Biphenotypic Sarcomas with Myogenic and Neural Differentiation Express the Ewing’s Sarcoma EWS/FLI1 Fusion Gene

Poul H. B. Sorensen, Hiroyuki Shimada, Xian F. Liu, Jerian F. Lim, Gilles Thomas, and Timothy J. Triche

Department of Pathology and Laboratory Medicine, British Columbia’s Children’s Hospital/University of British Columbia, Vancouver, British Columbia, Canada V6H 3V4 [P. H. B. S., J. F. L.]; Department of Pathology and Laboratory Medicine, Children’s Hospital of Los Angeles/University of Southern California, Los Angeles, California 90027 [H. S., X. F. L., T. J. T.]; and Laboratoires de Génétique des Tumeurs, Institut Curie, Paris, France [G. T.]

ABSTRACT

Accurate diagnosis of primitive childhood sarcomas continues to be a formidable problem because these malignancies generally demonstrate very little morphological evidence of their tissue of origin. One of these tumor classes, the Ewing’s sarcoma family of peripheral primitive neuroectodermal tumors (pPNETs), are thought to have a neural histogenesis based on evidence of neuroectodermal differentiation. Greater than 95% of pPNETs carry t(11;22) or t(21;22) chromosomal translocations which fuse the EWS gene from chromosome 22q12 in-frame with either FLI1 from chromosome 11q24 or ERG from chromosome 21q22. The pPNETs are considered to be histogenetically distinct from rhabdomyosarcomas, myogenic tumors lacking these EWS gene fusions and hypothesized to derive from immature skeletal muscle precursors. In the present study, we describe a unique set of childhood soft tissue sarcomas that show both neural and myogenic differentiation. These biphenotypic tumors express myogenic regulatory factors and muscle-specific antigens and also show neuroectodermal differentiation with ultrastructural evidence of neurosecretory granules and expression of neural-associated genes. Northern analysis and reverse transcriptase PCR reveal expression of EWS/FLI1 gene fusions in all biphenotypic sarcomas analyzed. Chimeric EWS/FLI1 transcripts and fusion proteins in these tumors are identical to those described for pPNETs. Our results provide evidence for a class of biphenotypic childhood sarcomas with myogenic and neural differentiation and suggest that these tumors may be related to the Ewing’s sarcoma family of pPNETs.

INTRODUCTION

Primitive sarcomas occurring in childhood have traditionally presented significant diagnostic challenges due to their relative lack of distinctive morphological features. One group, the SRCTs of childhood, are composed predominantly of poorly differentiated small round cells and include the Ewing’s sarcoma family of pPNETs, embryonal and alveolar subtypes of RMS, neuroblastoma, and others (1). These tumors may show minimal or no evidence of their histological cell of origin; therefore, they are often difficult to distinguish from each other based on morphological features.

The identification of tumor-specific fusion genes resulting from chromosomal translocations in human malignancies is not only yielding profound insights into oncogenesis but also holds great promise for tumor diagnosis. Two translocations, the t(11;22)(q24;q12) and t(21;22)(q22;q12), characterize pPNETs and are considered specific for this tumor class. The translocations fuse the 5’ portion of the EWS gene on chromosome 22q12 to either the FLI1 gene on 11q24 (2, 3) or the ERG gene on 21q22 (4–6). Chimeric proteins expressed by the fusion genes are capable of cell transformation and appear to act as aberrant transcription factors (3). Identification of EWS/FLI1 and EWS/ERG fusion transcripts by the RT-PCR has formed the basis of a sensitive and specific diagnostic test for pPNETs (7). Gene fusions have been identified and characterized in other pediatric sarcomas. The t(2;13)(q35;q14) and t(1;13)(p36;q14) translocations of alveolar RMS result in the expression of PAX3/FKHR (8) and PAX7/FKHR (9) fusion transcripts, respectively, while the recently described intra-abdominal DSRCT (10) has been shown to express a gene fusion involving EWS and the WT1 tumor suppressor gene located at chromosomal band 11p13 (11). Detection of these genetic abnormalities thus provides a potentially powerful modality for the diagnosis of primitive childhood sarcomas, and classification based on their identification may ultimately provide a more accurate predictor of clinical behavior than current morphological classification systems.

