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Case Report

5′ *ALK* Amplification in Neuroblastoma: A Case Report

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Abstract
Neuroblastoma is the most common cancer in infants younger than 12 months of age, occurring with an incidence of 1 in 100,000 children. The clinical outcome of neuroblastoma ranges from spontaneous regression to treatment-resistant progression and/or metastasis, and accounts for 8–10% of childhood cancer deaths. Segmental chromosomal aberrations, as well as *MYCN* and *ALK* amplification, are among factors contributing to an unfavorable genomic profile and high-risk disease classification. Here, we describe a 5-year-old male who presented with a large right renal neuroblastoma tumor having lung and liver metastases. Fluorescence in situ hybridization analysis indicated the presence of >20 copies of the 5′ region of the *ALK* gene in 26% of cells examined. Subsequent copy number assessment did not confirm *ALK* amplification, but revealed a gain of exons 2–5 of *ALK*, consistent with increased copy number for the 5′ region of the *ALK* gene. Subsequent array analysis showed the presence of other unfavorable prognostic genomic features, including segmental gain of the 17q region and amplification of the long arm of chromosome 12 harboring *CDK4* and *MDM2,* both reported to be poor prognostic indicators in patients with atypical clinical features in neuroblastoma. Taken together, this report illustrates the importance of careful interpretation of aberrant FISH findings and subsequent use of orthogonal methods to clarify the presence of genomic alterations to successfully determine potential treatment targets.

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Introduction

Neuroblastoma is the most common cancer in infants younger than 1-year of age, with a median age at diagnosis ranging from 17 to 22 months [1]. Neuroblastoma has an incidence of 1 in 100,000 children, with 650 new cases diagnosed annually in the United States accounting for 8–10% of all pediatric cancer deaths [2, 3]. Neuroblastoma originates from embryonic neural crest cells, and can arise anywhere along the sympathetic nervous system. Typical metastatic sites include regional lymph nodes and bone marrow by means of the hematopoietic system. However, metastases have also been found in the liver, most notably in patients with stage 4S tumors [1]. Encapsulated lower-stage neuroblastomas can be surgically excised with minimal complication. Higher-stage tumors often infiltrate local organ structures, nerves, and vessels, and are largely unresectable. Dose-intensive chemotherapy, external-beam radiotherapy to primary tumor and resistant metastatic sites, myeloablative chemotherapy with autologous hematopoietic stem-cell rescue, Isotretinoin with anti-GD2 Immunotherapy, and targeted kinase inhibition are means of treatment in this group of high-risk patients [1]. The clinical outcome of neuroblastoma ranges from spontaneous regression to treatment-resistant progression, metastasis, and death [2, 4] with a 5-year overall survival of >90% in the low-risk group and approximately 40–50% in high-risk group patients [1, 2]. The broad range of phenotypic heterogeneity in neuroblastoma is owed in part to the diverse genetic makeup of this tumor, and emphasizes the importance of identifying the underlying genetic components that are most amenable to treatment [3, 5].

The Anaplastic Lymphoma Kinase (*ALK*) Receptor Tyrosine Kinase gene encodes a receptor tyrosine kinase that belongs to the insulin receptor superfamily. Germline *ALK* is mutated or amplified in approximately 50% of familial neuroblastoma cases [5], while somatic *ALK* mutation or amplification occurs in up to 15% of neuroblastomas [1]. Genetic alterations of *ALK* mostly lead to constitutive activation of the tyrosine kinase domain of the gene [2, 6, 7], conferring a worse prognosis compared to cases lacking alterations [8]. Successful targeted inhibition of *ALK* with crizotinib or 2nd-generation *ALK* inhibitors in patients harboring *ALK* mutation or amplification is well known [5, 8, 9], but to our knowledge, amplification limited to the 5′ region of the *ALK* gene has not been reported.

Case Presentation

The subject of this study is a 5-year-old male with a large retroperitoneal tumor involving the right adrenal gland and superior pole of the right kidney with liver and lung metastases. Histopathologic examination of a biopsy specimen revealed neuroblastoma, undifferentiated subtype, with a high mitotic-karyorrhectic index (Fig. 1). Findings were consistent with unfavorable histology (International Neuroblastoma Pathology Classification). Additional pleomorphic and anaplastic features were noted. Tissue with an estimated tumor percentage of 90% was submitted for molecular testing. Immunohistochemistry performed as part of the diagnostic workup revealed positive staining for synaptophysin, CD56, PGP9.5, and chromogranin. Staining for CD99, desmin, S100, CK AE1/3, pooled CK, inhibin, calretinin, and melan A was negative.

