J Neurooncol (2014) 119:17–26
DOI 10.1007/s11060-014-1456-8

LABORATORY INVESTIGATION

Alternative lengthening of telomeres in neuroblastoma cell lines is associated with a lack of *MYCN* genomic amplification and with p53 pathway aberrations

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Received: 7 November 2013 / Accepted: 19 April 2014 / Published online: 3 May 2014
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Abstract Alternative lengthening of telomeres (ALT) is a telomerase-independent telomere length maintenance mechanism that enables the unlimited proliferation of a subset of cancer cells. Some neuroblastoma (NB) tumors appear to maintain telomere length by activating ALT. Of 40 NB cell lines, we identified four potential ALT cell lines (CHLA-90, SK-N-FI, LA-N-6, and COG-N-291) that were telomerase-negative and had long telomeres (a feature of ALT cells). All four cell lines lacked *MYCN* amplification and were p53 non-functional upon irradiation. Two of these cell lines (CHLA-90 and SK-N-FI) were positive for C-circles (telomeric DNA circles) and ALT-associated promyelocytic leukemia nuclear bodies, both of which are phenotypic characteristics of ALT. Mutation of *ATRX* (associated with ALT in tumors) was only found in CHLA-90. Thus, the ALT phenotype in NB may not be limited to tumors with *ATRX* mutations but is associated with a lack of *MYCN* amplification and alterations in the p53 pathway.

Keywords Neuroblastoma · Telomere · Telomerase · ALT · p53 · ATRX

Introduction

Telomeres are specialized nucleoprotein complexes of repetitive DNA sequence found at eukaryotic chromosomal ends and are essential for maintaining genomic stability [1, 2]. Due to the inability of conventional DNA polymerases to fully replicate the 3′ ends of linear DNA (end-replication problem), telomeres progressively shorten with each cell division [3]. When telomeres become critically short, cells

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undergo p53-dependent senescence or apoptosis, thus preventing unlimited cellular proliferation [4].

Cancer cells overcome this barrier and become immortalized by activating one of the two known telomere maintenance mechanisms: telomerase or alternative lengthening of telomeres (ALT). The telomerase enzyme complex contains the human telomerase reverse transcriptase (hTERT) subunit and human telomerase RNA (hTR or hTERC) [5, 6]. Telomerase catalyzes the synthesis of hexameric (TTAGGG) DNA repeats using hTR as template and is present in 80–90 % of human malignancies [7]. In contrast, ALT is a telomerase-independent mechanism where telomeric DNA is replicated via homologous recombination using DNA as template [8–10] and is much more prevalent in tumors of mesenchymal and neuroepithelial origin [11]. Phenotypic characteristics of ALT cells include long and heterogeneous telomere lengths [12], presence of ALT-associated promyelocytic leukemia (PML) nuclear bodies (APBs) [13], and abundant extrachromosomal telomeric repeats (ECTR) [14]. C-circles (CCs) are partially double-stranded circular ECTR, where the C-rich strand is complete and the G-rich strand is gapped, and have been shown to be ALT-specific [15]. In the CC assay, CCs act as primers in rolling circle amplification to generate long linear telomeric products [15]. Inactivation of the tumor suppressor p53 (TP53) by a viral oncoprotein or a *TP53* mutation is extremely common in ALT cell lines [11]; p53 inhibits homologous recombination and it is thought that loss of p53 function permits the activation of ALT. Mutations or structural alterations in alpha thalassemia/mental retardation syndrome X-linked (*ATRX*) or death domain-associated protein (*DAXX*) were found in a subset of pancreatic neuroendocrine tumors with ultra-bright telomere signals, a hallmark of ALT, suggesting that ATRX and DAXX loss-of-function may facilitate ALT activation [16]. The *ATRX* gene encodes a large helicase protein that is involved in chromatin remodeling [17]. Cooperating with DAXX, ATRX deposits the histone H3 variant, H3.3, at telomeres and other G-rich repetitive elements in the genome [18].

ATRX alterations have also been reported recently in three different studies of neuroblastoma (NB) tumors [19–21]. NB is a malignant pediatric tumor of the sympathetic nervous system and is the most common extra-cranial solid tumor in children [22]. Current evidence suggests that 10–20 % of NB tumors have elongated telomeres and/or APBs (supporting the presence of ALT in NB) and that these tumors have very poor outcome [23, 24]. Furthermore, NB tumors with *ATRX* mutations were found to have long telomeres and ultra-bright telomere signals [19]. Of interest, all *ATRX*-mutated NB tumors reported to date lacked *MYCN* amplification [19–21], a marker of high-risk disease in NB [25].

In order to study the impact of ALT in NB, we sought to identify tumor-derived NB cell lines harboring the ALT phenotype. Of 40 NB cell lines, we identified four telomerase-negative NB cell lines that had long telomeres, with two cell lines demonstrating the presence of CCs and APBs. All four cell lines lacked *MYCN* amplification and displayed aberrations in the p53 pathway. Hence, these cell lines provide valuable laboratory models to understand telomerase-independent telomere maintenance mechanisms in NB and will be useful tools for preclinical therapeutic studies, especially those targeting telomere maintenance.

