FOXO1–FGFR1 fusion and amplification in a solid variant of alveolar rhabdomyosarcoma

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Rhabdomyosarcoma is the most common pediatric soft tissue malignancy. Two major subtypes, alveolar rhabdomyosarcoma and embryonal rhabdomyosarcoma, constitute 20 and 60% of all cases, respectively. Approximately 80% of alveolar rhabdomyosarcoma carry two signature chromosomal translocations, t(2;13)(q35;q14) resulting in *PAX3–FOXO1* fusion, and t(1;13)(p36;q14) resulting in *PAX7–FOXO1* fusion. Whether the remaining cases are truly negative for gene fusion has been questioned. We are reporting the case of a 9-month-old girl with a metastatic neck mass diagnosed histologically as solid variant alveolar rhabdomyosarcoma. Chromosome analysis showed a t(8;13;9)(p11.2;q14;9q32) three-way translocation as the sole clonal aberration. Fluorescent *in situ* hybridization (FISH) demonstrated a rearrangement at the *FOXO1* locus and an amplification of its centromeric region. Single-nucleotide polymorphism-based microarray analysis illustrated a co-amplification of the *FOXO1* gene at 13q14 and the *FGFR1* gene at 8p12p11.2, suggesting formation and amplification of a chimerical *FOXO1–FGFR1* gene. This is the first report to identify a novel fusion partner *FGFR1* for the known anchor gene *FOXO1* in alveolar rhabdomyosarcoma.

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Rhabdomyosarcoma accounts for 4–10% of pediatric malignancies. Four pathological subtypes have been traditionally defined: alveolar rhabdomyosarcoma, embryonal rhabdomyosarcoma, botryoid rhabdomyosarcoma, and pleomorphic rhabdomyosarcoma.^{1,2} Alveolar rhabdomyosarcoma and embryonal rhabdomyosarcoma have distinct clinical manifestations, biological behavior, genetic alterations, and account for ~20 and ~60% of all cases, respectively.¹ Alveolar rhabdomyosarcoma is most often seen in older children and is associated with a

poor outcome, while embryonal rhabdomyosarcoma primarily occurs in children younger than 4 years of age and is usually associated with a better prognosis.² A rare solid variant of alveolar rhabdomyosarcoma has been described that is often morphologically indistinguishable from poorly differentiated embryonal rhabdomyosarcoma and may be confused with other 'small, round, blue cell tumors'.³

A study of a large pediatric cohort of 171 patients with rhabdomyosarcoma by the children's oncology group (COG) has demonstrated the role of genetics in the development of rhabdomyosarcoma.⁴ In the subtype of alveolar rhabdomyosarcoma, approximately 80% of cases harbored two signature chromosomal translocations, t(2;13)(q35;q14) in 60%, and t(1;13)(p36;q14) in 20% of cases. These translocations resulted in the formation and overexpression of chimerical genes *PAX3–FOXO1*

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(FOXO1, also known as *FKHR*) and *PAX7-FOXO1*, respectively.⁴ Chromosome rearrangements not involving the *FOXO1* locus have also been described in both alveolar rhabdomyosarcoma and embryonal rhabdomyosarcoma.⁵ In embryonal rhabdomyosarcoma, gains of all or portions of chromosomes 2, 7, 8, 11, 12, 13, 17, 19, and 20, with a particularly high-level gain of chromosome 8 material are consistently observed,^{6–8} and loss of heterozygosity (LOH) on the short arm of chromosome 11 (11p15.5) is frequently detected, suggesting that imprinting might be involved.⁹ In both alveolar rhabdomyosarcoma and embryonal rhabdomyosarcoma, amplification of genes such as *MYCN*, *MDM2*, *CDK4* and *IFG-R1* are often observed.^{10–14} Despite the usually sporadic occurrence of rhabdomyosarcoma, it has also been reported in various congenital disorders, including cancer predisposition syndromes and genetic disorders with multi-organ system defects such as Li-Fraumeni syndrome, Beckwith–Wiedemann syndrome, neurofibromatosis type 1, Costello syndrome, Nijmegen breakage syndrome, Rubinstein–Taybi syndrome, Dubowitz syndrome, multiple endocrine neoplasia type 2A, Roberts syndrome, and Duchenne muscular dystrophy.^{15–17} Subtype classification and gene fusion status closely correlate with the prognosis.¹⁸ However, a question remains, whether alveolar rhabdomyosarcoma cases without *PAX3/7-FOXO1* fusion are truly negative for any gene fusion.^{18–21} In this study, we describe the clinicopathological assessment, cytogenetic test results, and single nucleotide polymorphism (SNP)-based whole-genome microarray evaluation of a solid variant embryonal rhabdomyosarcoma with a novel *FGFR1-FOXO1* fusion and amplification.

