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Clinical features of *ATRX* or *DAXX* mutated neuroblastoma

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ABSTRACT

Purpose: Previously, we reported that alternative lengthening of telomere (ALT) may be a biomarker for chemosensitivity and late recurrence in neuroblastoma (NBL). In this study, alterations of *ATRX* or *DAXX*, which both encode chromatin remodeling proteins in telomeric region, and their relationship to ALT were examined in NBLs.
Methods: Our previous report on 121 NBLs revealed 11 NBLs with elongated telomeres by ALT. In these NBLs, *ATRX* or *DAXX* gene alterations were identified using next-generation sequencing and compared to clinical and other biological factors.
Results: In 11 ALT cases, *DAXX* mutations were detected in one case, and *ATRX* alterations were detected in 10 cases. Except for one case, no *DAXX* or *ATRX* alterations were detected in 110 tumors with normal or shortened telomeres. *MYCN* amplification was not detected in *ATRX* altered tumors. In ALT cases, three infants showed *ATRX* deletions, and all seven cases detected after 18 months of age showed poor prognosis.
Conclusions: In NBLs, ALT was caused by *ATRX* or *DAXX* alterations. *ATRX* altered cases without *MYCN* amplification detected at greater than 18 months showed poor prognosis, suggesting that *ATRX* or *DAXX* alterations are a particular NBL subtype. Since these tumors showed chemo-resistance and late recurrence, complete resection in a surgical approach should be performed to improve patient prognosis.

More than 90% of neuroblastomas (NBLs) are diagnosed within the first five years of age and exhibit different clinical behaviors such as life-threatening progression, and spontaneous regression or maturation. Since most NBLs produce catecholamines, vanillylmandelic acid (VMA) and homovanillic acid (HVA) are detectable in urine. In Japan, mass-screening of catecholamine metabolites to detect earlier stage NBLs in infants showed that the annual incidence increased more than two-fold prior to screening implementation, whereas both incidence of advanced NBLs in older children and cumulative mortality rate of NBLs were reduced significantly [1]. Therefore, NBLs consist of heterogeneous subtypes and the unfavorable subtypes increase in the older children.

Molecular and biological analyses revealed several distinguishable NBL subtypes with alterations in *MYCN*, *ALK*, *PHOX2*, *PTPN11*, *ATRX*, and *NRAS*[2,3]. *MYCN* amplification and hemizygous deletions of chromosomes 1p and 11q are highly recurrent and associated with poor prognosis in NBLs [4]. Heritable mutations in *ALK* or *PHOX2B* account for the majority of familial NBLs [5,6]. One distinguishable NBL characteristic, ALT (Alternative Lengthening of Telomere), was associated with unfavorable NBLs in older children without *MYCN* amplification [7–9]. Cheung et al. found *ATRX* loss-of-function mutations and deletions associated with NBLs in adolescents and young adults [10]. In this paper, we focused on *ATRX* and *DRXX* alterations in association with ALT activated tumors.

1. Materials and methods

1.1. Samples

Approximately 500 NBL cases, whose tumors were obtained prior to any treatment, were diagnosed at Hiroshima University Hospital or affiliated hospitals in Japan over the past two decades. As shown in our previous study [9], 121 cases that were followed for more than 2 years and had high quality isolated DNA and RNA were selected for further study. Mean age at initial diagnosis was 22.2 months (range, 0-168 months). Among these cases, 67 were detected by mass-screening in Japan at 6 months of age [1]. Clinical stages and histological findings were determined according to the International Neuroblastoma Staging System (INSS) [11] and the International Neuroblastoma Pathological Classification (INPC) [12]. Written informed consent was obtained from all subjects or from their parents before surgery and this study was approved by the Institutional Review Board of Hiroshima University (I-RINRI-Hi-No.20). NBLs were routinely examined for *MYCN* amplification using fluorescent *in situ* hybridization or qualitative PCR analysis, and DNA ploidy using flow-cytometry analysis.

Patients of any age with INSS 1 or 2 disease, and those less than 12 months old with INSS 3 or 4S disease were treated with either surgery or both surgery and chemotherapy. Patients 12 months or older with INSS 3 and 4 disease were typically treated according to the

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Japanese Neuroblastoma Study Group protocols [13]. In the cases with INSS 4 tumor or INSS 3 *MYCN* amplified tumor, most patients, except for some infants, underwent myeloablative chemotherapy followed by bone marrow transplantation.

1.2. Affymetrix platform

Array experiments were done according to standard protocols for Affymetrix GeneChip Mapping SNP 6.0 arrays (Affymetrix, Inc., Santa Clara, CA), which can detect genomic gains or deletions leading to LOH [14]. These arrays were scanned with the Affymetrix GeneChip Scanner 3000 using GeneChip Operating System 1.2 (Affymetrix). Genotype calls and intensity of the single nucleotide polymorphisms (SNP) were processed by GeneChip DNA Analysis Software. Individual SNP copy intensity and regions in the *ATRX* gene were evaluated with the Affymetrix Genotyping Console Workflow.

1.3. ATRX and DAXX mutation analysis

Target genes including *ATRX* and *DAXX* were chosen for next-generation sequencing. Primers for each gene were designed using DesignStudio (Illumina Inc., San Diego, CA). Using the Nextera Custom Enrichment system (Illumina), sample libraries were generated from 50 ng of DNA. Using a paired-end sequencing approach, 112 target genes were sequenced. Single nucleotide variations and deletions were identified using previously described methods [15].