Materials and methods

Cell lines

A total of 40 human NB cell lines established from 34 different patients (12 lines were pre/post therapy pairs from the same patients) were analyzed in this study (Table 1). All NB cell lines were established in the laboratory of one of the authors (Dr. C Patrick Reynolds), except for LA-N-5 and LA-N-6 (obtained from Dr. Robert Seeger), SK-N-BE(1) and SK-N-BE(2) (obtained from Dr. June Biedler), and SK-N-FI and SK-N-RA (obtained from Dr. Lawrence Helson) [26–28]. We used 6 matched cell line pairs for our analyses: CHLA-15 and CHLA-20, CHLA-122 and CHLA-136, SK-N-BE(1) and SK-N-BE(2), CHLA-144 and CHLA-145, SMS-KCN and SMS-KCNR, and COG-N-294 and COG-N-321. In each of these pairs, the cell lines were established from the same patient and therefore shared the same STR signature. Cell lines were cultured in either RPMI-1640 (Thermo Scientific Hyclone, Waltham, MA, USA) with 10 % fetal bovine serum (FBS) (GIBCO Life Technologies, Grand Island, NY, USA) or Iscove's modified Dulbecco's medium (Thermo Scientific Hyclone) with 1× ITS (BD Pharmingen, Franklin Lakes, NJ, USA), 20 % FBS and 4 mM L-Glutamine (CellGro, Manassas, VA, USA). All cell lines were tested as mycoplasma free. Cell line identities were confirmed with short tandem repeat (STR) genotyping using the AmpF STR Profiler Plus kit (Applied Biosystems, Grand Island, NY, USA) [29] and verified against the Children's Oncology Group cell line and xenograft STR database (www.COGcell.org).

Quantitative PCR

Genomic DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA, USA) and total RNA using the RNeasy Mini Kit (Qiagen). Reverse transcription was performed with High Capacity Reverse Transcription Kit (Applied Biosystems). Target genes were quantified

Table 1 Summary of the 39 NB cell lines established from 34 unique patients

Cell line	Phase of therapy	*hTERT*[a]	Telomerase activity	TC	CC	APB	*MYCN* genomic amp.	p53 dysfunction
CHLA-90	PD-BMT	–	–	18.6	159	+	–	+
SK-N-FI	PD	–	–	14.0	196	+	–	+
LA-N-6	PD	0.4	–	35.6	0	?	–	+
COG-N-291	PD	4.0	–	31.5	0	?	–	+
CHLA-136*	PD	8.2	+	2.3	0	–	+	–
CHLA-20*	Dx	7.8	+	5.3	0	ND	–	–
CHLA-53	PD, PM	+++	ND	11.8	0	ND	+	ND
SMS-SAN	Dx	+	+	10.2	0	–	+	–
CHLA-61	PD, PM	+++	ND	5.8	6.9	ND	–	ND
CHLA-11	PD-BMT, PM	+++	ND	<5	0	ND	+	ND
CHLA-12	PD-BMT, PM	+++	ND	<5	0	ND	+	ND
CHLA-15*	Dx	+++	ND	5–10	0	ND	–	–
CHLA-42	Dx	+	+	5–10	0	–	–	–
CHLA-52	PD, PM	+++	ND	5–10	0	ND	+	ND
CHLA-60	PD, PM	+++	ND	5–10	0	ND	–	ND
CHLA-79	PD-BMT	+++	ND	5–10	0	ND	+	–
CHLA-122*	Dx	+	+	5–10	1.1	–	+	–
CHLA-144*	Dx	+++	ND	5–10	0	ND	–	ND
CHLA-145*	PD	+	ND	5–10	0.4	ND	+	ND
CHLA-171	PD, PM	+	+	<5	0	–	+	+
CHLA-172	PD-BMT	+++	+	5–10	0	–	+	+
CHLA-225	PD, PM	++	+	5–10	0	–	+	+
CHLA-247	PD	+++	ND	<5	0.2	ND	+	ND
COG-N-253	PD	+	ND	5–10	0	ND	+	ND
COG-N-294*	Dx	++	ND	<5	0	ND	+	+
COG-N-316	DX	+++	ND	<5	0.1	ND	–	ND
COG-N-321*	PD	+	ND	<5	0.4	ND	+	+
COG-N-347	DX	+	+	<5	0.1	–	+	ND
COG-N-399	PD, PM	++	ND	<5	0	ND	+	ND
COG-N-415	PD	+++	ND	<5	1.0	ND	+	+
COG-N-421	PD, PM	+	ND	5–10	0	ND	+	ND
COG-N-440	PD, PM	+++	ND	<5	0	ND	+	ND
LA-N-5	Dx	+++	+	<5	0	–	+	–
SK-N-BE(1)*	Dx	+	+	5–10	0	–	+	–
SK-N-BE(2)*	PD	+	+	5–10	0	–	+	+
SK-N-RA	PD	+++	ND	5–10	0.6	ND	–	–
SMS-KAN	Dx	+	ND	5–10	0	ND	+	–
SMS-KCN*	Dx	++	+	5–10	2.9	–	+	–
SMS-KCNR*	PD	+	+	5–10	0	–	+	–
SMS-LHN	PD	++	+	5–10	0	–	–	–

amp amplification, *PD* progressive disease, *BMT* bone marrow transplant, *PM* post-mortem, *Dx* diagnosis, *TC* telomere content, *CC* C-circle, *ND* not done

