Aberrant CDK4 Amplification in Refractory Rhabdomyosarcoma as Identified by Genomic Profiling

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Rhabdomyosarcoma (RMS) is the most commonly occurring type of soft tissue tumor in children, but it is less common in adults, accounting for only 2–5% of all soft tissue sarcomas. Given the rarity of this disease, very little information is available on the most appropriate treatment strategy for adult RMS patients. We performed genomic analysis of RMS cells derived from a 27-year-old male patient whose disease was refractory to treatment. A peritoneal seeding nodule from the primary tumor, pleural metastases, malignant pleural effusion, and ascites obtained during disease progression, were analyzed. Whole exome sequencing revealed 23 candidate variants, and 10 of 23 mutations were validated by Sanger sequencing. Three of 10 mutations were present in both primary and metastatic tumors, and 3 mutations were detected only in metastatic specimens. Comparative genomic hybridization array analysis revealed prominent amplification in the 12q13–14 region, and more specifically, the CDK4 proto-oncogene was highly amplified. ALK overexpression was observed at both protein and RNA levels. However, an ALK fusion assay using NanoString technology failed to show any ALK rearrangements. Little genetic heterogeneity was observed between primary and metastatic RMS cells. We propose that CDK4, located at 12q14, is a potential target for drug development for RMS treatment.

Rhabdomyosarcoma (RMS) is the most commonly occurring type of soft tissue tumor in children, but it is less common in adults, accounting for only 2–5% of all soft tissue sarcomas. Given the rarity of this disease, very little information is available on the most appropriate treatment strategy for adult RMS patients. Patients with unresectable or metastatic RMS have an extremely low cure rate and a poor prognosis. A substantial improvement in survival has been achieved with the introduction of intensive chemotherapy regimens, which are usually based on pediatric oncology clinical trials on RMS. However, survival rates for patients with metastatic disease remain disappointing, and the prognosis is dismal in patients with a poor response to salvage chemotherapy. Thus, identification of novel therapeutic targets in RMS is urgently needed in order to improve treatment outcomes for this aggressive type of tumor.

Major histologic subtypes of RMS include embryonal RMS (ERMS) and alveolar RMS (ARMS). Despite advances in therapy, patients with the ARMS histological variant of RMS have a 5-year survival of less than 30%. ARMS presents with distinctive chromosomal translocations that result in specific fusion gene products, the most prevalent of which are PAX3–FOXO1 (55%) and PAX7–FOXO1 (22%). Reciprocal translocation of chromosomes 2 and 13 results in a PAX3-FKHR fusion gene in ARMS, which fuses the region of the gene encoding the DNA-binding domain of the transcription factor PAX3 with that encoding the transactivation domain of the transcription factor FKHR in-frame. However, at least 25% of ARMS cases lack such translocations, suggesting that ARMS is not a single disease, but a heterogeneous group of conditions with a common phenotype. Moreover, studies on the gene expression profile of RMS have proposed new molecular classifications and have revealed that a specific gene expression signature potentially determines tumor behavior as well as treatment outcome. ALK is one of the targets of interest, given that ALK alterations are relatively common in RMS, although the function of its gene product remains unknown.
Here, we report the clinical application of genomic profiling in identifying potential novel genetic mutations in patients with relapsed and chemotherapy-refractory alveolar RMS.

**Results**

**Case presentation.** A 27-year-old man presented with a complaint of left upper quadrant abdominal pain that had lasted for 3 years. Computed tomography (CT) and positron emission tomography (PET) scans showed multiple malignant masses, involving the pancreas and left upper abdominal wall, and pleural seeding was also noted (Fig. 1A). Pathological examination of the abdominal wall mass showed thin fibrous septae lined by small round blue cells in an alveolar growth or solid pattern; the cells appeared to lack cohesion and had hyperchromatic nuclei and scant cytoplasm. The tumor cells were diffusely positive for CD99, desmin, and WT1 and showed scattered focal positivity for cytokeratin. Ki-67 staining revealed high proliferative activity of the tumor cells. The FKHR break-apart fluorescence in situ hybridization (FISH) showed separate green and red signals, confirming FKHR rearrangement (Supplementary Fig. S1).

