New somatic TERT promoter variants enhance the Telomerase activity in Glioblastoma

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Abstract
The catalytic activity of human Telomerase Reverse Transcriptase (TERT) compensates for the loss of telomere length, eroded during each cell cycle, to ensure a correct division of stem and germinal cells. In human tumors, ectopic TERT reactivation, most frequently due to hotspot mutations in the promoter region (TERTp), i.e. c.1-124 C > T, c.1-146 C > T, confers a proliferative advantage to neoplastic cells. In gliomas, TERTp mutations (TERTpmut) mainly occur in oligodendrogloma and glioblastoma. We screened, for TERTp hotspot mutations, 301 adult patients with gliomas and identified heterozygous mutations in 239 cases: 94% of oligodendroglioma, 85% of glioblastoma, and 37.5% of diffuse/anaplastic astrocytoma. Besides the recurrent c.1-124 C > T and c.1-146 C > T, two cases of glioblastoma harbored novel somatic TERTp variants, which consisted of a tandem duplications of 22 nucleotides, i.e. a TERTp c.1-100_1-79dup and TERTp c.1-110_1-89, both located downstream c.1-124 C > T and c.1-146 C > T. In silico analysis predicted the formation of 119 and 108 new transcription factor’s recognition sites for TERTp c.1-100_1-79dup and TERTp c.1-110_1-89, respectively. TERTp duplications (TERTpdup) mainly affected the binding capacity of two transcription factors’ families, i.e. the members of the E-twenty-six and the Specificity Protein/Krüppel-Like Factor groups. In fact, these new TERTp-dup significantly enhanced the E-twenty-six transcription factors' binding capacity, which is also typically increased by the two c.1-124 C > T/c.1-146 C > T hotspot TERTpmut. On the other hand, they were distinguished by enhanced affinity for the Krüppel proteins. The luciferase assay confirmed that TERTpdup behaved as gain-of-function mutations causing a 2,3-2,5 fold increase of TERT transcription. The present study provides new insights into TERTp mutational spectrum occurring in central nervous system tumors, with the identification of new recurrent somatic gain-of-function mutations, occurring in 0.8% of glioblastoma IDH-wildtype.

Keywords: TERT, Gliomas, Gain-of-function mutation, ETS and Krüppel transcription factors

Introduction
The abnormal reactivation of human Telomerase Reverse Transcriptase (TERT) is a common hallmark of human solid tumors. Although it may be caused by several mechanisms, i.e. methylation, mutations, rearrangements/fusions, and DNA copy number amplifications, TERT promoter (TERTp) methylation, and gain-of-function mutations are the most frequent [2, 28]. In particular, two recurrent hotspot mutations are respectively located at -124 (TERTp-124) and -146 (TERTp-146) base pairs (bp), from the TERT ATG start site [2, 10–12, 28]. Both mutations, generated from a cytidine to thymidine dipyrimide transition (C > T), are usually heterozygous, mutually exclusive, and produce
TERT promoter mutations (TERTp\textsuperscript{mut}) typically occur in tumors that arise from low self-renewal tissue, such as melanomas, thyroid, hepatobiliary carcinoma, and central nervous system (CNS) tumors, with a variable frequency, that range from 15 to 90% of cases, in diverse histological subtypes [10, 14, 28]. In CNS tumors, TERTp\textsuperscript{mut} are typically associated with glioblastoma (GBM) (70–80%) and oligodendroglioma (ODG) (60–70%), whereas their frequency decreases in other glioma subtypes, such as diffuse/anaplastic astrocytoma (DA/AA) (30–40%), medulloblastoma (20–30%), and meningioma (about 7%) [10, 25, 27]. Although the clinical value of TERTp\textsuperscript{mut}, in refining the diagnostic classification of gliomas, is widely accepted [6], its role as prognostic/predictive biomarker is still largely debated. TERTp\textsuperscript{mut} have been associated with a poor disease outcome in GBM IDH\textsuperscript{wt} (GBM IDH\textsuperscript{wt}), but there is no full agreement on its impact on DA/AA [6, 15, 16, 22, 24, 29].