* Matched pair cell line

[a] + low (10-100), ++ medium (100-1,000), +++ high (>1,000)

using TaqMan primer and probe sets and the TaqMan gene expression master mix (Applied Biosystems). The assay IDs are: *GAPDH* (Hs03929097_g1), *TERT* (Hs00972656_m1), *TERC* (Hs03454202_s1), *RNAseP* (4401631), and *MYCN* (Hs02718426_cn) (Applied Biosystems). PCRs were performed in triplicate on an ABI Prism 7900 HT Fast Real

Time PCR System (95 °C for 10 min, and 40 cycles of 95 °C for 15 s, 60 °C for 60 s). Data was generated using SDS 2.3 and RQ Manager 1.2 software from the manufacturer.

C-circle assay and telomere quantitative PCR

Rolling circle amplification of CCs in genomic DNA was performed as described previously [15, 30]. The qPCR was performed in triplicate for both telomere and single copy gene (SCG) quantification as previously described [30]. VAV2, which encodes a guanine nucleotide exchange factor, served as our SCG as this has been previously been validated as an appropriate SCG for NB [31]. All PCRs were performed on an ABI Prism 7900 HT Fast Real Time PCR System (95 °C for 10 min, and 40 cycles of 95 °C for 15 s, 56 °C for 15 s, and 72 °C for 1 min).

Terminal restriction fragment (TRF) analysis

Telomere length was measured by TRF Southern blot analysis as previously described [32]. Briefly, genomic DNA digested with HinfI and RsaI restriction enzymes (4 U/μg DNA) (New England Biolabs (NEB)) and 25 ng/μg RNase (Roche) was resolved on a 1 % agarose gel using pulsed-field gel electrophoresis. The denatured gel was hybridized to an end-labeled $^{32}P\text{-}(CCCTAA)_4$ telomeric probe. Mean TRF length was calculated as previously described [30].

Telomere repeat amplification protocol (TRAP)

The TRAP assay was performed as described previously [32]. Lysates were prepared from equal cell numbers. PCR amplification products were separated by 10 % non-denaturing, polyacrylamide gel electrophoresis. Gels were stained with SYBR Green I (Molecular Probes, Mulgrave, Australia) and visualized using a Typhoon imager (GE Healthcare, Rydalmere, Australia). Telomerase activity (TA) was indicated by the presence of a 6 base pair ladder.

TP53 and *ATRX* sequencing

TP53 sequencing has been previously published for many of the lines in this study, including three of the four ALT cell lines by PCR amplification (primers synthesized by Integrated DNA Technologies) and sequencing of regions of the *TP53* gene identified as mutated by the Affymetrix Genechip [28, 33]. *TP53* sequencing of COG-N-291 was carried out in the same manner, with gel-purified PCR products and subjected to Sanger sequencing at the DNA Core Facility of the University of Southern California. All ATRX exons were amplified by PCR as described [34]. PCR products were purified with the QIAquick PCR Purification Kit (Qiagen) and subjected to Sanger sequencing at the Australian Genome Research Facility.

ATRX immunoblot and immunohistochemistry

ATRX immunoblotting was performed on whole cell extracts using standard procedures. Equal amounts of protein lysates were resolved on Novex 4–12 % Bis-Tris Gels (Invitrogen), transferred to PVDF membrane (Life Technologies), and probed with rabbit anti-ATRX (HPA001906) (Sigma Aldrich, St Louis, MO, USA) and mouse anti-β-actin antibody (Santa Cruz, Dallas, TX, USA). Chemiluminescence detection was performed using Novex ECL Chemiluminsecent Substrate Reagents (Life Technologies). For immunohistochemistry, cells were fixed in ice-cold methanol:acetone (1:1), incubated with rabbit anti-ATRX antibody (A301-353A) (Bethyl Labs, Montgomery, TX, USA), followed by Impress Reagent anti-rabbit IgG and Vector Hematoxylin QS (Vector Labs).

APB assay

Cells grown in chamber slides were fixed with 100 % cold methanol, incubated with mouse anti-PML (PG-M3) antibody (sc-966) (Santa Cruz) and Alexa Fluor 488 goat anti-mouse secondary antibody (Invitrogen), cross-linked with 4 % paraformaldehyde, and dehydrated with ethanol. Telomere DNA was detected using fluorescence in situ hybridization with the 5′ labeled Cy3-(5′ CCCCTAA 3′) peptide nucleic acid probe (Applied Biosystems). Images were captured on an Olympus fluorescence microscope using SPOT image analysis software. APB was defined as the co-localization of telomere DNA foci and PML [13].

p53 functional analysis

Cells exposed to 10 Gy of X-ray irradiation (RS 2000 Biological Irradiator) (Rad Source) were fixed in cold 90 % ethanol 16 h after irradiation. Fixed cells were incubated with FITC mouse anti-p53 (DO-7) (BD Pharmingen) and Alexa Fluor488 conjugated rabbit anti-p21 Waf1/Cip1 (12D1) antibody (Cell Signaling Technology) in triplicate. p53 and p21 levels were quantitated using flow cytometry (BD LSR II Flow Cytometer) (BD Pharmingen). A cell line was considered p53 functional when p53 induction resulted in ≥ 2-fold increase in p21. CHLA-20 cell line (*TP53* wild-type and functional) was used as positive control and SK-N-BE(2) (*TP53*-mutated and non-functional) as negative control [33].

