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Is the *EWS/FLI-1* Fusion Transcript Specific for Ewing Sarcoma and Peripheral Primitive Neuroectodermal Tumor?

A Report of Four Cases Showing This Transcript in a Wider Range of Tumor Types

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The presence of t(11;22) (q24;q12) is often considered diagnostic of Ewing sarcoma and peripheral primitive neuroectodermal tumor. We report four cases, all of which possessed this translocation as detected by reverse transcriptase polymerase chain reaction and confirmed by sequencing with or without fluorescent* in situ *hybridization, but none of which were Ewing sarcoma or peripheral primitive neuroectodermal tumor by histological criteria. Two were polyphenotypic tumors and two were mixed embryonal and alveolar rhabdomyosarcomas. Only one case was positive for MIC2 by immunohistochemistry and only in a rare cell. Two cases (one polyphenotypic tumor and one rhabdomyosarcoma) had double minute chromosomes with >100 copies of the* MDM2 *gene. The presence of the t(11;22) (q24;q12) translocation should probably not be considered diagnostic of Ewing sarcoma and peripheral primitive neuroectodermal tumor in the absence of supporting histological evidence. The presence of this translocation in Ewing sarcoma and peripheral primitive neuroectodermal tumor has been taken as evidence that these two tumors are related. Extending this relationship to include some polyphenotypic tumors and some rhabdomyosarcomas may not be justified unless additional evidence is gathered. Pathologists and oncologists will need to decide whether treatment regimens for tumors are better based on phenotype rather than genotype when these two profiles are seemingly in conflict. (Am J Pathol 1996, 148:1125–1138)

With the advent of each technical advance in pathology, the accuracy of categorizing small round cell tumors of childhood has increased. Electron microscopy and immunohistochemistry are now routinely used to help diagnose the vast majority of cases of neuroblastoma, rhabdomyosarcoma, lymphoma, Ewing sarcoma, and peripheral primitive neuroectodermal tumor (pPNET). This is more than an academic exercise as treatment regimens for these tumors vary considerably. The more recent arrival of molecular genetic and cytogenetic techniques to the pathology laboratory has resulted in an additional increase in diagnostic accuracy, as well as providing prognostic determinants and an increased understanding of tumor pathogenesis and relationships between individual tumor types. Certain genetic translocations have come to be associated with specific childhood tumors. For instance, the t(2;13) (q35;q14), which fuses the *PAX3* gene on chromosome 2 to the *FKHR* (also known as *ALV*) gene on chromosome 13 is associated with alveolar rhabdomyosarcoma[1] and the t(11;22) (q24;q12), which fuses the *FLI-1* gene on chromosome 11 to the *EWS* gene on

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chromosome 22 is associated with Ewing sarcoma and pPNET.[2–4] A different t(11;22) translocation at (p13;q12) which fuses the *EWS* gene with the *WT1* gene on chromosome 11 is associated with the intra-abdominal small cell desmoplastic tumor.[5,6] Although originally identified in karyotypic spreads, more recently, some of these genetic alternations have been detected using the reverse transcriptase polymerase chain reaction (RT-PCR).[7–9] The t(11;22) (q24;q12) translocation is seen in up to 85% of cases of Ewing sarcoma and pPNET[2–4] and is said to be specific for these two tumors.[10] Earlier studies based on phenotype had suggested that these tumors were related histogenetically,[3,11] and the identification of a genetic mutation common to both of these tumors provides additional evidence to support this concept. From this has emerged the current thinking that pPNET, although neural in nature, should be managed in a similar fashion to Ewing sarcoma, rather than as neuroblastoma, another childhood neural tumour, from which pPNET differs in both phenotype and genotype.

As any diagnostic test becomes increasingly used, its accuracy and limitations gradually become known. We have identified four cases not diagnosed as either Ewing sarcoma or pPNET, which unexpectedly also possessed the same t(11;22) (q24;q12) translocation as detected by RT-PCR as Ewing sarcoma and pPNET tumors. These four cases form the basis of this report and raise the questions of genotype *versus* phenotype in the classification of pediatric tumors and, in turn, the most appropriate management of such cases.

Case Histories

Case 1

This girl presented at 5 years of age with a 6-month history of progressive weakness in the left arm, associated with loss of muscle bulk and pain in the left neck and shoulder region. Examination revealed a firm supraclavicular mass fixed in the posterior triangle of the neck. The left arm demonstrated weakness of all major muscle groups with normal sensation. Radiological studies (chest x-ray, computerized tomography (CT) and magnetic resonance imaging scan) revealed a tumor in the axilla that extended proximally into the epidural space at the level of C3-C4 and the presence of multiple pulmonary nodules. Bone scan and bone marrow biopsy were normal. At surgery, the tumor was noted to displace the spinal cord and to extend along the brachial plexus, permitting only a partial resection. The patient was treated with five cycles of a 5-day course composed of vincristine, actinomycin D, and cyclophosphamide alternating with five cycles of a 2-day course of ifosphamide and VP-16 (etoposide). Between the fourth and fifth cycles of chemotherapy, she received 2000 cGy of radiation to the whole chest and 5000 cGy to the left shoulder. After completion of chemotherapy, additional surgical debulking of the tumor was performed (still leaving residual tumor) along with resection of two lung metastases. She was then given six cycles of cisplatinum, adriamycin, and VM-26. The patient is currently alive with tumor 20 months from diagnosis, with no increase in tumor size.

Case 2

This boy presented at 37 months of age with the sudden onset of abdominal pain and shock. He was noted to have a very large abdominal mass and free fluid in the abdomen. A CT scan identified a large nonhomogeneous retroperitoneal mass contiguous with the liver. Limited laparotomy revealed a friable mass, which was biopsied. He was treated with eight cycles of chemotherapy consisting of vincristine, cyclophosphamide, doxorubicin, ifosphamide, and etoposide, given together over a 48-hour period. After four cycles, a dramatic reduction in the mass size occurred, and surgical re-exploration was undertaken. The mass was resected in total with the exception of a small portion at the porta hepatis. Pathological examination revealed similar histology to that demonstrated in the original biopsy. He subsequently received 3000 cGy irradiation to the whole abdomen and has remained clinically well. However, a CT scan done 4 months after completion of his irradiation demonstrated recurrence in the site of the known residual tumor after surgery. This was subsequently resected but with involved surgical margins. He is currently 12 months from diagnosis with no detectable disease.