The pPNETs are a family of poorly differentiated tumors that include Ewing’s sarcoma, peripheral neuroepithelioma, and Askin tumor. These tumors are thought to have a neural cell of origin based on limited neuroectodermal development, and the different family members show varying degrees of neural differentiation (reviewed in Ref. 1). They display ultrastructural evidence of primitive neuroepithelium and neurosecretory granules and show immunoreactivity for several neural-associated markers, including neurofilament proteins (12). In addition, neural differentiation can be induced in these tumors by treatment with retinoic acid and nerve growth factors (13, 14). The pPNETs are considered to be clinically and histogenetically distinct from RMS, malignancies with skeletal muscle differentiation hypothesized to derive from immature muscle precursor cells (reviewed in Ref. 1). These tumors do not display neural features, containing instead tumor rhabdomyoblasts with immunoreactivity for skeletal muscle-associated antigens including desmin and muscle-specific actin. Ultrastructurally, they show muscle-specific cytoplasmic intermediate filaments and primitive Z-discs (in more differentiated tumors). These tumors express the MyoD family of basic helix-loop-helix regulatory factors, including MYOD1, myogenin, MRF4, and MYF5 (reviewed in Refs. 15 and 16), and this has been used as a diagnostic criterion for RMS (17, 18). These myogenic regulatory factors are thought to control the initiation and maintenance of myogenic differentiation by binding to muscle-specific enhancers and by activating the transcription of genes involved in skeletal muscle development (15, 16).

Soft tissue sarcomas have been described which show morphological evidence of both myogenic and neuroectodermal differentiation (1, 19). DSRCT demonstrates multilineage differentiation with epithelial, neural, and skeletal muscle immunophenotypic features (10). Other entities remain poorly defined, but at least some of these cases may represent examples of malignant ectomesenchymoma (20–22). Malignant ectomesenchymomas are predominantly pediatric soft tissue tumors characterized by the presence of neural sarcomatous elements in addition to one or more malignant mesenchymal components, including RMS. Molecular studies may be useful to clarify the relationship of these tumors to other neural and myogenic soft tissue...
sarcomas. In the present study, we describe a set of childhood tumors that were originally diagnosed as primitive forms of RMS based on morphological evidence of myogenic differentiation. These tumors expressed myogenic regulatory factors and demonstrated immunoreactivity for muscle-specific antigens. However, ultrastructural analysis revealed both myogenic and neural features, and molecular studies demonstrated expression of neural-associated genes. We show here that \textit{EWS/FLI1} fusion transcripts and chimeric \textit{EWS/FLI1} proteins, identical to those of pPNETs, are expressed in these biphenotypic tumors. Our results suggest that a subset of biphenotypic sarcomas with myogenic and neural features may be members of the pPNET family of childhood tumors, a finding which could have important implications in the diagnosis and treatment of this group of childhood malignancies.

**MATERIALS AND METHODS**

**Cell Lines.** The cell lines TC-131 (23), TC-206, TC-212, and CTR (24); A204 (25), Rhl8 (26), TC-32, TC-71, A4573, and 6647 (23, 27); and IMR-32 and SAN-2 (28) have been described previously. Other cell lines were established at Children's Hospital of Los Angeles (TTC-442, TTC-487, TTC-516, and TTC-547), NIH (TC-147, TC-174, TC-253, and TC-280), or St. Jude's Hospital of Los Angeles tumor bank. Electron microscopy and immunocytochemistry were performed according to standard procedures.

