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Accessibility
**TERT promoter mutations are highly recurrent in SHH subgroup medulloblastoma**

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**Abstract** Telomerase reverse transcriptase (TERT) promoter mutations were recently shown to drive telomerase activity in various cancer types, including medulloblastoma. However, the clinical and biological implications of TERT mutations in medulloblastoma have not been described. Hence, we sought to describe these mutations and their impact in a subgroup-specific manner. We analyzed the TERT promoter by direct sequencing and genotyping in 466 medulloblastomas. The mutational distributions were determined according to subgroup affiliation, demographics, and clinical, prognostic, and molecular features. Integrated genomics approaches were used to identify specific somatic copy number alterations in TERT promoter-mutated and wild-type tumors. Overall, TERT promoter mutations were identified in 21 % of medulloblastomas. Strikingly, the highest frequencies of

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TERT mutations were observed in SHH (83%; 55/66) and WNT (31%; 4/13) medulloblastomas derived from adult patients. Group 3 and Group 4 harbored this alteration in <5% of cases and showed no association with increased patient age. The prognostic implications of these mutations were highly subgroup-specific. TERT mutations identified a subset with good and poor prognosis in SHH and Group 4 tumors, respectively. Monosomy 6 was mostly restricted to WNT tumors without TERT mutations. Hallmark SHH focal copy number aberrations and chromosome 10q deletion were mutually exclusive with TERT mutations within SHH tumors. TERT promoter mutations are the most common recurrent somatic point mutation in medulloblastoma, and are highly enriched in adult SHH and WNT tumors. TERT mutations define a subset of SHH medulloblastoma with distinct demographics, cytogenetics, and outcomes.

Keywords TERT promoter mutations · SHH pathway · Adult · Medulloblastoma

Introduction

Medulloblastoma is a highly malignant embryonal brain tumor located in the posterior fossa [6, 29, 33, 35]. While this tumor comprises the most common malignant brain tumor in children, it only accounts for approximately 1% of primary CNS tumors in adults [18, 20]. The current consensus recognizes four core molecular subgroups (WNT, SHH, Group 3, and Group 4) with distinct molecular, demographic, clinicopathological, and prognostic characteristics [5, 15, 16, 26, 27, 37, 38, 41, 42]. The defining features of medulloblastoma subgroups differ dramatically according to age at diagnosis [15, 27, 41]. Specifically, Group 3 tumors are largely confined to non-adults, SHH tumors are most frequent in infants and adults, while WNT and Group 4 medulloblastomas are mostly observed in pediatric cohorts [15, 24, 27, 38, 41]. Particularly within SHH tumors, age-associated heterogeneity was observed regarding the transcriptional characteristics, somatic copy number alterations (SCNA), and the prognostic implications of biomarkers [15, 18, 38, 40]. Delineation of tumorigenic features characteristic for these age-related
differences, particularly within SHH tumors, are highly desirable to understand these clear biological and prognostic discrepancies.

Telomere maintenance is fundamentally important to normal self-renewing stem cells and cancer cells [3, 7, 9, 14, 22]. It has been suggested that tumors derived from cell populations with low self-renewal capacity generally rely on alterations that restore telomerase activity, while epigenetic mechanisms maintain telomerase activity in tumor types derived from self-renewing stem cells [13]. The identification of recurrent telomerase reverse transcriptase (TERT) promoter mutations in 21% of 91 medulloblastomas [13] is intriguing, since other mechanisms converging on increased telomerase activity including alternative lengthening of telomeres (ALT) [8] or mutations affecting the ATRX/DAXX complex are excessively uncommon in medulloblastoma [12, 25, 32, 34, 39]. Although TERT mutations have been reported in several cancers [2, 10, 11, 13, 19, 43], their putative association with distinct biological behavior and clinical or even prognostic characteristics has not been comprehensively studied. The initial analyses of TERT mutations in medulloblastoma [12] mainly cataloged the mutational frequency rather than correlating the molecular and clinical features of these mutations in a subgroup-specific manner.

In this study, we analyzed a representative set of 466 medulloblastomas for TERT promoter mutations. Subsequently, we correlated the mutational distribution with clinicopathological features, outcome, and molecular characteristics in a subgroup-specific manner. We demonstrate that TERT promoter mutations comprise the most recurrent mutation in adult SHH tumors identified to date and potentially define distinct prognostic subgroups in SHH and Group 4 medulloblastoma patients.