Based on the histology, immunohistochemistry (IHC), and FISH results, ARMS was diagnosed, and alternating cycles of vincristine, doxorubicin, and cyclophosphamide (VDC) and ifosfamide and etoposide (IE) were administered every 3 weeks.

After completing a 1-year course of cytotoxic chemotherapy, the patient achieved near complete remission, with disappearance of the multiple masses and pleural seeding (Fig. 1B). On the basis of a tumor board discussion involving a multi-modality team for sarcoma, the residual peritoneal seeding nodules were surgically resected. At the time of surgery, the resected seeding nodules were snap frozen and immediately stored at \(-80^\circ C\) for molecular analysis. The pathologic examination of the resected peritoneal seeding nodules verified the diagnosis of ARMS.

Postoperative follow-up abdominal pelvis CT and chest CT demonstrated no evidence of malignancy. However, 3 months after surgical resection, the patient was found to have developed a chest...
Genomic profiling and somatic mutation. DNA from the primary tumor (sample #1) was sequenced, revealing 23 candidate variants: SCN1B, PPP1R3A, GRID2, APBA2, ZNF142, ZYG11A, RBFOX1, TCF7L1, NARF, KIAA0182, TEX13B, MUC2, LRR3C, GRHL3, MUC16, TTR, UBA1, FEN1, ELAC2, NBEAL1, DSCAML1, PCDHA4, and POLR3C (Table 1). Only the MUC16 mutation was detected in blood, with 3.4% allele frequency. Amino acid substitutions were predicted to arise from some of the point mutations in each gene, and these in turn were predicted to have a substantial phenotypic effect based on the SIFT score (a SIFT score of 0 indicates a deleterious effect, a score > 0.05 indicates a damaging effect, and a score > 0.05 suggests that the substitution can be tolerated). These protein mutations were also predicted to have considerable functional impact based on the FI score as determined by Mutation Assessor (http://mutationassessor.org) (an FI score < 0.8 is considered neutral; 0.8 < FI score ≤ 1.9 indicates low impact; 1.9 < FI score ≤ 3.5 indicates medium impact; and FI score > 3.5 indicates high impact). Among the variants detected in exome sequencing from the tumor specimen, those in SCN1B, PPP1R3A, GRID2, APBA2, ZNF142, ZYG11A, RBFOX1, TCF7L1, TEX13B, and DSCAML1 were validated by Sanger sequencing, and the details of these 10 candidate genes are provided in Table 2.

The primary tumor (sample #1), pleural metastases (sample #2), and malignant cells from ascites (sample #4) were all found to carry point mutations in SCN1B, PPP1R3A, and ZYG11A. However, APBA2, ZNF142, and RBFOX1 mutations, although not present in the primary tumor, were detected in both metastatic (chemotherapy refractory) specimens. TCF7L1, TEX13B, and DSCAML1 mutations, which were detected during exome sequencing, were not confirmed in subsequent Sanger sequencing of the primary tumor or any metastatic specimens. Although the mutation in GRID2 was not seen in the primary tumor due to failure of the sequencing reactions, the mutation was confirmed in both metastatic samples.

Table 1 | Somatic variants detected in rhabdomyosarcoma (RMS; primary tumor, sample #1) by whole exome sequencing