It is worth noting, however, that DA/AA IDH\textsuperscript{wt} (DA/AA IDH\textsuperscript{wt}) harboring genomic abnormalities typically associated with GBM, i.e. TERTp mutations, or EGFR amplification, or gain of whole chromosome 7 in combination with monosomy of chromosome 10, have a clinical outcome similar to, or only slightly longer, than GBM [4]. Thus, the cIMPACT NOW (Update 3) recommended to use one of these molecular criteria to classify this subgroup of astrocytomas as “diffuse astrocytic glioma, IDH\textsuperscript{wt}, with molecular features of glioblastoma, WHO grade IV” and to revise the classification of DA/AA IDH\textsuperscript{wt}, accordingly [4].

Herein, we report two new TERTp mutations that were identified in two patients with GBM IDH\textsuperscript{wt}. Both these new variants originated from the duplication of a stretch of 22 nucleotides at TERTp (TERTp\textsuperscript{dup}) and, although slightly different, shared an overlapping sequence of 12 nucleotides. We demonstrated the somatic nature of one of these TERTp\textsuperscript{dup} and that, enhancing the binding affinity for ETS transcription factors (TFs), they both elicit the TERT transcription, thus widening the spectrum of recurrent gain-of-function mutations of TERTp in GBM.

Case presentation
Cohort
The study was carried out on a cohort of 301 patients, affected by primary CNS tumours, and referred to our laboratory during the last 10 years (Table 1). There were 175 males and 126 females (ratio 1.4:1) with a median age of 64 (range age: 20-86). According to the WHO 2016, the diagnosis was: grade II DA IDH\textsuperscript{wt} (6 cases) and DA IDH-mutant (DA IDH\textsuperscript{mut}) (10 cases); grade III AA IDH\textsuperscript{wt} (6 cases) and AA IDH\textsuperscript{mut} (10 cases); grade IV GBM IDH\textsuperscript{mut} (241) and GBM IDH\textsuperscript{mut} (10); grade II/III ODG (15). Three patients had a diagnosis of uncommon glioma (Table 1). The study was approved by Institutional Bioethics Committee (University of Perugia and Santa Maria della Misericordia Hospital of Perugia-Italy, Protocol no.2843/16); all patients gave informed consent for sample collection and molecular analyses, in agreement with the Declaration of Helsinki.

Index cases
A 71-year-old male (UPN#131) had a left frontal lesion of 24 mm diameter, partially infiltrating the corpus callosum; the second case (UPN#171), a male of 78 years, presented with a right frontal lesion. Histopathology and immunohistochemistry were consistent with a diagnosis of GBM IDH\textsuperscript{wt}, in both patients. In case UPN#131, neoplastic cells showed marked cytoplasmic and nuclear pleomorphism; there was a discrete number of atypical mitotic figures, widespread necrosis, a diffuse GFAP positivity (100%), and few neoplastic elements (20%) with strong nuclear TP53 stain. Case UPN#171, was characterized by striking atypia of neoplastic cells, diffuse necrosis, vascular proliferation, strong and diffuse positivity for GFAP and nuclear TP53 (>70%) (Fig. 1). No IDH1/IDH2 hotspot mutations were detected, while both cases showed MGMT promoter methylation. Monosomy of chromosome 10 co-occurred with EGFR amplification (UPN#131) or with gain of the whole chromosome 7 (UPN#171).

Materials and methods
TERT promoter mutational analysis
Genomic DNA was extracted from Formalin-Fixed Paraffin-Embedded (FFPE) tumor tissue and from peripheral blood (PB) by QIAamp DNA FFPE and AllPrep DNA/RNA kits, respectively, following the manufacturer’s instructions (QIAGEN, Milan, Italy). Hotspot TERTp\textsuperscript{mut} were investigated by Sanger sequencing using ABI 3500 Genetic analyzer instrument (Applied Biosystems, Monza, Italy). Primers were reported in Table S1 (Additional file 1: Table S1) and referred to GRCh37 genomic coordinate system (NM_000005.9, for regulatory core promoter 274 bp) (www.ncbi.nlm.nih.gov/gene [20], www.ensembl.org/Homo_sapiens [7]). Sequences’ alignments and their analyses were supported by Clustal Omega (www.ebi.ac.uk/Tools/msa/clustalo), Ensembl (http://www.ensembl.org/Homo_sapiens) [7], and
COSMIC (https://cancer.sanger.ac.uk/cosmic) websites [5].