Case 3

This girl presented at 20 months of age with a 4-week history of lumps on her neck and lumbar regions, associated with regression of developmental milestones, weight loss, lethargy, fever, and refusal to walk. A year earlier, she had been noted to have an interscapular mass that was diagnosed clinically as a lipoma. Physical examination revealed enlarged neck nodes bilaterally and two left-sided subcutaneous nodules, one of which was in continuity with a mass in the interscapular area. A large bulging mass with ill-defined margins was also noted in the lower lumbar area. CT scan and magnetic resonance imaging confirmed the

cervical lymphadenopathy, subcutaneous nodules, and the interscapular lesion. There was also a large paraspinal mass with bone destruction at vertebral levels L1-L3 and intraspinal extension. The chest CT scan revealed two lung nodules, and a bone scan showed multiple metastatic sites. The patient underwent an open biopsy of the interscapular mass as well as a needle biopsy of the paraspinal mass. She was started on chemotherapy that included two cycles of ifosphamide and VP-16 (etoposide) for 2 days followed by two cycles of VAC chemotherapy (vincristine on days 1 and 5, actinomycin D for 5 days, and cyclophosphamide for 5 days). She received a total of 12 courses (6 + 6) of this chemotherapy and achieved an almost complete response with minimal residual soft tissue densities in the paraspinal and interscapular areas. She underwent a second-look operation that demonstrated persistence of the tumor. Palliative radiotherapy was given to areas of known disease but shortly after she developed progressive disease and died 14 months from diagnosis.

Case 4

This girl presented at 4½ years of age with a 3-week history of swelling in her right parotid area. Physical examination revealed a mass of approximately 3 cm at the angle of the right jaw without any cervical or pre-auricular lymphadenopathy. The remainder of the examination was unremarkable. CT scan of the head, neck, and chest revealed only the presence of the soft tissue mass in the area of the right parotid gland. Bone scan and bilateral bone marrow aspirate and biopsies were normal. An excisional biopsy was performed and, subsequently, she was started on a chemotherapy protocol consisting of ifosphamide and VP-16 for 2 days followed by two cycles of vincristine plus cyclophosphamide on day 1, in addition to adriamycin continuous infusion on days 1 and 2. She also underwent radiation therapy to the tumor bed at a total dose of 4500 cGy. She is currently continuing on the same chemotherapy protocol until she completes a total of 12 courses (6 + 6). At present, she has no evidence of recurrent disease 9 months from diagnosis.

Materials and Methods

Pathology

For light microscopy, tissue was fixed in 10% buffered formalin and embedded in paraffin. Sections were cut at 4 μm. For immunohistochemistry, paraffin-embedded sections were stained for the following antigens by the indirect immunoperoxidase technique: low molecular weight keratin (monoclonal, prediluted; Becton Dickinson, Mountain View, CA), neuron-specific enolase (polyclonal, 1/200 dilution; Dako, Glostrup, Denmark), and S-100 protein (polyclonal, 1/200 dilution; Dako). The following antigens were stained by the avidin-biotin technique: vimentin (monoclonal, 1/100 dilution; Sigma Chemical Co., St. Louis, MO), smooth muscle actin (monoclonal, 1/50 dilution; Dako), desmin (monoclonal, 1/20 dilution; Dako,), CD57 (Leu 7; monoclonal, 1/5 dilution; Becton Dickinson), MIC2 (monoclonal, 1/50 dilution; Signet Labs, Dedham, MA), and epithelial membrane antigen (monoclonal, 1/10 dilution; Dako). For electron microscopy, tissue was fixed in a 4% paraformaldehyde/1% glutaraldehyde mixture, post-fixed in osmium tetroxide, and embedded in an Epon-Araldite mixture. Sections were cut at 50 nm, stained with uranyl acetate and lead citrate, and viewed on a Philips 400 electron microscope.

Southern Blotting

Each tumor sample was digested with proteinase K, and the DNA was extracted with phenol and chloroform and precipitated with ethanol as previously reported.[12] The DNA was digested with *Eco*RI (Boehringer Mannheim, Mannheim, Germany), and the DNA concentration was measured using a spectrophotometer. For *MYCN* gene copy determination, DNA from fresh human tonsil and a human neuroblastoma cell line (IMR32) known to contain 50 copies of *MYCN*,[13] both digested with *Eco*RI, were used as negative and positive controls, respectively. *MYCN* levels were assessed using a 1.0-kb *Eco*RI-*Bam*HI human *MYCN* cDNA insert from pNB-1 (Oncor, Gaithersburg, MD). This probe is specific for exon 2 of *MYCN*. The human J_κ probe, which represents the joining region of the immunoglobulin-κ light chain gene located at chromosome 2p14, served as the control for DNA loading. The probe insert is 1.8 kb long and hybridizes to a 9.4-kb germline band in *Eco*RI-digested human DNA. The *MDM2* gene probe was kindly provided by Dr. B. Vogelstein and is the *Xho*I fragment of the human colon cDNA (bp 1 to 949).[14] The probe hybridizes to bands at 6.6, 4.4, and 2.0 kb in *Eco*RI-digested human DNA. The Jκ probe was labeled by nick translation whereas pNB-1 and *MDM2* cDNA fragment probes were labeled using the random primer method as described previously.[12] For Southern blot analysis,

serial dilutions of restriction-endonuclease-digested DNA samples were size fractionated on 0.8% Tris-borate/EDTA agarose gels and transferred to nylon membranes (Boehringer Mannheim). After prehybridization with Hybrisol (Oncor, Gaithersburg, MD), the membranes were sequentially hybridized with the *MYCN*, Jκ, and *MDM2* probes. The membranes were washed to a final stringency of 0.2X saline sodium citrate (SSC) at 60°C for 15 minutes and exposed for 24 to 48 hours at −70°C to Kodak XAR-5 film (Rochester, NY) using intensifying screens. The *MYCN*, Jκ, and *MDM2* hybridization signals on the autoradiograms were quantitated by densitometric scanning using a Molecular Dynamics computing densitometer model 300AV (Molecular Dynamics Corp., Sunnyvale, CA). The *MYCN* and *MDM2* signals of each sample were normalized by comparison with the Jκ signal in each sample lane and by 10- to 100-fold dilution of tumor DNA relative to normal DNA on additional blots when amplification was detected.

Reverse Transcriptase Polymerase Chain Reaction

Polyadenylated RNAs were extracted using the Quick Prep mRNA purification kit from Pharmacia-LKB Biochemical Co. (Uppsala, Sweden) and were reverse transcribed using oligo dT (Bio-Rad Laboratories, Richmond, CA). For detection of the t(11;22) (q24;q12) translocation, cDNA was amplified using 11.3 (*FLI-1*) and 22.3 (*EWS*) primers.[7] Thirty cycles of amplification were performed with each cycle consisting of denaturation at 94°C for 30 seconds, annealing at 65°C for 1 minute, and extension at 72°C for 2 minutes. For detection of the t(2;13) (q35;q14) translocation, cDNA (5 μl of the reverse transcription reaction) was amplified using 5′ *PAX3* and 3′ *FKHR* primers as previously described.[8] The cycling parameters were as follows: 94°C for 1 minute, 60°C for 1 minute, and 72°C for 3 minutes for 35 cycles. The amplified fragments were identified by gel electrophoresis and ethidium bromide staining. The positive controls for the t(2;13) (q35;q14) and t(11;22) (q24;q12) translocations were RNAs from the alveolar rhabdomyosarcoma cell line RD13[1] and a pPNET cell line,[15] respectively, which had previously been shown to possess these two translocations. The negative controls included a neuroblastoma cell line lacking both translocations, a no-template control and a no-reverse-transcriptase control.