Materials and methods

Case Report

A 9-month-old female was admitted with a chief complaint of left neck mass present for a month, and a left shoulder mass present for a week. Chest CT showed a 3 × 1.9 × 2-cm soft tissue mass in the region of the supraspinal muscle, and multiple enlarged cervical lymph nodes. The remainder of the physical examination was within the normal range. The patient underwent excisional biopsy of one of the cervical lymph nodes.

Pathology

Formalin-fixed, paraffin-embedded tissue sections were stained with hematoxylin–phloxine–safran for routine histological assessment. Immunohistochemical studies were conducted using an automated immunostainer (Thermo Scientific, Fremont, CA, USA). The following primary antibodies were used: desmin (clone D33), muscle-specific actin (clone HHF35), myogenin (clone F5D), cytokeratin cocktail (clone AE1/AE3), smooth

muscle actin (clone 1A4), myosin (clone SMMS-1), CD99 (clone HO36-1.1), CD1a (clone MTB1), alpha fetoprotein (polyclonal), CD45 (clones 2B11 & PD7/26), and epithelial membrane antigen (EMA) (clone E29) at the dilution suggested by the manufacturer. All antibodies were obtained from Cell Marque (Hot Springs, AR, USA).

Conventional Cytogenetic Analysis

A piece of the resected lymph node was minced, cultured in conditioned RPMI 1640 medium supplemented with 25% fetal bovine serum for 72 h. Metaphase chromosomes for Giemsa banding pattern by trypsin digestion with Wright stain (GTW banding) were prepared according to standard procedures. In all, 20 metaphases were karyotyped with CytoVison system (Applied Imaging, Santa Clara, CA, USA), and karyograms were described according to the International System for Human Cytogenetic Nomenclature 2009.²² Coordinates of cells with chromosome aberrations were documented, and the slides were subsequently prepared for metaphase FISH analysis.

Interphase and Metaphase FISH Assays

Touch preparation slides were made from a frozen portion of the lymph node. Interphase FISH was undertaken using LSI *FOXO1 (FKHR)* dual color break-apart probe at 13q14 (Vysis, Abbott Park, IL, USA) and *FGFR1* dual color break-apart probe at 8p12p11.2 (LabCorp, Research Triangle Park, NC, USA). Metaphase FISH was performed on the destained GTW slides. Standard FISH protocol was followed. FISH images were captured and analyzed with a CytoVison system (Applied Imaging).

SNP-Based Microarray Analysis

Microarray assay was performed by LabCorp, using Affymetrix (Santa Clara, CA, USA) whole-genome-human SNP array 6.0 platform containing 1.8 million SNP and non-SNP markers. Genomic DNA extraction, probe labeling and hybridization were carried out following the manufacturer's instruction (Affymetrix). The publicly available HapMap set of 270 control individuals and an internal cohort of controls were implemented as control groups. Genotyping Console (GTC) 4.0 software (Affymetrix) was applied to assess copy number alterations and long contiguous stretches of homozygosity (LCSH) using the software default settings. The cut-off for a genomic imbalance was set up as 200 Kb for the deletion and 500 Kb for the duplication. NCBI Build 36.1 (hg18) was used as reference sequence.

