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Identification of Neuroblastoma Subgroups Based on Three-Dimensional Telomere Organization[1,2]

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Abstract

Using 3D telomere quantitative fluorescence *in situ* hybridization, we determined the 3D telomere organization of 74 neuroblastoma tissue samples. Hierarchical cluster analysis of the measured telomere parameters identified three subgroups from our patient cohort. These subgroups have unique telomere profiles based on telomere length and nuclear architecture. Subgroups with higher levels of telomere dysfunction were comprised of tumors with greater numbers of telomeres, telomeric aggregates, and short telomeres ($P < .0001$). Tumors with greater telomere dysfunction were associated with unfavorable tumor characteristics (greater age at diagnosis, unfavorable histology, higher stage of disease, *MYCN* amplification, and higher MYCN expression) and poor prognostic risk ($P < .001$). Subgroups with greater telomere dysfunction also had higher intratumor heterogeneity. MYCN overexpression in two neuroblastoma cell lines with constitutively low MYCN expression induced changes in their telomere profile that were consistent with increased telomere dysfunction; this illustrates a functional relationship between MYCN and 3D telomere organization. This study demonstrates the ability to classify neuroblastomas based on the level of telomere dysfunction, which is a novel approach for this cancer.

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Introduction

Neuroblastoma is the most common extracranial malignancy in children. This neoplasm is characterized by both clinical and molecular heterogeneity, and the prognostic risk calculation is multifactorial. The most important parameters predictive of an unfavorable outcome include *MYCN* amplification [1,2], MYCN protein overexpression [3,4], age greater than 18 months at diagnosis [5,6], loss of chromosome arm 1p [7,8] and 11q [9], and gain of chromosome arm 17q [10].

Neuroblastomas have been subgrouped according to their pattern of chromosomal instability: whereas some tumors exhibit numerical and few or no structural aberrations, others are dominated by structural rearrangements, including intrachromosomal rearrangements [11,12]. Chromosome instability has been linked to telomere length aberrations in many cancers, including neuroblastoma [12,13]. However, there have been conflicting findings on whether an increase, decrease, or unchanging telomere length is associated with a better outcome [13–15]. These inconclusive results may be due to the methods employed to measure telomere length, wherein only the average telomere length for each cell was determined.

Nuclear architecture is key to cellular function [16], and changes in nuclear architecture contribute to the pathogenesis and progression of cancer [17]. Our laboratory developed a method of analyzing the interphase nuclear organization of telomeres as a novel and more in-depth approach to study telomere length and telomere dysfunction in disease [18]. Our method uses 3D quantitative fluorescence *in situ* hybridization (Q-FISH) to label all telomeres in interphase nuclei while preserving the nuclear architecture of the sample. Our software measures multiple telomere parameters for each cell including the number of telomeres, the length of each telomere, the number of

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telomeric aggregates, the nuclear volume, and the spatial organization of the telomeres [18,19]. These measurements create a telomere profile unique to an individual sample. Increases in the number of telomeres, telomeric aggregates, and short telomeres are frequently associated with tumor compared to nontumor cells, aggressive forms of disease, and poor patient outcomes [19–22].

In this study, we examined for the first time the 3D telomere organization in 74 archived neuroblastoma tissue samples. Using hierarchical cluster analysis of the measured telomere parameters, we identified three tumor subgroups representing unique levels of telomere dysfunction. We found that tumors with greater telomere dysfunction were associated with unfavorable tumor characteristics including *MYCN* amplification and higher MYCN expression. To test the hypothesis that high levels of MYCN present in a subgroup of patients (11/74) lead to increased telomere dysfunction, we overexpressed MYCN in two neuroblastoma cell lines with constitutively low MYCN expression. This induced changes in the telomere parameters similar to those seen in high MYCN–expressing neuroblastoma tissue samples, demonstrating a functional relationship between MYCN expression and 3D telomere organization.