**In silico TERTp\textsuperscript{mut} functional analysis: JASPAR tool**

This bioinformatic tool estimates the binding affinity and the number of TFs binding sites for the input sequence provided in FASTA format. A relative threshold score of 80% and Δ relative score ≥ 0.05 (mutant’s relative score—wildtype’s relative score) were chosen to define the statistically significant changes induced by TERTp\textsuperscript{mut}, as previously reported [1]. The JASPAR CORE predicted the effects of the four different TERTp\textsuperscript{mut} that we detected in our patients, i.e. the two new TERTp\textsuperscript{dup}, the TERTp\textsuperscript{124}, and the TERTp\textsuperscript{146}, on TFs binding capacity (JASPAR CORE Collection 2020; http://jaspar.genereg.net, 8th version [8, 13]). JASPAR was also used to analyze two TERTp\textsuperscript{dup}, which have been previously reported in a case of MDS (c.1-110_1-101dup) and in a case of thyroid cancer (c.1-104_1-83dup) [21, 23]. According to JASPAR data, we used the Venn diagram to plot TFs for which a significant enhanced probability of binding capacity, or an

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**Table 1 Epidemiological and clinical features of our cohort of patients**

| Epidemiological-clinical data                                                                 | Total cohort |
|---------------------------------------------|--------------|
| **Gender**                                  | 301 pts      |
| Male                                        | 175 pts (58.1%) |
| Female                                      | 126 pts (41.9%) |
| MF                                          | 1.4          |
| **Age (years)**                             | 301 pts      |
| Range                                       | 20-86        |
| Median                                      | 64           |
| < 30 years                                  | 10 pts (3.3%) |
| ≥ 30 years                                  | 291 pts (96.7%) |
| **Diagnosis (WHO 2016)**                    | 301 pts      |
| Common Gliomas                              |              |
| Diffuse astrocytoma, IDH-wt (grade II)      | 6            |
| Diffuse astrocytoma, IDH-mut (grade II)     | 10           |
| Anaplastic astrocytoma, IDH-wt (grade III)  | 6            |
| Anaplastic astrocytoma, IDH-mut (grade III) | 10           |
| Glioblastoma, IDH-wt (grade IV)             | 241          |
| Glioblastoma, IDH-mut (grade IV)            | 10           |
| Oligodendroglioma, IDH-mut and 1p/19q-codeleted (grade II) | 7 |
| Anaplastic oligodendroglioma, IDH-mut and 1p/19q-codeleted (grade III) | 8 |
| Uncommon Gliomas                            |              |
| Pilocytic astrocytoma (grade I)             | 1            |
| Pleomorphic xanthoastrocytoma (grade II)    | 1            |
| Anaplastic pleomorphic xanthoastrocytoma (grade III) | 1 |
| **Anatomic location**                       | 301 pts      |
| Frontal                                     | 97           |
| Frontal-parietal                            | 15           |
| Frontal-temporal                            | 5            |
| Parietal                                    | 39           |
| Parietal-occipital                          | 10           |
| Temporal                                    | 84           |
| Temporal-parietal                           | 17           |
| Temporal-occipital                          | 3            |
| Occipital                                   | 9            |
| Cerebellar hemisphere                       | 4            |
| Corpus callosum                             | 2            |
| Thalamus                                    | 1            |
| Pituitary gland                             | 1            |
| Insular                                     | 1            |
| Multicentric                                | 13           |

*pts, patients; wt, wildtype; mut, mutant*
increase of the number of binding sites, was predicted (http://bioinformatics.psb.ugent.be/webtools/Venn/).