Materials and methods

Tumor material and patient characteristics

All tissues and clinicopathological information were serially collected in accordance with institutional review boards

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from various contributing centers to this study. Nucleic acid extractions were carried out as previously described [28]. The clinicopathological characteristics of the investigated patient cohort are outlined in Table 1. The median follow-up was 44.06 months (range 0.7–301.5 months).

Gene expression and copy number analysis

Subgroup affiliation was determined using nanoString limited gene expression profiling as previously described [31]. Somatic copy number alterations were assessed on the Affymetrix Single Nucleotide Polymorphism (SNP) 6.0 array platform in 418 of 466 cases to identify SCNAs specific for TERT mutant and wild-type tumors. Raw copy number estimates were obtained in dChip, followed by CBS segmentation in R as previously described [30]. Somatic copy number alterations were identified using GISTIC2 [21]. TERT expression levels were compared using r2 (www.r2.amc.nl). Differences in expression were tested using one-way ANOVA.

Sanger sequencing

Isolated DNA (25 ng) from all 466 tumors and 7 matched germline samples (25 ng) was amplified by PCR. PCRs contained 1 μl DNA template, 10 μM forward (5′-CAg CgC TgC CTg AAA CTC-3′) and reverse (5′-gTC CTg CCC CTT CAC CTT C-3′) TERT-specific primers, and 12.5 μl HotStar Taq Plus Master Mix (Qiagen, Gaithersburg, Maryland, USA) in a 25 μl total reaction volume. Cycle parameters comprised 95 °C × 15 min; 28 cycles of 98 °C × 40 s, 65 °C × 30 s, 72 °C × 1 min; 72 °C × 10 min. PCRs were carried out using the C1000 Thermal Cycler (BioRad, Hercules, CA, USA). PCR products were purified with the PureLink PCR Micro kit (Life Technologies, Burlington, ON, Canada). In all experiments, controls were included in the absence of DNA to rule out contamination by PCR products. Templates for Sanger sequencing were analyzed with forward (5′-CAG CGC TGC CTG A AA CTC-3′) and reverse (5′-GTC CTG CCC CTT CAC CTT C-3′) sequencing primers using dGTP Big-Dye Terminator v3.0 Cycle Sequencing Ready Reaction Kit (Life Technologies), and 5 % DMSO on the ABI3730XL capillary genetic analyzer (Life Technologies).

Genotyping assay

Two primers (forward primer, 5′-CAG CGC TGC CTG AAA CTC-3′; reverse primer, 5′-GTC CTG CCC CTT CAC CTT C-3′) were designed to amplify a 163-bp product encompassing C228T and C250T hotspot mutations in the TERT promoter—corresponding to the positions 124 and 146 bp, respectively, upstream of the ATg start site. Two fluorogenic LNA probes were designed with different fluorescent dyes to allow single-tube genotyping. One probe was targeted to the WT sequence (TERT

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WT, 5′-5HEX-CCC CTC CCg g-3′IABkFQ-3′), and one was targeted to either of the two mutations (TERT mut, 5′-56FAM-CCC CTT CCG G−3IABkFQ). Primer and probes were custom designed by Integrated DNA Technologies (Coralville, Iowa, USA) using internal SNP design software, and sequence homogeneity was confirmed by comparison to all available sequences on the GenBank database using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). Primers were optimized to avoid for hairpins and homo- and heterodimers. Primers and probes were obtained from Integrated DNA Technologies.

Real-time PCR was performed in 25 μl reaction mixtures containing 12.5 μl of TaqMan Universal Master Mix II with UNG (Applied Biosystems), 900 nM concentrations of each primer, 250 nM TERT WT probe, 250 nM TERT MUT probe, and 1 μl (25 ng) of sample DNA. Thermocycling was performed on the StepOnePlus (Applied Biosystems) and consisted of 2 min at 50 °C, 10 min at 95 °C, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

Analysis was performed using StepOne Software, version 2.1. Samples were considered mutant if they had CT values of ≤39 cycles. Each sample was verified visually by examining the PCR curves generated to eliminate false positives due to aberrant light emission. End-point allelic discrimination genotyping was performed by visually inspecting a plot of the fluorescence from the WT probe versus the MUT probe generated from the post-PCR fluorescence read.

