Research Article

MDM2 Amplification and PI3KCA Mutation in a Case of Sclerosing Rhabdomyosarcoma

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Received 9 March 2013; Accepted 24 April 2013

Academic Editor: Cyril Fisher

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A rare sclerosing variant of rhabdomyosarcoma characterized by prominent hyalinization and pseudovascular pattern has recently been described as a subtype biologically distinct from embryonal, alveolar, and pleomorphic forms. We present cytogenetic and molecular findings as well as experimental studies of an unusual case of sclerosing rhabdomyosarcoma. The primary lesion arose within the plantar subcutaneous tissue of the left foot of an otherwise healthy 23-year-old male who eventually developed pulmonary nodules despite systemic chemotherapy. Two genetic abnormalities identified in surgical and/or autopsy samples of the tumor were introduced into 10T1/2 murine fibroblasts to determine whether these genetic changes cooperatively facilitated transformation and growth. Cytogenetic analysis revealed a complex abnormal hyperdiploid clone, and MDM2 gene amplification was confirmed by fluorescence in situ hybridization. Cancer gene mutation screening using a combination of multiplexed PCR and mass spectroscopy revealed a PIK3CA exon 20 H1047R mutation in the primary tumor, lung metastasis, and liver metastasis. However, this mutation was not cooperative with MDM2 overexpression in experimental assays for transformation or growth. Nevertheless, MDM2 and PIK3CA are genes worthy of further investigation in patients with sclerosing rhabdomyosarcoma and might be considered in the enrollment of these patients into clinical trials of targeted therapeutics.

1. Introduction

Rhabdomyosarcoma (RMS) is subdivided into three major variants: embryonal, alveolar, and pleomorphic. Embryonal and alveolar subtypes are most common sarcomas of childhood and adolescence. Better clinical outcome is associated with botryoid and spindle cell variants of embryonal RMS. In particular, the spindle cell variant in childhood is considered to be of low malignant potential with excellent overall patient survival. Pleomorphic RMS is rare and highly aggressive adult sarcomas typically arising in the deep soft tissue of the extremities. Even rarer are recently described spindle cell and sclerosing variants of RMS in adults. Due to their rarity, the experience with the newer subsets is limited but appears to show poor outcome in adults. Sclerosing variant of RMS as a distinct entity was initially reported in three cases by Mentzel and Katenkamp in 2000 [1]. Histologically the tumor is characterized by polygonal to spindle-shaped neoplastic cells forming anastomosing cords in pseudovascular clefts and a highly sclerotic, hyalinized matrix. Rare rhabdomyoblasts can be seen and the skeletal muscle differentiation is evidenced by immunoreactivity for desmin, MyoD1, and myogenin. In a subsequent series of four additional cases, Folpe considered these tumors to be either
highly unusual variants of adult embryonal rhabdomyosarcoma or an entirely novel subcategory of rhabdomyosarcoma [2]. In these and other reported cases, lesions arose slightly more commonly within the distal extremities, but others have been observed in the head and neck [3], retroperitoneum, and scrotum [4]. There is no particular gender predominance in patients ranging in age from young children to older adults. With fewer than 30 cases reported, genetic analysis has been limited. To date, only six karyotypes [5–7] and one comparative genomic hybridization [8] have been reported showing aneuploidy with numerous chromosomal gains but no regional amplifications [5–7]. Reciprocal translocations typical of alveolar rhabdomyosarcoma, either t(1;13)(p36;q14) or t(2;13)(q35;q14), have not been present. In one case, typical of alveolar rhabdomyosarcoma, either t(1;13)(p36;q14) or t(2;13)(q35;q14), have not been present. In one case, comparative genomic hybridization revealed loss of chromosome 10q22, loss of chromosome Y, and trisomy 18 [8]. Recently, single nucleotide polymorphism genotyping of a sclerosing rhabdomyosarcoma revealed amplification within the 12q13-15 region, including the genes SAS, GLI1, CDK4, and MDM2 [9]. Herein we describe a case of sclerosing rhabdomyosarcoma studied by karyotyping, mutational screening of 53 cancer genes, and in vitro correlating analyses.