Nucleotide Sequencing

The amplified PCR products were purified by Magic PCR Preps DNA Purification System (Promega, Madison, WI) according to the manufacturer's instructions. The sequences were obtained by subcloning the PCR products into the TA cloning PCR vector (Invitrogen, San Diego, CA) and then using M13 universal primer and Sequenase (U.S. Biochemicals, Cleveland, OH), according to the manufacturer's instructions.

Cytogenetics

A sterile suspension of tumor cells was made by repeatedly slicing sections of tumor with scalpel blades. Two to five million cells were placed in 5 ml of minimal essential medium containing antibiotics and 10% fetal calf serum. Cells were harvested within 3 to 7 days of culture after treatment with colcemid (0.01 μg/ml) for 3 to 4 hours. Cell harvesting, G-banding, and photomicrography were carried out using standard procedures.[16]

Fluorescent in Situ *Hybridization (FISH)*

FISH was performed on cases 2 and 4 for which there was additional frozen material available. For each case, a 5-μm section was stained to locate tumor, following which tumor tissue was excised from the block and a cell suspension made. Normal lymphocyte cytogenetic controls were used in all FISH experiments to ensure that probe hybridization efficiency was adequate to detect chromosomal rearrangements associated with *EWS/FLI-1* fusion and that background signal was below levels that could lead to false positive hybridizations on nuclei. Cells were hypotonically treated with 0.56% KCl for 10 minutes, fixed in methanol/acetic acid 3:1, and then spread onto slides. The FISH method was based on published procedures.[17] The slides were denatured in 70% formamide in 2X SSC, followed by dehydration in an ethanol series. Probes for chromosomes 11 and 22 were those used by Selleri et al.[18] A total of 200 ng of the cosmid genomic probe 23.2 from chromosome 11q24 was labeled with digoxigenin dUTP by nick translation, whereas 200 ngs of the cosmid probe leukemia inhibitory factor gene from chromosome 22q12 was labeled with biotin-14 dUTP. The probes were placed on the slide under a coverslip ringed with rubber solution. Hybridization was carried out for 16 to 20 hours at 37°C. Washing consisted of three 5-minute washes in 50% formamide and 2X SSC, followed by three 5-minute

washes in 2X SSC at 45°C. The slides were then soaked in 3% bovine serum albumin, 0.1% Tween 20 in 4X SSC for 15 minutes at 37°C before incubating with anti-digoxigenin (Boehringer Mannheim) in fluorescein isothiocyanate (FITC)-conjugated avidin (Oncor) diluted 1:50 for 30 minutes at 37°C, followed by three 5-minute washes in phosphate-buffered detergent (Oncor) at 45°C. The signal was amplified by adding digoxigenin anti-mouse (Boehringer Mannheim) in anti-avidin (Oncor) diluted 1:50 for 15 minutes at 37°C, followed by three 5-minutes washes in phosphate-buffered detergent at 45°C. Next, 3% bovine serum albumin, 0.1% Tween 20 in 4X SSC was added for 15 minutes at 37°C, followed by rhodamine-conjugated anti-digoxigenin (Boehringer Mannheim) in FITC-conjugated avidin (Oncor) diluted 1:5 for 15 minutes at 37°C. This was washed off with phosphate-buffered detergent using three 5-minute washes at 45°C, and the preparations were counterstained and mounted with a 2:3 mixture of DAPI in antifade (Oncor). The photomicrographs were obtained using a Nikon Microphot-FXA epifluorescence microscope equipped with triple-band FITC/Texas red/4′,6-diamidino-2-phenylindole filters (Omega Optical, FL). Some images were captured by a cooled CCD camera (Photometrix, Tucson, AZ) and overlaid electronically using Gene Join Software (courtesy of Tim Rand and David Ward, Yale University, NH).

Comparative Genomic Hybridization

We used a modification[19] of the comparative genomic hybridization technique[20,21] to identify the chromosomal localization of any amplified sequences present in tumor DNA derived from case two. Briefly, equimolar amounts of denatured tumor DNA labeled with biotin and unlabeled size-fractionated human placental DNA were mixed together. The mixture was allowed to anneal for 1 hour in the presence of *COT1* DNA to block repetitive sequences. The mixture was then hybridized to normal chromosome metaphase spreads after detection with FITC-conjugated avidin. An amplified sequence was detected as a green fluorescent signal visualized by direct microscopic observation.

Results

Case 1

Pathology

The biopsies from the region of the brachial plexus and from the lung metastases appeared similar. Both contained a tumor composed of round to spindle-shaped cells arranged in sheets and small nests separated by varying amounts of extracellular matrix that ranged from myxoid to collagenous (Figure 1a). No better defined architectural arrangement was noted. There was a moderate degree of nuclear pleomorphism and frequent mitoses. Cytoplasm ranged from scanty to moderate in amount; no particular line of differentiation was apparent (Figure 1b). By immunohistochemistry, the tumor was diffusely positive for vimentin and focally positive for epithelial membrane antigen, actin, desmin, neuron-specific enolase, S-100 protein, and CD57 (Figure 1c). Only a rare cell was positive for MIC2, and this was cytoplasmic rather than surface in location (Figure 1d). Staining for the other antigens was negative. By electron microscopy, the tumor cells showed well formed junctions and occasional cytoplasmic processes. Abundant mitochondria and glycogen granules were noted. No neurosecretory granules or myofilaments were seen. Based on these studies, a diagnosis of polyphenotypic small cell tumor was made.

Genetic Studies

RT-PCR detected an *EWS/FLI-1* hybrid transcript implying the presence of a t(11;22) (q24;q12) translocation (Figure 2). This was confirmed by sequencing, which showed a 584-bp PCR product resulting from fusion of exon 10 of the *EWS* gene to exon 6 of the *FLI-1* gene (results not shown). No t(2;13) (q35;q14) hybrid transcript was detected. Cytogenetic studies were not successful. Southern analysis showed that neither the *MYCN* nor the *MDM2* genes were amplified (results not shown).

