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Absence of telomerase reverse transcriptase promoter mutations in neuroblastoma

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Abstract. Maintenance of telomere length is a critical hallmark of malignant transformation. While silenced in somatic cells, telomerase reverse transcriptase (TERT), the catalytic subunit of telomerase, is frequently overexpressed in malignant cells thereby maintaining their telomere length. Specific point mutations in the *TERT* promoter region have recently been identified in melanoma and other tumor entities resulting in high TERT expression. Neuroblastoma is the most common extracranial tumor of childhood, arising from neural-crest progenitor cells. TERT overexpression has been observed in the majority of neuroblastoma. Taking into consideration that *TERT* promoter mutations are frequently described in neural-crest-derived tumors such as melanoma, as well as a variety of other neuronal tumors, the present study analyzed the frequency of *TERT* promoter mutations in primary neuroblastoma and neuroblastoma cell lines. In 131 neuroblastoma primary tumors representing the whole spectrum of neuroblastoma, no *TERT* promoter mutations were detected. However, in 3 out of 19 neuroblastoma cell lines the previously described C228T *TERT* promoter mutation was present. In conclusion, the *TERT* promoter mutations are not a frequent mechanism of TERT overexpression in neuroblastoma.

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Introduction

Each mitotic cell division results in the shortening of telomeres (1). In somatic cells, subsequent shortening of telomeres eventually leads to senescence. The multiunit enzyme telomerase maintains telomere length by adding repetitive sequences to telomeres at each replicative cycle. However, the telomerase activity is absent in somatic cells. By contrast, malignant cells (re-)express telomerase or use an alternative mechanism to maintain telomere length, known as alternative lengthening of telomeres (ALT) (2).

Telomerase reverse transcriptase (TERT) is an essential part of the telomerase enzyme complex (3). Most recently, specific point mutations were described in the promoter region of the *TERT* gene in familial and sporadic melanoma (4). These point mutations lead to a cytosine to thymine exchange at the positions 228 or 250 upstream of the start codon of *TERT*, further referred to as C228T and C250T, respectively (3-5). Through these mutations, the *TERT* promoter acquires novel binding sites for the E twenty-six transcription factor, resulting in increased TERT expression (6). Subsequently, *TERT* promoter mutations were described in a variety of tumors, including several neuronal tumors as medulloblastoma (7) and glioma, as well as other tumor entities (4,5). Of note, the presence of *TERT* promoter mutations often correlated with aggressive disease and an adverse outcome.

Neuroblastoma is the most common extracranial tumor during childhood arising from the neural-crest (8,9). Only few recurrent mutations or genomic rearrangements have been described thus far (10). Among these are the genomic amplifications of *MYCN* and activating mutations of anaplastic lymphoma kinase. Most recently, two independent studies described ALT through mutations in the transcriptional

regulator α-thalassemia/mental retardation X-linked (*ATRX*) in a minor fraction of neuroblastomas (11,12). Although the ALT phenotype may not be limited to neuroblastomas with *ATRX* mutations, the majority of neuroblastomas express telomerase and in particular TERT (13). Of note, high TERT expression was detected in *MYCN*-amplified tumors, but even in *MYCN* single copy neuroblastomas a high TERT expression correlated with adverse outcome (13). By contrast, low or absent TERT expression was detected in favorable neuroblastomas and absence of telomerase activity has been proposed as a potential mechanism to explain the high rate of spontaneous regression of favorable neuroblastomas (14). While MYCN is known to induce *TERT* transcription (15,16) and *TERT* amplification has been described in few neuroblastoma cases (17), the mechanism that drives TERT expression remains elusive in the majority of neuroblastomas.

As *TERT* promoter mutations are frequent in neural-crest-derived melanoma, as well as in various neuronal tumors including medulloblastoma, the present study analyzed the frequency of *TERT* promoter mutations in neuroblastoma.