2. Materials and Methods

Representative 5-μm thick sections of formalin-fixed, paraffin-embedded tissue from the open biopsy specimen were stained with hematoxylin and eosin. For immunohistochemistry, three to four μm unstained paraffin-sections were prepared. Immunohistochemical staining was performed on Ventana (Tucson, AZ, USA) XT stainers. Primary antibodies are as follows: smooth muscle actin (1A4, Dako, Carpinteria, CA, USA), desmin (DE-R-II, Ventana, Tuscon, AZ, USA), myogenin (F5D, Dako), H-Caldesmon (h-CD, Dako), CD99 (H036-1.1, Cell Marque, Roklin, CA, USA), S100 (Polyclonal, Ventana), HMB45 (HMB-45, Dako), cytokeratin cocktail (AE1/AE3/PCK26, Ventana), EMA (E29, Cell Marque), CD54 (QBEnd/10, Ventana), and CD31 (IA10, Cell Marque). Ventana’s Ultraview diaminobenzidine chromogen kit was used for secondary antibodies and detection.

2.1. Cytogenetic Analysis. Fresh tumor in RPMI tissue culture medium (Invitrogen, Carlsbad, CA, USA) was sent to the Cytogenetics Laboratory within 2 hours from time of biopsy. The sample was enzymatically dissociated using Type 1 Collagenase (cat. no. LS004196; Worthington Biochemical, Lakewood, NJ, USA) at a final concentration of 1 unit/mL for 90 minutes. The dissociated cells were cultured in RPMI plus 15% fetal bovine serum (Irvine Scientific, Santa Ana, CA, USA) or a combination of this with Chang (Irvine Scientific) complete medium in closed tissue culture flasks for 5 and 7 days. Cell harvest and preparation of slides were performed according to standard laboratory protocol. Twenty-four G-banded (trypsin/Wright stain) metaphase cells were analyzed at approximately the 300 band level.

2.2. Fluorescence In Situ Hybridization. Fluorescence in situ hybridization was performed using Abbott probes for FOXOA1 (13q34) and MYCN (2p24.1) with a control probe for the 2 centromere (CEP 2) (Abbott Molecular, Des Plaines, IL, USA) and the Zytovision MDM2 (12q14.3-15) probe with a 12 centromere probe (CEN 12) as control (Zytovision, Bremerhaven, Germany). Hybridization methods were per manufacturer’s instructions and using a HYBridiTybridization system (Abbott Molecular, Des Plaines, IL, USA). Interphase cells were evaluated using a Nikon Eclipse E800 (Nikon Corporation, Tokyo, Japan). One hundred interphase cells were scored for the FOXOA1 and MYCN/CEP2 probes. Two hundred interphase cells were scored for the MDM2/CEN12 probe set. The cells were captured on a Zytovision computer system with a digital camera (Genetix-Applied Imaging, San Jose, CA, USA).

2.3. Mass Spectrometry-Based Mutation Detection. Multiplex mutation analysis was performed on the Mass Array system (Sequenom, San Diego, CA, USA) using a panel that screens for 647 mutations across 53 genes, exactly as previously described [10]. A PIK3CA H1047R mutation identified by this approach was confirmed by Sanger sequencing.

2.4. Cell Culture. 10T1/2 cells and 10T1/2-H1047R cells have been previously described [11]. Cells were cultured in 4.5 g/L glucose DMEM (Invitrogen) supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin (Sigma-Aldrich, St. Louis, MI, USA) in 5% CO2 in air at 37°C.

2.5. Vector Transfection. The MDM2 vector was kindly provided by Dai et al. (Department of Biochemistry and Molecular Biology, School of Medicine, Oregon Health and Science University) [12]. Transient transfections were carried out using Lipofectamine 2000 (Invitrogen/Life Technologies, Grand Island, NY, USA). pcDNA3 empty vector (Dharmacon-Thermo Fisher Scientific, Waltham, MA, USA) was used as control.

2.6. Immunoblotting. 10T1/2 cells were lysed in radioimmunoprecipitation assay (RIPA) buffer or NP40 buffer containing both protease and phosphatase inhibitor (Sigma-Aldrich). The lysates were homogenized and centrifuged at 8000 g for 10 minutes. The resulting supernatants were used for immunoblot analysis by mouse anti-β-actin (cat. A1978; Sigma), mouse anti-MDM2 (cat. sc-965; Santa Cruz Biotechnology, Dallas, TX, USA), rabbit anti-Akt (cat. no. 9272; Cell Signaling, Danvers, MA, USA) or rabbit anti-phospho-Akt (Ser473) (cat. no. 4060; Cell Signaling Technology, Beverly, MA, USA).