Case 2

Pathology

The original biopsy, the residual intra-abdominal tumor, and the recurrent tumor all appeared similar; much of the residual tumor was necrotic, whereas the recurrent tumor was only focally necrotic. The tumor was composed of extremely pleomorphic cells that tended to be arranged randomly but with focal fascicular and nesting growth patterns (Figure 3a). The nuclei ranged from small and ovoid to large and convoluted, with intranuclear pseudoinclusions of cytoplasm. Some multinucleated giant tumor cells were also seen (Figure 3b). Frequent mitoses including atypical ones were readily identified. Cytoplasm ranged from scanty to moderate in amount; some of

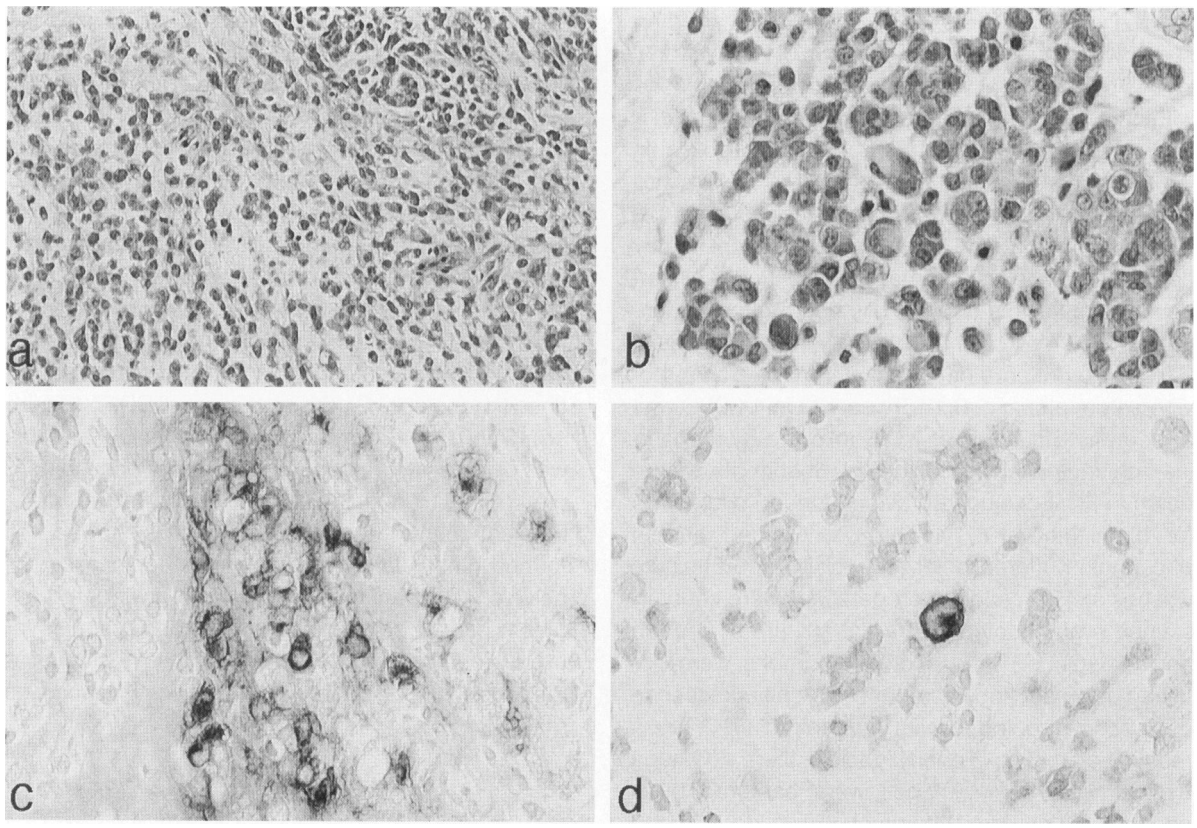

Figure 1. a: *Photomicrograph of the tumor from case 1 showing a poorly differentiated malignancy composed of small primitive cells arranged singly and in loosely cohesive nests (magnification, ×175).* b: *Tumor cells at higher magnification (×350).* c: *Immunoperoxidase stain for CD57 showing focal cytoplasmic positivity (×350).* d: *Immunoperoxidase stain for MIC2 also showing rare cytoplasmic positivity (×350).*

the larger cells possessed brightly eosinophilic cytoplasm, suggesting rhabdomyoblastic differentiation. Immunohistochemical studies showed the tumor to be diffusely positive for vimentin, keratin (Figure 3c), and desmin (Figure 3d) and focally positive for neuron-specific enolase, S-100 protein, and CD57. Staining for the other antigens tested including MIC2 was negative. Electron microscopy revealed cells with primitive cell junctions and occasional intermediate filaments. No tonofilaments or thick and thin myofilaments were noted. Some cell processes were found, but no neurosecretory granules were seen. Based on these observations, a diagnosis of anaplastic polyphenotypic tumor was made.

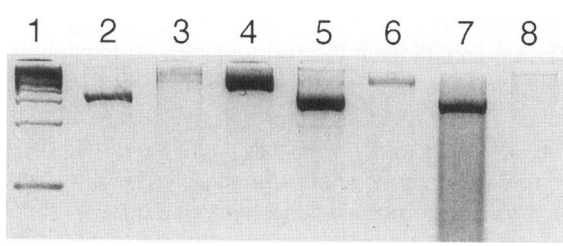

Figure 2. *RT-PCR analysis of the* EWS/FLI-1 *hybrid transcripts resulting from the t(11;22)(q24;q12) translocation. RT-PCR products were separated on a 4% agarose gel followed by ethidium bromide staining. Patient samples from case 1* (lane 4), *case 2* (lane 6), *case 3* (lane 5), *and case 4* (lane 7) *were run with a 100-bp ladder marker* (lane 1), *EW2 cell line, which possesses the t(11;22)(q24;q12) translocation* (lane 2), *NUB7 cell line, which lacks the t(11;22)(q24;q12) translocation* (lane 3), *and a no-template control* (lane 8). *A no-reverse-transcriptase control was also negative (result not shown).*

Genetic Studies

RT-PCR performed on the original specimen detected an *EWS/FLI-1* hybrid transcript (Figure 2). Sequencing showed a 649-bp product fusion of exon 10 of the *EWS* gene to exon 5 of the *FLI-1* gene (results not shown). No t(2;13) (q35;q14) hybrid transcript was detected. Cytogenetic studies showed a mixture of diploid and aneuploid cells with chromosome numbers in the range of 120 to 148 (near hexaploid). The aneuploid cells possessed a large number of double minute chromosomes reflecting gene amplification, as well as a deletion of 1p22 and extra material on 19p. Chromosomes 11 and 22 appeared normal. Nevertheless, by FISH, a translocation between chromosomes 11 and 22 was detectable (Figure 4). The *MYCN* gene was not found to be