To validate the identified *ATRX* and *DAXX* mutations by Sanger sequencing, PCR primers were designed for each exon of these genes according to previous reports [16] and each exon was amplified from genomic DNA using exon-specific primers. Sequencing products were purified using Centri-Sep Spin Columns (Princeton Separations, USA) and then prepared for analysis on the ABI 3100-Avant Genetic Analyzer (Applied Biosystems, USA) according to the manufacturer's instructions.

1.4. Telomere analysis

Telomere length was estimated as the length of terminal restriction fragments (TRFs) by Southern as previously described [17]. Briefly, genomic DNA was digested with *Hinf*I, separated by electrophoresis on 0.8% agarose, and hybridized with a 5'-end [^{32}P]-labeled $(TTAGGG)_4$ probe. Signal peaks were estimated as the TRF length. Length of 3'-overhang (3'-OH), single strand of the telomere end, was measured by a telomere-oligonucleotide ligation assay (T-OLA) [18,19]. Normalized 3'-OH intensity was then calculated in comparison with that of HeLa cells (defined as 1.0), which were run on each gel.

1.5. Quantification of telomerase activity

Extraction of telomerase protein and evaluation of activity were done by the TRAP (telomeric repeat amplification protocol) assay as described [20,21]. Levels of telomerase activity, expressed as Total Product Generated (TPG) units, were quantified by examining the ratio of the fluorescein intensity of the entire TRAP ladder. Since telomerase activity levels in fetal adrenal gland tissue were under 10.0 TPG, telomerase activity levels were divided into four categories: undetectable (TPG < 1.0), low ($1.0 \leq$ TPG < 10.0), moderate ($10.0 \leq$ TPG < 100.0), and high (TPG > 100.0).

1.6. Statistical analysis

Chi-square test or Mann–Whitney's U test was used to examine the significance of the comparisons of telomerase activity, telomere length, and clinicopathological factors. A log rank test was used to evaluate survival rates. Statistical significance was defined as $p < 0.05$.

2. Results

2.1. Single nucleotide polymorphism (SNP) analysis data using Affymetrix platform

SNP signals around *ATRX* and *DAXX* regions were analyzed by the SNP 6.0 array in 121 tumors including 11 tumors with elongated telomeres. Large deletions in *ATRX* were detected in 8 tumors (6.6%) (Fig. 1) and no deletions in *DAXX* were identified. The *ATRX*-deleted cases included 5 males and 3 females and 7 of these cases had elongated telomeres. The minimum overlapping deleted region contained exon 5 to exon 10, which encodes a predicted nuclear localization signal (Fig. 1).

2.2. ATRX and DAXX sequencing

We performed on discovery of the *ATRX* and *DAXX* gene alterations on the cohort of the remaining 114 cases without deleted *ATRX* gene using the next-generation sequencing and/or Sanger method. A *DAXX* mutation was detected in only one case with elongated telomere. This mutation results in a frame-shift of *DAXX* (A470 indel). *ATRX* mutations were also detected in three cases with elongated telomeres. Two were missense mutations (Q929E and A1690D) and the remaining one was a nonsense mutation (E555*). Neither *DAXX* nor *ATRX* mutations were detected in the cases without elongated telomeres.

2.3. Telomere length, 3'-OH length, and telomerase activity in NBLs

We previously reported no correlation between telomere length and telomerase activity, but did find a significant correlation between telomere length and 3'-OH length [10]. In this study, we identified *DAXX* or *ATRX* alterations (4 mutations and 7 deletions) in all NBLs with elongated telomeres (>15 kb) and a long length of 3'-OHs (>1.5). On the other hand, no alterations of *DAXX* or *ATRX* were detected in the remaining 110 cases, except for one case. Therefore, *DAXX* or *ATRX* alterations might contribute to ALT activation in NBLs. The *ATRX* deleted case without elongated telomeres also had elongated 3'-OH (Table 1).

2.4. Clinical and biological features of cases with ATRX and DAXX alterations

For patients detected either clinically or in large scale screens, clinical features of all cases with detected *ATRX* and *DAXX* mutations are shown in Table 1. Survival rate for patients with high telomerase activity had already been shown to be less than for those with other tumors ($P < 0.0001$) (Fig. 2) [9]. However, tumors without high telomerase activity did not usually show good prognosis. In this study, we analyzed the correlation between patient outcome and *ATRX* alterations of the tumor. Since high telomerase activity is well known as a poor prognostic marker, we analyzed prognosis of patients with tumors that did not have high telomerase activity (Fig. 2). The *ATRX* altered cases showed significantly worse prognosis.

Among the *ATRX or DAXX* altered cases, 8 INSS 4 tumors showed poor outcomes except for one infant case (Table 1). In these deceased cases, survival periods were more than 2 years and relatively long as compared with the deceased cases without *ATRX* or *DAXX* alterations. These cases with these aberrations, except for one, responded poorly to myeloablative chemotherapy. Moreover, the patient with complete remission also relapsed soon afterwards, and died of the disease. *ATRX* or *DAXX* altered NBLs diagnosed at older ages did not have high proliferative capacities, but showed poor outcomes due to chemoresistance. Although these alterations were very rare in tumors without elongated telomeres, one screening-detected case showed the *ATRX* deletion. Clinical feature of this *ATRX* deletion case without elongated telomeres included a ranking of INSS1, complete resection by surgery, but death by recurrence 25 months after initial diagnosis.