Gene Ontology and Data Mining

Gene ontology analysis was conducted using UCSC genome browser (<http://genome.ucsc.edu>).

Functional annotation, pathway hunting, and network construction were performed with Ingenuity Pathway Analysis (IPA) (<http://www.ingenuity.com>) (Ingenuity, Redwood City, CA, USA). VISTA enhancer browser (<http://enhancer.lbl.gov>) and ECR browser (<http://ecrbase.dcode.org>) were used to search for regulatory elements.

Results

Histopathological study of the lymph node established the diagnosis of metastatic solid variant of alveolar rhabdomyosarcoma. The neoplastic cells had small amounts of lightly eosinophilic cytoplasm and prominent 1–2 centrally located nucleoli

(Figure 1a). In some areas, especially toward the periphery, the tumor cells stood like a picket fence at the edge of an empty 'alveolar' space. Rare foci of tumor necrosis were present. The tumor cells showed strong diffuse nuclear positive staining for myogenin and focal positive staining for muscle-specific actin (HHF-35) and desmin immunohistochemical stains. The tumor cells also showed membranous and cytoplasmic staining for CD99 and focal membranous and cytoplasmic positivity for EMA and pancytokeratin (Figure 1b-d). They were negative for myosin and CD45. Conventional cytogenetic and FISH studies identified chromosome rearrangements involving *FOXO1* and suggested a novel fusion partner. GTW-banding analysis, performed on unstimulated tumor cells,

Figure 1 Histopathology and immunohistochemistry of the alveolar rhabdomyosarcoma, solid-variant, metastatic to a lymph node. (a) Sheets of poorly differentiated small round blue tumor cells with prominent nucleoli and scant, pale eosinophilic cytoplasm (hematoxylin–phloxine–saffron staining, $\times 400$). (b) Strong, positive membranous, and cytoplasmic staining for muscle-specific actin (HHF-35, $\times 400$). (c) Strong, positive nuclear staining for myogenin ($\times 400$) and (d) strong, cytoplasmic and membranous positive staining for desmin ($\times 400$).

Figure 2 Abnormal cytogenetic findings in the tumor cells. (a) Representative karyogram of a three-way translocation 46,XX,t(8;13;9)(p11.2;q14;q32) observed in 10 of 20 metaphases analyzed. Arrows indicate aberrant chromosomes. (b) Metaphase spread of the same cell karyotyped in panel a. (c) Partial karyograms of seven representative abnormal cells, with no structural changes shown on chromosomes 1 and 2, while chromosomes 8, 9 and 13 display an apparently balanced three-way translocation. Chromosome numbers are listed on the bottom. (d) FISH using dual color break-apart probe for *FOXO1* demonstrating one normal fusion signal (yellow), one split signal for the telomeric region of *FOXO1* (red), and ~20 copies of the split signal for the centromeric region of *FOXO1* (green). These results indicate a rearrangement at *FOXO1* locus, as well as an amplification of its centromeric region.

demonstrated an apparently balanced three-way translocation, 46,XX,t(8;13;9)(p11.2;q14;q32), identified as the sole clonal abnormality in 10 of 20 metaphases analyzed (Figure 2a-c). Rearrangement at 13q14 suggested that *FOXO1* might be involved. However, loci of the characteristic fusion partners *PAX3* at 2q35 and *PAX7* at 1p36 seemed not affected, suggesting in this case a novel fusion partner for *FOXO1* (Figure 2c). The dual color break-apart FISH probe for *FOXO1* used on interphase nuclei to further characterize the cytogenetic aberrations demonstrated one pair of juxtaposed yellow signals for *FOXO1* locus, one red signal for the telomeric region of *FOXO1*, and 10–20 copies of green signals for the centromeric region of *FOXO1*, indicating

that one allele of the *FOXO1* gene was rearranged and its centromeric region subsequently amplified (Figure 2d). FISH analysis of de-stained G-banded metaphase cells confirmed translocation of the telomeric region of *FOXO1* to the long arm of chromosome 9, and amplification of the centromeric region of *FOXO1*, with the caveat that the amplification signal could represent a chimerical gene consisting of an unknown DNA sequence at the novel translocation locus 8p11.2 identified by chromosome analysis.