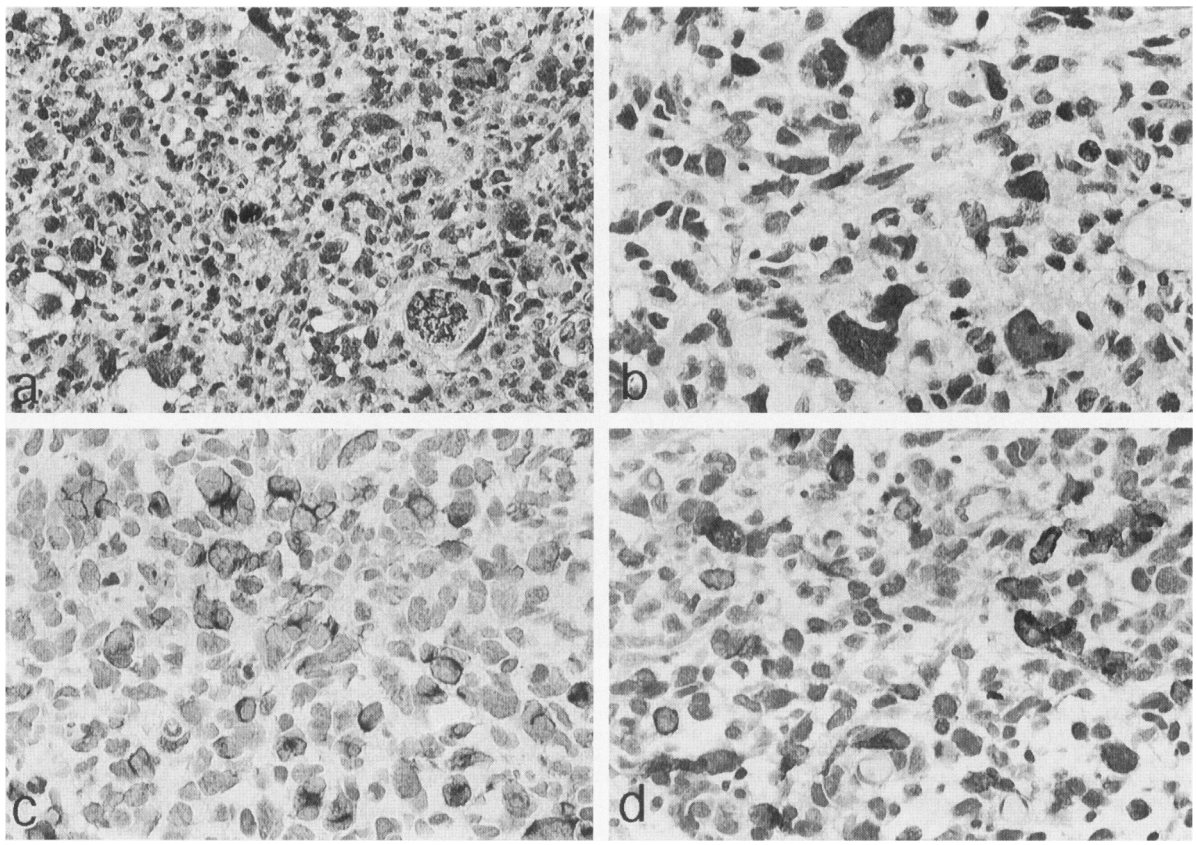

Figure 3. a: *photomicrograph of the tumor from case 2 showing a malignancy composed of very pleomorphic cells including small cells with scanty cytoplasm mixed with bizarre multinucleated tumor cells (magnification, ×175).* b: *Tumor cells at higher magnification (×350).* c: *Immunoperoxidase stain for low molecular weight keratin showing cytoplasmic staining of tumor cells, including both the small and large cell populations (×350).* d: *Immunoperoxidase stain for desmin showing similar results (×350).*

amplified (results not shown). Comparative genomic hybridization studies indicated the amplified genetic material localized to chromosome bands 12q13–14 in the region of the *MDM2* gene (Figure 5). Southern blots of tumor DNA with the *MDM2* cDNA probe showed that this gene was amplified at a copy number of >100-fold (Figure 6).

Case 3

Pathology

The original specimen from the paraspinal muscle showed a tumor-infiltrating skeletal muscle composed of ovoid to elongated cells with hyperchromatic nuclei and variable amounts of cytoplasm. Frequent mitoses were seen. Some cells possessed more abundant, brightly eosinophilic cytoplasm and eccentric nuclei, in keeping with rhabdomyoblastic differentiation. Glycogen was plentiful. Two distinct growth patterns were seen. Some areas had predominantly spindle-shaped cells separated by loose myxoid extracellular material (Figure 7a). Other foci showed nests of more rounded cells separated by connective tissue septa (Figure 7b). Some of these nests were solid, whereas others showed loss of centrally placed cells. By immunohistochemistry, the tumor was positive for desmin, actin, and vimentin (Figure 7c) but not for the other antigens tested including MIC2. Electron microscopy showed cells with abundant glycogen and paranuclear aggregates of thick and thin myofilaments. Some cells showed primitive sarcomeric structures with Z-band material (Figure 7d). Based on these results, a diagnosis of mixed embryonal and alveolar rhabdomyosarcoma was made. The recurrent tumor biopsied from the same region 1 year later showed only the embryonal component. The immunohistochemical profile was unchanged.

Genetic Studies

RT-PCR performed on both specimens detected an *EWS/FLI-1* hybrid transcript indicating the presence