**RNA and DNA Analysis.** Poly(A\(^+\)) or total RNA was isolated from cell lines as described previously (29). Northern analysis was performed according to standard methods (29) using 2 fig of poly(A\(^+\)) or 20 fig of total RNA for isolated as described previously (30). RT-PCR detection of \textit{EWS/FLI1} fusion transcripts was performed as reported previously using total RNA as starting material (7). Oligonucleotide primers used included 11.3 (FLU) and 22.3 (EWS; Refs. 2 and 7). RT-PCR screening for neural-associated gene expression was performed on DNase I (Pharmacia)-treated total RNA using the manufacturer's protocols. Primers for \textit{CAT} included 5'-GACATCCTTTTCTG-CATCT-3' (sense) and 5'-GGCTTCTCCTCAGTGGG-3' (antisense); for \textit{CA}, they included 5'-GCCAGCTCTCGCTTCTTG-3' (sense) and 5'-GGCTACAGCTCCCTCCAC-3' (antisense); and for \textit{N}SE, they included 5'-TTCTGAAGTCCGGCTAAATACA-3' (sense) and 5'-GAAGAGGGAAAGTGAGTGATCGG-3' (antisense). Amplified products were analyzed by electrophoresis using 2% agarose gels and stained with ethidium bromide.

**Immunoprecipitation Analysis.** Antisera were raised to a glutathione S-transferase-\textit{EWS/FLI1} fusion polypeptide consisting of EWS amino acids 245–264 (2) fused to FLI1 amino acids 240–369 (31). A 450-bp \textit{BamHI}-PstI fragment from a EWS/FLI1 fusion cDNA isolated from a tumor cell line containing an 11;22 translocation (7) was subcloned into the expression vector pGEX-2T (Pharmacia), fractionated by SDS-polyacrylamide gel electrophoresis, and analyzed by autoradiography.

**RESULTS**

**Biphenotypic Tumors with Myogenic and Neuroectodermal Differentiation.** Previous studies have documented the existence of childhood sarcomas with multilineage differentiation (1), and preliminary studies in our laboratory indicated that a subset of childhood tumors with myogenic phenotype also express primitive neural features. Therefore, we screened a series of tumors and corresponding cell lines of myogenic phenotype features for evidence of neuroectodermal differentiation. Five cases were identified; pathological features of the five cell lines examined in detail, TC-131, TC-174, TC-206, TC-253, and TTC-547, are summarized in Table 1. Primary tumors frozen in liquid nitrogen were obtained from the Children's Hospital of Los Angeles tumor bank. Electron microscopy and immunocytochemistry were performed according to standard procedures.

<table>
<thead>
<tr>
<th>Age/sex</th>
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<th>TC-206</th>
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<td>t(11;12;22)(q24;q14;q12)</td>
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4 IHC, immunohistochemical studies performed on primary tumors; findings maintained in cell lines (data not shown). ++, strong staining in > 50% of cells; +, strong staining in 10–50% of cells.

5 EM, electron microscopy performed on primary tumors.
Fig. 1. Pathology of biphenotypic tumors. A, histological section of original tumor tissue from case TTC-547 showing primitive rhabdomyoblastic cells separated by fibrous septae. B, electron micrograph of original tumor tissue from case TTC-547 demonstrating muscle-specific cytoplasmic actin-like filaments (arrow). C, electron micrograph of the same cell as in (B), showing dense-core neurosecretory granules (arrows).

WS/FUI (ILION IN lill'lll NOI VIC SARCOMAS demonstrated cytoplasmic immunoreactivity for muscle-specific desmin and actin, which was maintained in cell lines (data not shown). Control pPNETs were negative. Ultrastructurally, original tumor tissue from each case showed cytoplasmic actin-like filaments characteristic of primitive RMS (1), as shown for case TTC-547 in Fig. 1B. Moreover, neuroectodermal differentiation in these tumors could be demonstrated ultrastructurally by the presence of primitive neurites and dense core neurosecretory granules (33), shown in Fig. 1C for case TTC-547. Fig. 1, B and C, represent electron micrographs taken from the same cell, indicating the divergent differentiation of these tumor cells. Each tumor also showed immunoreactivity for the neural antigen, neurofilament protein (Ref. 34; Table 1), corroborating the biphenotypic nature of these tumors.