Statistical analysis

Survival time according to TERT mutational status was assessed using the Kaplan–Meier estimate and a log-rank test. Comparisons of binary and categorical patient characteristics between subgroups and cohorts were performed using the two-sided Fisher’s exact test or Chi-squared test. Continuous variables were analyzed using the Mann–Whitney U test. p values < 0.05 were considered statistically significant. Multivariate Cox proportional hazards regression was used to adjust for additional covariates using the survival R package (v.2.36). All other statistical analyses were performed using StataSE 12 (Stata Corp. College Station, TX, USA) and Graphpad Prism 5 (La Jolla, CA, USA).

Results

Characteristics of TERT-mutated medulloblastomas

We performed Sanger sequencing on a clinically well-annotated medulloblastoma cohort (n = 466), reflecting the spectrum of demographics and histological subtypes of the disease (Table 1; Supplementary Figure 1A). Our results were verified using a Taqman-based genotyping assay that detects both of the most highly recurrent TERT promoter mutations (C228T and C250T). Since both mutational hotspots are located in highly homologous sequences, C228T and C250T mutations result in an identical binding sequence for the mutation-specific probe (CCCgAggG; Supplementary Figure 1B). A total of 21% of medulloblastomas harbored TERT mutations (Fig. 1a). In line with a previous report, these mutations were enriched in older patients (Table 1; p < 0.0001), all mutations were heterozygous, and none of the available matched germline controls displayed this mutation [13]. Interestingly, we found that TERT-mutated medulloblastomas present less
frequently with metastatic dissemination at diagnosis compared to TERT wild-type tumors (p = 0.03).

**TERT** mutations are specifically enriched in SHH medulloblastomas

In a subgroup-specific analysis, we revealed that **TERT** mutations were significantly enriched in SHH tumors (80/213; 38%; p < 0.0001) compared to WNT (6/53; 11%) and Group 3 (2/50; 4%) or Group 4 tumors (8/150; 5%). **TERT** mutations in both WNT and SHH medulloblastomas were positively correlated with age. **TERT** mutations were significantly enriched in adult patients (Fig. 1c, d, both p < 0.0001). Increasing age was not associated with increased mutational frequency in either Group 3 or Group 4 tumors (n.s.). While histopathological features were similar between **TERT**-mutated and wild-type tumors across subgroups, we observed that classic histology was more commonly observed in **TERT** mutant SHH tumors, and desmoplastic histology in wild-type SHH tumors (Fig. 1e; p = 0.04), respectively.

**Prognostic implications of **TERT** mutations**

When medulloblastoma patients across all subgroups were stratified by **TERT** mutational status, we observed no significant differences in survival (Fig. 2a; p = 0.45). Further after normalizing the subgroup composition to reported subgroup ratios, a statistical difference was still not revealed (data not shown; p = 0.36) [1, 15, 26, 41]. However, when **TERT** mutational status is re-analyzed in a subgroup-specific manner, several important survival associations are observed. **TERT** mutations had no prognostic impact within WNT tumors (Fig. 2b; p = 0.17). However, a significant association between **TERT** promoter mutations and outcomes was noted in SHH and Group 4 medulloblastomas. Specifically, the 5-year overall survival of SHH tumors with and without **TERT** mutations was 77.6 ± 7%
and 64.1 ± 5.1 %, respectively (Fig. 2c; \( p = 0.04 \)). In contrast to the improved prognosis of TERT mutant SHH tumors, we observed the inverse pattern in Group 4 tumors where the 5-year overall survival for patients without and with TERT mutations was 73.3 % ± 4.3 % and 62.5 % ± 17.1 % (Fig. 2d; \( p = 0.04 \)). Similar to the unfavorable prognosis of TERT mutations in Group 4 tumors, both of the patients with TERT-mutated Group 3 tumors died after 7 and 45 months of follow-up, respectively (Supplementary Table 1). Thus, we conclude that TERT mutations define distinct prognostic patient cohorts in a subgroup-specific fashion with good prognosis in SHH and poor prognosis in Group 4 medulloblastomas.