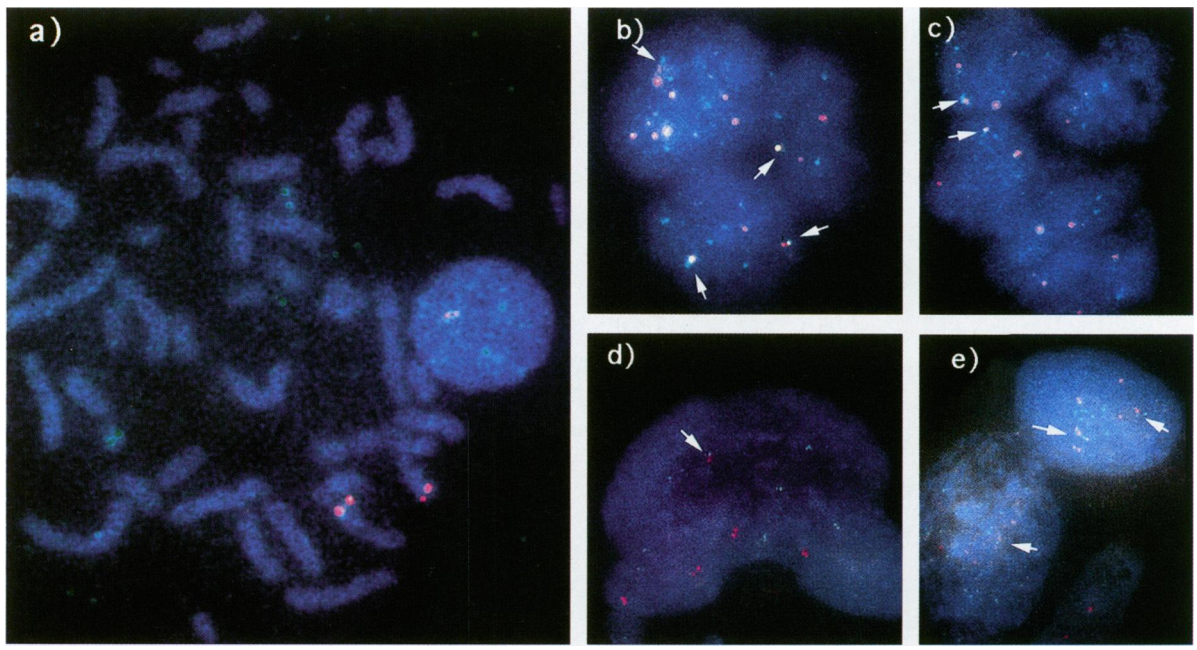

Figure 4. *Two-color FISH analysis of normal lymphocyte control cytogenetic preparation* (**a**), *case 2* (**b** *and* **c**), *and case 4* (**d** *and* **e**) *to indicate the presence of the t(11;22)(q24;q12) translocation. The leukemia inhibitory factor cosmid probe tagged with fluorescein (yellow-green) identifies chromosome 22q12 and the cosmid probe 23.2 tagged with rhodamine (red) hybridizes to chromosome 11q24. All preparations have been counterstained with 4',6-diamidino-2-phenylindole (blue). Tumor cell preparations were generated from frozen tumor made into cell suspensions. In cases 2 and 4, the presence of the translocation* (**arrows**) *in nuclei is detected by close juxtaposition of the rhodamine and fluorescein signals or in some cases an orange fusion signal. Additional signals for other chromosomes 11 and 22 are also apparent, in keeping with the highly aneuploid karyotype (85 to 200 chromosomes/nucleus) in both tumors.*

of a t(11;22) (q24;q12) translocation (Figure 2). Sequencing showed a 332-bp PCR product resulting from fusion of exon 7 of the *EWS* gene to exon 6 of the *FLI-1* gene (results not shown). No t(2;13) (q35; q14) hybrid transcript was detected. Cytogenetic studies were unsuccessful. Southern analysis could be carried out only on a later specimen and showed that neither the *MYCN* nor the *MDM2* genes were amplified (results not shown).

Case 4

Pathology

The specimen consisted of a portion of the parotid gland and a tumor mass measuring overall 3.5 × 3 × 2 cm. The tumor was similar in appearance to case 3 and was composed of small round to elongated cells with enlarged moderately pleomorphic nuclei and

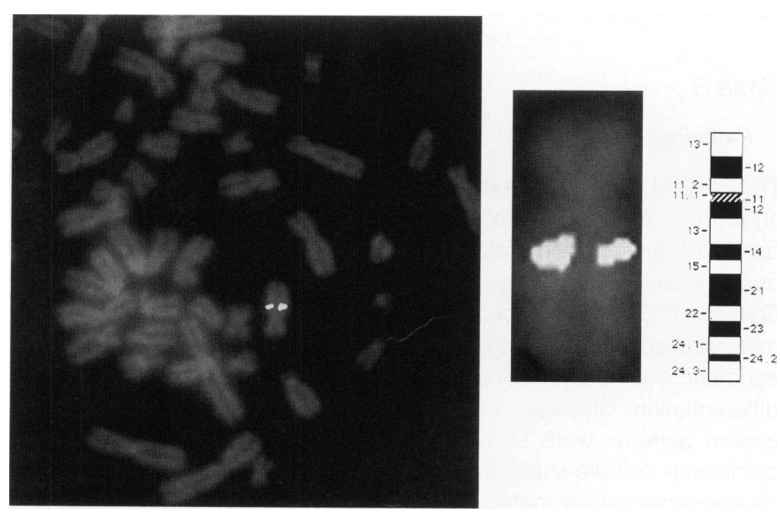

Figure 5. *Partial metaphase banded with 4',6-diamidino-2-phenylindole showing a strong hybridization signal at 12q12–13 (the location of* MDM2 *gene).*

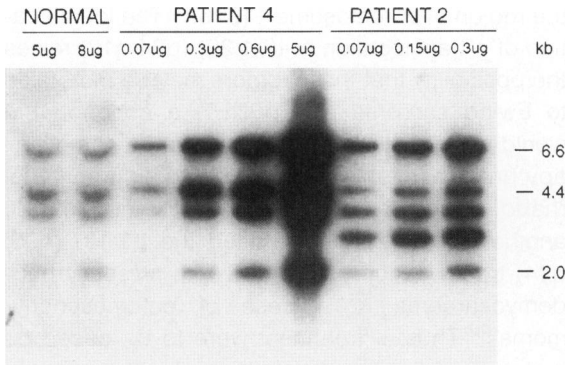

Figure 6. *Southern analysis of* MDM2 *gene.* Eco*RI-digested genomic DNA from cases 2 and 4 and one normal control was separated by agarose gel electrophoresis and transferred to a nylon membrane. The membrane was then hybridized with the* MDM2 *cDNA probe.*

frequent mitoses. Two different patterns of growth were noted. For approximately one-half of the tumor, the cells were arranged in a loose streaming pattern separated by myxoid extracellular material. Elsewhere, the tumor cells were arranged in solid nests separated by connective tissue septa. No convincing line of differentiation was appreciated. Nevertheless, by immunohistochemistry, the tumor was diffusely positive for desmin and vimentin and focally for actin. No staining for the other antigens including MIC2 was observed. Ultrastructural analysis detected primitive cell junctions and cytoplasmic aggregates of intermediate filaments. No myofilament or neurosecretory granules were found. Based on these results, a diagnosis of rhabdomyosarcoma with mixed embryonal and solid alveolar morphology was made.

Genetic Studies

RT-PCR detected an *EWS/FLI-1* hybrid transcript (Figure 2), and sequencing demonstrated a 332-bp PCR product with fusion of exon 7 of the *EWS* gene to exon 6 of the *FLI-1* gene (results not shown). No t(2;13) (q35;q14) hybrid transcript was detected. Cytogenetic studies performed after 7 days of culture

