Duplication of ALK F1245 missense mutation due to acquired uniparental disomy associated with aggressive progression in a patient with relapsed neuroblastoma

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Abstract. Recent genome-wide analysis of neuroblastoma (NBL) revealed amplification and heterozygous mutation of anaplastic lymphoma kinase (ALK) are responsible for onco-genicity, frequently observed during relapses. A 3-year-old girl with relapsed high-risk NBL had a heterozygous ALK F1245L mutation at diagnosis, which became homozygous due to uniparental disomy (UPD) of the entire chromosome 2, confirmed by single nucleotide polymorphism array and variant allele frequency of this mutation. The ALK inhibitor, crizotinib, failed to control the tumor and the patient died of the disease. Further genomic analysis using targeted capture sequencing for 381 genes related to pediatric cancers identified more alterations acquired at relapse, such as TSC complex subunit 2 and protein tyrosine phosphatase receptor type D. In addition to these several acquired mutations, this extremely rare duplication of ALK mutation might explain the aggressive clinical course after relapse, because acquired UPD, resulting in the duplication of an oncogenic mutation, has been reported for various neoplasms. Although a clinical benefit of ALK inhibitors in patients with NBL has not been confirmed yet, a treatment based on the ALK mutation status will be promising in future using more potent next-generation ALK inhibitors.

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Introduction

Neuroblastoma (NBL) is a common solid tumor in children that originates from primordial neural crest cells (1). Despite multidisciplinary therapies, patients with high-risk NBL have a dismal prognosis. Recent genome-wide analyses have revealed anaplastic lymphoma kinase (ALK) gene alterations, amplification, and activating heterozygous mutations in the tyrosine kinase domain (TKD) in approximately 8% of primary NBLs (2-5). ALK mutations, often detected in heterozygous, are involved in cell proliferation and invasion and more frequently occur in high-risk and relapsed cases (1,6), while homozygous ALK mutations are quite rare (4,7). The discovery of ALK alterations provides the first tractable oncogenic target in NBL and facilitates the clinical application of ALK inhibitors, such as crizotinib (8). Here we present the case of a female patient with relapsed high-risk NBL harboring a heterozygous ALK F1245L mutation at the time of the initial diagnosis. This mutation became homozygous at relapse due to uniparental disomy (UPD), which was associated with aggressive tumor behavior after relapse. We further analyzed genetic alterations that may explain the molecular mechanism for NBL relapse.

Materials and methods

Samples. Biopsy samples at primary and relapse were subjected to this analysis after receiving written, informed consent according to protocols approved by the Human Genome, Gene Analysis Research Ethics Committee of the University of Tokyo and St. Luke’s International Hospital. No matched normal sample was available. Genomic DNA of each sample was isolated by NucleoSpin DNA RapidLyse kit (Macherey-Nagel GmbH & Co., Düren, Germany) according to manufacturer’s protocol.

Direct sequencing. Direct sequencing of ALK was performed according to manufacturer’s protocol with the use of an ABI
3500 Genetic Analyzer (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The following set of primers were used: ALK‑24_F: GCTGCCCATGTTTACAGAATGC; ALK‑24_R: TCTACGGAGGACTGGCTAC; ALK‑25_F: CTGGTGATGCTGACATGTC; ALK‑25_R: CATCCTACAATCAATGCTC; ALK‑26_F: AATCTCAGTGATGGCCGTTG; ALK‑26_R: GGAGATGATGTAAGGCAAGC.

**Targeted capture sequencing.** Targeted capture was performed using a SureSelect custom kit (Agilent Technologies, Inc., Santa Clara, CA, USA) according to the manufacturer’s protocol (9‑11). The custom bait library was designed to include: i) all coding exons of 367 genes; ii) untranslated regions and introns of 16 genes (CD274, CD150, CD30, CD262, TERT), FoundationOne (Foundation‑10, Agilent Technologies, Inc., Santa Clara, CA, USA) using a SureSelect custom kit (Agilent Technologies, Inc., Santa Clara, CA, USA) with a combination of vincristine and irinotecan, was performed to detect mutations, and a F1245L mutation was adopted and filtered by SNP array analysis was performed using GeneChip Human Mapping Cytoscan HD (Affymetrix; Thermo Fisher Scientific, Inc.). Genome‑wide detection of copy number abnormalities or allelic imbalances was processed using Copy Number Analysis for Affymetrix GeneChip (CNAG)/Allele‑Specific Copy Number using Anonymous References (AsCNAR) v3.5.1 software (28).