Materials and methods

Primary tumor samples. Primary tumor samples and patient data were obtained from the German Society of Pediatric Oncology and Hematology Tumor Bank and Neuroblastoma Study Center (Cologne, Germany), respectively. All the patients were registered with the German neuroblastoma study and written informed consent was obtained. A tumor content of ≥60% was confirmed by a pathologist. DNA was isolated from ~20 mg of snap-frozen tissue obtained prior to cytotoxic treatment using the Puregene Blood Core kit B (Qiagen, Hilden, Germany), according to the manufacturer's instructions.

Cell culture. Neuroblastoma cell lines BEC(2), CHP134, IMR-32, IMR-5-75, KCN, KCN(R), Kelly, LAN1, LAN5, LAN6, NGP, NLF, SH-EP, SH-SY5Y, SK-N-AS, SK-N-BE, SK-N-FI and SK-N-SH were cultured in RPMI-1640 supplemented with 10% fetal calf serum, 5% penicillin (100 U/ml) and streptomycin (100 µg/ml) until reaching a confluence of 70%. Cells were collected and DNA was isolated using the QIAamp DNA kit (Qiagen). All the cell lines were authenticated by short tandem repeat genotyping performed by DSMZ (Braunschweig, Germany) or IDEXX GmbH (Ludwigsburg, Germany).

Polymerase chain reaction (PCR) and sequencing. DNA (100 ng) was used for the PCR reaction. To each PCR reaction, *TERT* forward (ACGAACGTGGCCAGCGGCAG) and reverse primers (CTGGCGTCCCTGCACCCTGG) were added to amplify a 474-base pair (bp) long region of the *TERT* promoter (390-bp upstream and 80-bp downstream of the start codon). The PCR program used was as follows: 95˚C for 180 sec, followed by 95˚C for 30 sec, 62˚C for 45 sec and 72˚C for 60 sec repeated 35 times, and a final amplification step at 72˚C for 600 sec. Sanger sequencing was performed by Sequence Laboratories Göttingen GmbH (Göttingen, Germany).

Table I. Patient data of the 131 sequenced primary neuroblastomas tumors.

Characteristics	Values, n
Patients	131
Gender	
Female	47
Male	84
Stage (INSS)	
1	29
2a	12
2b	7
3	19
4	50
4S	14
MYCN status	
Non-amplified	96
Amplified	33
Not detectable	2
1p36 status	
Wild-type	90
Deletion	24
Imbalance	12
Not detectable	5
Age at diagnosis, years	
<1	36
1-2	50
>2	45

INSS, International Neuroblastoma Staging System.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was isolated from cells using the RNeasyMini kit (Qiagen) and cDNA synthesis was performed using the SuperScript® RT kit (Invitrogen Life Technologies, Darmstadt, Germany). *TERT* expression was monitored using the QuantiTect Primer Assay™ (Qiagen). Expression values were normalized to the geometric mean of *GAPDH* (18). Data analysis and error propagation were performed using the qbasePLUS software version 1.5 (http://www.biogazelle.com).

Pyrosequencing. To determine the presence of the mutant C228T allele or the wild-type allele using pyrosequencing, the following primers were used to amplify a 151-bp amplicon: Biotinylated forward primer 5'-BIOTIN-CTTCACCTTCCA GCTCCGC-3', and reverse primer 5'-CGCTGCCTGAAA CTCGC-3'. The PCR products were analyzed by pyrosequencing using the primer 5'-GAGGGGCTGGGAGGG CCC-3', on the PyroMark Q96 MD system according to the manufacturer's instructions (Qiagen) and as previously described (19). Results were analyzed using PyroMark MD 1.0 software (Biotage, Uppsala, Sweden).

Genome-wide single-nucleotide polymorphism (SNP) analysis. SNP array experiments were performed according to the standard protocol for Affymetrix CytoScanHD arrays

Table II. Location of the Affymetrix CytoScanHD array probes located within or near the *TERT* gene. The probes lying within the TERT gene showed a loss of heterozygousity for SH-EP, SH-SY5Y and SK-N-SH. Probes lying outside of the TERT gene showed a heterozygous status.