**In vitro TERTp<sup>mut</sup> functional study: luciferase assay**

To study the effect of TERTp<sup>mut</sup> on the expression of TERT, a luciferase assay was done for the TERTp<sup>dup</sup> detected in case UPN#171, the TERTp<sup>-146</sup> (UPN#205), and the TERTp<sup>-124</sup> (UPN#216). The TERTp<sup>dup</sup> of case UPN#131 could not be studied due to lack of material. A TERTp wildtype (TERTp<sup>wt</sup>) construct, already available in the laboratory, was also used as reference (Additional file 2: Table S2) [21]. TERT core promoter (310 bp) was amplified with specific primers reported in Table S3 (Additional file 3: Table S3), introducing cleavage sites for BglII (forward) and HindIII (reverse) restriction enzymes. Then, TERTp<sup>mut</sup> constructs were inserted in pGEM-T easy plasmid (Promega, Madison WI, USA) and cloned in Electromax DH10BT1 cells (Invitrogen, Milan, Italy) to increase the amount of mutant DNA. Finally, the inserts were subcloned in pGL4.10[luc2] vectors (Promega, Madison WI, USA) upstream of LUC2 gene, encoding for luciferase enzyme of Photinus Pyralis and resequenced. An empty pGL4.10[luc2] vector was also used as negative control. Luciferase assay was performed using the GBM U87-MG cell line, maintained in Dulbecco’s Modified Eagle Medium (Thermo Fisher Scientific, Monza, Italy) with 10% fetal bovine serum, and 0.5% streptomycin/penicillin at 37 °C/5% CO<sub>2</sub>. U87-MG cells were seeded in a 6-multiwell plate (3 × 10<sup>5</sup> cells/ml), co-transfected with 3 µg of modified pGL4.10[luc2] plasmids and with 1:10 of pGL4.74[hRluc/TK], a vector containing the luciferase gene of Renilla Reniformis, by Viafect Transfection Reagent (Promega Madison WI, USA). After 24-h incubation, cells were lysed and fluorescence emission was assessed using Dual-Glo Luciferase assay kit (Promega) following manufacturer’s instructions. All experiments were performed in triplicate, in three independent experiments.
Results

New somatic TERT promoter variants

TERT\textsuperscript{pmut} were detected in 239/301 cases (79.4\%), including 14/15 ODG (93\%), 12/32 DA/AA (37.5\%), and 213/251 GBM (84.8\%) (Additional file 4: Table S4). In GBM (\textasciitilde123) and DA/AA (\textasciitilde12), TERT\textsuperscript{pmut} were prevalent in IDH\textsuperscript{wt} cases (209/241 GBM IDH\textsuperscript{wt} vs 4/10 GBM IDH\textsuperscript{mut}; 10/12 DA/AA IDH\textsuperscript{wt} vs 2/20 DA/AA IDH\textsuperscript{mut}) (Chi square, P<0.001) (Additional file 5: Table S5). Thus, in agreement with the diagnostic criteria recommended by the cIMPACT-NOW (Update 3), the 10 DA/AA IDH\textsuperscript{wt} with TERT\textsuperscript{pmut} were referred to as "diffuse astrocytic glioma, IDH\textsuperscript{wildtype}, with molecular features of glioblastoma, WHO grade IV" [4].

In GBM TERT\textsuperscript{pmt} there was a significant enrichment of cases harbouring EGFR amplification (46% vs 17%) (Chi square, P=0.0001) and/or monosomy 10/PTEN deletions (84% vs 37.5%) (Chi square, P<0.0001). Likewise, EGFR amplification or gain of whole chromosome 7 occurred in 25/29 (86%) in TERT\textsuperscript{pwt} cases and 18/53 (34%) for TERT\textsuperscript{pmut} (Fig. 2a, b) (Additional file 6: Table S6). Other recurrently involved TFs in TERT\textsuperscript{pdup} variants were the Specificity Protein/Krüppel-Like Factor (Sp/KLF) family, i.e. 19/65 (29\%) in TERT\textsuperscript{p110-89} and 16/53 (30\%) in TERT\textsuperscript{p110-89}, and the more than 3 adjacent zinc finger factors family (12/65 in TERT\textsuperscript{p100-79} and 7/53 TERT\textsuperscript{p110-89} (Additional file 7: Table S7).