SNP microarray analysis detected a co-amplification of *FOXO1* gene at 13q14 and *FGFR1* gene at 8p12-p11. Whole-genome screening for copy number variations using Affymetrix 6.0 microarray

platform showed co-amplification of two genomic segments. The first amplification demonstrated approximately 12 copies of a 446-Kb segment (Chr8: 38 304 468–38 750 291) on the short arm of chromosome 8 at p12p11.23 encompassing 293 makers. This segment contains the full-length genomic regions of two reference genes, fibroblast growth factor receptor 1 (*FGFR1*; NM_023110.2) and leucine zipper-EF-hand containing transmembrane protein 2 (*LETM2*; NM_001199660.1), and part of the gene Wolf-Hirschhorn syndrome candidate 1-like 1 (*WHSC1L1*; NM_017778.2) (Figure 3a). The second amplification demonstrated approximately 12 copies of a 255-Kb segment (Chr13: 39 782 030–40 037 118) on the long arm of chromosome 13 at q14.11 encompassing 208 markers. This segment overlaps the centrometric end of forkhead box O1 (*FOXO1*; NM_002015.3) (Figure 3b). Breakpoints of the above two fragments are consistent with the three-way translocation previously diagnosed by chromosome analysis. In combination with the karyogram and FISH findings, the co-amplification seen on microarray suggested formation of *FOXO1-FGFR1* fusion due to chromosome translocation and subsequent amplification of the resultant chimerical product. Using dual color break-apart probe for *FGFR1*, amplification of the intact locus represented by 10–20 copies of fused yellow signals was observed in all of the interphase nuclei analyzed (Figure 3c). No copy number change was detected for *PAX3* or *PAX7* by microarray study.

In addition, two submicroscopic microdeletions undetectable using current state-of-art cytogenetic techniques were identified by the microarray analysis. The first microdeletion was 2.4 Mb in length (Chr11: 78 493 948–80 917 971), located on the long arm of chromosome 11 at q14.1. The proximal end of this deletion overlaps the promoter and several exons of *ODZ4* gene (NM_001098816.2), which is the human homolog of the *odd Oz* gene in *Drosophila* (Supplementary Figure 1a). This deletion could presumably disrupt the transcription of *ODZ4*, but functional information available in the literature is very limited for this gene. Yet, as the major part of this deletion is a non-coding sequence, functional elements regulating gene expression over long distance could reside inside this gene desert. However, no enhancers were found in this area by searching VISTA enhancer browser (<http://enhancer.lbl.gov/>). Comparative genome analysis was done by aligning this 2.4-Mb human DNA sequence with corresponding regions in rhesus macaque, dog, opossum, rat, mouse, chicken, frog, zebrafish, and fugu genomes (<http://ecrbase.dcode.org/>). In all, 11 fragments were seen evolutionally conserved between human and chicken and one fragment conserved between human and frog, (Supplementary Figure 1b) suggesting that these identified DNA fragments might be non-coding regulator elements. The second finding was a 283-Kb intragenic deletion

(ChrX: 32 100 786–32 383 598) across several exons of *DMD* gene on chromosome X at p21.1, gene responsible for X-linked Duchenne muscular dystrophy. The significance of both deletions in this patient's malignancy is unclear.

The final cytogenetic diagnosis for this patient's tumor is therefore written as 46,XX,t(8;13;9)(p11.2;q14;q32)[10]/46,XX[10].nuc ish amp(*FGFR1*) [200],(5'*FOXO1* × 2, amp(3'*FOXO1*))(5'*FOXO1* con 3'*FOXO1* × 1)[90/100]/(*FOXO1* × 2)(5'*FOXO1* sep 3'*FOXO1* × 1)[3/100].arr Xp21.1(32 100 786–32 383 598) × ~1,8p12p11.23(38 304 468–38 750 291) × ~12,11q14.1(78 493 948–80 917 971) × 0–1,13q14.11(39 782 030–40 037 118) × ~12.

