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The prognostic impact of *TERT* promoter mutations in glioblastomas is modified by the rs2853669 single nucleotide polymorphism

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Human hotspot *TERT* promoter (*TERTp*) mutations have been reported in a wide range of tumours. Several studies have shown that *TERTp* mutations are associated with clinicopathological features; in some instances, *TERTp* mutations were considered as biomarkers of poor prognosis. The rs2853669 SNP, located in the *TERT* promoter region, was reported to modulate the increased *TERT* expression levels induced by the recurrent somatic mutations. In this study we aimed to determine the frequency and prognostic value of *TERTp* mutations and *TERT* rs2853669 SNP in 504 gliomas from Portuguese and Brazilian patients. *TERTp* mutations were detected in 47.8% of gliomas (216/452). Glioblastomas (GBM) exhibited the highest frequency of *TERTp* mutations (66.9%); in this glioma subtype, we found a significant association between *TERTp* mutations and poor prognosis, regardless of the population. Moreover, in a multivariate analysis, *TERTp* mutations were the only independent prognostic factor. Our data also showed that the poor prognosis conferred by *TERTp* mutations was restricted to GBM patients

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carrying the rs2853669 A allele and not in those carrying the G allele. In conclusion, the presence of *TERTp* mutations was associated with worse prognosis in GBM patients, although such association depended on the status of the rs2853669 SNP. The status of the rs2853669 SNP should be taken in consideration when assessing the prognostic value of *TERTp* mutations in GBM patients. *TERTp* mutations and the rs2853669 SNP can be used in the future as biomarkers of glioma prognosis.

What's new?

Cancer cells avoid senescence in part by reactivating telomerase (TERT), a ribonucleoprotein that replenishes shortening telomeres. Here, the authors discover a positive association between TERT promoter mutations and unfavorable prognosis in glioblastoma patients from Portuguese and Brazilian origin. This association was only observed in patients with a specific allelic background (AA) in a TERT polymorphism (rs2853669) recently linked to enhanced *TERT* mRNA levels. The authors recommend considering the allelic status of rs2853669 when assessing the prognostic value of TERT promoter mutations in glioblastoma patients.

Gliomas are the most common malignant primary brain tumour in adults.[1,2] According to the WHO classification, astrocytoma is the most frequent histological subtype of glioma, followed by oligodendroglioma and oligoastrocytoma.[1] Gliomas can be divided in 4 malignancy grades, from WHO grade I to WHO grade IV. Pilocytic astrocytomas (WHO grade I) are considered benign tumours and occur more commonly in children. Diffuse astrocytomas (WHO grade II) arise in young adults and, despite being low-grade gliomas, frequently evolve to higher grade malignancies, such as anaplastic astrocytomas (WHO grade III) and glioblastomas (GBM; WHO grade IV).[1] Oligodendrogliomas and oligoastrocytomas can be classified as low-grade or high-grade/anaplastic tumours (WHO grade II and III, respectively). GBM is not only the most malignant, but also the most frequent brain tumour. The current gold-standard therapy for GBM patients combines temozolomide with radiotherapy, with an overall survival of about 15 months.[3] Therefore, the prognosis of GBM patients is still dismal, highlighting the need for identifying and consolidating biomarkers that help in the management of the patients.

Achieving cancer cell immortalization depends on the ability to circumvent the telomere erosion sensing mechanisms and evade senescence or cell death. Two major pathways are used by cancer cells for telomere maintenance: reactivation of telomerase—a ribonucleoprotein protein consisting of molecules each of human telomerase reverse transcriptase (TERT) and telomerase RNA (TERC) —that elongates telomeres by adding 5′-TTAGGG-3′ tandem repeats to the tips of the chromosomes, or engagement in a nontelomerase dependent mechanism, the "alternative lengthening of telomeres" (ALT).[4,5] Reactivation of telomerase is reported in up to 90% of human cancers,[6] although the underlying mechanisms have only recently started to be revealed.

