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*TERT* promoter mutation and aberrant hypermethylation are associated with elevated expression in medulloblastoma and characterise the majority of non-infant SHH subgroup tumours

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**Running title:** *TERT* alterations in medulloblastoma

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To the editor:

The childhood brain tumour medulloblastoma comprises four molecular disease subgroups (MB\textsubscript{WNT}, MB\textsubscript{SHH}, MB\textsubscript{Group3} and MB\textsubscript{Group4}). However, large-scale whole-exome sequencing investigations have not identified defining genetic lesions for the non-MB\textsubscript{WNT} subgroups [8,11]. Recent studies reported in this journal and others [1,3,6,7,9] have identified frequent TERT promoter mutations and aberrant DNA methylation in CNS malignancies, suggesting an important mechanism in tumour development (Figure 1a). In medulloblastoma, Castelo-Branco et al.[3] reported a high frequency of TERT promoter methylation, while Killela et al.[6] described TERT promoter mutations which Koelsche et al.[7] and Remke et al.[9] subsequently reported were most frequent in adult MB\textsubscript{SHH}, but rarer in childhood tumours. However, while TERT mutations have been associated with elevated expression in other cancers [1, 5], and account for a proportion of MB\textsubscript{SHH}, the relative contribution of TERT methylation alterations has not yet been investigated alongside mutational analysis. Moreover, relationships between TERT promoter methylation and gene expression are unclear; the positive association reported across multiple malignancies by Castelo-Branco et al.[3] is contradicted by the inverse association described by Arita et al.[1] in TERT wild-type adult gliomas.

We therefore sought to clarify the role of TERT alterations in medulloblastoma, by assessing the frequency of TERT promoter hot-spot mutations [1,6,7,9], aberrant methylation of the critical cg11625005 TERT promoter CpG residue [3], and TERT expression, in our tumour series. We show a common, subgroup-specific, involvement of TERT mutations in MB\textsubscript{SHH} alongside a wider involvement of TERT methylation across the MB subgroups, both associated with elevated TERT expression. Notably, in the non-infant MB\textsubscript{SHH} patient group aged 4 and over at diagnosis within our cohort, we show these genetic and epigenetic aberrations occur in both childhood and adult tumours, and in a mutually exclusive fashion, representing a defining molecular alteration in >75% of this patient group.

TERT promoter mutations occurred at high frequency in both childhood (14/41 (34%)) and adult (8/11 (73%)) MB\textsubscript{SHH} (Figure 1b) in our cohort, more common than any coding mutation reported in these groups to date (TP53, 30%; PTCH1, 27%; DDX3X, 18%; all other genes, <6% (n=33, data from cancer.sanger.ac.uk). The age distributions of mutated (4.7-15.5 years)
and non-mutated (5.2-15.4 years) childhood patients did not differ significantly (p=0.27; Mann-Whitney U test). TERT mutations were tumour-specific where germline DNA was available for comparison (n=4) and exclusive to non-infant MB<sub>SHH</sub> in our investigations. Mutations were not found in MB<sub>WNT</sub> (n=16; age range, 4.7-16.8 years), MB<sub>Group3</sub> (n=16; 1.5-16.1 years) or MB<sub>Group4</sub> (n=20; 2.4-15.8 years) from infants and children, or in tumours from infant MB<sub>SHH</sub> (<4.0 at diagnosis; n=17; 0.2-3.5 years), consistent with the rarity of mutations in these subgroups reported by Remke <i>et al.</i> [9].

Aberrant TERT promoter methylation at cg11625005 was a feature of all medulloblastoma molecular subgroups, but varied significantly in level and incidence between tumour groups (p=9x10<sup>-6</sup>, ANOVA); aberrant hypermethylation (with respect to normal cerebellar levels; n=17, foetal to 67 years) was observed in 63% (10/16) MB<sub>WNT</sub>, 69% (11/16) MB<sub>Group3</sub> and in 10% (2/20) MB<sub>Group4</sub>, while MB<sub>SHH</sub> tumours (36%; 16/44 hypermethylated) showed greatest variation (Figure 1c). Notably, TERT hypermethylation showed significant age-dependent associations within the MB<sub>SHH</sub> group (0% (0/6) >16 years; 52% (11/21) 4-16 years; 29% (5/17) <4 years; p=0.05, χ<sup>2</sup> test). Moreover, TERT promoter mutation and aberrant methylation at cg11625005 were mutually exclusive in non-infant MB<sub>SHH</sub> within our cohort (Figure 1d) suggesting methylation alterations contribute significantly to TERT alteration in this group, and possible common mechanistic effects. Aberrant hypermethylation was detected in 11/17 non-mutated vs. 0/10 mutated non-infant MB<sub>SHH</sub> tumours assessed (p=0.001; Fisher’s exact test (Figure 1d)).