To confirm myogenic differentiation in these biphenotypic tumors, we examined cell lines for expression of the myogenic regulatory genes MYF5, myogenin, and MYOD1 by Northern analysis (Fig. 2A). TC-131, TC-174, TC-206, TC-253, and TTC-547 as well as RMS control cell lines expressed MYF5. Expression varied among the cell lines, with TC-131, TC-253, and TTC-547 consistently showing lower levels. Myogenin expression was not observed in TC-131 and TC-253 but was clearly evident in TC-174, TC-206, and TTC-547. Interestingly, MYOD1 expression was virtually undetectable in any of the biphenotypic tumor cell lines, while expression was observed in all control RMS cell lines except Rh18. As shown in Fig. 2B, MYOD1 expression could not be induced by transfer to differentiation medium (2% horse serum), known to promote myogenic differentiation (15). Therefore, all five biphenotypic cell lines expressed MYF5, three of five expressed MYOGENIN, and none expressed MYOD1. These findings were not due to the maintenance of primitive muscle cells in culture, as identical results were obtained using both early and late passage cell lines.
EWS/FLI1 Gene Rearrangements in Biphenotypic Tumors. The t(11;22), present in 85% of pPNETs, fuses the EWS gene to FLI1 (2,3). Cytogenetic analysis of the biphenotypic cell lines revealed alterations of band 22q12 in each case, and each had a t(11;22) or variant (Table 1). This, along with evidence for neuroectodermal differentiation, prompted us to investigate whether the EWS/FLI1 gene fusion might be present in these tumors. We first screened TC-131, TC-174, TC-206, TC-253, and TTC-547 for EWS gene rearrangements by Southern blot analysis using genomic probes for the EWS locus. All five cell lines were rearranged at EWS using the indicated restriction enzymes as well as at least two others, while control RMS cell lines showed only germline bands (Fig. 4 and data not shown). Therefore, EWS is rearranged in these biphenotypic tumors. In addition, the Rh18 cell line was also rearranged at EWS in these studies (Fig. 4). Subsequent cytogenetic analysis of Rh18 did not reveal a t(11;22) but did demonstrate a t(10;22) involving band 22q12 where EWS is located (data not shown). Interestingly, this cell line also lacked expression of MYODI (Fig. 2), while it did express MYF5 and myogenin.

EWS/FLI1 Fusion Gene Expression in Biphenotypic Tumors. To determine whether EWS is fused to FLI1 in biphenotypic sarcomas, we screened cell lines for expression of EWS/FLI1 fusion transcripts by RT-PCR using EWS and FLI1 oligonucleotide primers. This assay, used as a diagnostic test for pPNETs (7), detects several different amplification species (2,7). These include 328-bp type 1 products, which represent in-frame fusion of exon 7 of EWS to exon 6 of FLI1, 395-bp type 2 products (fusion of EWS exon 7 to FLI1 exon 5), and 581-bp type 3 products (fusion of EWS exon 10 and FLI1 exon 6; Ref. 5). As shown in Fig. 5A, type 1 products were observed in TC-131, TC-174, TC-206, and TTC-547, while TC-253 showed a

All pPNET cell lines tested were uniformly negative for expression of these genes (Fig. 2 and data not shown).