Data analysis

Telomere qPCR data was analyzed using the two standard curves method [30, 35]. The telomere content (TC) and CC of a sample was relative to that of the SK-N-FI cell line with an arbitrary value of 14 and 195, respectively [30]. The delta–delta C_t method was to quantitate mRNA expression (*GAPDH* as reference) and *MYCN* copy number (*RNAseP* as SCG). *hTERT* mRNA level was relative to that of LA-N-5 cell line (known telomerase-positive cell line) [36] with an arbitrary value of 1,000. Relative *hTERT* level of <10 was considered as very low, 10 to <100 as low, 100 to <1,000 as medium, and ≥1,000 as high. *MYCN* amplification was defined as ≥10 gene copies per diploid genome [37]. Medians were compared using the Mann-Whitney U test and proportions by Fisher's Exact test.

Results

Lack of telomerase activity in four NB cell lines with long telomeres

Forty NB cell lines were first screened for *hTERT*, which encodes the catalytic component of telomerase. Relative *hTERT* mRNA levels ranged from 0.35 to 57,856 with a mean of 3,718. Two cell lines (CHLA-90 and SK-N-FI) were found to be *hTERT*-negative and four cell lines had very low (<10) *hTERT* mRNA (LA-N-6, 0.35; COG-N-291, 4.0; CHLA-136, 8.2; CHLA-20, 7.8) (Table 1). Since ALT cells are known to have long telomeres [12] and abundant CCs [15], we next measured TC, indicative of long telomeres, and CC levels using a telomere PCR-based assay in these 40 cell lines. The two *hTERT*-negative cell lines (CHLA-90 and SK-N-FI) and two of the very low *hTERT* cell lines (LA-N-6 and COG-N-291) were considered to have long telomeres with a relative TC above the previously described ALT cut-off value of 12.0 (Fig. 1) [30]. Telomere length was also measured by TRF Southern blot analysis in the four cell lines with high TC and in another two cell lines with low TC (SK-N-BE(2) and LA-N-5). The mean TRF length of CHLA-90, SK-N-FI, LA-N-6, COG-N-291, SK-N-BE(2), and LA-N-5 were 19.1, 19.4, 37.8, 31.8, 4.1, and 3.5 kb, respectively. Only two (CHLA-90 and SK-N-FI) of the four cell lines with long telomeres and negative/very low *hTERT* expression were CC-positive (CC level above cut-off of 7.5) (Fig. 1). CHLA-136 and CHLA-20, the other cell lines with very low *hTERT*, had very short telomeres (TC 2.3 and 5.3, respectively) and no CCs. In addition, there were three cell lines with borderline TC (CHLA-53, 11.8; SMS-SAN, 10.2) or CC levels (CHLA-61, 6.9) close to but below the ALT cut-off value

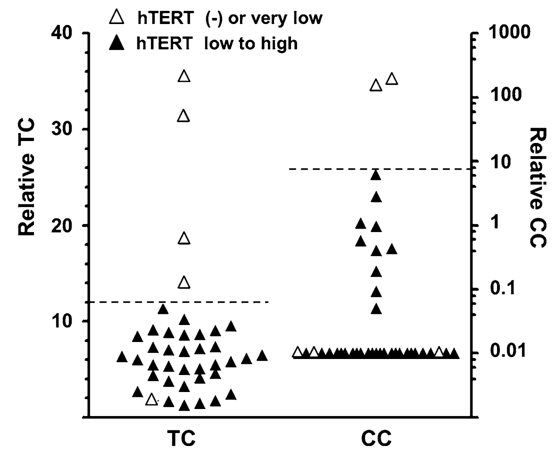


Fig. 1 Analysis of telomere content (TC) and C-circle (CC) level in 40 tumor-derived NB cell lines using a telomere qPCR-based ALT detection assay. Previously described ALT cut-off values (*dashed line*) for TC (>12) and CC (>7.5) were applied [30]. Two *hTERT*–negative (CHLA-90 and SK-N-FI) and two very low *hTERT* (COG-N-291 and LA-N-6) cell lines had TC above the ALT cut-off value. CHLA-90 and SK-N-FI were also positive for CC

(Fig. 1; Table 1). All three of these cell lines expressed *hTERT*.