Two seminal papers reported a high frequency of *TERT* promoter (*TERTp*) mutations in familial and sporadic forms of melanoma.[7,8] The mutations were present in the promoter region of the *TERT* gene and largely clustered in two hotspots positions, located upstream of the ATG site, at positions −146 bp (c.−146:G > A) and −124 bp (c.−124:G > A).[8] *TERTp* mutations generate a new consensus binding site for ETS/TCFs transcription factors (CCGGAA) and lead to a 2- to 4-fold increase of the *TERT* promoter activity.[7,8] Recently, it was also shown that GABP is the critical ETS transcription factor activating *TERT* expression in the context of these highly recurrent promoter mutations.[9] We and others extended the search to other tumour models and reported the presence of these recurrent somatic mutations in tumours of the central nervous system (CNS), bladder, liver, thyroid (follicular cell-derived tumours), skin and tumours originated in tissues with low rates of self-renewal.[10–14] Gliomas are among the tumours harbouring the highest frequency of *TERTp* mutations[10,13,15–21]; in GBM, such frequency can be as high as 84% but elevated frequencies are also found in low and high-grade oligodendroglial tumours.[6,10,13]

Recent studies reported that the rs2853669 A > G single nucleotide polymorphism (SNP), located −245 bp upstream of the *TERT* gene ATG site, modulates the increased *TERT* mRNA expression levels induced by the recurrent *TERTp* somatic mutations.[22] The less common G allele reverts the biological effect of *TERTp* mutations and also modifies the outcome in patients with bladder cancer and gliomas.[23–25]

In this study we investigated the frequency and prognostic value of *TERTp* mutations and *TERT* rs2853669 SNP in gliomas. The analysis was performed in two series of gliomas—one composed of Portuguese patients ($n = 298$) and another composed of Brazilian patients ($n = 206$) —as a whole or separately.

Material and Methods

Tumor samples

Representative formalin-fixed paraffin-embedded (FFPE) samples from 504 gliomas were retrieved from the pathology archives of Portuguese institutions (Centro Hospitalar São João, Hospital Pedro Hispano and Hospital de Braga) and Brazilian hospitals (Hospital de Câncer de Barretos and

Hospital das Clínicas da Faculdade de Medicina de Ribeirão Preto). All tumours were reviewed and classified according to the WHO classification of CNS tumours.[1] Of the 504 samples, 298 were from Portugal and 206 were from Brazil. Supporting Information Table S1 summarizes the distribution of samples per histotype in both series. The only major difference concerns the percentage of pilocytic astrocytomas, which was higher in the Brazilian series than in the Portuguese series. Part of the results reported in this study, in particular the frequency of *TERTp* mutations in 118 gliomas, has been previously published elsewhere.[13]

The information on gender, age at diagnosis, tumour location, Karnofsky Performance Status and radio/chemotherapy is summarized in Table 1. All the procedures described in this study were in accordance with national and institutional ethical standards and previously approved by Local Ethical Review Committees.

Genotyping characterization

DNA from FFPE tissues was retrieved from 10 μm cuts, after careful macrodissection of tumour area, ensuring the presence of >75% of neoplastic cells, as well as absence of necrosis and microvascular proliferation. DNA extraction of the Portuguese and Brazilian samples was performed using the Ultraprep Tissue DNA Kit (AHN Biotechnologie, Nordhausen, Germany) and Qiagen's QIAamp® DNA Micro Kit (Qiagen, Venlo, The Netherlands), respectively, following the manufacturer's instructions. DNA was further quantified by NanoDrop® 2000 (Thermo Scientific, Waltham) and stored at −20°C until further genetic analysis.

The c.−146:G > A and c.−124:G > A hotspot *TERTp* mutations were screened by PCR followed by direct Sanger sequencing as previously described.[13] PCR for *TERTp* mutation analysis was performed with the primer pair Fw: 5′-CAGCGCTGCCTGAAACTC-3′ and Rw: 5′-GTCCTGCCCCTTCACCTT-3′ resulting in a PCR product of 235 bp, which contained the sites chr5.hg19:g.1295228C > T and chr5.hg19:g.1295250C > T, corresponding to the c.−124:G > A and c.−146:G > A mutations, respectively. PCR amplification of the genomic DNA (25–100 ng) was performed using the Qiagen Multiplex PCR kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's protocol, using Q solution. Sequencing reactions were performed with the ABI Prism BigDye Terminator Kit (Life Technologies, Carlsbad) and the fragments were run in an ABI prism 3100 and 3500 xL Genetic Analyser (Life Technologies, Carlsbad).

The sequencing reaction was performed in forward direction. An independent PCR amplification/sequencing, both in a forward and reverse direction, was performed in positive samples or samples that were inconclusive. For genotyping the rs2853669 SNP, the electropherogram analysis of the same 235 bp PCR product allowed the genotyping of rs2853669 SNP. Additionally, in a subset of GBM ($n = 85$) we also used the TaqMan SNP genotyping assay ID: C___8773290_10 (Life Technologies, Carlsbad, USA). The procedure was performed by real time PCR according to manufacturer's instructions in an ABI Prism 7500 Fast system (Life Technologies, Carlsbad).