To assess the potential mechanistic contributions of promoter mutation and methylation to TERT gene expression, we next assessed their association within our cohort using expression data generated by RNA-seq. TERT methylation and expression showed a significant positive and linear relationship (p=0.001; Pearson’s correlation test) in TERT wild-type tumours across all medulloblastoma subgroups, while all TERT mutant tumours (all MB<sub>SHH</sub> > 4 years (Figure 1b)) displayed high TERT expression in the absence of hypermethylation (Figure 1e).

Non-coding TERT promoter alterations, encompassing mutually-exclusive mutation and aberrant hypermethylation, both associated with elevated TERT expression, are therefore a defining feature for the majority (>75%) of non-infant MB<sub>SHH</sub> in our cohort, indicating a key
mechanism in their molecular pathogenesis. Moreover TERT hypermethylation and deregulation, in the absence of mutation, suggests a wider involvement across the other medulloblastoma molecular subtypes, notably frequent in MB_{WNT} and MB_{Group3}, but less so in MB_{Group4}, which now mandates further investigation.

Finally, our findings raise the potential importance of additional non-coding and/or epigenetic regulatory alterations in medulloblastoma, which have hitherto been overlooked by exome sequencing studies [8]. Despite current nomenclature, MB_{SHH} is not solely defined by SHH pathway activation and SHH is likely to contribute alongside other frequently disrupted pathways, with TERT alterations representing the most common identified to date. We believe these findings have important implications for future diagnosis, research and targeted therapy of a significant proportion of medulloblastoma patients.
**Figure legend**

**Figure 1.** *TERT* non-coding mutations, aberrant DNA hypermethylation, and expression in medulloblastoma.  

**a.** The *TERT* promoter region, showing positions of mutational hotspots and methylated regulatory CpG site, relative to the translational start site.  

**b.** Numbers and frequencies (%) of *TERT* mutations in medulloblastoma subgroups (subgroups determined as previously described [4,10]). Mutations were frequently and exclusively detected in the non-infant MB\textsubscript{SHH} subgroups ($X^2$ test between MB\textsubscript{SHH} age groups shown). The three different mutations are colour coded (pink, purple and gold).  

**c.** *TERT* promoter CpG site cg11625005 DNA methylation levels ($\beta$-value, assessed by Illumina Human Methylation 450K array [2]), are shown for normal cerebella (grey), MB\textsubscript{SHH} (by age; red), MB\textsubscript{WNT} (blue), MB\textsubscript{Group3} (yellow) and MB\textsubscript{Group4} (green). Black horizontal line, upper 99% confidence interval of mean cerebellar methylation levels, above which tumours were classed as aberrantly hypermethylated. ‘p’ value, one-way analysis of variance between tumour groups shown.  

**d.** *TERT* promoter methylation in MB\textsubscript{SHH} tumours from patients ≥4 years old at diagnosis. CpG site cg11625005 DNA methylation levels ($\beta$-value) are shown for *TERT* mutated ($n=10$) and wild-type non-infant MB\textsubscript{SHH} ($n=17$) and normal cerebella ($n=17$, grey). Specific mutations are colour coded as above (b). The mean methylation level was significantly higher in wild-type tumours compared to either mutated tumours ($p=0.0006$) or the normal cerebellum ($p=0.00001$) (Student’s t-test). Black horizontal line, see above (c).  

**e.** *TERT* expression (by RNA-seq; further details given in Supplementary Table 1) versus *TERT* promoter CpG site cg11625005 DNA methylation levels and mutation status in 51 medulloblastomas. Subgroup assignment is coloured as above (c). Mutated tumours, bold outlined boxes; wild-type tumours, circles. Dashed line, linear regression of methylation vs. expression in *TERT* wild-type tumours with associated ‘p’ values (Pearson’s correlation). VSD, variance-stabilised transform of normalised read counts aligned to ENSG00000164362. All tumour-specific data is summarised in Supplementary Table 1.
References


Figure 1
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