The Venn diagram showed a close inter-relationship between all TERT\textsuperscript{pmut} mutations. Namely, all TERT\textsuperscript{pmut} mutations shared an increase of the binding affinity, or the number of binding motifs, for 19 common TFs (Fig. 3a), including 18 ETS members (ETS1, ETS2, ERG, ELK1, ETV6, FLI1, ELK4, SPIB, ELF1, ELF3, ETV4, ETV1, FEV, EHF, ETV5, ELF5, SPI1, and GABPA) and TEAD1 (Fig. 3a; Additional file 8: Table S8). The Venn diagram also showed that the new TERT\textsuperscript{pdup} were characterized by the exclusive involvement of 30 common TFs. Specifically, there were 16 Sp/KLF members, i.e. KLF2, KLF3, KLF4, KLF5, KLF6, KLF10, KLF11, KLF14, KLF15, KLF16, SP1, SP2, SP3, SP4, SP8, SP9, and EGR1, (Fig. 3a, Additional file 8: Table S8) and 14 TFs that belong to 9 different families (Fig. 3a, Additional files 7 and 8: Tables S7 and S8). Matching our TERT\textsuperscript{pdup} with the two cases of TERT\textsuperscript{p110-89} previously reported (Additional files 9 and 10: Tables S9 and S10) [21, 23], JASPAR predicted that all variants determined an increase of binding sites for 21 common TFs, and confirmed that the Sp/KLF family was the most frequently involved (14/21) (Fig. 3b, Additional file 11: Table S11).

In vitro analysis confirms the increasing of TERT transcriptional activity induced by its promoter mutations

In vitro luciferase assay was carried out to evaluate whether the new TERT\textsuperscript{p110-89} variant induced an increase of TERT transcriptional activity, enhancing its expression, similarly to TERT\textsuperscript{p124} and TERT\textsuperscript{p146} [12, 21]. In Table S12 (Additional file 12: Table S12) we reported raw data referred to the fluorescence emission values, expressed in Relative Luciferase Activity (RLA), of both Photinus Pyralis and Renilla Reniformis luciferase enzymes, for all samples. Our experiments demonstrated that all three variants caused a significant increase of TERT transcription by 2.3-2.5 fold than wildtype (TERT\textsuperscript{p110-89} vs TERT\textsuperscript{pwt}: P<0.0001; TERT\textsuperscript{p124} vs TERT\textsuperscript{pwt}: P<0.00315; TERT\textsuperscript{p146} vs TERT\textsuperscript{pwt}: P<0.0001; Mann–Whitney U test) (Fig. 4). On the other hand, no differences on the levels of TERT expression were present between the diverse TERT\textsuperscript{pmut} variants, indicating they may all behave as gain-of-function mutations, likely exerting the same consequences on TERT transcription.

In silico analysis predicts TERT\textsuperscript{pmut} effects

In silico analysis predicted that both TERT\textsuperscript{pdup} created new binding sites, i.e. 119 for TERT\textsuperscript{p100-79} and 108 for TERT\textsuperscript{p110-89}, which were respectively recognized by 65 and 53 TFs. Instead, TERT\textsuperscript{p124} and TERT\textsuperscript{p146} were predicted to increase the binding affinity for 40 and 43 sites, and to enhance the probability of binding for 28 and 29 TFs, respectively (Additional file 6: Table S6). Although all TERT\textsuperscript{pmut} affected the binding sites for diverse families of TFs, the ETS group emerged as one of the most frequently involved: 18/65 (28\%) in TERT\textsuperscript{p100-79}, 18/53 (34\%) for TERT\textsuperscript{p110-89}, 23/28 (82\%) in TERT\textsuperscript{p124}, and 25/29 (86\%) in TERT\textsuperscript{p146} (Fig. 2c, Additional file 7: Table S7).
Abnormal genomic events that alter telomere elongation are common in gliomas. Particularly, mutually exclusive mutations affect the \textit{TERT} or the \textit{ATRX} chromatin remodeler (\textit{ATRX}) genes, a critical regulator of telomere homeostasis by chromatin remodeling [9].

Our studies, on a cohort of 301 patients, confirmed previous data on the incidence and distribution of \textit{TERTp}\textsuperscript{mut} in diverse subtypes of CNS tumors. As expected, we found that \textit{TERTp}\textsuperscript{mut} were highly recurrent in ODG and GBM, and less frequent in DA/AA (Additional file 4: Table S4). \textit{TERTp}\textsuperscript{mut} were significantly enriched in GBM \textit{IDH}\textsuperscript{wt} cases (83%) (Chi square, \(P < 0.001\)) (Additional file 55: Table S5), where they mainly occurred together with \textit{EGFR} amplification (Chi square, \(P = 0.001\)) and/or monosomy 10/\textit{PTEN} deletions (Chi square, \(P < 0.0001\)). Similarly, in DA/AA, \textit{TERTp}\textsuperscript{mut} were highly recurrent in \textit{IDH}\textsuperscript{wt} cases, thus allowing the reclassification of 83% of these subgroup of astrocytomas as “diffuse astrocytic glioma,