Discussion

Histological classification of the tumor and identification of genetic aberrations have significant clinical applications in rhabdomyosarcoma. Although *PAX-FOXO1* gene fusion is a characteristic feature for the diagnosis of alveolar rhabdomyosarcoma, translocations involving *PAX7* often indicate a better outcome in patients with metastatic disease than that associated with *PAX3*.⁴ Yet, 20% of alveolar rhabdomyosarcoma are negative for *PAX-FOXO1* rearrangements, and it has remained unclear whether those cases are truly 'fusion-negative' or carry chromosomal aberrations at unsuspected novel gene loci,²³ as in the case of the t(2;2)(p23;q53) and t(2;8)(q35;q13) described recently, respectively, resulting in fusion genes *PAX3-NCOA1* and *PAX3-NCOA2*.²⁴

PAX proteins are highly specific transcriptional factors crucial for the differentiation of myogenic and neurogenic progenitor cells during early embryonic development.²⁵ In the 80% alveolar rhabdomyosarcoma cases that are *PAX-FOXO1* positive, *PAX3* and *PAX7* DNA-binding domains and *FOXO1* transactivation domain are fused. *PAX3-FOXO1* or *PAX7-FOXO1* chimerical transcripts are overexpressed and activate downstream transcription from *PAX* protein-binding sites more effectively than their wild-type counterparts. Altered biological behaviors such as dysregulation of angiogenesis, promotion of cell growth, and anti-apoptosis prohibiting differentiation are induced.^{26,27} *FGFR1* is a tyrosin kinase receptor gene located on the short arm of human chromosome 8 at p12p11. Activating mutations in *FGFR4*, a family member of *FGFR1*, were recently detected in 7.5% of rhabdomyosarcoma including both embryonal rhabdomyosarcoma and *PAX3-FOXO1*-positive alveolar rhabdomyosarcoma tumors.²⁸ Overexpression of *FGFR4* was previously found to be associated with advanced stage in rhabdomyosarcoma and particularly alveolar rhabdomyosarcoma.²⁹ Of note, array comparative genomic hybridization (aCGH) on a cohort of 128 primary rhabdomyosarcoma samples showed *FGFR1* amplification in 11% *PAX-FOXO1*-negative alveolar rhabdomyosarcoma cases and 6%

Figure 3 Snapshot of the GTC karyoview and FISH assay depicting copy number alterations detected by affymetric 6.0 SNP microarray analysis. (a) An amplification of 12 copies on the short arm of chromosome 8 at p12-p11.23, spanning approximately 446 Kb. Two full-length reference genes, *FGFR1* and *LETM2*, and one partial gene *WHSC1L1* are located inside the amplified area. (b) A second amplification of 12 copies on the long arm of chromosome 13 at q14.11, spanning approximately 255 Kb. This amplified segment overlaps the centromeric region of *FOXO1*. (c) Amplification of *FGFR1* locus was visualized on 100% interphase nuclei analyzed from the tumor by FISH study with dual color break-apart probes, 10–20 fusion signals were seen. Centromeric region of *FGFR1* locus was labeled in red and telomeric region of *FGFR1* locus was labeled in green, the yellow fusion signal indicates an intact *FGFR1* locus. A few split signals represent artifact staining.

embryonal rhabdomyosarcoma cases, but not in alveolar rhabdomyosarcoma patients positive for *PAX-FOXO1*.¹⁹

SNP arrays technology applied to frozen tumor tissue allowed the patient's entire genome to be screened for copy number variation and the amplified fragment, and the unknown *FOXO1* fusion partner was characterized. Microarray has been widely used in clinical diagnosis of developmental disorders and in some cases of hematological malignancies, but rarely in solid tumors.³⁰ As opposed to the 5–10-Mb resolution level of conventional chromosome analysis, this array platform allowed detection of submicroscopic copy number alterations (deletions, duplications, amplifications) and loss of LOH at a submegabase resolution. The results obtained would benefit from additional confirmation, ruling-out unlikely constitutional changes in the peripheral blood.