ALK Kinase Domain Sequencing

DNA extraction from formalin-fixed paraffin embedded (FFPE) tissue was performed using the AllPrep FFPE kit (Qiagen, Germantown, MD; Cat# 80234), per manufacturer's instruction. PCR amplification and Sanger sequencing of the *ALK* kinase domain (exons 21–28) was performed (*ALK* genomic reference sequence (Ensembl: ENSG00000171094;

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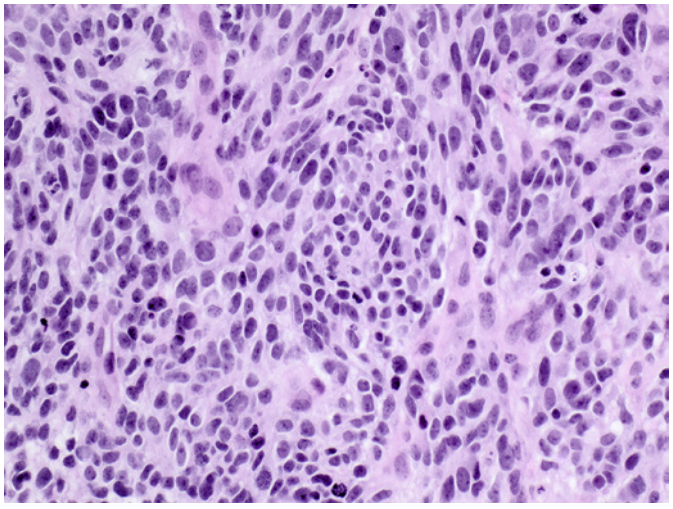

Fig. 1. Biopsy histology demonstrates sheets of round to spindled neoplastic cells with a high nuclear to cytoplasmic ratio and minimal cytoplasm. There is nuclear pleomorphism with variably sized nuclei and occasional prominent nucleoli, with scattered more prominently enlarged bizarre nuclei. There are frequent mitoses and apoptoses. H&E. Original magnification, ×400.

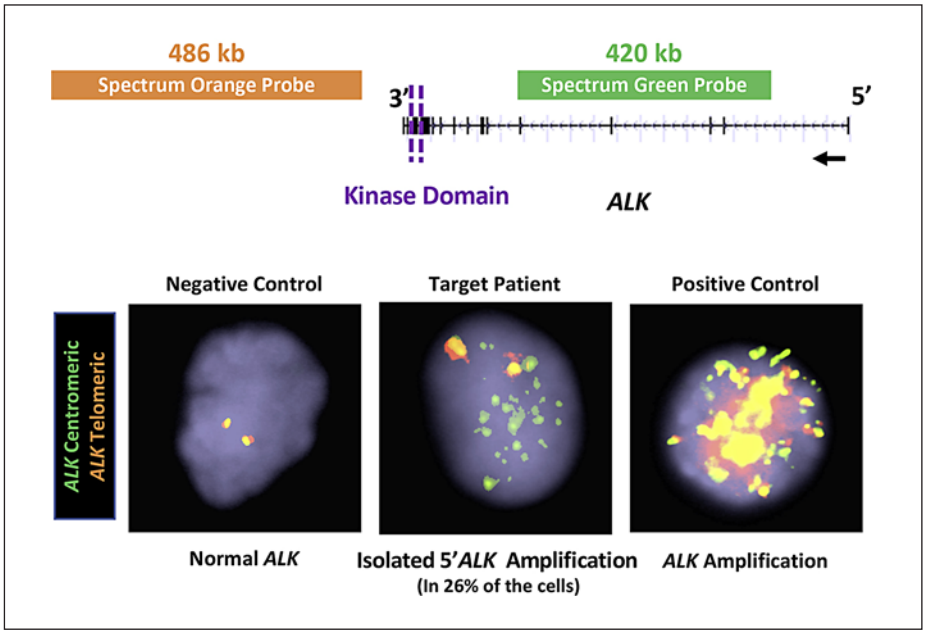

Fig. 2. Isolated amplification of the 5′*ALK* region. Top panel: schematic view of *ALK* gene. Spectrum orange probe: chromosome 2: 28889680–29375856; spectrum green probe: chromosome 2: 29602379–30022356. All the coordinates are based on GRCh37/hg19. Lower panel: Comparison of FISH patterns. ALK, Anaplastic Lymphoma Kinase (*ALK*) Receptor Tyrosine Kinase; FISH, fluorescence *in situ* hybridization.

reference transcript: ENST00000389048 [Ensembl]/NM_004304 [RefSeq]). No clinically significant variants were identified.