**Fig. 2** Schematic representation of \textit{TERTp} mutations: \textbf{a} \textit{TERT} promoter electropherogram in case UPN#131. The arrow indicates the start point of the c.1-100_1-79dup. \textbf{b} \textit{TERT} promoter electropherogram in case UPN#171. The arrow indicates the start point of the c.1-110_1-89dup. \textbf{c} Overview of all \textit{TERTp} variants detected in our cases. Upper arrow: wildtype \textit{TERT} core promoter with the normal location of ETS binding sites. The vertical black lines indicate the genomic positions of \textit{TERTp} variants. Lower arrow: positions and types of \textit{TERTp} variants and their predicted effects on transcription factors binding sites.
IDH-wildtype, with molecular features of glioblastoma, WHO grade IV” [4].

Besides the two known TERTp\textsuperscript{124} and TERTp\textsuperscript{146} variants, we uncovered two new TERTp variants in two cases of GBM IDH\textsuperscript{wt} (UPN#131 and UPN#171). These novel TERTp\textsuperscript{mut} consisted of a 22 nucleotide tandem duplication, sharing a duplicated region of 12 nucleotides, from 1–100 to 1–89, from the ATG start site. Hitherto, somatic TERTp\textsuperscript{dup} has been reported in three human tumors. The first one, a duplication of 41 nucleotides in the TERT core promoter, was detected in a case of ODG [3]. Afterwards, TERTp\textsuperscript{dup} were found in a case of myelodysplastic syndrome (MDS) (c.1-110\_1-101dup) and in a case of papillary thyroid carcinoma (c.1-104\_1-83dup) [21, 23]. Published TERTp\textsuperscript{dup} as well as our cases, are located in the same core promoter region, that span 1-110/1-79 bp from the ATG start site. Furthermore, they are all located downstream TERTp\textsuperscript{124} and TERTp\textsuperscript{146}, i.e. at 13–23 nucleotides from TERTp\textsuperscript{124} and 35–45 nucleotides from TERTp\textsuperscript{146}, in a region that contains the binding sites for the TFs modulating TERT transcription. Interestingly, in silico analysis predicted these new TERTp\textsuperscript{dup} affect the transcriptional regulation of the gene through the creation of new binding sites for TFs that mainly belong to the ETS family (Fig. 2c, Additional file 7: Table S7). Likewise, an increased number of binding sites or an enhanced affinity for the ETS TFs, has been previously reported in a thyroid cancer harbouring a TERTp c.1-104\_1-83dup variant, and in cases bearing TERTp\textsuperscript{124} or TERTp\textsuperscript{146} mutations [3, 10, 23]. Bioinformatic analyses were consistent with the luciferase data showing a significant increase of TERT expression in cells transfected with the new TERTp\textsuperscript{110-89} variant as well as with the two recurrent TERTp\textsuperscript{mut}.

Then, we sought to assess the possible inter-relationship between the four diverse TERTp mutations using the Venn diagram (Fig. 3a). All four TERTp variants were predicted to share an increase binding capacity for 18 ETS members (Fig. 3a; Additional file 8: Table S8), which included GABPA, a putative oncogene in GBM. Namely, in vitro studies on GBM cell lines have demonstrated that this transcription factor is needful in mediating the transcriptional reactivation of TERT dependent from TERTp\textsuperscript{124} or TERTp\textsuperscript{146} [3, 10, 19]. Besides ETS TFs, all TERTp variants affected the binding capacity for TEAD1, a protein that belongs to TEF-1-related factors family, and that has been demonstrated to act as a putative oncogene in GBM, favoring cell infiltration in vitro/in vivo models [26].