Materials and Methods

Patient Samples

A total of 74 primary neuroblastoma tissue samples, 5 μm in thickness, were obtained from the Health Sciences Centre (Winnipeg, Manitoba, Canada) ($n = 16$) and Children's Oncology Group (COG) ($n = 58$). All of the tumor samples were derived from untreated patients. Hematoxylin and eosin–stained sections were used to identify tumor areas. The experimenters were blinded to the tumor characteristics and outcome data until after the experiments and measurements were complete. After decoding this information, it was discovered that only 31 out of the 74 patients had clinical follow-up, and therefore survival analyses for the whole cohort was not feasible.

Patients were classified according to the International Neuroblastoma Staging System (INSS) [23] and divided into clinical-genetic risk groups using the COG risk scoring system [7]. The *MYCN* amplification status was determined by FISH for the COG samples. The *MYCN* amplification status of the Manitoban samples was provided to us by the Health Sciences Centre (Winnipeg, Manitoba, Canada). MYCN protein expression was determined for all samples by tissue immunofluorescence. Tumor characteristics of the study cohort are shown in Table 1.

This study was approved by Pathology Access Committee for Tissue (12-0048), Health Science Centre Research Impact committee (2012:187), CancerCare Manitoba Research Resource Impact Committee (92-2012), and Research Ethics Board (H2012:391).

Cell Lines

The established neuroblastoma cell lines SHEP and GIMEN were a gift from Manfred Schwab (The German Cancer Research Center, Heidelberg, Germany). The cells were cultured in RPMI 1640 with 10% fetal bovine serum with 1% l-glutamine, 1% sodium pyruvate, and 1% penicillin-streptomycin (Life Technologies Inc., Burlington, Ontario, Canada) at 37°C in a humidified atmosphere and 5% CO_2.

MYCN Transfection of SHEP and GIMEN Neuroblastoma Cell Lines

SHEP and GIMEN cell lines were transfected with *MYCN* pUHD 10-3, a gift from Manfred Schwab (The German Cancer Research Center, Heidelberg, Germany), which contains the entire *MYCN* coding sequence under the control of an hCMV minimal promoter. Before transfection, 6×10^5 SHEP and 2×10^5 GIMEN cells were seeded per well and grown to near confluence. Twenty-four hours postseeding, cells were cotransfected with TransIT-X2 (MIR6004; Mirus Bio LLC, Madison, WI) and pmaxGFP (Lonza, Allendale, NJ) in a ratio of reagent:vector DNA of 2:1, as per the company's protocol. Cells were cultured in the absence of serum for the first 12 hours. The transfection efficiency was 40% at 15 hours when the cells were sterile sorted by GFP expression. The sorted transfected cells were put back into culture and harvested at 72 hours for 3D nuclei fixation. Mock-transfected cells were used as a control.

Table 1. Neuroblastoma Cohort Characteristics

	Cases (*n*)
Stage	
1	13
2	11
3	8
4	30
4S	11
Unknown	1
Histology	
Favorable	28
Unfavorable	35
Unknown	11
Age at diagnosis	
<18 months	41
>18 months	33
MYCN amplification	
No	63
Yes	11
MYCN expression	
Low	43
Medium	20
High	11
COG risk score	
Low	36
Intermediate	8
High	28
Unknown	2

3D Nuclei Fixation

Cells were harvested from the cultures of SHEP and GIMEN and underwent 3D fixation to prepare nuclei for Q-FISH experiments. 3D nuclear fixation was performed according to the protocol published by Solovei et al. [24].

Immunofluorescence

MYCN immunofluorescence, imaging, and analysis were performed on the neuroblastoma tissue samples and SHEP and GIMEN cell lines as previously described [25]. The MYCN antibody, a gift from Manfred Schwab, is described in Wenzel et al. (1991) and was used at a titer of 1/2000. The slides were imaged using an AxioImager Z2 microscope (Carl Zeiss, Toronto, Ontario, Canada), an AxioCam HR charge-coupled device (Carl Zeiss) with a 63×/1.4 oil objective lens (Carl Zeiss), and DAPI and Cy3 filters (Carl Zeiss). Cy3 exposure times were kept constant between cell lines and between tissue samples. Thirty cells per experimental replicate were analyzed for the cell lines, and 100 cells were analyzed in each tumor sample. We used the following ranges to classify MYCN expression: low as <2000 relative fluorescence units (RFU), medium as 2000 to 4999 RFU, and high as ≥5000 RFU.