We next examined telomerase activity (TA) in the two *hTERT*-negative and three *hTERT* very low cell lines, as well as in 13 other *hTERT*-positive cell lines. Consistent with the negative or very low *hTERT* expression, TA was absent in the four cell lines with high TC (CHLA-90, SK-N-FI, LA-N-6 and COG-N-291) (Fig. 2a; Table 1). TA was detected in the remaining 14 cell lines, including CHLA-136 with very low *hTERT* and TC. Hence, we identified four telomerase-negative cell lines that also had high TC and mean TRF length, suggesting ALT-based telomere length maintenance as defined by high TC (>12) and very low *hTERT* (<10). Furthermore, we were able to grow each of the four telomerase-negative cell lines for >40 passages and found no significant changes in TC by telomere qPCR (data not shown). We also measured *hTR* mRNA (RNA component of telomerase) and found no significant difference in *hTR* levels between the four telomerase-negative cell lines and the 14 telomerase-positive cell lines ($P = 0.4$) (Fig. 2b).

Correlation of APB and CC in NB cell lines

To further elucidate the ALT status of the four telomerase-negative cell lines with high TC, we examined these cells for the presence of ALT-associated promyelocytic leukemia nuclear bodies (APBs), a well-established marker of ALT [11, 13]. We found that only CHLA-90 and SK-N-FI that were CC-positive were also positive for APBs, which were present in 30 % of the cells (Fig. 3a). In LA-N-6 and COG-

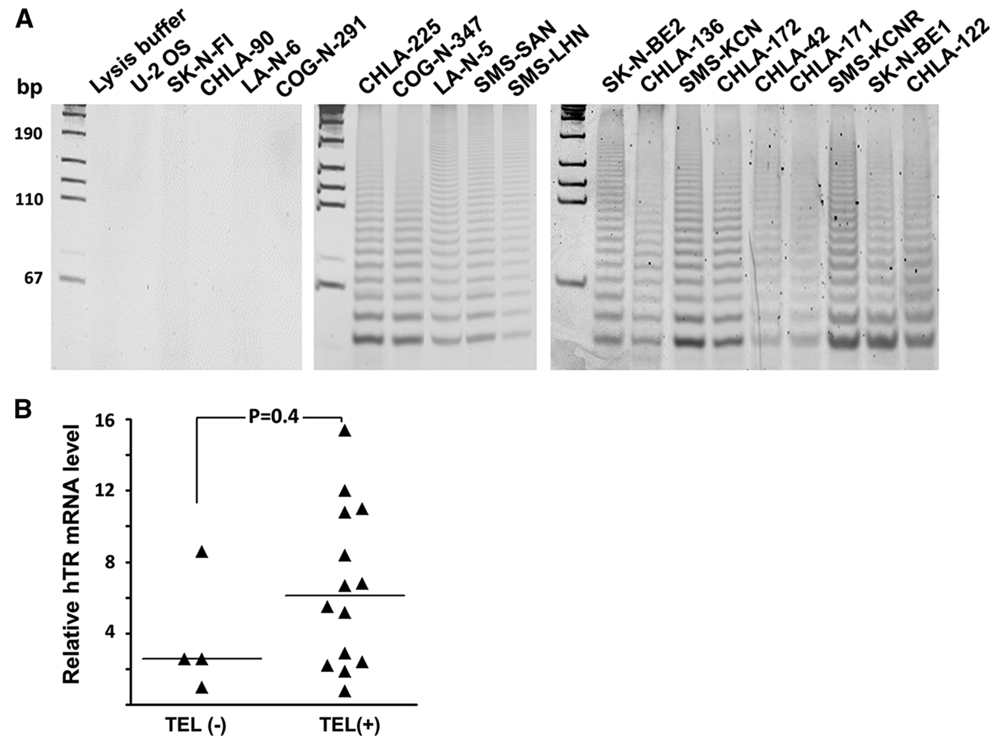


Fig. 2 Telomerase activity and hTR expression in 18 NB cell lines. **a** TRAP assay was performed in the five *hTERT*-negative or very low cell lines (CHLA-90, SK-N-FI, LA-N-6, COG-N-291, and CHLA-136) and 13 *hTERT*-positive cell lines. U-2 OS, an ALT+ osteosarcoma cell line, was used as negative control. Telomerase activity was indicated by the presence of a 6 base pair ladder. **b** *hTR* (human telomerase RNA) mRNA expression was measured by RT-qPCR and segregated by telomerase activity as detected by TRAP. *TEL* telomerase

N-291, which were CC-negative, we found occasional co-localizations of telomere DNA with very small PML bodies in <3 % of the cells. The 14 telomerase-positive cell lines did not have APBs (Table 1). Hence, CHLA-90 and SK-N-FI represent typical ALT cell lines, given the high TC and the presence of APBs and CCs. However, the equivocal APBs and absent CCs in LA-N-6 and COG-N-291 may represent non-classical ALT. Prior work showing some NB tumors with elongated telomeres being CC- and APB-negative [30] suggests that CCs and APBs may be absent in a minority of ALT+ NB tumors and cell lines. Alternatively, the undetectable CCs in LA-N-6 and COG-N-291 may represent very low levels of CCs that are below the threshold of detection by telomere qPCR.