To demonstrate molecular evidence of neural differentiation, we analyzed the five biphenotypic cell lines for human neurofilament gene expression by Northern analysis. We used a cDNA probe cocktail that detects the 44.5-kb neurofilament heavy (NF-H) gene transcript as well as the 2.5- and 4.0-kb major transcripts of neurofilament light (NF-L) (35,36). As shown in Fig. 3A, all five biphenotypic tumors expressed the NF-H transcript and at least one of the major transcripts of NF-L. Similar expression was observed in two neuroblastoma and two pPNET cell lines, while three RMS cell lines lacking EWS/FLI1 fusion transcripts did not express these genes. As a further test for neural phenotype, we (37) and others have previously used cDNA sequence data to develop RT-PCR-based assays to screen for the expression of neural-associated gene expression, including the cholinergic cell-specific gene, CAT (38-40), CGA, the major protein of dense core neurosecretory granules (41,42), and NSE (43). As shown in Fig. 3B, the five biphenotypic tumors, a neuroblastoma cell line, and two pPNETs all expressed the 135-bp CAT product, while RMS controls were negative. For CGA expression, we used primers that amplify both 485- and 583-bp fragments, as demonstrated for the SAN-2 neuroblastoma cell line (Fig. 3B, Lane k). All five biphenotypic tumors and the two pPNET cell lines were positive for 485- or 583-bp fragments (or both), while RMS controls were negative. All cell lines were positive for a 333-bp fragment of NSE, known to be widely expressed (1), which was useful to confirm the presence of intact RNA in all samples tested. The above RT-PCR results were confirmed by probing blotted amplification products with cloned PCR fragments obtained from the SAN-2 neuroblastoma cell line for each assay (data not shown).
type 3 amplified product. The two pPNET cell lines, TC-32 and TC-71, also expressed type 1 fusion transcripts, while the remaining RMS were negative (the presence of intact cDNA for the negative cases was confirmed by amplification using control primer sets). These results were confirmed by hybridizing the PCR products with an EWS oligonucleotide probe (Ref. 7; data not shown) and could be reproduced when several independently derived cryopreserved passages of each cell line were tested. Notably, the Rh18 cell line was negative for EWS/FLII gene expression by RT-PCR, consistent with cytogenetic evidence of a t(10;22) rather than a t(11;22). We are currently studying this cell line to determine if EWS is fused to a different gene (on 10q22) in Rh18.

Because the five biphenotypic sarcomas described here were originally diagnosed as alveolar or primitive RMS, we tested these cell lines for PAX3/FKHR or PAX7/FHKR fusion gene expression by RT-PCR using methods described previously (8, 9). All five cell lines were negative for the expected 409-bp PAX3/FKHR product (8) or the 695-bp PAX7/FHKR product (9), in contrast to positive control cell lines or tumor samples (data not shown). The Rh18 cell line was similarly negative for PAX3/FKHR or PAX7/FHKR products by RT-PCR. We also used established methods (11) to test these tumors for expression of EWS/WT1 fusion transcripts derived from the t(11;22)(p13;q12) of DSRCT, since this tumor is known to show multilineage differentiation. While the expected PCR product (11) was present in a positive control DSRCT, all five cell lines were negative (data not shown).

To examine whether EWS/FLII fusion transcripts produced in biphenotypic tumors differ from those observed in pPNETs, we cloned and sequenced PCR products from TC-131, TC-174, TC-206, TC-253, and TTC-547. Sequence analysis of products from TC-131, TC-174, TC-206, and TTC-547 revealed in-frame fusions between exon 7 of EWS and exon 6 of FLI-1 as described for type 1 fusions of pPNETs (Ref. 2; data not shown). The TC-253 product demonstrated an in-frame type 3 fusion between exon 10 of EWS and exon 6 of FLI-1 as previously reported for pPNETs (Ref. 5; data not shown). These data confirm that RT-PCR products detected in these biphenotypic cell lines represent amplification of EWS/FLII fusion transcripts and demonstrate that junctional sequences are identical to those observed in pPNETs.

We next determined whether EWS/FLII fusion transcripts identified by RT-PCR were functional. Northern blots of biphenotypic cell lines hybridized with an EWS cDNA probe demonstrated aberrant transcripts in addition to the expression of germline EWS (data not shown). To detect EWS/FLII chimeric proteins, immunoprecipitation experiments were performed using antisera raised to an EWS/FLII polypeptide fragment (4). As shown in Fig. 5B, immunoprecipitation of total cell lysates revealed a 68-kDa band corresponding to the EWS/FLII fusion protein (3). The size of this protein was identical to that of an in vitro translated EWS/FLII fusion protein (Fig. 5B, Lane b), in which exon 10 rather than exon 7 of EWS is fused to exon 6 of FLII, showing an 80-kDa band. This protein is predicted to contain an additional 84 amino acids compared to the type 1 protein (2). These bands were not detected in lysates from negative control RMS TTC-487 or CTR cells, which lack EWS/FLII fusion transcripts. A 90-kDa species present in all cell lines tested corresponds to germline EWS.