| No | Ch | position | gene name | ref | var | vac | tr | vrf | vac | tr | vrf | func. Impt | fi score | prediction | score |
|----|----|---------|-----------|-----|-----|-----|----|-----|-----|----|-----|-----------|----------|------------|-------|
| 1  | 19 | 355224413| SCN1B     | A   | G   | 0   | 35 | 0.0%| 11  | 21 | 52.4%| low       | 1.865     | D          | 0     |
| 2  | 7  | 113519206| PPP1R3A   | A   | T   | 0   | 102| 0.0%| 66  | 142| 46.5%| neutral   | 0         | D          | 0.03  |
| 3  | 4  | 94411875 | GRID2     | C   | A   | 0   | 61 | 0.0%| 31  | 67 | 46.3%| D          | 0         | D          | 0     |
| 4  | 15 | 29364353 | APBA2     | G   | A   | 0   | 62 | 0.0%| 23  | 53 | 43.4%| medium    | 2.28      | D          | 0.05  |
| 5  | 2  | 219513493| ZNF142    | C   | T   | 0   | 24 | 0.0%| 9   | 21 | 42.9%| low       | 0.875     | T          | 0.3   |
| 6  | 1  | 53347155 | ZYG11A    | G   | T   | 0   | 53 | 0.0%| 18  | 43 | 41.9%| D          | 0         | D          | 0     |
| 7  | 16 | 75638302 | RBFOX1    | C   | A   | 0   | 74 | 0.0%| 37  | 93 | 39.8%| medium    | 2.595     | D          | 0     |
| 8  | 2  | 85533345 | TCF7L1    | G   | A   | 0   | 23 | 0.0%| 6   | 19 | 31.6%| medium    | 2.25      | T          | 0.05  |
| 9  | 17 | 180459492| NARF      | A   | G   | 0   | 23 | 0.0%| 8   | 28 | 28.6%| neutral   | 0.455     | D          | 0.03  |
| 10 | 16 | 85698723 | KIAA0182  | T   | A   | 0   | 24 | 0.0%| 4   | 16 | 25.0%| low       | 1.32      | D          | 0.01  |
| 11 | X  | 107224952| TEX13B    | G   | A   | 0   | 47 | 0.0%| 12  | 54 | 22.2%| low       | 1.5       | T          | 0.06  |
| 12 | 11 | 11093735 | MUC2      | C   | T   | 0   | 26 | 0.0%| 4   | 18 | 22.2%| low       | 1.5       | T          | 0.23  |
| 13 | 21 | 45877015 | LRR3C     | A   | C   | 0   | 28 | 0.0%| 4   | 20 | 20.0%| low       | 0.865     | T          | 0.11  |
| 14 | 1  | 24663626 | GRHL3     | A   | C   | 0   | 26 | 0.0%| 4   | 22 | 18.2%| low       | 1.67      | D          | 0     |
| 15 | 19 | 9005714  | MUC16     | A   | C   | 2   | 58 | 3.4%| 8   | 44 | 18.2%| low       | 1.935     | T          | 0.13  |
| 16 | 18 | 29197855 | TTR       | G   | C   | 1   | 28 | 0.0%| 4   | 23 | 17.4%| low       | 1.545     | D          | 0     |
| 17 | X  | 47069419 | UBA1      | G   | C   | 4   | 41 | 0.0%| 6   | 36 | 16.7%| medium    | 3.34      | D          | 0     |
| 18 | 11 | 61563225 | FEN1      | T   | G   | 0   | 29 | 0.0%| 5   | 32 | 15.6%| medium    | 2.83      | D          | 0     |
| 19 | 17 | 12892642 | ELAC2     | A   | C   | 0   | 26 | 0.0%| 4   | 26 | 15.4%| low       | 1.87      | D          | 0     |
| 20 | 2  | 204045181| NBEAL1    | A   | C   | 0   | 72 | 0.0%| 9   | 66 | 13.6%| high      | 4.49      | D          | 0     |
| 21 | 11 | 117342607| DSCAML1   | A   | C   | 0   | 37 | 0.0%| 5   | 37 | 13.5%| medium    | 2.905     | D          | 0     |
| 22 | 5  | 140188268| PCDHA4    | T   | G   | 0   | 39 | 0.0%| 5   | 37 | 13.5%| medium    | 1.39      | D          | 0.04  |
| 23 | 1  | 145601821| POLR3C    | A   | C   | 0   | 39 | 0.0%| 5   | 37 | 13.5%| low       | 1.39      | D          | 0.04  |

Ch, chromosome; ref, reference; var, variant; vac, variant allele count; tr, total read; vrf, variant read frequency; D, damaging; T, tolerated; SIFT, Sorting Tolerant From Intolerant (Nucleic Acids Res 2003;31:3812).

Nos. 1, 2, 3, 4, 5, 6, 7, 8, 11, and 21: somatic mutations that were validated by Sanger sequencing (Table 2).

No. not mentioned above: somatic mutations that were not validated by Sanger sequencing.
most of the tumor cells. Given the high level of ALK protein expression, ALK RNA overexpression was also detected in the pleural metastasis specimen (sample #2), as expected (Fig. 3B).