Probe ID	Probe start position	Zygosity status			Comment
		SK-N-SH	SH-EP	SH-SY5Y	
S-4ELKX	1240103	Heterozygous	Heterozygous	Heterozygous	Probe downstream of *TERT* gene
S-4OOCR	1267356	Homozygous	Homozygous	Homozygous	Probe inside of *TERT* gene
S-4FACZ	1269006	Homozygous	Homozygous	Homozygous	Probe inside of *TERT* gene
S-4IBBK	1269365	Homozygous	Homozygous	Homozygous	Probe inside of *TERT* gene
S-3MNHV	1299087	Heterozygous	Heterozygous	Heterozygous	Probe upstream of *TERT* gene

Chromosomal position of *TERT*: Chr.5.1253167-1295047; Chromosomal position of the C228T mutation: Chr.5.1295228. *TERT*, telomerase reverse transcriptase.

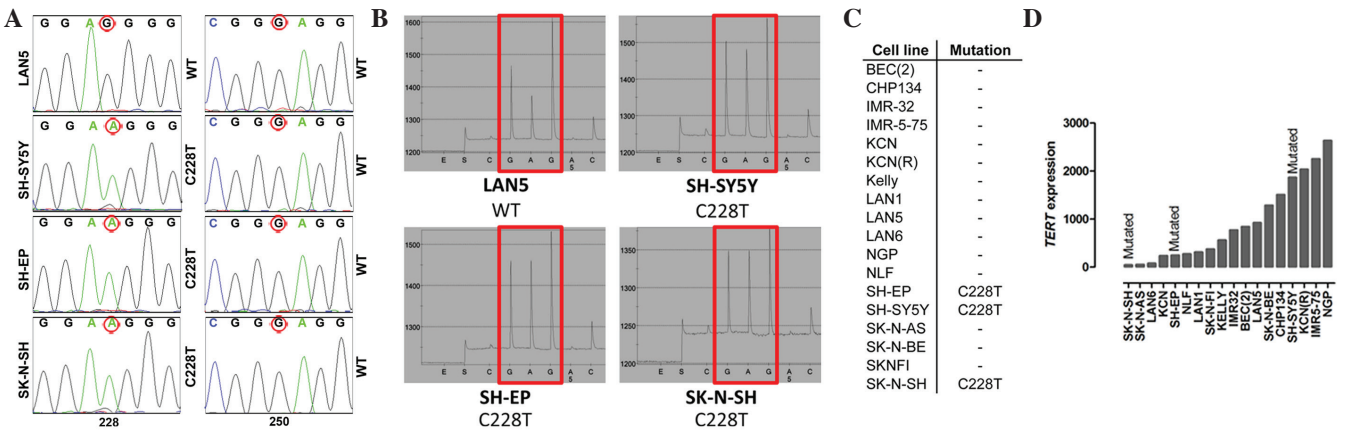

Figure 1. (A) Electropherogram examples of the cell lines SH-SY5Y, SH-EP and SK-N-SH harboring the C228T nucleotide exchange. By contrast, LAN5 shows the wild-type (WT) sequence at the mutation hotspots. (B) Representative pyrograms obtained for WT LAN5 cells, as well as SK-N-SH, SH-EP and SH-SY5Y cells, harboring the C228T telomerase reverse transcriptase (*TERT*) promoter mutation. Pyrograms were generated by sequential addition of the single-nucleotide polymorphism-specific nucleotides shown beneath each trace. Note that each peak corresponds to one or more identical bases added to the synthesized DNA-strand and that the height of each peak is correlated to the number of sequential nucleotides of the same type. (C) List of the neuroblastoma cell lines sequenced for *TERT* promoter mutations (C228T=guanine to adenine nucleotide exchange upstream of the *TERT* promoter at position-228). (D) TERT expression scaled to the lowest expression found in SK-N-SH (*TERT* expression=1). The cell lines that carry a *TERT* promoter mutation are marked with 'mutated'.