**EWS/FLII Expression in Primary Tumors.** To rule out that EWS/FLII fusion transcripts observed in the above cell lines represent an artifact of in vitro cell culture conditions, we performed RT-PCR on original patient material from biphenotypic tumors. Frozen primary
DISCUSSION

We demonstrate here that a subset of childhood sarcomas with both myogenic and neuroectodermal differentiation contain a chromosomal alteration resulting in the formation of the EWS/FLI1 fusion gene. The EWS/FLI1 gene fusion was described as a specific feature of the Ewing's sarcoma family of pPNETs (2, 3), resulting from the t(11;22) found in 85% of these tumors (44). EWS is a ubiquitously expressed gene of unknown function, while FLI1 (31, 45, 46) is a member of the ETS gene family of transcription factors. The EWS/FLI1 chimeric protein consists of the glutamine-rich amino terminal portion of EWS fused to the carboxy terminal DNA binding domain of FLI1 (2, 3). This molecule efficiently transforms NIH3T3 cells, and this transformation ability requires both EWS and FLI1 domains (3). Recent studies indicate that a second translocation in pPNETs, the t(21;22)(q22;q12), fuses EWS with another ETS family transcription factor, ERG, located on chromosome 21q22 (4–6). The chimeric EWS/FLI1 product (and likely EWS/ERG) appears to function as an oncoprotein by acting as an aberrant transcription factor (47). Chimeric EWS/FLI1 transcripts and fusion proteins in the tumors described in the present study were indistinguishable from those found in pPNETs, suggesting that the EWS/FLI1 oncoprotein plays a similar role in biphenotypic tumors.

The findings presented here provide a unique example of identical gene fusions occurring in two phenotypically distinct solid tumor types. While pPNETs display exclusively neuroectodermal differentiation with no evidence of skeletal muscle development (reviewed in Ref. 1), the biphenotypic tumors described here also demonstrated clear morphological, immunocytochemical, and ultrastructural evidence of myogenic differentiation. All biphenotypic cell lines expressed MYF5 and three of five expressed myogenin. These factors, along with MYOD1 and MRF4, constitute the MyoD family of myogenic regulatory factor genes (15, 16). Constitutive expression of each factor is sufficient to convert a variety of nonmyogenic cells to skeletal muscle cells (15). Expression of this gene family is restricted to skeletal muscle and myogenic cell lines, and among human tumors, has only been described in entities with skeletal muscle differentiation (17, 48, 49). Neuroectodermal differentiation in biphenotypic tumors was demonstrated by immunoreactivity for neurofilament proteins, as well as by ultrastructural identification of dense core neurosecretory granules and primitive neurites, which have not been described in RMS. These structures are characteristic of cells of neural crest origin and are found in neuroblastomas and pPNETs (reviewed in Refs. 1 and 33). In addition, all five cell lines as well as pNET and neuroblastoma controls, but not conventional RMS controls, expressed multiple human neurofilament genes which encode the major intermediate filaments of neural cells (12). Biphenotypic tumors, along with pPNETs and neuroblastomas, but not RMS controls, also expressed CAT, specific for cholineric cells (38) and CGA, which encodes the major protein component of neurosecretory granules (41). Interestingly, MYOD1 expression was not detected in any of the biphenotypic tumor cell lines tested and could not be induced by growth in differentiation medium. MyoD1 may be dispensable for the initial stages of myogenesis, possibly due to functional redundancy among the myogenic regulatory factors (50, 51). Transgenic mice lacking MyoD1 express other myogenic regulatory factors, and skeletal muscle formation appears to be normal (51). The EWS/FLI1 oncprotein itself may inhibit MYOD1 expression, as has been demonstrated for other oncogene or proto-oncogene products including Ras and c-Fos (52) as well as c-Jun (53). Lack of expression of MYOD1 may be a unique feature of these biphenotypic tumors.