Statistical analysis

Statistical analysis was conducted with SPSS version 22.0 (IBM, Armonk). Fisher's exact test and Student's *t test* (unpaired, two tailed) were used when appropriate. The predictive value of *TERTp* mutations and other factors (age, gender and Karnofsky Performance Status) were assessed using univariate and multivariate logistic regression models. Survival curves were plotted by the Kaplan-Meier method with the log-rank statistics. Multivariate survival analysis was performed using Cox regression. Results were considered statistically significant if $p < 0.05$.

Results

Altogether, we detected the presence of *TERTp* mutations in 216 out of 452 gliomas (47.8%). In 52 cases, the low quantity or quality of DNA precluded the evaluation of *TERTp* mutations. The most common mutation was the c.−124:G > A, detected in 154 cases (71.3%), whereas the c.−146:G > A was found in 62 cases (28.7%). *TERTp* mutations were more frequently detected in glioblastoma (66.9%), followed by anaplastic oligodendroglioma (51.5%), grade II oligodendroglioma (46.3%), oligoastrocytoma (40.0%), diffuse astrocytoma (15.2%), anaplastic astrocytoma (10.0%) and pilocytic astrocytoma (7.2%; Supporting Information Table S2). In 22 cases, we had access to the material from the primary tumour (initial surgery) and the recurrence. When comparing primary *vs*. recurrence, all cases showed concordant results regarding the presence of *TERTp* mutations, with the exception of one anaplastic oligodendroglioma, in which we detected the c.−124:G > A *TERTp* mutation in the recurrence but not in the primary tumour (Supporting Information Table S3).

TERTp mutations in astrocytomas

TERTp mutations were detected in all astrocytoma subtypes, with increasing frequencies along more malignant grades: 7.2% (4/55) in pilocytic astrocytomas (WHO grade I), 15.2% (7/46) in diffuse astrocytomas (WHO grade II), 10.0% (1/10) in anaplastic astrocytomas (WHO grade III) and 66.9% (141/211) in GBM (WHO grade IV) (Supporting Information Table S2). Among diffuse astrocytomas, *TERTp* mutations were significantly associated with older age at diagnosis (Supporting Information Table S4). The low frequency of *TERTp* mutations in grade I and III astrocytic tumours precluded further statistical analyses.

TERTp mutations and GBM clinicopathological features

The highest frequency of *TERTp* mutations was detected in GBM, with a similar frequency in Portuguese patients (66.4%) and Brazilian patients (67.5%). When pooling Portuguese and Brazilian patients together, the presence of *TERTp*

Table 1. Clinicopathological features of gliomas across different histological subtypes

Clinical features[1]	Pilocytic astrocytoma ($n = 67$)	Diffuse astrocytoma ($n = 50$)	Anaplastic astrocytoma ($n = 10$)	Glioblastoma ($n = 243$)	Oligodendro-glioma ($n = 55$)	Anaplastic oligodendro-glioma ($n = 67$)	Oligoastrocy-toma ($n = 12$)
Gender							
Female	36 (53.7%)	22 (45.8%)	1 (10.0%)	88 (37.4%)	20 (37.0%)	29 (43.3%)	6 (50.0%)
Male	31 (46.3%)	26 (54.2%)	9 (90.0%)	147 (62.6%)	34 (63.0%)	38 (56.7%)	6 (50.0%)
Age							
Mean (range)	14 (1–76)	37 (4–78)	45 (30–62)	57 (18–82)	42 (2–79)	49 (9–81)	35 (14–58)
Tumor Location							
Frontal	3 (4.5%)	11 (25%)	5 (55.6%)	42 (18.2%)	20 (42.6%)	21 (35.0%)	4 (36.4%)
Temporal	0	10 (22.7%)	2 (22.2%)	59 (25.5%)	8 (17.0%)	11 (18.3%)	0
Parietal	3 (4.5%)	4 (9.1%)	1 (11.1%)	43 (18.6%)	10 (21.3%)	14 (23.3%)	6 (54.5%)
Occipital	2 (3%)	3 (6.8%)	0	13 (5.6%)	0	3 (5.0%)	0
Multiple	0	6 (13.6%)	1 (11.1%)	65 (28.1%)	6 (12.8%)	9 (15.0%)	1 (9.1%)
Other	59 (88.1%)	10 (22.7%)	0	9 (3.9%)	3 (6.4%)	2 (3.3%)	0
KPS							
<70	6 (12.8%)	1 (7.7%)	1 (20%)	48 (31.4%)	0	3 (10.0%)	0
≥ 70	41 (87.2%)	12 (92.3%)	4 (80%)	105 (68.6%)	24 (100%)	27 (90.0%)	6 (100%)
Radio/chemotherapy							
Yes	10 (16.9%)	9 (20.5%)	5 (50%)	132 (62.3%)	21 (45.7%)	35 (59.3%)	6 (50%)
No	49 (83.1%)	35 (79.5%)	5 (50%)	80 (37.7%)	25 (54.3%)	24 (40.7%)	6 (50%)