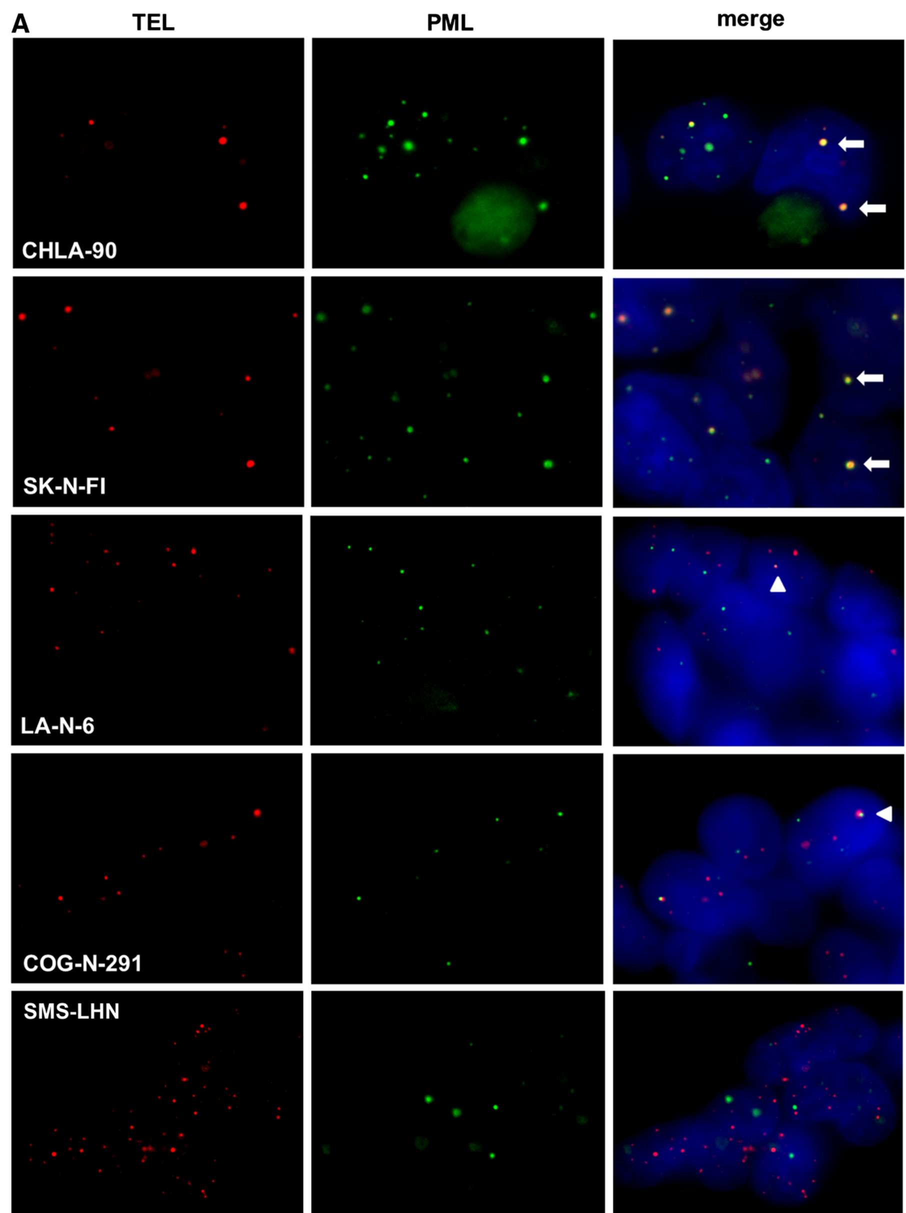
MYCN amplification and ATRX status

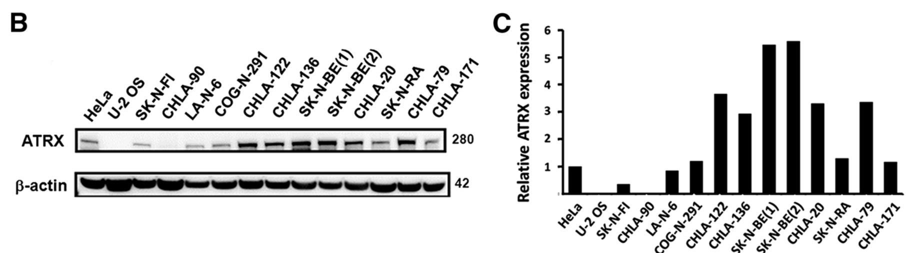
ATRX alterations resulting in loss of protein expression are closely related to ALT [16, 38] and have recently been reported to be mutually exclusive with *MYCN*-amplification in NB tumors [19–21].

The four ALT NB lines were found to lack *MYCN* amplification (Table 1). We next sequenced the *ATRX* gene and found only one cell line (CHLA-90) had a partial deletion of exon 9. CHLA-90 lacked detectable ATRX protein consistent with *ATRX* deletion (Fig. 3b–d). The other three cell lines (SK-N-FI, LA-N-6, and COG-N-291) were *ATRX* wild-type (wt) and had intact nuclear expression of ATRX by immunohistochemistry (IHC) (Fig. 3d), but also had relatively less ATRX protein levels compared to most of the telomerase-positive lines on immunoblots (Fig. 3c). Given that there is no functional assay for ATRX at this time, it is unclear whether the low expression of ATRX seen in SK-N-FI, LA-N-6, and COG-N-291 is related to the ALT phenotype, especially when some telomerase-positive cell lines (SK-N-RA, CHLA-171, HeLa) also had low ATRX levels. However, these data do indicate that ALT can develop in NB carrying wt *ATRX*.