The findings presented here that the EWS/FLI1 gene fusion occurs in both biphenotypic sarcomas and in pPNETs suggests that these two classes of tumors are related. It is not clear why one set of tumors expressing the EWS/FLI1 gene fusion demonstrates only a neuroectodermal phenotype, while another set also shows myogenic features. One possibility is that expression of this chimeric gene somehow influences neural differentiation, such that neuroectodermal phenotype is a general feature of tumors with this gene fusion. In this context, the muscle phenotype observed in biphenotypic sarcomas may be either a function of additional genetic alterations or a reflection of the differentiation capacity of the cell of origin of these malignancies. Soft tissue sarcomas (in addition to DSRCT) have been described which show both skeletal muscle and neural differentiation (1, 19). Malignant ectomesenchymomas occur primarily in childhood and are composed histologically of tumor cells with neural differentiation as well as one or more malignant mesenchymal elements, including RMS (20–22). They are thought to arise from pluripotential embryonic neural crest tissue, or ectomesenchyme, which is known to be widely dispersed in human tissues (54). Experimental studies have demonstrated that these cells are pluripotential, being capable of differentiating not only into neuroectodermal tissue but also along mesenchymal lineages, including skeletal muscle (54, 55). The biphenotypic tumors described here may thus represent examples of malignant ectomesenchymomas in which neural development is very primitive and previously unrecognized. In this context, the pPNETs, which are also thought to have a neural crest histogenesis

7 Y. Hachitanda, C. Aoyama, J. K. Sato, and H. Shimada. The most primitive form of malignant ectomesenchymoma: an immunohistochemically and ultrastructurally defined entity, manuscript in preparation.
(13, 14), may be derived from a distinct but related cell of origin that retains neuroectodermal features but has lost the capacity for myogenic differentiation.

Our data indicate that a subset of childhood sarcomas with myogenic differentiation may be histogenetically distinct from conventional RMS, tumors which are thought to arise from immature muscle precursor cells (reviewed in Ref. 1). RMS are the most commonly diagnosed soft tissue sarcomas in children. However, primitive forms of this tumor class may be more heterogeneous than appreciated previously, and the differential diagnosis of primitive RMS appears to overlap with other nosological entities. Myogenic immunophenotype is known to be a feature of DSRCT, a tumor of multilineage differentiation that appears to be a distinct tumor class associated with a characteristic gene fusion (10, 11). Other rare primitive sarcomas thought to be distinct from RMS have been described that demonstrate evidence of muscle differentiation, but these remain poorly characterized and difficult to classify (1, 19). The biphenotypic tumors described here were all initially diagnosed as alveolar or primitive RMS on the basis of morphological and immunophenotypic features. However, we failed to detect PAX3/FKHR (8) or PAX7/FKHR (9) fusion transcripts described for alveolar RMS or EWS/WTI fusion transcripts found in DSRCT (11), observing instead pNET-associated EWS/FUSI gene fusions. These tumors, possibly representing primitive malignant ectomesenchymomas related to the pNET family, therefore, provide a further example of a primitive childhood sarcoma with myogenic differentiation. Preliminary studies in our laboratory suggest that these sarcomas may comprise 10% or more of childhood tumors with a primitive RMS phenotype. As described in the present study, these tumors can be distinguished by expression of the EWS/FUSI gene fusion, and so it will be important to determine the incidence and prognostic significance of this tumor entity. The findings here provide a further argument that classification of many forms of childhood solid tumors should be considered from a molecular genetic as well as from a morphological perspective.

ACKNOWLEDGMENTS

We thank C. T. Denny and S. L. Lessnick of the University of California (Los Angeles, CA) and J. Peters at Children's Hospital of Los Angeles for their contributions to this study.

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