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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

Janet C. Lindsey¹, Ed. C. Schwalbe¹, Sandeep Potluri¹, Simon Bailey¹, Daniel Williamson¹ and Steven C. Clifford¹*

¹Northern Institute for Cancer Research, Newcastle University, Royal Victoria Infirmary, Newcastle upon Tyne, U.K.

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

*Correspondence to: Steven C. Clifford, Northern Institute for Cancer Research, Newcastle University, Sir James Spence Institute Level 5, Royal Victoria Infirmary, Newcastle upon Tyne, NE1 4LP, U.K.  Tel:  +44 (191) 2821319, Fax:  +44 (191) 2821326, e-mail: steve.clifford@ncl.ac.uk

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To the editor:

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

We therefore sought to clarify the role of \text{TERT} alterations in medulloblastoma, by assessing the frequency of \text{TERT} promoter hot-spot mutations [1,6,7,9], aberrant methylation of the critical cg11625005 \text{TERT} promoter CpG residue [3], and \text{TERT} expression, in our tumour series. We show a common, subgroup-specific, involvement of \text{TERT} mutations in MB\text{SHH} alongside a wider involvement of \text{TERT} methylation across the MB subgroups, both associated with elevated \text{TERT} expression. Notably, in the non-infant MB\text{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.

\text{TERT} promoter mutations occurred at high frequency in both childhood (14/41 (34%)) and adult (8/11 (73%)) MB\text{SHH} (Figure 1b) in our cohort, more common than any coding mutation reported in these groups to date (\text{TP53}, 30%; \text{PTCH1}, 27%; \text{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 MBshh in our investigations. Mutations were not found in MBWnt (n=16; age range, 4.7-16.8 years), MBgroup3 (n=16; 1.5-16.1 years) or MBgroup4 (n=20; 2.4-15.8 years) from infants and children, or in tumours from infant MBshh (<4.0 at diagnosis; n=17; 0.2-3.5 years), consistent with the rarity of mutations in these subgroups reported by Remke et al. [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^-6, ANOVA); aberrant hypermethylation (with respect to normal cerebellar levels; n=17, foetal to 67 years) was observed in 63% (10/16) MBWnt, 69% (11/16) MBgroup3 and in 10% (2/20) MBgroup4, while MBshh tumours (36%; 16/44 hypermethylated) showed greatest variation (Figure 1c). Notably, TERT hypermethylation showed significant age-dependent associations within the MBshh group (0% (0/6) >16 years; 52% (11/21) 4-16 years; 29% (5/17) <4 years; p=0.05, χ² test). Moreover, TERT promoter mutation and aberrant methylation at cg11625005 were mutually exclusive in non-infant MBshh 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 MBshh 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 MBshh > 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 MBshh 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\textsubscript{WNT} and MB\textsubscript{Group3}, but less so in MB\textsubscript{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\textsubscript{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 SHH subgroups ($\chi^2$ test between MB 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 SHH (by age; red), MB WNT (blue), MB Group3 (yellow) and MB 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 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 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


Mutation rate (%)

- 4-16 yrs
- <4 yrs

Mutated Wild-type Cerebellum

Unmethylated

Methylated

TERT TERT

Normal

n = 10

n = 17

n = 17

n = 21

n = 16

n = 16

n = 20

p = 0.0006

p = 0.00001

Methylation Level

SHH medulloblastoma

4 yrs

0.0

0.2

0.4

0.6

0.8

1.0

a

Position relative to ATG start site

-58

-124

-146

-633

C228T

C228A C250T

c

Methylated

Hyper-methylated

Median Expression

2

r = 0.247

p = 0.001

c

Methylation Level

Unmethylated

Methylated

Cerebellum

Normal

SHH

4-16 yrs

SHH<4 yrs

WNT

Group 3

Group 4

p = 9x10^-6

C228T C228A C250T

0 20 40 60 80 100

Figure 1
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