, may be upregulated over the disease course, possibly in response to therapeutic interventions (data not shown), which is consistent with other publications that have noted that prior therapies can affect telomerase activity in other cell types [39–41]. Although our non-brainstem HGG cohort included diagnostic and relapsed tumor specimens from patients who had undergone radiotherapy and chemotherapy, a comparison of TERC or *hTERT* expression levels from diagnosis versus relapse specimens did not reveal statistically significant differences, albeit in a small, unpaired cohort.

Our results suggest that telomerase inhibition may be a promising therapeutic approach for children with HGG, including those with DIPG. Imetelstat (Geron, Menlo Park, CA, USA) is a telomerase inhibitor that binds to the template region of TERC has demonstrated in vitro and in vivo activity in GBM [40, 41] and has undergone Phase I–II trials in adults with advanced solid tumors [42]. Since telomerase is active in a variety of pediatric cancers, imetelstat may be an effective drug for pediatric malignancies [34, 43–50]. The Children's Oncology Group has recently completed a Phase I trial of imetelstat in children with relapsed solid tumors. Elucidation of the telomere maintenance mechanisms used by pediatric HGG tumors is important in determining subpopulations of children with HGG who are most likely to benefit from treatment with telomerase inhibitors.

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