Two lung cancer cell lines, NCIH3122 and NCIH2228, were used as positive controls for EML4–ALK fusion and A549 cells were used as the negative control. ALK-fusion lung cancer only overexpresses the 3′-ALK mRNA (NCIH3122 and NCIH2228), whereas sarcoma overexpresses the full-length mRNA. The mean of 3′-ALK expression of ALK+ lung tumor is approximately 500, whereas this reached approximately 3000 in the sarcoma. Despite ALK overexpression at the protein and RNA levels, ALK amplification was not observed in the tumor cells of this patient.

Next, we screened for the presence of ALK fusion partners using a NanoString assay; however, the ALK fusion assay failed to show any ALK rearrangements (Fig. 3C). ALK rearrangement was also not detected by FISH (Fig. 3A-3).

### Discussion

Patients with recurrent RMS usually present with a rapidly deteriorating condition and have markedly limited options in terms of chemotherapy9–20. In this study, we found that the majority of somatic mutations found during exome sequencing of primary tumor tissue were also observed in metastatic tumor tissue and metastatic cells in ascites samples, although Sanger sequencing revealed genetic alterations involving several genes, such as APBA2, ZNF1142, and RBFOX1, only in the metastatic samples. These results led to 2 important conclusions: First, there is little genetic heterogeneity between primary and metastatic RMS cells at least in terms of mutational spectra, reflecting relatively little genetic evolution during the course of metastasis. This is consistent with the results of recent similar studies on melanoma21, breast22, and pancreatic cancers23, in which genomic profiling for both primary and metastatic sites was performed. Second, we confirmed that malignant cells isolated from body fluid can be used for genomic profiling, as their genome is nearly identical to that of the resected tumor specimen. This may be especially important in clinical practice because body fluid can be obtained relatively easily using a bedside procedure. Of the 23 candidate genes found during exome sequencing, we selected 9 mutated genes (APBA2, RBFOX1, TCF7L1, MUC16, UBA1, FEN1, NBEAL1, DSCAML1, and PCDHA4) for which substantial functional impact was predicted (medium or high functional impact; FI score > 1.9), with or without damaging/deleterious phenotypic effects based on the SIFT score (≤0.05), and assessed their clinical relevance to RMS.

However, we could not find any pre-existing evidence that these genetic alterations contribute to RMS development.

The aCGH array used in this study revealed prominent amplification in the 12q13 and 12q14 regions. Although we found that many genes within this chromosome 12 region were amplified, CDK4 amplification was of particular interest because it is known to play a pivotal role in the oncogenic process24, and perhaps more importantly, the corresponding proteins are potential drug targets25. Its overexpression is frequently observed in well-differentiated and dedifferentiated liposarcomas26–28; consequently, a clinical trial of the CDK4 inhibitor (PD0332991) for CDK4-amplified tumors has been conducted. In both phase I and phase II trials, the CDK4 inhibitor has proven effective in C, and a randomized phase 3 trial is being conducted. In both phase I and phase II trials, the CDK4 inhibitor has proven effective in C, and a randomized phase 3 trial is being considered by researchers29. Amplification of 12q13–q14 and CDK4 in RMS has been reported previously30,31, as has amplification of MYCN, and both of these genes are known to be involved in RMS tumorigenesis32. However, these genes are associated with distinct expression profiles and clinical parameters. MYCN overexpression occurs more frequently in cases in which 2p24 amplification is present, whereas CDK4 overexpression is associated with 12q13–14 amplification33. In addition, 12q13–14 amplification was significantly associated with poor clinical outcomes, such as short failure-free and overall survival, compared to that seen in cases with...
2q24 amplification\(^3\)\(^3\). In the RMS case studied here, we confirmed the presence of a 12q13–14 amplification and showed that CDK4 is one of the genes overexpressed in this chromosomal region.