2.7. Soft-Agar Colony Formation Assay. 10T1/2 cells were plated at 6 x 103 cells per well in a 6-well plate with soft-agar with DMEM and 10% fetal bovine serum (SeepagueAgarose; cat. 50101, Lonza, Allendale, NJ, USA). MDM2 vector or empty vector was transfected to the cells before plating the top layer. After the cells were plated 14 days, colonies were counted.
2.8. In Vitro Growth Assay. 10T1/2 cells were plated at $2 \times 10^3$ cells of each cohort per well in a 96-well plate. MDM2 vector or empty vector was transfected to the cells before plating the top layer. After cell incubations, in vitro growth was assayed using CellTiter 96 AQUEous One Solution Cell Proliferation Assay system (Promega, Madison, WI, USA) and SpectraMax M5 luminometer (Molecular Devices, Sunnyvale, CA, USA).

3. Results

3.1. Case Report, Histopathological Diagnosis, and Cytogenetics Analysis. An otherwise healthy 23-year-old man presented with a 6-month history of an enlarging mass within the plantar aspect of his left foot. MRI revealed an 8 cm mass infiltrating the plantar surface, extending through the dorsal...
surface, and circumferentially involving the 2nd through 4th metatarsals (Figure 1(a)). Open biopsy was performed and light microscopy revealed heavily hyalinized matrix with poorly formed alveolar spaces lined by round hyperchromatic cells (Figure 1(b)). Some cells had scant eosinophilic cytoplasm and other areas showed spindling of tumor cells. Mitotic activity was easily identified. Immunohistochemical staining highlighted skeletal muscle differentiation with strong, diffuse positivity for desmin, and patchy nuclear myogenin positivity (Figures 1(c) and 1(d), resp.). There was scattered nuclear MDM2 immunoreactivity within several cells (Figure 1(e)), whilst a CDK4 immunostain was negative (data not shown). In concert with the marked stromal sclerosis, these results were consistent with the pattern previously reported for sclerosing rhabdomyosarcoma.

Cytagenetic analysis of tumor from the original biopsy revealed fourteen of twenty-four metaphase cells to have a complex hyperdiploid (near-triploid) karyotype. These cells contained approximately 20 to 50 double minutes, shown by FISH (below) to include amplification of the MDM2 gene. The karyotype, described according to Folpe et al. (2009) [13], was 71–95<3n>, XX–Y, +1, +add(1)(p13); +2, +5, +7, +7, +8, +8, 14, +16, +17, +add(18)(q22), add(19)(q13.3); x2, +22, +22, +22, +20–50dmin (Figure 1(g)).

Interphase fluorescence in situ hybridization (FISH) showed that 15.5% of 200 nuclei had amplification of MDM2. These results confirmed amplification of MDM2 in the form of the double minutes observed in the metaphase karyotype, as well as the presence of multiple copies of chromosome 12 (Figure 1(g)). FISH results for MYCN and for the FOXO1A break-apart probe were within normal limits (data not shown).

Staging studies, including operative sampling of left inguinal and iliac lymph nodes due to an abnormal PET scan, did not reveal evidence of metastatic disease. The patient underwent systemic chemotherapy with vincristine, dactinomycin, and cyclophosphamide as a part of a clinical trial. He was offered a below-the-knee amputation, but he declined in favor of radiation. He received a total dose of 50.4Gy/28 fractions and had an excellent response in the foot. Unfortunately, he developed pulmonary metastases immediately upon the completion of chemotherapy. Subsequent therapies included irinotecan with temozolomide, doxorubicin with ifosfamide, and sorafenib, none of which resulted in clinical response. He also underwent surgical resection of a massive and symptomatic right lower lung metastasis and received several courses of palliative radiation therapy. The patient expired 25 months after diagnosis.

A complete autopsy was performed twenty seven hours after death. Tumor masses were present in all lung lobes, nearly replacing the right lung. There were multiple metastases in both liver lobes, adjacent to esophagus, in the right adrenal, and in the right frontal cortex of the brain, as well as invading the chest wall, diaphragm, and multiple vertebrae. Fresh tissue was obtained sterilely for culture and frozen aliquots from the right chest, left upper lobe, and liver as well as normal skin and skeletal muscle (frozen only) immediately after opening of the body. Histologically the tumor was similar to that in the prior tumor biopsy with somewhat more cytologic atypia and a range of 30 to 90% necrosis (Figure 2).