Fluorescence in situ Hybridization with Probes for MYCN and ALK

MYCN fluorescence in situ hybridization (FISH) analysis performed at the originating institution showed no amplification. Subsequent FISH analysis was performed on paraffin-embedded tissue using *ALK* break apart probe specific to the 2p23.2 region (LPS 019; CytoCell, Tarrytown, NY, USA). FISH revealed the presence of >20 copies of the 5′ region of the *ALK*

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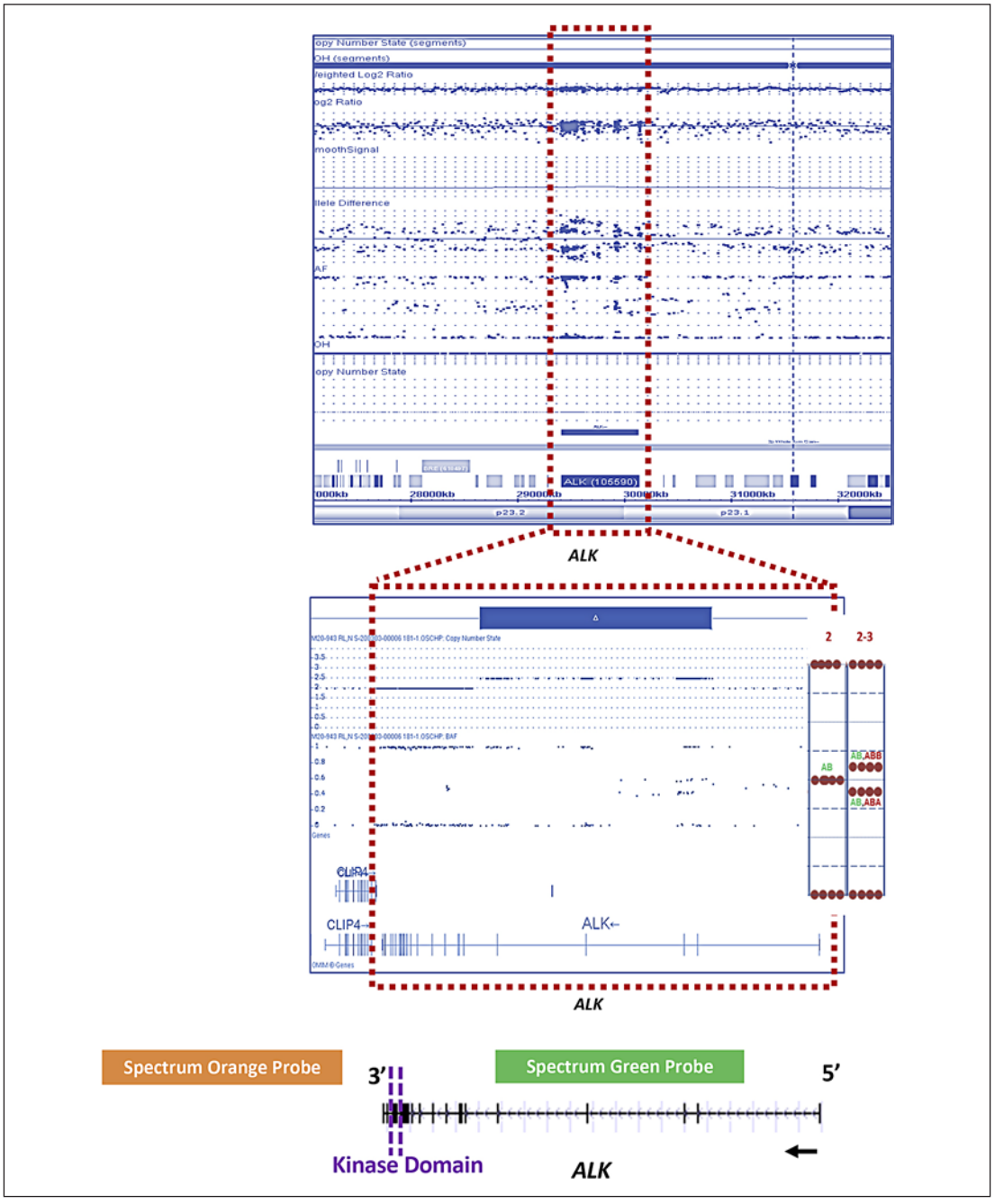