ALT NB cell lines display p53 pathway aberrations

Although *TP53* mutations in NB are rare at diagnosis, alterations in the p53/MDM2/p14(ARF) pathway have been reported to be frequent at relapse [39, 40]. MDM2, which targets p53 for degradation, is inhibited by p14(ARF) [41]. Since all four telomerase-negative NB cell lines were established at the time of progressive disease, and there is a strong association between ALT and loss of p53 function [11], we assessed p53 mutational and functional status in the cell lines. CHLA-90 and SK-N-FI that were both CC- and APB-positive were known to carry *TP53* mutations while LA-N-6 (CC-negative) was reported to be *TP53* wt [28]. However, LA-N-6 has been shown to carry a homozygous deletion of the *CDKN2A* gene that encodes p14(ARF) [42] potentially resulting in impaired p53 function. We sequenced *TP53* in COG-N-291 and found wt sequence with a codon 72 polymorphism (P72R). This particular polymorphism has been shown to markedly





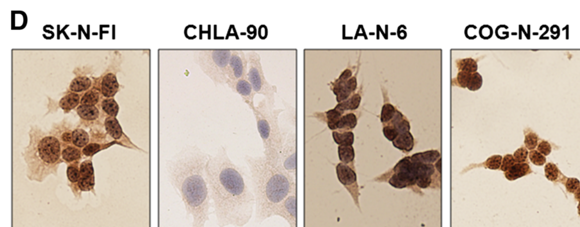


Fig. 3 APB and ATRX status in telomerase-negative NB cell lines. **a** The APB assay involving combined PML immunofluorescence and telomere fluorescence in situ hybridization is shown in the four telomerase-negative cell lines (CHLA-90, SK-N-FI, LA-N-6, COG-N-291) and in a telomerase-positive cell line (SMS-LHN). CHLA-90 and SK-N-FI are positive for APBs, with telomeric DNA foci co-localizing with PML body (*white arrow*). LA-N-6 and COG-N-291 have only occasional co-localization of telomere with very small PML foci (*white triangle*). SMS-LHN is a telomerase-positive cell line that is APB-negative. **b** ATRX immunoblot of the four telomerase-negative cell lines and eight telomerase/*hTERT*-positive cell lines. HeLa (telomerase+) and U-2 OS (ALT+) cells were used as positive and negative controls, respectively. U-2 OS is known to be ATRX-negative [38]. **c** Quantitative analysis for ATRX as assessed by immunoblotting shown in (**b**). ATRX expression is relative to that of HeLa cells. **d** Immunohistochemical analysis showed that ATRX was present in the nucleus of SK-N-FI, COG-N-291, and LA-N-6, but absent in CHLA-90

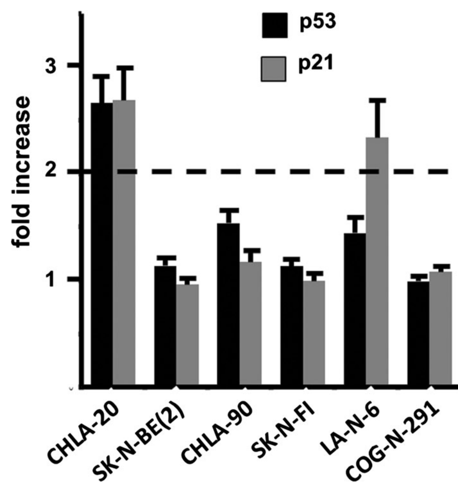


Fig. 4 Telomerase-negative cell lines with long telomeres are p53 non-functional. p53 function was assessed by p53 and p21 induction using flow cytometry. p53 functional was defined as concurrent 2-fold increase in p53 and p21 levels in cells exposed to 10 Gy of X-ray irradiation compared with non-irradiated cells. CHLA-20, a known *TP53* wt and functional NB cell line, was used as positive control and SK-N-BE(2), a known *TP53* mutant and non-functional line, was used as negative control. The assays were performed in triplicate. *Error bars* display standard error of the mean

lower the ability of p53 to induce cell-cycle arrest after DNA-damaging treatment [43]. To verify whether these genetic changes were associated with loss of p53 function, we quantified induction of p53 and its downstream target p21 following X-ray irradiation. All four cell lines were considered p53 non-functional (<2-fold increase in p53 and p21). While we observed a >2-fold increase in p21 in LA-N-6, there was no change in p53 levels after irradiation, suggesting p53-independent induction of p21 (Fig. 4). Since p53 dysfunction was found in all four cell lines, we asked whether p53 dysfunction was more common in ALT than in ALT-negative NB cell lines. We have previously reported p53 function in 17 of the 36 non-ALT cell lines in this study [28, 33] and have unpublished data of 4 additional non-ALT cell lines (Table 1). Seven (33 %) of these 21 cell lines were found to be p53 non-functional (Table 1). To avoid sampling bias, we treated the paired cell lines as "one" cell line, and found six (35 %) of these 17 cell lines to be p53 non-functional. Hence, p53 dysfunction was significantly more frequent in ALT than in ALT-negative NB cell lines (100 vs. 35 %; $P = 0.035$).