Although TERTp\textsuperscript{124} and TERTp\textsuperscript{146}, and the new TERTp\textsuperscript{100-79} and TERTp\textsuperscript{110-89} variants, shared the same effects on the binding capacity for ETS members, the latters were characterized by the exclusive involvement of 30 TFs, mainly belonging to Sp/KLF family (Fig. 3a, Additional files 7 and 8: Tables S7 and S8). Sp/KLF TFs are involved in a plethora of cellular processes ranging from proliferation and differentiation, pluripotency and apoptosis, in normal and tumoral tissues [17].

Altogether these data support the hypothesis that the recruitment of ETS family TFs plays a pivotal role in mediating the reactivation of TERT transcription in human tumors bearing different types of TERTp\textsuperscript{mut}. However, they also indicate that slight differences mark TERTp\textsuperscript{dup} variants, whose activities appear to be
also dependent from Krüppel-related factors. Indeed, among the 21 TFs shared by all TERTpdup (Fig. 3b), 14 belonged to Sp/KLF family (67%) as reported in Tables S10 and S11 (Additional files: 10 and 11). Hence, the precise definition of mutation-specific profiles would strengthen the definition of TERT-dependent oncogenesis mechanisms.

Our study contributes to enrich the spectrum of recurrent somatic TERTpdup variants reporting, for the first time, two new gain-of-function mutations, i.e. TERTp-100-79 and TERTp-110-89, in 0.8% of GBM IDHwt cases. These new mutations can be reliably detected by diagnostic assays used to investigate hotspot TERTp-124 and TERTp-146. Although the assessment of TERTp mutational status is not an essential diagnostic criterion, it can be a relevant information to assist histological diagnosis [18]. As a matter of fact, the status of TERTp, together with IDH mutations and 1p/19q co-deletion, classify gliomas in 5 distinct subcategories, i.e. triple negative, triple positive, cases with IDH/TERT mutations, and cases with a unique mutation (either IDH or TERT), that are typified by unique demographic, clinical and biological characteristics [6]. Moreover, TERTpmut has been proposed as one of the most relevant molecular marker to stratify DA/AA IDHwt [4]. Thus, we consider that molecular testing of TERTp mutations should be included in the clinical work-up of GBM and DA/AA in order to provide a precise diagnosis: prospective multicentric studies, on large cohort of patients, will clarify the value of TERTp mutations as prognostic marker.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s40478-020-01022-4.

Additional file 1: Table S1. Primer set used for Sanger sequencing.
Additional file 2: Table S2. Samples used for in vitro luciferase assay.
Additional file 3: Table S3. Primer set used to create constructs for luciferase assay.
Additional file 4: Table S4. Incidence and distribution of TERTp variants in the main glioma subgroups.
Additional file 5: Table S5. Incidence and distribution of TERTp variants in glioma subtypes (according to WHO 2016 guidelines).
Additional file 6: Table S6. JASPAR analysis for the TERTp c.1-124 C>T, c.1-146 C>T and the new TERTpdup(c.1-110_1-89dup; c.1-110_1-89dup).
Additional file 7: Table S7. Transcription Factors predicted to be involved in TERTp variants.
Additional file 8: Table S8. Transcription Factors predicted to be involved in different TERTp variants.
Additional file 9: Table S9. JASPAR analysis for the two published TERTp duplications c.1-110_1-101dup and c.1-104_1-83dup [ref. 21, 23].
Additional file 10: Table S10. Transcription factors predicted to be involved in the TERTpdup c.1-110_1-101dup and c.1-104_1-83dup [ref. 21, 23].
Additional file 11: Table S11. Transcription factors predicted to be involved in all TERTp duplications.
Additional file 12: Table S12. Luciferase assay: raw data.

Abbreviations

TERT: Telomerase Reverse Transcriptase; TERTp: TERT promoter; TERTpdup: TERT promoter duplication; TERTpmut: TERT promoter mutation; IDH: isocitrate dehydrogenase; GBM: glioblastoma; ODG: oligodendroglioma; DA: diffuse astrocytoma; AA: anaplastic astrocytoma; GBM IDHwt: glioblastoma IDH-wildtype; DA IDHwt: diffuse astrocytoma IDH-wildtype.

**Fig. 4** Luciferase assay. The histogram reports the relative luciferase activities (RLA) of TERTp wildtype and for the variants c.1-110_1-89dup, c.1-124 C>T, and c.1-146 C>T. p value refers to probability obtained using Mann–Whitney U test.
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