Genotyping performed on tumor from the autopsy revealed a PIK3CA exon 20 H1047R (catalytic domain) mutation in the primary tumor, lung metastasis, and liver metastasis. No other mutations were identified in the genes listed in Table 2.

3.2. In Vitro Growth Analysis of Combined MDM2 Amplification and PIK3CA-Activated Mutation. To investigate the effect of combined MDM2 amplification and the PIK3CA activating mutation, 10T1/2-PIK3CA H1047R fibroblasts transfected with an MDM2 overexpression construct versus empty vector control were studied. As expected, the MDM2 vector induced MDM2 protein overexpression (Figure 3(a)). Similarly, 10T1/2 cells harboring the PIK3CA H1047R mutation showed higher Akt phosphorylation compared with control 10T1/2 cell (Figure 3(a)). Next, we compared anchor-independent growth and anchorage-dependent proliferation of 10T1/2 control cells and 10T1/2-H1047R cells with or without the MDM2 vector. The PIK3CA H1047R mutation induced anchor-independent growth, but MDM2 transient transfection could not alone induce anchor-independent growth or accelerated proliferation in control 10T1/2 cells (Figures 3(b) and 3(c)). These results suggest lack of synergy between MDM2 amplification and PIK3CA mutation with respect to the tumor cell-autonomous growth characteristics tested.

4. Discussion

In contrast to childhood RMS, the adult variants are believed to be highly aggressive irrespective of the morphologic subtype. The pleomorphic variant of RMS, exclusively seen in adults, has been extensively studied. Recently described variants in adults include spindle cell and sclerosing RMS. One of the limitations of understanding biology of these new entities is the overall rarity of such cases. It has also been noted that about 15–20% cases of adult spindle cell RMS show morphological overlap with the sclerosing variant [14, 15]. This observation suggests close relationship between the two subsets and raises the possibility of morphological variation of a single-tumor type. Future molecular and genetic studies will help address this issue, but for now these two variants are co-classified as a new distinct subset of adult RMSs [16].

The sclerosing variant of rhabdomyosarcoma presents a significant diagnostic challenge. From its hyalinizing pseudovascular appearance and sometimes focal and dot-like desmin and myogenin expression, these tumors could easily be mistaken for variants of angiosarcoma, extraskeletal myxoid chondrosarcoma, osteosarcoma, or sclerosing epithelioid fibrosarcoma.

Sclerosing rhabdomyosarcoma is an aggressive sarcoma. Among the original three cases reported by Mentzel and Katenkamp, one exhibited progressive pulmonary metastases despite systemic chemotherapy [1]. In one of the four cases reviewed by Folpe et al., there were extensive intra-abdominal and lymph node metastases, and this patient expired within
Figure 2: Disseminated disease at necropsy. (a) Cross-section of liver demonstrating metastatic nodules. (b) Histological section of lung with metastatic sclerosing rhabdomyosarcoma. Hematoxylin-eosin, insert original magnifications ×200.

Table 1: Summary of reported cytogenetic abnormalities in sclerosing rhabdomyosarcoma.

| Author                  | Year | Cases | Notes                                                                 |
|-------------------------|------|-------|----------------------------------------------------------------------|
| Mentzel and Katenkamp   | 2000 | 3     | No ARMS fusion transcripts due to t(1;13) or t(2;13) found by RT-PCR  |
|                         |      |       | Cases 1 and 3: inadequate RNA for RT-PCR. Case 2: no evidence of     |
|                         |      |       | PAX3/FOXO1A or PAX7/FOXO1A fusion gene by RT-PCR. Case 4: not tested |
|                         |      |       | due to the absence of nonembolized tissue                            |
| Folpe et al.            | 2002 | 4     | 4 cases negative for ARMS fusion transcripts (PAX3- and PAX7-FOXO1A) |
|                         |      |       | by RT-PCR                                                              |
| Vadgama et al.          | 2004 | 1     | No ARMS fusion transcripts by RT-PCR                                   |
| Chiles et al.           | 2004 | 13    | Tumor cells negative for FOXO1A-disrupting translocation by FISH; karyotype: 44–49,XX,+del(1)(p22)[2],+11,+16[5],+18[12],+21[3],−22[cp13] |
|                         |      |       | Case 1: normal. Case 2: complex structural and numerical abnormalities |
|                         |      |       | with numerous double minutes in most cells; case 3: less complex, with |
|                         |      |       | balanced translocation 46,XX,t(5;20)(q31;p13)                         |
| Croes et al.            | 2005 | 1     | CGH: loss of 10q22, loss of chromosome Y, gain of chromosome 18 (trisomy) |
| Zambrano et al.         | 2006 | 3     | SNP array: complex aneuploid pattern including gains and losses of whole |
|                         |      |       | chromosome and an amplification of 12q13-15. FISH analysis showed     |
|                         |      |       | amplification of HMGA2 and MDM2                                       |
| Kuhnen et al.           | 2006 | 1     | SNP array: complex aneuploid pattern including gains and losses of whole |
| Bouron-Dal Soglio et al. | 2009 | 1     | chromosome and an amplification of 12q13-15. FISH analysis showed     |