**Results**

**Aggressive clinical course in a relapsed NBL patient.** A 3‑year‑old girl was diagnosed with high‑risk NBL of the left adrenal gland with bone and marrow metastases. Neuron specific enolase (NSE) and urine vanillylmandelic acid (VMA) levels were elevated (215 µg/l and 8.4 mg/l, respectively), and MYCN amplification was present. Immunohistochemical analyses revealed tumor cells positive for ALK. The patient received five courses of chemotherapy followed by high dose chemotherapy (busulfan and melphalan) with autologous hematopoietic stem cell transplantation, surgical gross total resection of the primary tumor, and radiation therapy to the tumor bed (19.8 Gy). The combination therapy successfully induced complete remission. However, 12 months after the diagnosis, she suffered from local tumor recurrence at the primary site. Two cycles of topotecan and cyclophosphamide (TC) and three of ifosfamide, cisplatin, and etoposide (ICE) gradually reduced the tumor size. After a fourth course of ICE, the tumor rapidly grew and urgent radiotherapy, with a combination of vincristine and irinotecan, was required. After obtaining approval from the institutional review board, crizotinib was administered for 28 days. Unfortunately, the tumor progressed and she succumbed to the disease (Fig. 1).

**Molecular analysis of paired diagnosis‑relapse samples.** After obtaining informed consent from her guardians, DNA was extracted from biopsy samples of the adrenal tumor both at diagnosis and after relapse. Direct sequencing was performed to detect ALK mutations, and a F1245L mutation (c.T3733C; TTC>TCC) was identified in both the sample at diagnosis and relapse, which is a common activating mutation in NBL. Intriguingly, the relapse sample exclusively contained the mutated allele, whereas the diagnosis sample clearly showed a heterozygous change (Fig. 2A). In accordance with this, SNP array analysis in the relapse sample revealed acquired UPD of chromosome 2, including the ALK locus, in which the wild‑type allele was likely to be lost by chromosomal mis‑segregation with duplication of the mutated allele (Fig. 2B and C). We also detected several shared copy number alterations in both the diagnosis and relapse samples, including a focal amplification of 2p24.2‑24.3 comprising the MYCN...
locus (Fig. 3). We further analyzed other genetic alterations, which might have been associated with disease recurrence and aggressiveness, using targeted capture sequencing (TCS) for 381 genes related to pediatric cancers (U-Tokyo Onco-panel v1). Because only tumor samples were analyzed, candidate mutations were filtered referring to our previous study (25), where we have confirmed the somatic mutations detected in TCS by using matched normal samples. We identified four and ten variants in the diagnosis and relapse samples, respectively (Tables I and II). All mutations detected at diagnosis were also detected in relapse sample. The \textit{ALK} F1245L mutation was detected in both the diagnosis (variant allele frequency (VAF)=0.47) and relapse samples (VAF=0.83), which is consistent with other results showing duplication of \textit{ALK} at relapse. Furthermore, alterations in \textit{TSC2} (p.P1092fs), \textit{PTPRD} (translocation to chromosome 6), \textit{AXL} (p.A549S), \textit{ERG} (p.S315X), and \textit{EZH2} (p.E204X) were acquired at relapse (Fig. 4).

**Discussion**

The \textit{ALK} mutation hotspots are located within the intracellular TKD at codons F1174, R1275, and F1245, accounting for 85% of the \textit{ALK} mutations found in NBL (2-5). Germline \textit{ALK} mutations, such as R1275, have also been identified as the major cause of hereditary NBL; however, their penetrance has been considered relatively low (29-31). In contrast, other hotspot mutations (F1174 and F1245) are rarely observed in familial cases. These F1174 and F1245 germline mutations have been linked to severe neurocognitive defects and abnormalities (30), which is consistent with reports that mutations in F1174 and F1245 have strong effects on the nonphosphorylated \textit{ALK} TKD activity (5). Because both F1174 and F1245 are located in the region inhibiting ligand-independent activation, they impair self-inhibition and enhance the tyrosine kinase activity (32).