Abbreviations: KPS, Karnofsky Performance Status.
[1]The number of patients analysed for each clinical feature may differ from the total number of patients within each histological subtype due to missing information.

Table 2. Clinicopathological features of GBM according to *TERTp* mutation status

	Entire series			Portuguese series			Brazilian series		
Clinical features[1]	*TERTp* mut patient ($n = 141$)	*TERTp* WT patients ($n = 70$)	p values	*TERTp* mut patient ($n = 85$)	*TERTp* WT patients ($n = 43$)	p values	*TERTp* mut patients ($n = 56$)	*TERTp* WT patients ($n = 27$)	p values
Gender			$p = 0.283$			$p = 0.483$			**$p = 0.012$**
Female	51 (37.0%)	30 (44.8%)		32 (39.0%)	13 (32.5%)		19 (33.9%)	17 (63.0%)	
Male	87 (63.0%)	37 (55.2%)		50 (61.0%)	27 (67.5%)		37 (66.1%)	10 (37.0%)	
Age			**$p < 0.001$**			**$p = 0.004$**			**$p < 0.001$**
Mean (Range)	61 (25–82)	50 (18–80)		60 (41–79)	51 (18–77)		62 (25–82)	47 (18–80)	
Tumour Location			**$p = 0.044$**			$p = 0.644$			**$p = 0.007$**
Frontal	18 (13.3%)	16 (24.6%)		14 (17.7%)	9 (23.1%)		4 (7.1%)	7 (26.9%)	
Temporal	41 (30.4%)	12 (18.5%)		19 (24.2%)	9 (23.1%)		22 (39.3%)	3 (11.5%)	
Parietal	23 (17.0%)	13 (20.0%)		14 (17.7%)	8 (20.5%)		9 (16.1%)	5 (19.2%)	
Occipital	6 (4.4%)	5 (7.7%)		5 (6.3%)	2 (5.1%)		1 (1.8%)	3 (11.5%)	
Multiple	45 (33.3%)	15 (23.1%)		25 (31.6%)	8 (20.5%)		20 (35.7%)	7 (26.9%)	
Other	2 (1.5%)	4 (6.2%)		2 (2.5%)	3 (7.7%)		0	1 (3.8%)	
KPS			$p = 0.552$			$p = 0.575$			$p = 0.621$
<70	28 (29.8%)	14 (35.0%)		16 (36.4%)	9 (47.4%)		12 (24.0%)	5 (23.8%)	
≥ 70	66 (70.2%)	26 (65.0%)		28 (63.6%)	10 (52.6%)		38 (76.0%)	16 (76.2%)	
Rad./Ch. therapy			$p = 0.395$			$p = 0.822$			$p = 0.623$
Yes	78 (61.9%)	39 (68.4%)		44 (62.9%)	21 (67.7%)		34 (60.7%)	18 (69.2%)	
No	48 (38.1%)	18 (31.6%)		26 (37.1%)	10 (32.3%)		22 (39.3%)	8 (30.8%)	

Abbreviations: KPS, Karnofsky Performance Status; *TERTp* mut, *TERT* promoter mutated; *TERTp* WT, *TERT* promoter wild-type; Rad./Ch.therapy, Radio or Chemotherapy; Bold, statistically significant.
[1]The number of patients analysed for each clinical feature may differ from the total number of patients within each *TERTp* category due to missing information.