Survival analysis restricted to specific age groups

As TERT mutations are predominantly observed in non-infant medulloblastomas, we evaluated the prognostic implications of these promoter mutations across all four medulloblastoma subgroups in an age-dependent manner. TERT mutational status across subgroups had no prognostic impact among patients older than 3 years of age at diagnosis (Fig. 3a; \( p = 0.59 \)). Interestingly, the prognostic impact of TERT mutation was more pronounced in the non-infant SHH population with a 5-year overall survival of 76.9 % ± 7.6 % and 59.3 % ± 6.9 % of non-infants with and without TERT promoter mutations, respectively (Fig. 3b; \( p = 0.019 \)). These prognostic implications were similar in adult medulloblastoma patients and in the adult SHH subgroup (Supplementary Figure 2). In a subset of 76 SHH cases with known TP53 mutational status [44], we revealed that TP53 mutations identify non-infant SHH tumors with a particularly poor prognosis, while in contrast TERT mutations identify a subsets with good prognosis (Fig. 3c; \( p = 0.047 \)). Mutations of both TERT and TP53 were observed in 4/12 SHH tumors (Supplementary Table 2). Non-infant Group 4 showed an inverse prognostic association with poor outcome of TERT-mutated cases (Fig. 3d; \( p = 0.024 \)). Lastly, we analyzed the overall survival of SHH patients under a multivariate Cox proportional hazards model comprising age at diagnosis, TERT mutational status, M-stage, and histology. In addition to the known prognostic significance of M-stage (\( p < 0.001 \)) and histology (\( p = 0.02 \)), we revealed that TERT status continued to be associated with good prognosis (HR 0.17, CI 0.04–0.69, \( p = 0.01 \)), independent of other prognostic factors including age at diagnosis (\( p = 0.35 \)).

Distinct somatic copy number alterations of TERT-mutated medulloblastomas

To identify additional genetic features associated with these distinct demographic and clinical differences, we evaluated broad and focal copy number alterations according to subgroup affiliation and TERT promoter mutations. Notably, only 1/6 (17 %) of TERT-mutated WNT tumors harbored monosomy 6, while this alteration is observed in approximately 80 % of TERT wild-type medulloblastomas of the WNT subgroup (Fig. 4a; \( p = 0.005 \)). Loss of chromosome 2 and 10q loss were significantly enriched in TERT wild-type SHH tumors, while 3q loss was more frequently observed in their TERT mutant counterparts (Fig. 4b). Previously described focal alterations characteristic for SHH tumors including amplification of MYCN/GLI2/CDK6/ YAP1/PPM1D, and deletions targeting PTCH1/CDKN2A/ CDKN2B/PTEN were largely confined to TERT wild-type SHH medulloblastomas, while TERT mutant SHH (Fig. 5) and Group 4 (Supplementary Figure 3) showed very few focal SCNAs. Consistent with the higher frequency of TERT mutations in SHH tumors, we observed increased TERT expression in the SHH subgroup compared to Group

### Table 2: Clinicopathological and molecular characteristics of SHH medulloblastoma according to TERT mutational status

| Characteristic  | TERT MUT | TERT WT | \( p \) value |
|---------------|---------|---------|--------------|
| Age (years)   |         |         |              |
| Median        | 25.00   | 3.00    | <0.0001*     |
| Range         | 0.66–49.00 | 0.24–52.00 | \( p = 0.005 \) |
| NA            | 1       | 0       |              |
| Gender        |         |         |              |
| Male          | 46      | 80      | 0.77*        |
| Female        | 31      | 48      |              |
| NA            | 3       | 5       |              |
| Histology     |         |         |              |
| MBEN          | 3       | 4       | 0.04*        |
| Desmoplastic  | 10      | 44      |              |
| Classic       | 34      | 47      |              |
| LC/A          | 10      | 16      |              |
| NA            | 23      | 22      |              |
| M-stage       |         |         |              |
| M0            | 46      | 87      | 0.84*        |
| M1-3          | 10      | 22      |              |
| NA            | 24      | 24      |              |
| TP53 status   |         |         |              |
| MUT           | 4       | 8       | 1*           |
| WT            | 38      | 71      |              |
| NA            | 38      | 54      |              |

* Female, LC/A large cell/anaplastic, M male, MB medulloblastoma, MBEN medulloblastoma with extensive nodularity, NA not available (data were excluded from statistical comparison)

** Bold values indicate \( p < 0.05 \)

* Mann–Whitney U test

* Fisher’s exact test

* Chi-square test
4 tumors in two independent gene expression profiling studies ($p < 0.001$; Supplementary Figure 4). Furthermore, we observed $TERT$ amplification in two tumors included in the entire cohort of 1,088 previously studied tumors [30]. Both of these cases with $TERT$ amplification were SHH-driven medulloblastomas with wild-type $TERT$ status, which were derived from pediatric patients who were both alive after 15 and 83 months of follow-up (Supplementary Figure 5). Thus, broad and focal SCNAS underline that $TERT$ mutations define a genetically distinct subset within SHH tumors and possibly within the WNT and Group 4 tumors.