The 2.4-Mb deletion in 11q14 overlaps the 5' end of the *ODZ4* gene, which is the human homolog of the *odd Oz* gene in *Drosophila* with limited functional information in the literature. The majority of this deleted fragment is a gene desert that could harbor regulatory elements (eg enhancers, silencers, insulators, or locus control regions (LCRs)), defects on which could lead to misexpression of the target genes.³¹ Unfortunately, we were unable to find any experimentally validated enhancer by searching VISTA enhancer browser (<http://enhancer.lbl.gov>). Although more than 95% of human genome does not code proteins, non-coding regions evolutionarily conserved across vertebrate genomes could suggest regulatory functions.³² When we aligned the 2.4-Mb fragment with the corresponding regions in rhesus macaque, dog, opossum, rat, mouse, chicken, frog, zebrafish, and fugu genomes (<http://ecrbase.dcode.org>), deeply conserved fragments were seen between human, chicken, and frog (Supplementary Figure 1b). However, the significance of this deletion for this patient's malignancy remains unclear.

Mutations in *DMD* gene, which were also detected, are at the root cause of the X-linked Duchenne muscular dystrophy.³³ Interestingly, *DMD* gene and other members in the dystrophin-associated glycoprotein (DAG) complex have also been studied in rhabdomyosarcoma but no clear interpretation is available at present, yet it is interesting to note that knockout mice with dystrophin or α -sarcoglycan deficiency spontaneously develop alveolar rhabdomyosarcoma or embryonal rhabdomyosarcoma in aged individuals; however, we could find only two Duchenne muscular dystrophy patients with rhabdomyosarcoma reported in the literature, which may be a coincidence.^{16,17}

Solid variant of alveolar rhabdomyosarcoma is rare and sometimes mimics other malignancies.³⁴ The 12 cases in the literature since 1994 including the current one are summarized in Supplementary Table 1.^{34,39–46} Fusion gene status was known

for 11 patients: eight had *PAX3-FOXO1*, one had *PAX7-FOXO1*, and one (the current one) had *FGFR1-FOXO1* fusion. Only one patient was negative for identifiable genetic aberration. Myogenin, desmin, vimentin, and actin were all positive for most of the cases.^{39–46}

Genetic events in rhabdomyosarcoma detected by conventional cytogenetics, FISH, or whole-genome microarray studies affect multiple genes. Manifestation of rhabdomyosarcoma have also been described in several single gene disorders.³⁵ Moreover, signaling pathways such as p53 pathway is tightly involved in the tumorigenesis of rhabdomyosarcoma, as introduction of the fused *Pax-Foxo1* is not sufficient to induce the tumor in mice unless other genetic modifications such as p53 mutations were also included.^{21,36–38} Interestingly, online bioinformatic tool IPA revealed interrelationship between most of the collected 31 genes including those genes responsible for developmental disorders (Supplementary Table 1, Supplementary Figure 2).

In summary, using integrated cytogenetic and genomic approaches, we have identified a chromosomal rearrangement that resulted in the formation and amplification of a novel *FOXO1-FGFR1* chimerical product in an infant girl with solid variant alveolar rhabdomyosarcoma. Considering the unusual early onset of the tumor, our finding may be a characteristic of a new rhabdomyosarcoma subgroup among the so-called 'fusion-negative rhabdomyosarcoma'. As opposed to other newly identified translocation loci in alveolar rhabdomyosarcoma such as *NCOA1* and *NCOA2*, *FGFR* family members have established roles in tumorigenesis. The identification of *FGFR1* will help further clarify the molecular mechanisms for rhabdomyosarcoma development, and may also lead to targeted therapies for this type of tumor. We suggest that microarray analysis be performed on all sarcomas with histological evidence of alveolar features, but negative for an identifiable *PAX-FOXO1* translocation.

Disclosure/conflict of interest

The authors declare no conflict of interest.

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