Among the few karyotypes reported, no characteristic numerical alteration of the chromosomal complement has emerged (Table 1). None of the cases have shown reciprocal translocation t(1;13)(p36;q14) or t(2;13)(q35;q14), which are characteristic of alveolar rhabdomyosarcoma.

Bouron-Dal Soglio et al. reported a highly complex aneuploid pattern by SNP array, including whole chromosome trisomy 5, 7, 8, 11, 15, 16, 20, and 22; tetrasomy 4 and 18; and monosomy 1, 2, 3, 9, 10, 13, 14 [9]. Their analysis revealed amplification at region 12q13-15 which included the HMGA2 and MDM2 genes but not CDK4/SAS/GLI. Both the initial and recurrent lesions possessed identical amplifications. Their pattern was similar to an analysis of 38 well-differentiated/dedifferentiated liposarcomas which revealed overexpression of both HMGA2 and MDM2 in all cases, but with an inconsistent amplification of CDK4 in 13% of cases [17]. Amplification and overexpression of MDM2, CDK4, GLI, and SAS genes of the 12q13-15 region has been associated with other sarcomas such as well-differentiated and dedifferentiated liposarcoma, leiomyosarcoma, and a significant subset of alveolar and embryonal rhabdomyosarcomas [18] and even sclerosing epithelioid fibrosarcoma [19].

Ours is the second reported case of sclerosing rhabdomyosarcoma in which MDM2 amplification has been demonstrated [9]. The histological sections did not show areas resembling spindle cell RMS, and hence was a pure variant. Despite some clinical similarities to alveolar RMS, including age range and frequent location within the extremities, translocations involving the FOXO1A (also referred to as FOXOA10) gene have been absent. Our case further confirms sclerosing RMS as a distinct diagnostic entity and implicates MDM2 amplification in the pathogenesis of this tumor. This may aid in the diagnosis of future cases. Aside from the potential diagnostic significance of this finding, enhanced MDM2 expression or related p53 abnormalities, if present, may render sclerosing RMS vulnerable to an ever-expanding array of small molecule inhibitors. For example,
Figure 3: (a) Western blotting of MDM2, Akt, phosphoAkt, and β-actin. Total cell lysates were isolated 48 h after transfection. (b) Soft-agar colony formation assay (means ± SD, n = 3). Black line shows significant difference (P < 0.05). (c) Relative proliferation rates of 10T1/2 cells and 10T1/2-H1047R cells transfected with MDM2 vector or empty vector, as measured by MTS assay (means ± SD, n = 3).

Table 2: Genes screened by solid tumor sequenom panel.

| Genes | Genes | Genes | Genes | Genes | Genes | Genes | Genes | Genes | Genes |
|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| AKT1  | CTNNB1 | FGFR1 | GNAS  | KRAS  | NRAS  | PIK3R5 | STAT1 |       |       |
| AKT2  | EGFR  | FGFR2 | HRAS  | MAP2K1| NTRK1 | PKHD1 | TEC   |       |       |
| AKT3  | ERBB2 | FGFR3 | IDHI   | MAP2K2| NTRK2 | PRKCB1|       |       |       |
| ALK   | ERCC6 | FGFR4 | IDH2   | MAP2K7| NTRK3 | RAF1  |       |       |       |
| BRAF  | FBX4  | FOXL2 | IGFIR  | MET   | PDGFRA| RET   |       |       |       |
| CDK4  | FBXW7 | GNAI1 | KDR    | MYC   | PIK3R1| SMO   |       |       |       |
| CSF1R | FES   | GNAQ  | KIT    | NEK9  | PIK3R4| SOS1  |       |       |       |
Author's Contribution

Conflict of Interests

Acknowledgments

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