Fig. 3. Copy number assessment of the *ALK* gene. Top panel: Affymetrix OncoScan panel of chromosome 2. Lower panel: zoomed panel of the *ALK* region. ALK, Anaplastic Lymphoma Kinase (*ALK*) Receptor Tyrosine Kinase.

gene (spectrum green probe) in 26% of cells examined, suggestive of partial amplification of the *ALK* gene, with less than four copies of the 3′ region of the *ALK* gene (spectrum orange) (Fig. 2). The sequence of the FISH probes flank the *ALK* kinase domain; it was therefore unclear whether the observed amplification included the *ALK* kinase domain (critical region). To obtain higher resolution for additional copy number assessment of the amplified region, microarray analysis was performed on extracted DNA.

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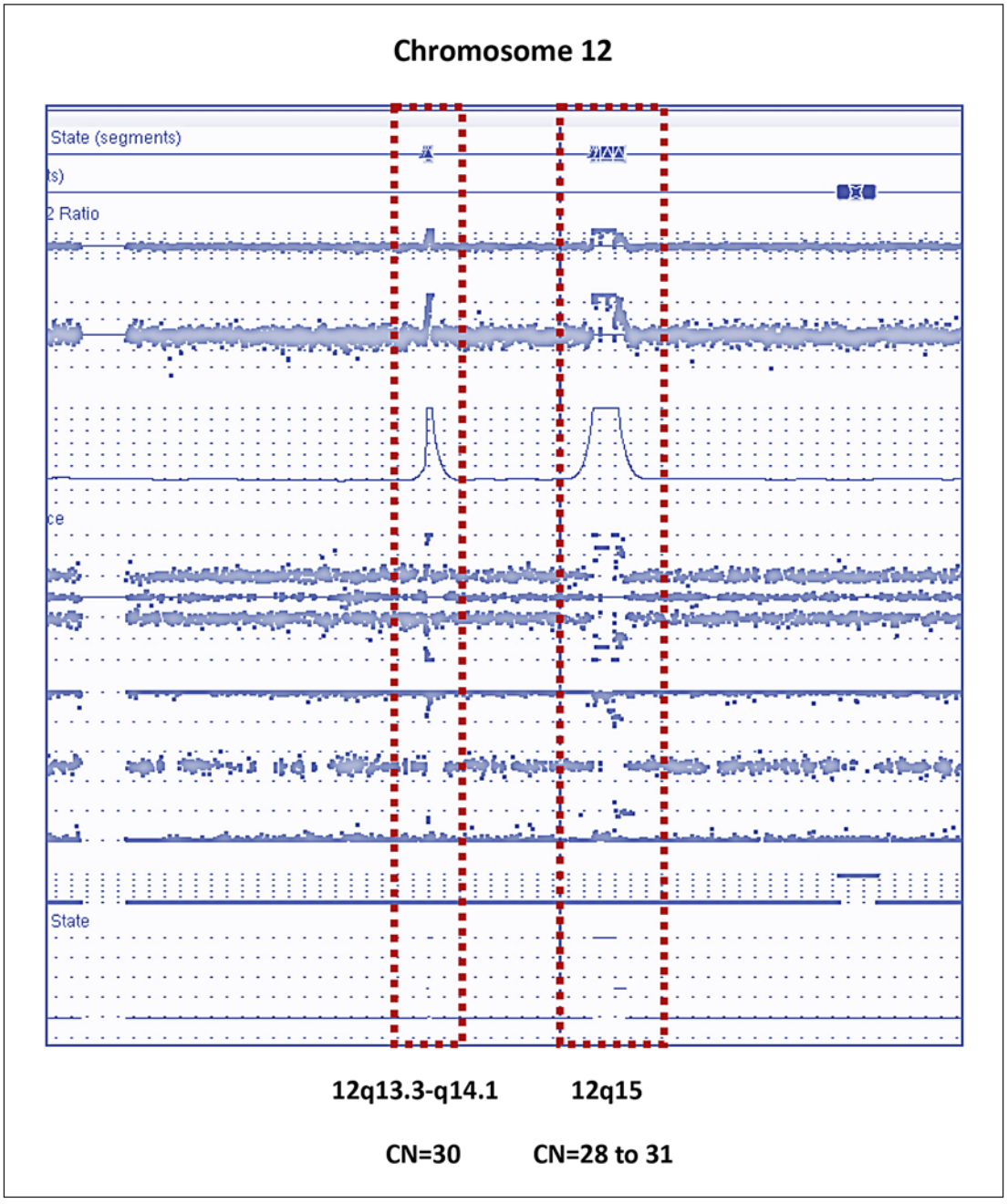

Fig. 4. Copy number assessment of chromosome 12. CN, copy number.

