

# Long telomeres in Peripheral Blood Leukocytes are associated with an increased risk of neuroblastoma

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## ABSTRACT

*Telomere dysfunction is an important feature of tumorigenesis. Prior epidemiological studies have associated shorter telomere length in peripheral blood leukocytes with the development of many pediatric malignancies. However, the association between telomere length and the risk of neuroblastoma remains unclear. In this matched case-control study, we determine the relative telomere length (RTL) in the peripheral blood leukocytes from 20 neuroblastoma cases and 40 apparently healthy controls, and evaluated the association between RTL and neuroblastoma risk. By using median RTL in the healthy controls as a cutoff, children who had longer telomeres were at an increased risk of developing a solid tumor [odds ratio [OR] 9.45; P = 0.0037]. When participants were categorized according to quartile RTL values of controls, a significant dose-response relation was observed ( $\chi^2$ - 8.64; P < 0.01) between longer RTL and increased risk of neuroblastoma. Our pilot study provides evidence that RTL in peripheral blood leukocytes could potentially be used as a biomarker for neuroblastoma risk.*

**Keywords:** Neuroblastoma, Telomeres, Pediatric solid tumor, Northeast India.

## Introduction

Neuroblastoma is the most common extracranial solid tumor in childhood and is derived from the primitive cells of the sympathetic nervous system. Complex patterns of genetic aberrations cooperate to determine its heterogeneous clinical characteristics (Costa & Seuánez, 2018). Interestingly, disease regression is common among most infants with minimal therapy, even with metastasis. But, older patients frequently have metastatic disease that grows relentlessly, in spite of the most intensive multimodality therapy (Brodeur, 2003). The genetic mechanisms underlying this altered behavior is not clearly understood but may be attributes to acquired somatic variations (Esposito et al., 2018)

Telomeres are specialized structures which are composed of TTAGGG DNA repeats and found at the end of all eukaryotic linear chromosomes. They shorten with each cell division and this process ultimately triggers cellular senescence. In about 80% to 90% of human tumors, cancer cells evade this crisis and become immortalized by expressing telomerase – a ribonucleoprotein DNA polymerase that plays a key role in telomere synthesis. It has been reported that telomeric dysfunction is associated with increased mutation rate and genomic instability (Hackett, Feldser, & Greider, 2001). Also in case of neuroblastoma, frequent structural rearrangements are detected near the telomerase gene (Lopez et al., 2017). In view of this, we conducted a pilot research to measure the telomere length from the peripheral blood leukocyte in twenty neuroblastoma cases and forty age and sex matched controls, and evaluated the association of telomere length with susceptibility to neuroblastoma.

## Materials and methods

### Ethical statement

This study was reviewed and approved by the Institutional Ethical Committee.

### Study population

This was a hospital-based matched case-control study. The patients were successively enrolled from those who visited the pediatric surgery departments at Assam Medical College and Hospital, Dibrugarh and Gauhati Medical College and Hospital, Guwahati. Patients above the age of 14 years were excluded. We recruited 20 neuroblastoma cases who had received no prior chemotherapy or radiotherapy and 40 apparently healthy children (two control subjects for each case) as controls, matched with each case by gender and age. None of the participants reported of any familial history of cancer in at least one first degree relative.

## Telomere length assessment by Real-time polymerase chain reaction

We isolated genomic DNA from participant's peripheral blood leukocytes using the Wizard genomic DNA purification kit (Promega) following the manufacturer's protocol. The relative telomere length (RTL) was determined based on a modified version of the real-time quantitative polymerase chain reaction (PCR) method originally described by Cawthon (Cawthon, 2009). The protocol measured the ratio of the copy number of telomeric DNA (T) to that of the single-copy gene (S), in our case human  $\beta$ -globin to produce a relative, unit-less measurement of telomere length (T/S ratio) for each sample. Two PCR master mixes were prepared, each consisted of 4  $\mu$ L of 2X Power SYBR Green Master Mix (Applied Biosystems), 0.5  $\mu$ L of each telomere (or HbG) specific primers (stock concentration 2  $\mu$ mol/L) and 3  $\mu$ L of nuclease free water. The primer sequences were as follows: telomere primers, 5'-GGC TTG CCT TAC CCT TAC CCT TAC CCT TAC CCT TAC CCT-3' (Tel 2b); 5'- CGG TTT GTT TGG GTT TGG GGG TTT GGG TT-3' (Tel 3b); human HbG primers, 5'- GCT TCT GAC ACA ACT GTG TTC ACT AGC-3' (HBG1); 5'-CAC CAA CTT CAT CCA CGT TCA CC-3' (HBG2). We added 8  $\mu$ L of the T master mix to the first 48 wells and 8  $\mu$ L of the S master mix to the rest; for a 96-well plate setup. Three 2  $\mu$ L aliquots of appropriately diluted DNA sample/ reference DNA/ negative control sample were added to wells with T or S master mix. Negative controls were included in each assay for quality control. The reference DNA sample consisting of pooled case DNA was serially diluted over a two-fold range to construct a five-point standard curve. The plates were analysed on a StepOne Plus real-time PCR system (Applied Biosystems). The cycling conditions were 95°C for 10 min followed by 10 seconds at 95°C, 10 seconds at 60°C and 11 seconds at 72°C for a total of 45 cycles. The R<sub>2</sub> for the telomere and HBG standard curves were  $\geq 0.98$ , with acceptable standard deviation set at  $\leq 0.5$  (for the Ct values). Samples with less than two valid telomere (or HbG) Ct values were repeated.