We detected a heterozygous \textit{ALK} F1245 mutation at diagnosis that became homozygous at relapse in the present case. Homozygous \textit{ALK} mutations have been rarely reported as far (4,7). This duplication at relapse may lead to growth advantage of tumor cells and be associated with disease progression. In fact, acquired UPD, resulting in the duplication of an oncogenic mutation, has been reported for various neoplasms. We have previously shown that the aggressive transformation of juvenile myelomonocytic leukemia is associated with the duplication of the oncogenic \textit{KRAS} due to acquired UPD (33). Further, a previous report has revealed that the homozygous \textit{ALK} F1174S mutation due to acquired UPD during the disease course may be responsible for the very rapid progress of NBL and resistance to chemotherapy (7). An enhanced oncogenic potential has also been reported in homozygous \textit{EGFR} mutations on TKD in patients with non-small cell lung cancer, particularly in metastatic cases (34). Thus, homozygous TKD mutations are associated with an aggressive tumor progression in patients. In addition to the strong effects of the F1245 mutation on the \textit{ALK} TKD activity, the bi-allelic presence of the \textit{ALK} oncogenic mutation due to acquired UPD may have conferred further aggressiveness to the tumor during the relapse in this case.

In our comprehensive analysis, in addition to the \textit{ALK} F1245 mutation and \textit{MYCN} amplification, we also detected somatic oncogenic alterations comprising \textit{PTPRD} (chromosomal rearrangement) and \textit{TSC2} (p.P1092fs) at relapse. \textit{PTPRD} is a member of a large family of protein tyrosine phosphatases.
Figure 2. (A) ALK (NM_004304) F1245L mutation status by direct sequencing at diagnosis (heterozygous) and after relapse (homozygous). Copy number analysis using a SNP array for chromosome 2 including the (B) ALK gene region in diagnosis and (C) relapse samples. Red dots at the top represent the signal from each probe (raw data), and blue line indicates the moving average of red dots. Red and green lines at the bottom represent allele-specific copy number. UPD was observed in the relapse sample. UPD, n iparental disomy.

Figure 3. Results of SNP array analysis in (A) primary sample and (B) relapsed sample. Colorful dots at the top represent signal of each probe (raw data). Red and green lines at the bottom show allele specific copy number.
and functions as a tumor suppressor by destabilizing aurora kinase A (35). The low expression of PTPRD is associated with high-risk NBL with MYCN amplification (36). TSC2 is a tumor suppressor gene that is responsible for tuberous sclerosis, which is an autosomal dominant disorder characterized by the formation of hamartomas in various organs. The inactivation of TSC2 results in TSC1-TSC2 dissociation and markedly impairs the ability of TSC2 to inhibit mTOR signals, leading to cell proliferation and disease progression (37). A TSC2 mutation has not been reported in patients with NBL. However, because alterations of PTPRD and TSC2 occurred in a heterozygous setting, the functional relevance of these genes in the tumor progression of our case remains unclear.

We also identified a truncated mutation of EZH2 (p.E204X),

Table I. Mutation calls of targeted capture sequencing.