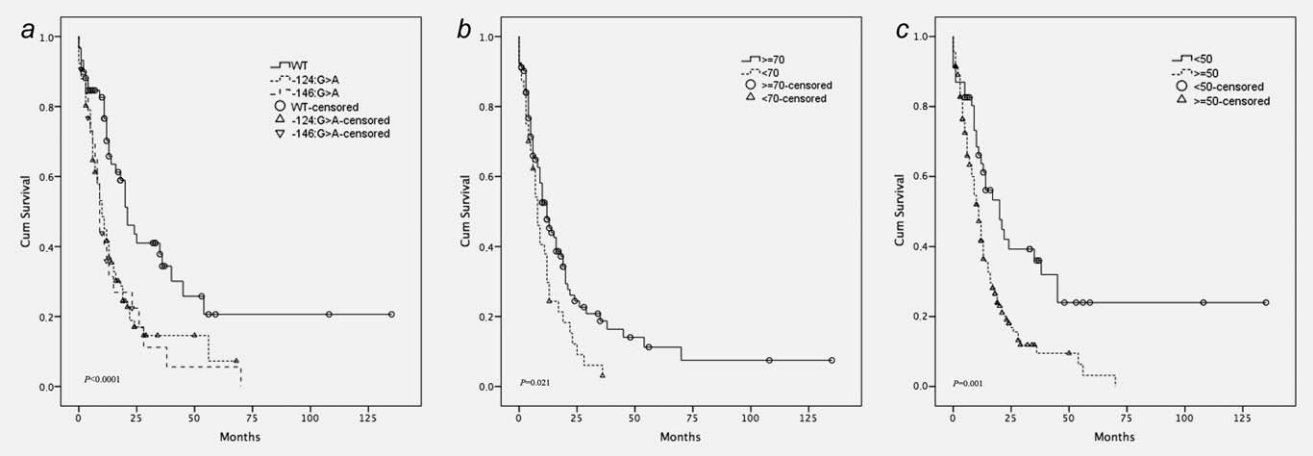

Figure 1. Overall survival of GBM patients according to *TERTp* mutation status (*a*), Karnofsky Performance Status (KPS; *b*) and age at diagnosis (*c*).

Table 3. Univariate and multivariate analysis of prognostic factors for overall survival in GBM patients

	Univariate analysis			Multivariate analysis		
Clinical features	***p* values**	**HR**	**95% CI**	***p* values**	**HR**	**95% CI**
KPS	**0.027**	1.581	1.054–2.371	0.122	1.435	0.908–2.266
Age (continuous)	**0.000**	1.026	1.013–1.039	**0.048**	1.019	1.000–1.039
***TERTp* mutation**	**0.000**	2.136	1.428–3.193	**0.027**	1.851	1.078–3.204

Abbreviations: KPS: Karnofsky performance status; HR: hazard ratio; CI: confidence interval; Bold: statistically significant.

mutations was significantly associated with older age at diagnosis and different tumour location: GBM with *TERTp* mutations were more frequently located in the temporal lobe, while GBM without *TERTp* mutations were more evenly distributed, albeit being more frequently located in the frontal lobe (Table 2). Following GBM stratification according to their origin (Portuguese or Brazilian), the significant association of *TERTp* mutations with older age at diagnosis was retained in both groups, while the association with tumour location was kept only in the Brazilian patients, with the same trend observed in Portuguese patients (Table 2). Within Brazilian patients, *TERTp* mutations were also associated with male patients (Table 2).

Using Kaplan-Meier analysis, we observed that GBM patients harbouring *TERTp* mutations had significantly shorter survival than GBM patients without *TERTp* mutations (median 10.0 *vs.* 21.0 months; Log-rank test $p < 0.0001$; Fig. 1*a*). No differences in survival were found between patients with the c.−124:G > A mutation and those with the c.−146:G > A mutation (Fig. 1*a*). The correlation between *TERTp* mutations and worse survival was preserved when Portuguese (median 13.0 *vs.* 21.0 months; Log-rank test $p = 0.008$) and Brazilian (median 6.0 *vs.* 21.0 months; Log-rank test $P = 0.01$) patients were analysed separately (Supporting Information Figs. S1 and S2, respectively). In Kaplan-Meier analysis, patients with a Karnofsky Performance Status <70 (median 8.0 *vs.* 12.0 months; Log-rank test $p = 0.021$) and patients aged ≥50 years (median 11.0 *vs.* 20.0 months; Log-rank test $p = 0.001$) were also associated with significantly shorter survival (Figs. 1 *b*and 1*c*, respectively). The multivariate Cox regression models of overall survival, using *TERT* mutational status, age at diagnosis and preoperative Karnofsky Performance Status (<70 *vs.* ≥70) as covariates, showed that *TERTp* mutations remained significantly associated with worse survival (HR 1.851, 95% CI 1.078–3.204, $p = 0.027$; Table 3), while age at diagnosis (computed as a continuous variable) showed a borderline value (HR 1.019, 95% CI 1.000–1.039, $p = 0.048$; Table 3).