### Statistical analysis

Telomere length as a continuous variable was analysed using the Student *t* test. Telomere lengths were also analyzed as categorical variables by setting a cutoff point at the median, tertile, or quartile value in the control group. The association between neuroblastoma risk and PBL's telomere length was estimated using conditional univariate logistic regression with age and sex as matching variables to determine the crude odds ratios (OR) and their 95% confidence interval (CI). All statistical tests were two-sided, and associations were considered statistically significant at  $P < 0.05$ . All statistical tests were done using IBM SPSS Statistics 21 (SPSS Inc., Chicago, USA).

### Results

In total, 20 patients with neuroblastoma and 40 healthy controls were included in this study. The mean  $\pm$  SD ages of the cases and controls were  $4.53 \pm 3.12$  years and  $4.46 \pm 3.38$  years, respectively.

**TABLE 1** Association between Relative Telomere Length in Peripheral Blood Leukocytes and Neuroblastoma Risk

Genotype	Cases		Controls		Crude OR	<i>P</i>
	N	%	N	%		
<b>By median</b>						
Short	3	15.0	17	42.5	Ref.	
Long	17	85.0	23	57.5	9.45	0.037*
<b>By tertile</b>						
First	0	0.0	13	32.5	Ref.	
Second	4	20.0	14	35.0	8.38	
Third	16	80.0	13	32.5	33.00	
Overall trend: $\chi^2 = 12.18$ ; $P = 0.0005^*$						
<b>By quartile</b>						
First	0	0.0	8	20.0	Ref.	
Second	3	15.0	9	22.5	6.26	
Third	3	15.0	13	32.5	4.41	
Fourth	14	70.0	10	25.0	23.48	

Overall trend:  $\chi^2 = 8.64$ ;  $P = 0.003^*$ \* indicates  $P$  value of statistical significance;

Patients in the case group had significantly longer RTL than controls ( $0.99 \pm 0.21$  for cases vs  $0.84 \pm 0.06$  for controls;  $P = 0.0001$ ). We then implemented the conditional logistic regression analysis adjusting for age and sex. When participants were classified according to the median RTL value in controls, it was observed that longer RTL was significantly associated with a 9.45-fold increased risk of neuroblastoma ( $P = 0.037$ ). When participants were classified into three groups according to tertile values of RTL in healthy controls, we observed a significant dose-response relation between longer RTL and increased neuroblastoma risk ( $P_{\text{trend}} < 0.001$ ).

Similarly, when participants were classified into four groups according to quartile values of RTL in healthy controls, there was again a significant dose-response relation. When the first (shortest) quartile was used as the reference group, the ORs for the second, third, and fourth quartiles were 6.26, 4.41 and 23.48 respectively ( $P_{\text{trend}} = 0.003$ ) (Table 1).

## Discussion

In this pilot study, we showed that longer RTL in peripheral blood leukocytes was associated with a significantly increased risk of neuroblastoma. In the conditional logistic regression analysis, individuals with longer telomeres exhibited a nine fold increased risk of neuroblastoma and there was a significant dose-response effect.

Several epidemiological studies have investigated the association between leukocyte telomere length and cancer risk, and the association appears to depend on the type of cancer (Xie et al., 2013). Studies have revealed that children with cancer have shorter telomeres in peripheral blood lymphocytes (Tabori, Nanda, Druker, Lees, & Malkin, 2007). Shorter telomeres have been associated with the risk of rhabdomyosarcoma, retinoblastoma and medulloblastoma (Parker et al., 2012), low-grade pediatric gliomas (Tabori et al., 2006) and Ewing sarcoma (Avigad et al., 2007). Shorter telomeres have also associated with the risk of neuroblastomas (Dagg et al., 2017). In contrast, the current study demonstrated that longer RTL in leukocytes was associated with increased risk of neuroblastoma. Although these findings are contrasting, such intratumoral telomeric length heterogeneity has been reported earlier (Dagg et al., 2017; Garcia-Martin et al., 2017) and may be associated with the genetic landscape of the tumor. For instance, Ohira et al. reported a high prevalence of non-silent somatic mutations among aggressive MYCN-non-amplified neuroblastomas (Ohira et al., 2017). Further, most of the high risk neuroblastomas reportedly harbor *TERT* rearrangements and *ATRX* mutations that are involved in telomere maintenance through telomerase activity and alternative lengthening of telomeres which consequently effects telomere length (Duan & Zhao, 2018; Lopez et al., 2017).

In conclusion, through this pilot study, we found that longer RTL in peripheral blood leukocytes could potentially be used as a biomarker of the risk of neuroblastoma. Some limitations of our study must be considered. As the numbers of observations were quite small, our findings may be regarded as preliminary and further validation of our results is warranted.

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