Cytogenomic Microarray Analysis

Cytogenomic microarray analysis using the Affymetrix OncoScanTM copy number variation (CNV) FFPE platform was performed to interrogate over 220,000 SNP markers with a median probe density of 19 kb across the genome and 2.5 kb per probe within 232 genes of high clinical relevance in cancer, allowing loss of heterozygosity (LOH) or CNV resolution down to 50 kb (Affymetrix Oncoscan, Santa Clara, CA, USA; https://www.thermofisher.com/order/catalog/product/902695). Analysis was limited to segmental aberrations of chro-

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mosome regions of known prognostic significance in neuroblastoma including copy number loss or LOH of 1p, 3p, 4p, and 11q, and gain of 1q, 2p, and 17q [10]. Segmental aberrations were defined as altered regions of >1 Mb but less than a whole chromosome arm, or a region of homozygosity >5 Mb, assumed to be LOH in the specific regions of loss (1p, 3p, 4p, 11q). Data interpretation was based on available resources including information from the UCSC Genome Browser (http://genome.ucsc.edu), the Database of Genomic Variants (http://projects.tcag.ca/variation/), literature searches and internal copy number variation databases. Genomic positions are given in reference to GRCh37 (hg19) [11].

Segmental gain of 17q was observed in the setting of a diploid chromosome copy number, consistent with a less favorable genomic profile. Of particular interest was a small region of low-level copy number gain with an average of 2.5 copies per cell, including exons 2–5 of the *ALK* gene on chromosome 2, comprising the 5′ segment of the *ALK* coding region (Fig. 3). Copy number gain of several contiguous regions within 12q was also identified, including chr12:69,017,408-70,446,300 (average of 30 copies per cell) and a region extending to chr12:71,211,437 (average of 5 copies per cell) (Fig. 4).

Discussion and Conclusion

Despite significant improvements in the diagnosis and management of neuroblastoma, our understanding of the critical drivers of neuroblastoma is ongoing. Several targeted therapies have been designed and established for *ALK* positive neuroblastomas via multiple clinical trials (NCT03126916, NCT03107988, and NCT01742286); determining the appropriate diagnostic testing to identify potentially actionable lesions is essential for treatment success.

In this case of undifferentiated neuroblastoma, we demonstrate that isolated amplification of 5′ *ALK* does not necessarily correlate to pathogenic *ALK* amplification. Cytogenomic microarray analysis further clarified the copy number status of exons within *ALK*, and detected other unfavorable prognostic biomarkers including segmental gain of 17q and amplification within 12q. The latter harbors potential oncogenic target genes, including *CKD4* and *MDM2* [12].

While previous studies determined 3′ *ALK* amplification to be a negative prognostic marker associated with inferior survival in cancer patients [13–15], the current case demonstrates that amplification limited to the 5′ *ALK* region may result in complete response to neoadjuvant chemotherapy. Taken together, this report illustrates the need to carefully consider the position and coordinates of FISH probes when interpreting aberrant FISH patterns and emphasizes the utility of orthogonal diagnostic methods for clarifying results in order to provide the most optimal course of treatment.

Statement of Ethics

This study was conducted ethically in accordance with the World Medical Association Declaration of Helsinki. The guardians of the subject have given their written informed consent to publish this case.

Conflict of Interest Statement

The authors declare no competing interests.

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Author Contributions

Conceptualization: S.A., R.P., and S.C.R.; Methodology: S.A., C.D., M.H., R.P., and S.C.R.; Data interpretation: S.A., R.P., and S.C.R; Pathology slide review and interpretation: M.A.B., E.N.; Validation, Supervision: S.C.R.; Writing – Original Draft, Review & Editing: S.A. and S.C.R.; All authors critically reviewed and approved the final manuscript.

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