TERTp mutations in oligodendrogliomas

TERTp mutations were also frequently detected in oligodendrogliomas grade II (25/54; 46.3%) and III (34/66; 51.5%). Similar to what we observed for GBM, *TERTp* mutations were significantly associated with older age at diagnosis, both in grade II and III oligodendrogliomas (Supporting Information Table S5). In grade II patients, the presence of *TERTp* mutations was significantly associated with male patients, a finding not observed for grade III patients (Supporting Information Table S5). No association was found between *TERTp* mutations and tumour location in grade II and III oligodendrogliomas (Supporting Information Table S5). In contrast to

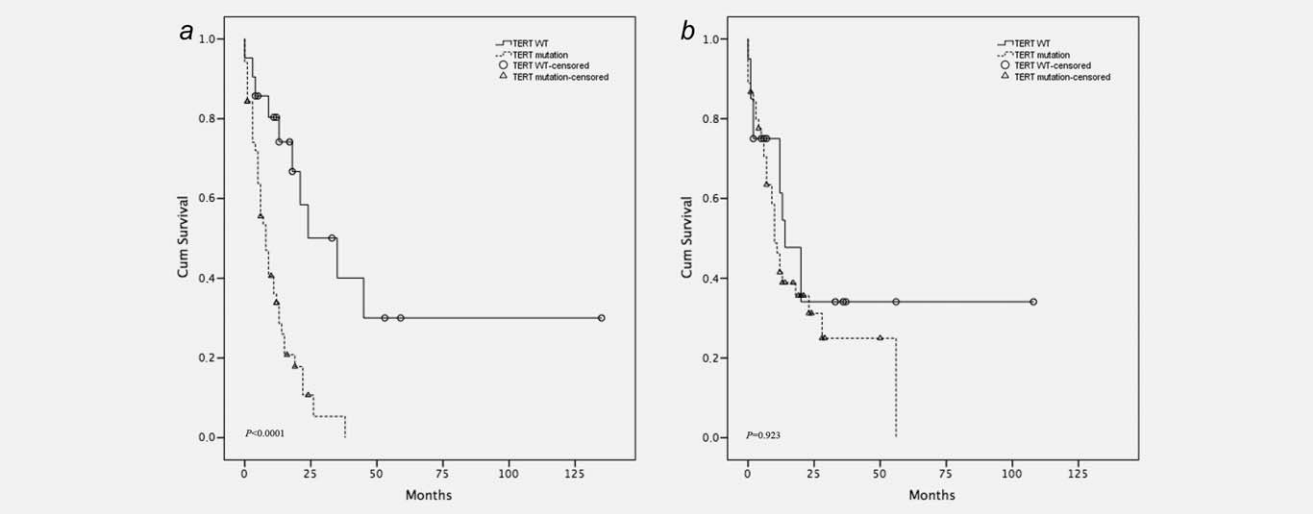

Figure 2. Overall survival according to *TERTp* mutation in GBM patients carrying the rs2853669 AA genotype (*a*) or carrying the rs2853669 GG or AG genotypes (G carriers; *b*).

Table 4. Univariate and multivariate analysis of prognostic factors for overall survival in GBM patients without the rs2853669 G allele

	Univariate analysis			Multivariate analysis		
Clinical features	***p* values**	**HR**	**95% CI**	***p* values**	**HR**	**95% CI**
KPS	**0.031**	2.079	1.070–4.041	0.312	1.435	0.712–2.889
Age (continuous)	**0.000**	1.061	1.033–1.090	**0.001**	1.060	1.025–1.096
***TERTp* mutation**	**0.000**	3.816	1.806–8.063	0.328	1.455	0.686–3.083

Abbreviations: KPS: Karnofsky performance status; HR: hazard ratio; CI: confidence interval; Bold: statistically significant.

the results in GBM patients, the presence of *TERTp* mutations was not associated with worse survival in grade II or grade III oligodendroglioma patients (Supporting Information Figs. S3 and S4, respectively).