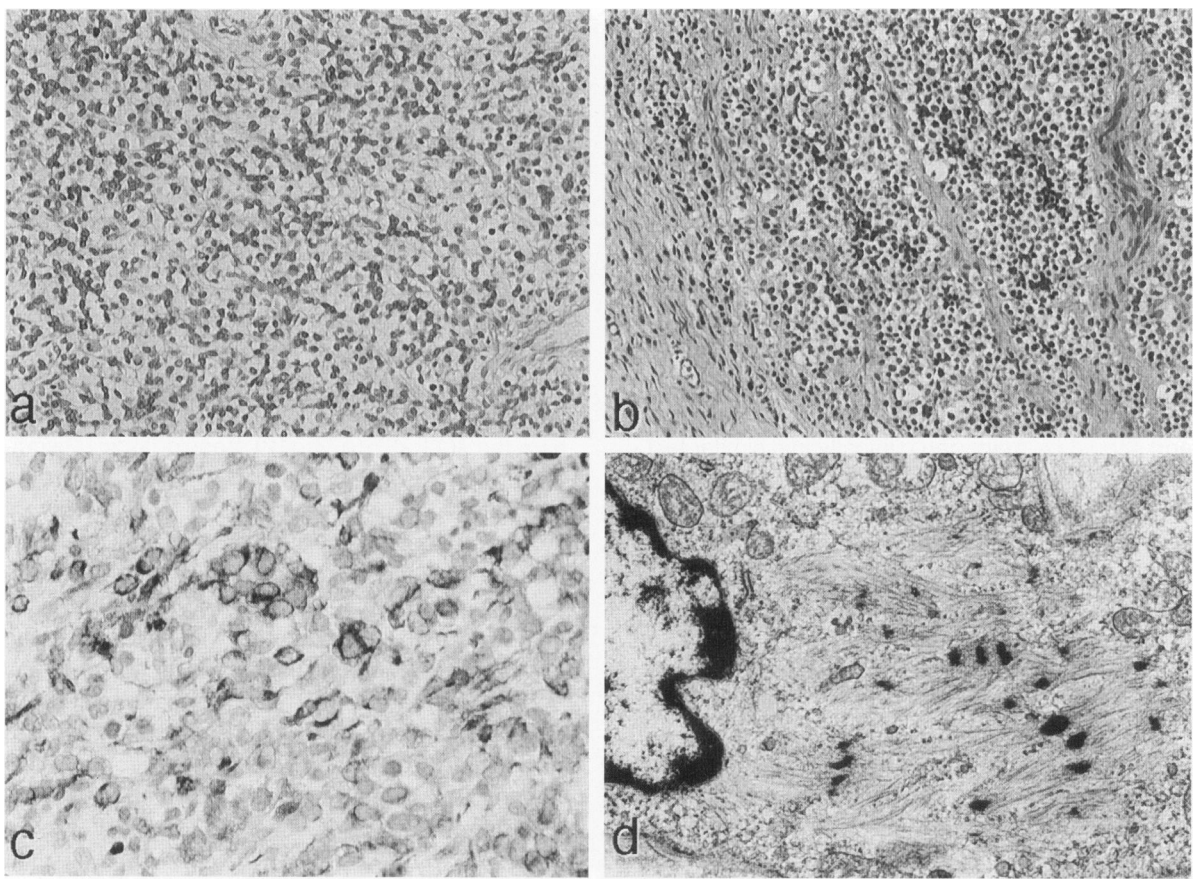

Figure 7. a: *Photomicrograph of the tumor from case 3 showing an area with the appearance of embryonal rhabdomyosarcoma, with cells separated by varying amounts of myxoid extracellular matrix. Many cells show obvious rhabdomyoblastic differentiation (magnification, ×175).* b: *Photomicrograph of the same tumor showing an area with the appearance of alveolar rhabdomyosarcoma, with nests of loosely cohesive, rounded tumor cells separated by fibrous septa (×175).* c: *Immunoperoxidase stain for desmin showing cytoplasmic positivity in tumor cells (×350).* d: *Electron micrograph showing primitive sarcomeres within a tumor cell (×6400).*

showed a highly abnormal karyotype with chromosome numbers in the 85 to 200 range. The chromosomes were unusually contracted, and the quality of the preparation was therefore not adequate for determining whether subtle chromosomal rearrangements such as the t(11;22) (q24;q12) translocation associated with the *EWS/FLI-1* hybrid transcript were present. A consistent finding in all of the aneuploid cells was the presence of numerous double minute chromosomes. As well, a 13q+ was present in all abnormal cells. By FISH, a translocation between chromosomes 11 and 22 was observed (Figure 4). Southern analysis showed that the *MYCN* gene was not amplified (results not shown), but the *MDM2* gene was amplified >100-fold (Figure 6).

Discussion

In Ewing sarcoma and pPNET, a translocation at t(11;22) (q24;q12) can be identified in up to 85% of cases by classical cytogenetics[2–4] and up to 90% of cases by RT-PCR,[7,9,22] which is said to be specific for these two tumors.[10] This translocation brings together the *EWS* gene on chromosome 22 and the *FLI-1* gene on chromosome 11, such that a hybrid transcript results[7] with increased transcriptional activity.[23] The size of the transcript varies with the individual tumor and can range from 300 to 700 bp depending on the chromosome break site. No significance, however, has yet been associated with these variations. The remaining 10% of cases may have other translocations such as t(21;22).[24] The presence of this mutation in both of these tumors has been used to support the concept that they are histogenetically related[3,11] and should be managed clinically in a similar fashion. An extension of this concept is the idea that this particular translocation defines the Ewing family of tumors.[22] We have tested a series of 16 cases of Ewing sarcoma and pPNET for the presence of this translocation by RT-PCR and found similar results (results not shown).

The presence of this translocation has also been noted, however, in a case diagnosed as a rhabdomyosarcoma with both myogenic and neural features[9,25] and in five of five cases diagnosed as malignant ectomesenchymoma.[26] The original description of this tumor is one that expresses a rhabdomyoblastic phenotype with focal neuroectodermal features[27] and thus is a type of polyphenotypic tumor. It has been suggested that ectomesenchymoma is related to embryonal rhabdomyosarcoma based on loss of heterozygosity in the region of chromosome 11p15.[28] The identification of a translocation at t(11;22) (q24;q12) raises the possibility that these tumors may be more akin to Ewing sarcoma and pPNET, a concept that would suggest that a Ewing sarcoma chemotherapy protocol might be preferable to an embryonal rhabdomyosarcoma protocol. On the other hand, another recent study identified the t(2;13) (q35; q14) translocation associated with alveolar rhabdomyosarcoma in a case of ectomesenchymoma.[29] Thus, if treatment were to be based on genotype rather than phenotype, depending upon which genetic abnormality is given priority, three different treatment protocols could be used to treat ectomesenchymoma.