Discussion

The underlying biology of adult medulloblastomas remains poorly understood. Next-generation sequencing studies have revealed a broad spectrum of novel, potentially tumorigenic mutations in the recent past, but none of these studies focused on adult medulloblastomas [12, 25, 32, 34, 39]. In addition, the vast majority of these mutations are not recurrent enough to stratify patients into distinct clinical and prognostic subgroups.

In this study, we demonstrate that $TERT$ promoter mutations, initially described in melanoma [10, 11], comprise the most recurrent mutation described so far across medulloblastoma subgroups, with a particular enrichment in older patient cohorts. These somatic mutations are especially common in older patients with SHH tumors (83 %) and to a lesser extent in adults with WNT medulloblastomas (11 %). Based on the transcriptional heterogeneity of SHH tumors in infant and adult patients, we suspect that the adult cluster mainly comprised $TERT$-mutated medulloblastomas [24]. According to the initial classification of tumor types with $TERT$ mutations at frequencies over 15 % ($TERT$-high) vs. below this threshold ($TERT$-low) [13], our report suggests distinct baseline telomerase activity of the cell of origin in each of the subgroups (Group 3 > Group 4 > WNT >> SHH). Furthermore, the identification of
recurrent TERT promoter mutations makes a compelling argument that the increasing availability of whole-genome sequencing results may substantially add to a refined understanding of the mutational landscape of different biological and age-driven medulloblastoma subgroups, since earlier next-generation sequencing studies focusing on the protein-coding regions had not encompassed gene-regulatory regions including promoter mutations.

In this study, we demonstrate that the mutational status of the TERT promoter can segregate individuals with SHH across subgroups (a), in SHH tumors (b), in SHH tumors (TP53 mutated/wild-type) (c), and Group 4 (d). Survival differences were calculated using continuous log-rank tests.
and Group 4 medulloblastomas with distinct prognostic outcomes, while a prognostic impact of this mutation was not observed in glioblastomas [23]. Molecular mechanisms converging on TERT up-regulation were recently reported to be associated with dismal prognosis in pediatric brain cancers [4]. Our findings in Group 4 tumors with TERT mutations follow this pattern, while SHH tumors with TERT mutations comprise a prognostically favorable subgroup. Notably, survival curves of SHH tumors increasingly approximate with extended follow-up. We hypothesize that this pattern might be due to secondary malignancies and late relapses in older SHH tumors [36–38]. Since virtually all of the TERT promoter mutations encompass the mutational hotspots C228T and C250T, patient stratification can be carried out using a single PCR followed up with Sanger sequencing or with a single experiment using our newly designed Taqman-based genotyping assay. The latter assay is particularly suitable for routine clinical applications as it is highly sensitive and specific (5 ng DNA input is sufficient). Furthermore, our Taqman-based genotyping assay can be used on DNA derived from fresh-frozen and formalin-fixed paraffin-embedded tissue, since it only amplifies a short DNA fragment.

Both hotspot mutations C228T and C250T create an E-twenty-six (ETS) binding motif [10, 11] resulting in up-regulation of TERT expression at the mRNA level [2], which was not observed at the protein level in glioblastomas [43]. We now demonstrate that SHH tumors with TERT mutations are mostly mutually exclusive with those harboring 10q loss (p = 0.017) Notably, the relatively favorable prognosis of TERT-mutated SHH medulloblastomas may be explained by the relative lack of high-risk biomarkers [17, 18, 24, 44].

In summary, we describe the demographic, clinicopathological, and biological implications of TERT promoter mutations in a subgroup-specific fashion. This study underlines the dependence of adult WNT and SHH tumors to reacquire telomerase activity and suggests a potential prognostic utility of TERT mutational analysis in an era of individualized therapy.

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