TERTp mutations in oligoastrocytomas

We detected the presence of *TERTp* mutations in 40.0% (4/10) of oligoastrocytomas. The clinicopathological features did not differ between patients with and without *TERTp* mutations, although the sample was too small to allow definite conclusions (Supporting Information Table S4).

rs2853669 TERTp SNP in GBM

In light of previous reports showing that the rs2853669 −245:A > G SNP in the *TERTp* region modifies the prognostic value of *TERTp* mutations, we assessed the clinical value of *TERTp* mutations in GBM patients according to their rs2853669 background. We first screened the −245:A > G polymorphism in GBM patients and found that the G allele was present in 47% of GBM patients (77/164) and that the most common genotype was A/A (87/164; 53.0%), followed by A/G (61/164; 37.2%) and G/G (16/164; 9.8%) genotypes (Supporting Information Table S6). The genotype frequencies were similar in Portuguese and Brazilian series (Supporting Information Table S6). Kaplan-Meier analysis in GBM patients according to their rs2853669 background showed that the poor prognosis conferred by *TERTp* mutations was confined to patients without the G allele (median 8.0 *vs.* 35.0 months; Log-rank test $p < 0.0001$; Fig. 2*a*), whereas in patients carrying the G allele *TERTp* mutations were not significantly associated with worse prognosis (median 10.0 *vs.* 14.0 months; Log-rank test $p = 0.923$; Fig. 2*b*). Likewise, a Karnofsky Performance Status <70 (median 8.0 *vs.* 12.0 months; Log-rank test $p = 0.021$) and an age at diagnosis ≥50 years (median 11.0 *vs.* 20.0 months; Log-rank test $p = 0.001$) were significantly associated with shorter survival only in patients without the G allele (Supporting Information Figs. S5A and S6A, respectively). In the multivariate Cox regression models of overall survival (using *TERT* mutational status, age at diagnosis and preoperative Karnofsky Performance Status as covariates), restricted to patients without the G allele, only age at diagnosis remained significantly associated with shorter survival in multivariate analysis (Table 4). The presence of the G allele by itself was not associated with age, gender, tumour location or *TERTp* mutation (data not shown). Furthermore, the overall survival of GBM patients was identical when we compared patients carrying the G allele *vs.* those without the G allele, or when we compared patients carrying the AA, AG and GG genotypes (Supporting Information Figs. S7 and S8, respectively).

Discussion

The finding that telomerase is reactivated in the majority of human cancers denotes that it plays a crucial role in tumour development and/or progression. The identification of *TERTp* mutations has provided a genetic cause for telomerase reactivation in a subset of human cancers, namely in gliomas where *TERTp* mutations are particularly frequent. Furthermore, *TERTp* mutations were associated with increased *TERT* mRNA expression in gliomas[9,26] and also with decreased telomere length,[27] thus supporting the functional relevance of such mutations in cancer. Interestingly, Chiba *et al.*[28] showed that neural precursor cells and neurons, engineered to carry cancer-associated *TERTp* mutations, failed to repress *TERT* transcription upon induction of differentiation and showed robust telomerase activity, suggesting that *TERTp* mutations are sufficient to overcome the proliferative barrier imposed by telomere shortening, thus promoting immortalization.

In this study we confirmed that the highest frequency of *TERTp* mutations is detected in GBM, followed by oligodendroglial tumours, while low grade astrocytic tumours exhibited the lowest frequency of *TERTp* mutations. The *TERTp* mutation frequency detected in GBM from our series (66.9%) is lower than the one found in several studies,[10,16,21,29] while comparable to other series[17,24,30] and higher that some studies.[18,20,31] The lower percentage of *TERTp* mutations here reported could be related with the fact that we did not separate primary from secondary GBM; since secondary GBM show a significantly lower percentage of *TERTp* mutations,[18] this could hinder (and lower) the overall percentage of *TERTp* in GBM. The lower frequency of *TERTp* mutations in grade I to grade III astrocytomas may be explained by their lower malignancy grade or, alternatively, by the existence of mechanisms such as ALT that provide telomere maintenance. Supporting the latter hypothesis, *ATRX* mutations that are able to trigger ALT, have been reported in low-grade astrocytomas.[15,32]

Interestingly, we detected *TERTp* mutations in four out of 55 (9.1%) pilocytic astrocytomas, which are best known for showing molecular alterations in the MAPK pathway.[33–35] To our knowledge, this has not been previously reported in the literature, although most studies on this subject either do not present data regarding pilocytic astrocytomas, or have very few screened cases, with the exception of the study by Koelsche *et al.*,[20] who did not find *TERTp* mutations among 111 pilocytic astrocytomas. The four cases where we detected *TERTp* mutations (two with the c.−124:G > A and two other with the c.−146:G > A) did not present any particular features regarding age, gender or disease outcome; nevertheless, our findings warrant further studies in order to fully understand the relevance of *TERTp* mutations in pilocytic astrocytomas.