A study provided in vitro evidence for the successful pharmacologic inhibition of CDK4/CDK6 activity in myoblasts and RMS-derived cells\(^3\)\(^4\); in this study, most ARMS-and ERMS-derived cell lines and tumor samples expressed CDK4 and CDK6, and exposure of these cells to a CDK4 inhibitor caused G1 cell cycle arrest, which is closely associated with myogenic differentiation. Given that defective cell cycle control, which leads to failure of myogenic differentiation, is one of the notable characteristics of RMS-derived cells, it was not surprising that CDK4 inhibition with PD0332991 ultimately facilitated skeletal muscle differentiation. This finding suggests that CDK4 inhibition is a potential therapeutic strategy for RMS. However, there is a scarcity of data on the use of a CDK4 inhibitor in patients with RMS. Although it is therefore difficult to draw firm conclusions regarding the potential efficacy of this inhibitor, the need for novel therapeutics arising from the dismal prognosis in refractory RMS, together with the genetic profiling data presented here, warrant clinical trials on a CDK4 inhibitor in chemotherapy-refractory RMS patients.

In summary, our study revealed that there was little genetic heterogeneity between primary and metastatic RMS cells and suggested that malignant cells from body fluid can be used for genomic profiling of RMS patients. The RMS tumor in this case overexpressed ALK, but this was not associated with the amplification or translocation of this gene. Prominent amplification of the 12q13–14 region was also observed, and we propose that CDK4, located in 12q14, is a potential target for drugs in RMS.

**Methods**

**Ethics statement.** This study was approved by the SMC Institutional Review Board and was conducted in accordance with the 1996 Declaration of Helsinki. Written informed consent was obtained from the patient before genomic analyses were performed for research purposes.
IHC. Five-micrometer-thick tissue sections were deparaffinized in xylene, rehydrated, and heated to 100°C in citrate buffer (pH 6.0) for 5 min for non-enzymatic antigen retrieval. The sections were incubated with monoclonal mouse anti-human desmin antibodies (1:100 dilution; RLM30; Novocastra, Newcastle-upon-Tyne, UK) for 60 min at room temperature, followed by incubation with a 1:100 dilution of biotinylated goat anti-mouse IgG (Vector Laboratories, Burlingame, CA, USA) for 30 min at room temperature. The sections were stained with 5 μg/ml 3,3′-diaminobenzidine chromogen for 5–10 min and were then counterstained with methyl green (Vector Laboratories). For detection of muscle-specific actin, tissue sections were incubated with a 1:100 dilution of biotinylated goat anti-mouse IgG (Vector Laboratories). The sections were stained with 50 μg/ml 3,3′-diaminobenzidine chromogen for 5–10 min and were then counterstained with methyl green (Vector Laboratories).

**FISH.** FISH was performed using commercially available ALLK (Vysis LSI ALK Dual Color, Break Apart Rearrangement Probe; Abbott Molecular, Abbott Park, IL) and FKHR (Vysis LSI FKHR Dual Color, Break Apart Rearrangement Probe; Abbott Molecular) probes according to the manufacturer’s instructions. One hundred cells were analyzed in each case. FISH was considered positive when more than 15% of the tumor cells showed distinct red and green signals and/or a single red (residual 3′-end) signal. In cases of discrepant results, FISH from a second section was performed.

**Bio specimens processing and quality control.** Excised tumor tissues were divided into 2 pieces. One piece was embedded in optimal cutting temperature compound and used to prepare hematoxylin and eosin-stained frozen section slides. The other piece of tissue was snap frozen in liquid nitrogen and stored at −80°C. The tumor cell populations on the frozen section slide accounted for more than 60% of the total cell population; less than 10% were necrotic. Genomic DNA was extracted from snap frozen tissue and peripheral blood using the QiAmp DNA Mini Kit (Qiagen GmbH, Hilden Germany) according to the manufacturer’s instructions. DNA integrity was evaluated using 1% agarose gel electrophoresis. Tumor and normal DNA concentrations were measured using PicoGreen dsDNA Quantitation Reagent (Invitrogen, Carlsbad, CA). A minimum DNA concentration of 20 ng/μl was required for allele-specific PCR.