Discussion

Telomerase has been widely studied in NB tumors and high telomerase expression is associated with high risk disease [44, 45]. However, not all metastatic NB express TA [46], and elongated telomeres and APBs, both phenotypic characteristics of ALT, have been found in some NB tumors [23, 24]. These observations, together with the detection of *ATRX* alterations in NB tumors [19–21], supports the conclusion that a subset of NB utilizes ALT to maintain telomeres. NBs with elongated telomeres were also reported to have poor long-term survival despite myeloablative therapy [23]. We therefore sought to identify ALT-positive NB cell lines to serve as in vitro models for understanding telomere maintenance mechanisms in NB and for preclinical therapeutic studies.

Of the 40 lines that we tested, four had long telomeres in the absence of TA, suggesting ALT-based telomere length maintenance. Two (SK-N-FI and CHLA-90) of these four cell lines had APBs and abundant CCs, both of which are markers for ALT [13, 15]. The other two cell lines (LA-N-6 and COG-N-291) were equivocal for APBs and have undetectable CCs by telomere qPCR. Abundant CCs have been shown to be highly specific for ALT. Of the 19 ALT cell lines tested in a previous study, all 19 were positive for CCs, including two cell lines that lacked APBs [15]. However, the undetectable CCs in LA-N-6 and COG-N-291 may be related to the difficulty of telomere qPCR detecting very low levels of CC due to interference from the high levels of double-stranded TC. Hence, with equivocal APBs and absent or very low levels of CCs, the mechanism of ALT in LA-N-6 and COG-N-291 is uncertain, but there was no detectable decrease in TC by telomere qPCR over 40 passages in these two cell lines that lacked TA. Moreover, not all NB tumors with elongated telomeres are CC-positive [30] or harbor *ATRX* mutations [19].

Although *ATRX* mutations and/or loss of ATRX protein expression are frequently found in ALT cell lines, 13 % of ALT cell lines express ATRX [38]. Thus, the expression of ATRX in three of the cell lines (SK-N-FI, LAN-6 and COG-N-291) does not exclude ALT. While uniformly low ATRX levels were found in these three cell lines, similar low levels were also present in three (SK-N-RA, CHLA-171, HeLa) of the nine ALT-negative cell lines. In the absence of an ATRX function assay, it is unclear whether these NB cells that express relatively low levels of full-length ATRX are fully functional. However ATRX needs to be localized to the nucleus to function as a chromatin modifier and IHC in this study confirmed ATRX nuclear localization in these three cell lines, suggesting ATRX is likely to be functional. Other mechanisms other than loss of ATRX function may also be present to facilitate the development of ALT in NB. In addition to *hTERT* mRNA, we also examined hTR that has been reported to be undetectable in some but not all ALT cell lines [47] and hence lack of *hTR* would confirm ALT. All four NB cell lines were found to express *hTR,* but this does not exclude ALT as *hTR* expression is not inconsistent with ALT.

p53 deficiency is closely associated with ALT in both *in vitro* immortalized and tumor-derived cell lines [11]. In this study we found p53 dysfunction in all four ALT cell lines, which was significantly more frequent than ALT-negative NB cell lines, of which only 33 % were deficient in p53 function. Thus, in NB p53 loss-of-function is not a characteristic unique to ALT. Nevertheless, the strong association of ALT with p53 loss-of-function suggests that novel therapeutic approaches, especially p53-independent therapy, should be explored for this subset of NB tumors.

In conclusion, our study has identified four NB cell lines that are able to proliferate continuously in the absence of TA, suggesting an ALT-based telomere maintenance mechanism. All four cell lines have elongated telomeres, lack *MYCN* gene amplification, and have aberrations in the p53 pathway, with two (CHLA-90 and SK-N-FI) also demonstrating the presence of classical ALT phenotypic markers including CCs and APBs. To our knowledge there are less than 10 tumor-derived ALT lines that have been characterized before this study and none of which were derived from NB. Utilizing these lines as *in vitro* models will aid in understanding the mechanisms and biology of telomere maintenance in cancer cells. These cell lines will also enable defining drug sensitivity and resistance in ALT versus telomerase-positive NB and provide preclinical therapeutic models for a distinct subset of high-risk NB.

Acknowledgments This work was supported by a Grant from the National Cancer Institute (CA82830; C. P. Reynolds), Cancer Prevention & Research Institute of Texas (RP 110763; C. P. Reynolds), and Cure Cancer Australia Foundation (L. Lau); by a Cancer Institute of New South Wales Clinical Research Fellowship, Australia (07/CRF/105; L Lau) and National Health and Medical Research Council Early Career Fellowship, Australia (APP1012500; L. Lau).

Conflict of interest The authors declare that they have no conflict of interest.

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