Our cases 1 and 2 were also polyphenotypic by immunohistochemistry, yet were very different from each other in histology and clinical presentation, with neither one histologically typical of ectomesenchymoma. Perhaps case 1 may be thought of as related to an ectomesenchymoma, with a mixed neural and epithelial phenotype, but lacking muscle differentiation. The finding of a t(11;22) (q24;q12) translocation would then be in agreement with those of Sorensen et al.[26] In contrast, case 2 shares the same location and immunohistochemical profile as a different polyphenotypic tumor, the intra-abdominal small cell desmoplastic tumor, which occurs primarily in adolescent males.[30,31] These tumor cells typically co-express keratin and desmin and sometimes neural markers. Our case 2, however, has a very different histology and perhaps is an anaplastic variant of this tumor. However, the genetic findings in our case 2 are not those expected for an intra-abdominal small cell desmoplastic tumor. First, our case had the t(11;22) (q24;q12) translocation associated with Ewing sarcoma and pPNET and not the t(11;22) (p13;q12) associated with intra-abdominal small cell desmoplastic tumor.[5,6] Second, our tumor was composed of hexaploid cells that possessed double minute chromosomes. This latter finding is usually associated with neuroblastoma and retinoblastoma where it is a reflection of *MYCN* gene amplification.[32,33] For this reason, we determined *MYCN* gene copy in this case but found it to be diploid. We then performed comparative genomic hybridization to identify the chromosomal localization of any amplified sequences present in tumor DNA.[20,21] Our results indicated amplified genetic material present at chromosome bands 12q13–14 in the region of the *MDM2* gene (murine double minute).[14] We therefore screened Southern blots of tumor DNA with the *MDM2* cDNA probe

and confirmed amplification of *MDM2* with a copy number in excess of 100 copies per cell. The MDM2 protein binds to p53, thereby inactivating it.[14] Increased amounts of MDM2 could provide this tumor with a mechanism of overcoming the controlling influences on cell division exerted by p53 under normal circumstances. There is, however, no diagnostic significance of *MDM2* amplification as this has been documented in various sarcomas, including liposarcoma, malignant fibrous histiocytoma, and osteogenic sarcoma, and in gliomas[14,34] but only in rare cases of pediatric tumors including Ewing sarcoma, pPNET, and rhabdomyosarcoma.[35–38] Other genes in the same region such as *SAS*,[39] *GLI*,[40] and *CDK4*[34] were not investigated. Thus, although our case 2 shares some genetic findings as have been reported in Ewing sarcoma and pPNET, it differs greatly in its clinical presentation, histology, and immunohistochemical profile. For this reason, we classified it simply as an anaplastic polyphenotypic tumor.

Cases 3 and 4 were pure rhabdomyosarcomas by light microscopy, immunohistochemical profile, and electron microscopy. The only unusual histological feature, seen in both cases, was a mixture of embryonal and alveolar morphologies. No *PAX3-FKHR* fusion transcript was detected in either case by RT-PCR. The significance of this result is unclear as 1) only approximately 70% of cases of alveolar rhabdomyosarcoma possess this translocation,[25] 2) the material submitted for RNA extraction may not have represented the alveolar component of each tumor, and 3) the primers may not have been for the appropriate sequences. The last possibility is particularly true for case 4, in which there was an abnormality in chromosome 13q but none apparent for chromosome 2, suggesting some variant translocation might have occurred. For example, recently, some cases of alveolar rhabdomyosarcoma have been described with a t(1;13) translocation instead of t(2;13) (q35;q14).[41] Case 4 also had double minute chromosomes with >100 copies of the *MDM2* gene, a finding seen in rare cases of rhabdomyosarcoma as mentioned above.

It is worthwhile noting that none of the four tumors in this report were positive for MIC2 except for a rare cell in case 1. MIC2 has been found to be a reliable marker for Ewing sarcoma and pPNET with positive staining seen in >90% of cases. It has further been stated that, when the presence of the t(11;22) (q24;q12) translocation is combined with positive staining for MIC2, the accuracy of diagnosing Ewing sarcoma and pPNET approaches 100%.[22,42,43] In contrast, a lack of staining for MIC2 has been noted in polyphenotypic tumors studied by Swanson et al,[44] and this observation may provide a valuable diagnostic clue to separating these tumors from Ewing sarcoma and pPNET. Our results would tend to support this observation.

The finding of the Ewing sarcoma and pPNET t(11;22) (q24;q12) translocation in our four cases raises two issues: 1) the role of this translocation as a primary genetic alteration and 2) the diagnostic usefulness of this finding in patient management. It is possible that the presence of this translocation identifies a group of tumors that are all related, just as Ewing sarcoma and pPNET are believed to be.[22] Our cases 1 and 2 do not resemble Ewing sarcoma or pPNET at the clinical (the young ages of presentation, case 2 with an intra-abdominal location) or phenotypic level. Nevertheless, as both cases were ultimately classified simply as polyphenotypic tumors, it cannot be ruled out that they might be pathogenetically related to Ewing sarcoma and pPNET. If that is the case, our current definition of Ewing sarcoma and pPNET may be too narrow and may need to be redefined. At present, however, the presence of this translocation is probably necessary for a diagnosis of Ewing sarcoma and pPNET but is not sufficient on its own without confirming histology. The converse situation is more straightforward; in a case suspected to be Ewing sarcoma or pPNET that lacks the t(11;22) (q24;q12) translocation, this diagnosis is called into serious doubt.

The situation is different with tumors 3 and 4, both of which occurred in children younger than usual for Ewing sarcoma and which were rhabdomyosarcomas by conventional diagnostic criteria, although both were unusual in that they contained a mixture of embryonal and alveolar subtypes. The presence of a t(11;22) (q24;q12) translocation in rhabdomyosarcoma is an unusual event; in one review of 51 cases, none were found to possess this genetic change,[45] whereas a second review of 100 cases detected this change in 1 case of embryonal and 4 cases of alveolar rhabdomyosarcoma.[46] It is difficult to consider these 5 cases and our two tumors to be in the same family of tumors as Ewing sarcoma and pPNET, based purely on the presence of the t(11;22) (q24;q12) translocation. This does not imply, however, that this translocation does not play a critical pathogenetic role in these tumors, as studies have shown that the fusion product from the *EWS/FLI-1* hybrid transcript has transforming activity.[23] Thus, these two rhabdomyosarcomas, by sharing the t(11;22) (q24;q12) translocation with Ewing sarcoma and pPNET, may have followed one or more of the same key pathways to tumorigenesis, without necessarily

being related tumors. Whether this genetic change contributed to the mixture of embryonal and alveolar histologies in cases 3 and 4 is unknown.

The four cases in our study are examples in which the genotype (Ewing's) and phenotype (non-Ewing's) might appear to be in conflict. Treating by genotype would mean using a Ewing sarcoma protocol in these cases. This may be reasonable for cases 1 and 2 (and was used in case 2). For cases 3 and 4, however, this would mean that some cases of rhabdomyosarcomas would be treated as Ewing sarcoma and others as rhabdomyosarcoma, and to make this distinction, all cases would need to be examined for the presence of the t(11;22) (q24;q12) translocation. Pathologists and oncologists will need to decide whether treatment of a particular tumor will be based on genotype or phenotype when these profiles are seemingly discordant. We feel that, for the present time, identification of the t(11;22) (q24;q12) translocation by cytogenetics or RT-PCR should not be taken as absolute proof of a diagnosis of Ewing sarcoma or pPNET, in the absence of confirming histology to include positive staining for MIC2. In general, genetic change(s) in a tumor should probably be interpreted in conjunction with the clinical features, the histology, and the phenotypic profile to determine the best diagnosis and treatment.

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