No major differences were found between Portuguese and Brazilian patients, an observation that reinforces the relevance of the results obtained in the present study, independently of patient ethnic background.

Our results show that *TERTp* mutations are a major prognostic factor for shorter survival in GBM patients, independently of other known clinical prognostic factors, such as older age at diagnosis and poor Karnofsky Performance Status. These findings are in accordance with previous reports showing that *TERTp* mutations are an independent prognostic factor in GBM and in other tumour types.[10,21,26,36] Our results should, however, be interpreted with caution, as we were not able to assess the prognostic value of *IDH* mutations in GBM, due to the low number of mutated cases (6 out of 211) and to lack of clinical information. Heidenreich *et al.*[27] showed that the group of glioma patients with the combined presence of *IDH* and *TERTp* mutations had the best overall survival, while the worst overall survival was observed in patients carrying *TERTp* mutations only. Since *IDH* mutations are known to be major prognostic indicators in low- and high-grade gliomas,[21,30,37] our results require validation in larger datasets, with thorough genetic and clinical information, that provide statistical power to assess the combined prognostic significance of *TERTp* and *IDH* mutations.

Nonoguchi *et al.*[18] observed that only the *TERTp* c.−124:G > A mutation was predictive of shorter survival in GBM patients; this could be related with the finding that the *TERTp* c.−124:G > A leads to higher transcriptional activity of the *TERT* promoter when compared with the c.−146:G > A mutation,[38] although both mutations generate the same putative ETS-binding motif. This apparent discrepancy suggests a strong positional effect between the location of the ETS mutation and the core transcription initiation machinery, as suggested by Chiba *et al.*[28] Our results, however, did not replicate those of Nonoguchi *et al.*,[18] as we did not find differences in overall survival when comparing the c.−124:G > A with the c.−146:G > A mutation.

Our study also demonstrates that the prognostic value of *TERTp* mutations depends on the status of the rs2853669 −245A > G SNP. In a univariate analysis, the poor prognosis conferred by *TERTp* mutations seems to occur only in GBM patients that do not carry the G allele in position −245, while in those carrying the G allele in homozygosity or heterozygosity, *TERTp* mutations do not affect overall survival. This is in accordance with recent reports in GBM patients, in which it was also demonstrated a major influence of the rs2853669 SNP on the prognostic effect of *TERTp* mutations.[23,24,39] The modifier effect of the rs2853669 SNP may be related with a lower telomerase activity and *TERT* expression conferred by the G allele,[22] thereby blunting the effect of *TERTp* mutations. When we further performed a multivariate analysis, considering also the clinical variable age and Karnofsky Performance Status, we did not confirm this finding, probably due to the lack of statistical power and also to the absence of important clinical parameters, such as tumor resection and patient therapy and molecular features such as *IDH* mutations and 1p/19q loss. If confirmed in additional studies, the status of the rs2853669 SNP would be an important aspect to consider when evaluating *TERTp* mutations;

indeed, this SNP may explain some discrepancies observed in the literature regarding the prognostic value of *TERTp* mutations in GBM. Given the prognostic relevance of *IDH* mutations, it will also be crucial to understand how the rs2853669 alleles may modulate GBM outcome, taking into consideration not only *TERTp* mutations, but also *IDH* mutations and 1p/19q losses.

In line with previous reports, the presence of *TERTp* mutations was consistently associated with older age at diagnosis across gliomas with different histotypes and grades, with the exception of oligoastrocytomas. Although this might be expected, taking into consideration that older age is also associated with poor prognosis in GBM patients, the fact that *TERTp* mutations remain as poor prognostic factors in the multivariate analysis implies a role for *TERTp* mutations both in early and later stages of glioma development.

To our knowledge, we report for the first time that GBM location is significantly different when comparing *TERTp* mutant with *TERTp* WT GBM, namely an increased frequency of temporal lobe tumours in *TERTp* mutant GBM. In two reports that analyse tumour location according to *TERTp* mutation status, no significant differences were uncovered; however, the results of Chen *et al*. also show a trend for temporal lobe location in *TERTp* mutant GBM.[38]

In conclusion, we present evidence that *TERTp* mutations are a major indicator of poor survival in GBM, independently from other risk factors such as age and Karnofsky Performance Status. In addition, the effect promoted by *TERTp* mutations may be modified by a common SNP in the *TERT* promoter region, but studies in larger series are necessary to fully elucidate this finding.

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