| Sample  | Chr  | Start          | End            | Ref | Alt | Gene type | Amino acid change | V AF  |
|---------|------|----------------|----------------|-----|-----|-----------|-------------------|-------|
| Primary | 2    | 29436860       | 29436860       | A   | G   | SNV       | ALK               | 0.469 |
| Relapse | 2    | 29436860       | 29436860       | A   | G   | SNV       | ALK               | 0.825 |
| Relapse | 3    | 142253938      | 142253938      | G   | C   | SNV       | ATR               | 0.409 |
| Primary | 5    | 180043943      | 180043943      | C   | G   | SNV       | FLT4              | 0.425 |
| Relapse | 5    | 180043943      | 180043943      | C   | G   | SNV       | FLT4              | 0.496 |
| Relapse | 7    | 148525847      | 148525847      | C   | A   | Frameshift | TSC2             | 0.387 |
| Relapse | 16   | 2129420        | 2129420        | -   | G   |          | EZH2 stopgain     | 0.245 |
| Primary | 17   | 10361019       | 10361019       | C   | A   | Stopgain  | MYH4              | 1     |
| Relapse | 17   | 10361019       | 10361019       | C   | A   | Stopgain  | MYH4              | 0.7   |
| Relapse | 19   | 41754659       | 41754660       | CG  | TC  | SNV       | AXL stopgain      | 0.429 |
| Primary | 21   | 32493125       | 32493125       | C   | G   | SNV       | TIAM1 stopgain    | 0.506 |
| Relapse | 21   | 32493125       | 32493125       | C   | T   | Stopgain  | ERG               | 0.43  |
| Relapse | 21   | 32493125       | 32493125       | C   | G   | SNV       | TIAM1 stopgain    | 0.552 |

Chr, chromosome; SNV, single nucleotide variant; V AF, variant allele frequency.

Table II. Structural variant call of targeted capture sequencing.

| Sample  | Chr  | Position  | Direction | Chr  | Position  | Direction | Variant type | Gene 1 | Gene 2 | V AF  |
|---------|------|-----------|-----------|------|-----------|-----------|--------------|--------|--------|-------|
| Relapse | 6    | 138269463 | -         | 9    | 8338848   | -         | Translocation | PTPRD | 0.5745 |

Chr, chromosome; V AF, variant allele frequency.

Figure 4. Results of the genomic analysis and hypothetical model of aggressive progression in this case. A heterozygous ALK F1245 mutation was detected at diagnosis. This mutation became homozygous at relapse due to acquired UPD at chromosome 2, leading to both the duplication of the mutant allele and loss of the wild type allele. The duplication might be due to clonal evolution, acquired later in the process of relapse. UPD, uniparental disomy.
which is an essential component of the polycomb repressive complex 2. The pathogenic role of the truncated mutation of EZH2 is unclear because most EZH2 mutations reported in NBL cases were gain of function mutations mediating the epigenetic silencing of a tumor suppressor (38).

In the present case, all detected single nucleotide variants were observed in the major clone (VAF>0.35). None of acquired alterations at relapse were detected in primary sample even as the minor clone (VAF <0.35), although there was a technical limitation on our TCS, which cannot detect mutations with low allele frequency (VAF <0.05). Because no minor variants were detected, intratumor heterogeneity was not defined in the present case. Intratumor heterogeneity leads to selective outgrowth of clones that have a phenotypic advantage (39). The occurrence or presence of bi-allelic change of the ALK mutation due to acquired UPD and other acquired mutations such as TSC2 and PTPRD might be involved in tumor relapse and chemo-resistance in the present cases.

We report a patient with relapsed NBL harboring ALK F1245 mutation, which was heterozygous at diagnosis and became homozygous at relapse with resistance to crizotinib. This duplication of ALK mutation was extremely rare, and may be associated with aggressive behavior after relapse in this case. As previously reported, the ALK F1245 mutations would be suggested to be relatively crizotinib-resistant (5). Although a clinical benefit of ALK inhibitors in patients with NBL has not been confirmed, more potent next-generation ALK inhibitors have been developed and clinical trials to estimate their efficacy are currently underway (NCT03107988) (40,41). In future, a treatment based on the ALK mutation status will be promising.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors’ contributions

SK, DH, M Seki, M Sekig, JT and AM wrote the manuscript; SK, DH, M Seki, YY, AD, SH, YH, and AM collected and analyzed data; SK, M Sekig, and SM performed experiments; MK, SO, JT and AM conceived the study; JT designed the study. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of the University of Tokyo and St. Luke’s International Hospital.

Patient consent for publication

Prior written informed consent was obtained from the patient’s guardians.

Competing interests

The authors declare that they have no competing interests.

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