**Exome sequencing and analysis.** Genomic DNA was extracted from the blood and primary tumor (abdomen) of the RMS patient. Exon capture was performed using Agilent SureSelectXT Human All Exon (50 M), which includes all exons annotated in the consensus CDS (CCDS) database, as well as 10 bp of flanking sequence for each targeted region (http://www.genomics.agilent.com). The captured DNA fragments were sequenced with Illumine HiSeq2000, generating 100 bp × 2 paired-end reads. The clean reads were aligned against the human reference genome (hg19/GRCh37) using the Burrows-Wheeler Aligner (BWA). The alignment results were further processed sequentially using local realignment, duplicate read marking, and base quality recalibration by using the Picard (http://picard.sourceforge.net) and GATK (http://www.broadinstitute.org/gatk) pipeline software. Variant and germline calling were performed using jointSNVMix (http://code.google.com/p/joint-snv-mix/), and the somatic mutations observed in tumor tissue were annotated using ANNOVAR (http://www.openbioinformatics.org/annovar/).

**Quality control, sequence alignment, somatic variant calling, and annotation.** In the first quality control step, Cutadapt v.1.0 [1] removed adapter sequences from the input fastq sequence. After adapter trimming, FasT v.0.0.13 [2] filtered low-quality reads, such that base quality was more than 20 and the proportion of good-quality bases in each read was more than 50%. Finally, cmplFastq [3] classified paired-end reads and single-end reads. Classified fastq sequences were aligned to the human reference sequence (hg19) using the Burrows-Wheeler Aligner v.0.5.9 (BWA) [4], and then were merged to a BAM file. Subsequently, sequential cleanup processes, consisting of the addition or replacement of read groups, marking and removing duplicates, and fixing mate information were performed using Picard Tools v.1.69 [5].
The cleaned bam file was then sorted using Samtools v.0.1.18 [6] and the local realignment and base quality score recalibration were processed using the Genome Analysis Toolkit v.1.6–7 (GATK) [7].

Somatic mutations were designated into 3 categories: single nucleotide polymorphisms (SNPs), indels, and CNVs. We began by applying the joint_snv_mix_one model in JointSNVMix v.0.7.5 [8] in order to find point mutations, and used Annovar [9], Mutation Assessor [10], and SIFT [11] for annotation. Annovar performed filter-based annotation indicating mutations that are present in 1000 genome projects or dbSNP (snp135). It also performed gene-based annotation using Mutation Assessor to identify whether protein-coding changes caused by SNPs or CNVs are deleterious. We selected genes that were annotated as “medium or high functional impact” by Mutation Assessor and were predicted as “damaging” by SIFT. Indels were detected by the SomaticIndelDetector in GATK, following which Annovar gene-based annotation was used to describe the functional impact of somatic indels. CNVs were detected using ExomeCNV (R package) from the coverage file prepared using DepthOfCoverage in GATK. We used default parameters, except for the aforementioned software.

aCGH. Genomic DNA was extracted from the cells cultured from the primary tumor of the patient. aCGH was performed using the Agilent Human Genome CGH Microarray Kit 8 × 60 K, which contains approximately 45,000 probes.

**Figure 3 | ALK expression and fusion assay.** (A). ALK immunohistochemistry (2A-1, positive in the present case, 2A-2, negative control) and ALK FISH (2A-3) of the primary tumor: ALK protein overexpression was confirmed, but ALK rearrangement was not detected. (B). ALK RNA expression in tumor cells from pleural metastasis (sample#2): ALK RNA overexpression was detected. The ALK expression has been normalized to that of 4 housekeeping genes. Lung cancer cell lines (NCI-H3122, NCI-H2228, and A549) were used as controls for ALK RNA expression and EML-ALK fusion detection. (C). ALK fusion assay using NanoString: no EML4-ALK RNA was detected in the sarcoma specimen.
ALK fusion transcript assay. nCounter assays were performed in duplicate, according to the manufacturer’s instructions (NanoString Technologies, Inc, Seattle, WA, USA). Briefly, 500 ng of total RNA was hybridized to nCounter Sample Prep Station (NanoString Technologies, Inc). Cartridges containing immobilized and aligned reporter complexes were subsequently imaged on the nCounter Digital Analyzer (NanoString Technologies, Inc), set at 1155 fields of view. Reporter counts were collected using the nSolver analysis software version 1 in NanoString, normalized, and analyzed as described below. A detailed description of the assay is given elsewhere21.

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Author contributions
J.L. and Y.C. conceived the idea and designed the study. S.P. and J.L. collected and analyzed the study material. All authors reviewed the manuscript.

Additional information
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