(Affymetrix, Inc., Santa Clara, CA, USA). In brief, a 250-ng sample of genomic DNA was digested with NspI, ligated to adaptors, amplified by PCR, fragmented and biotin-labeled. The labeled samples were hybridized to Affymetrix CytoscanHD arrays, followed by washing, staining and scanning in the Affymetrix GeneChip Scanner 3000. Analysis was performed using the Affymetrix Chromosome Analysis Suite v2.1.

Results

TERT promoter mutations are absent in primary neuroblastomas. To determine the frequency of *TERT* promoter mutations in neuroblastoma, including the previously described C228T and C250T mutations, the respective *TERT* promoter region of 131 primary neuroblastomas was sequenced. These tumors represented the whole spectrum of neuroblastoma regarding stage, *MYCN* and 1p36 status (Table I). Of note, none of the analyzed neuroblastomas harbored a C228T or a C250T mutation in the *TERT* promoter region.

TERT promoter mutations in neuroblastoma cell lines. In addition to primary neuroblastomas, 19 neuroblastoma cell lines were analyzed. In 3 out of 19 analyzed cell lines (16%) a mutation of the *TERT* promoter was detected. The neuroblastoma cell lines SK-N-SH, SH-SY5Y and SH-EP harbor the C228T mutation (Fig. 1A-C). Of note, SH-SY5Y and SH-EP are subclones of SK-N-SH (20). SK-N-SH was established from a bone marrow biopsy of a 4-year-old girl (21,22). The presence of the C228T *TERT* promoter mutation in SK-N-SH, SH-SY5Y and SH-EP was independently validated using pyrosequencing (Fig. 1B). As the C228T mutation appeared to be homozygous in SK-N-SH, SH-SY5Y and SH-EP, Affymetrix Human SNP array 6.0 arrays were used to analyze zygosity. The 3 probes located within the *TERT* gene were homozygeous in all three cell lines, which could point to the presence of a small loss-of-heterozygosity region spanning the *TERT* gene (Table II). To analyze if the mutations in the *TERT* promoter correlate with increased *TERT* expression, the expression was analyzed in all the neuroblastoma cell lines using RT-qPCR. No correlation between mutations and *TERT* expression was detected (Fig. 1D).

Discussion

Analysis of the *TERT* gene promotor region in 20 neuroblastoma cell lines and 131 primary neuroblastoma tumors revealed mutations in 3 cell lines and no mutations in primary tumors.

Of note, the only 3 mutated cell lines, which harbor the C228T mutation, are SK-N-SH and its 2 subclones SH-EP and SH-SY5Y, further limiting the general indication of this finding. It is uncommon that *TERT* mutations occur homozygously, as detected in SK-N-SH, SH-EP and SY5Y. However, further analysis confirmed a loss-of-heterozygosity region in the region of the *TERT* gene, pointing to a hemizygous rather than a homozygous status of the C228T mutation in these cases. Taking into consideration the variable levels of TERT expression in SK-N-SH, SH-EP and SH-SY5Y cells, the functional indication of the C228T mutations in these cell lines remain questionable.

The absence of *TERT* mutations in primary neuroblastomas and the majority of analyzed cell lines is unexpected, as *TERT* expression is of pathogenic relevance in the majority of neuroblastomas (13) and *TERT* mutations are frequent in related neuroectodermal tumors, such as melanoma and medulloblastoma (4,7,23,24). However, the present results are in line with previous studies by Papathomas *et al* (25), who did not find *TERT* promoter mutations in 15 primary neuroblastomas and Killela *et al* (23), who identified only 2 *TERT* promoter mutations in 22 neuroblastomas.

The mechanisms by which *TERT* expression is induced, particularly in *MYCN* non-amplified high-risk neuroblastomas, remains elusive and warrants further studies. Potential alternative mechanisms underlying *TERT* induction in neuroblastoma include epigenetic changes, as well as mutations in more distant regulatory elements that were not analyzed in the present study.

Taken together, we conclude that *TERT* (core) promoter mutations are not relevant events in neuroblastoma pathogenesis.

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