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Short Communication

Detection of Chimeric Transcripts in Desmoplastic Small Round Cell Tumor and Related Developmental Tumors by Reverse Transcriptase Polymerase Chain Reaction

A Specific Diagnostic Assay

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Desmoplastic small round cell tumor is a recently described entity associated with fusion of the* EWS *and* WT1 *genes and with expression of a chimeric transcript. To investigate the structure and potential diagnostic utility of the detection of* EWS-WT1 *chimeric RNA in desmoplastic small round cell tumor, 12 examples of this entity and 49 other tumors that enter in its differential diagnosis were studied by reverse transcriptase polymerase chain reaction for the presence of* EWS-WT1, EWS-FLI-1, PAX3-FKHR, *and* PAX7-FKHR *chimeric transcripts.* EWS-WT1 *was detected in 11 of 12 desmoplastic small round cell tumors but not in any other tumor type studied, including 17 Wilms' tumors, 10 Ewing's sarcomas/primitive neuroectodermal tumors, 13 alveolar rhabdomyosarcomas, and 9 embryonal rhabdomyosarcomas. One desmoplastic small round cell tumor was found to have a variant* EWS-WT1 *chimeric product that included exon 8 of* EWS. EWS-FLI-1 *chimeric RNA was present in all Ewing's sarcoma/primitive neuroectodermal tumor and not identified in any other tumor types, including desmoplastic small round cell tumor.* PAX3/PAX7-FKHR *chimeras were present in 9 of 13 alveolar rhabdomyosarcomas but not in any other tumors. Detection of chimeric transcripts by reverse transcriptase polymerase chain reaction is a very specific aid in differential diagnosis of developmental tumors and further establishes desmoplastic small round cell tumor as a distinct entity. (Am J Pathol 1995, 147:1584–1591)

Small round cell tumors of childhood are a diagnostic challenge to the surgical pathologist. They constitute a family of biologically aggressive tumors occurring in children and adolescents that share several morphological features and are often difficult to distinguish from each other. Accurate diagnosis is essential to institute appropriate therapy and predict prognosis.

Consistent cytogenetic alterations have been identified in several members of this family, including a t(2;13)(q35;q14) translocation in alveolar rhabdomyosarcoma,[1] and a t(11;22)(q24;q12) translocation in Ewing's sarcoma/peripheral neuroectodermal tumor (PNET).[2] Recently, these chromosomal translocations have been characterized at the molecular

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level and found to result in *EWS-FLI-1* and *EWS-ERG* gene fusions in Ewing's sarcoma/PNET[3,4] and *PAX3-FKHR* or *PAX7-FKHR* in alveolar rhabdomyosarcoma.[5,6] Chimeric transcripts and gene fusions specific for each translocation can be detected in clinical samples by reverse transcriptase polymerase chain reaction (RT-PCR) and fluorescent *in situ* hybridization (FISH) and may prove to be a reliable and very useful diagnostic technique in the small round cell tumor family, because conventional karyotyping for these tumors is often unsuccessful.[3]

Desmoplastic small round cell tumor (DSRCT) is a recently recognized entity[7–9] that affects mainly children and adolescent males, usually in the form of widespread intra-abdominal growth not related to any organ system. Histologically, it is characterized by angulated nests of small round cells within an abundant desmoplastic stroma. The tumor cells show multiphenotypic differentiation, expressing epithelial, muscle, and neural markers.[7] DSRCT is associated with a unique chromosomal translocation t(11;22)(p13;q12)[10] that we have recently shown to involve the *EWS* and the *WT1* genes.[11,12] *EWS* is the breakpoint site of translocations associated with Ewing's sarcoma, and *WT1* is a gene altered in some Wilms' tumors. The translocation results in fusion of the two genes with expression of a chimeric *EWS-WT1* product that can be detected in tumor samples.

The aim of this study was to assess the specificity and sensitivity of the molecular detection of *EWS-WT1* chimeric transcripts by RT-PCR and its potential utility in the differential diagnosis of the DSRCT from other developmental tumors. We have analyzed 12 DSRCTs and 49 other developmental tumors associated with related genetic abnormalities and considered in the differential diagnosis of DSRCT, using a panel of primer pairs that distinguish specific chimeric transcripts.

Materials and Methods

Characteristics of the Tumors Analyzed

Study cases included 12 cases of DSRCT (9 males and 3 females; mean age, 19 years; range, 11 to 32 years). In 11 patients the tumor involved the abdominal cavity and in 1 patient it involved the pleura and posterior mediastinum. The diagnosis was based on established histopathological and immunohistochemical criteria.[9]

The remaining 49 tumors included 22 rhabdomyosarcomas (15 males and 7 females; mean age, 17.9 years; range, 2 to 47 years; 13 alveolar and 9 embryonal[13]); 17 Wilms' tumors (10 males and 7 females; mean age, 4.6 years; range, 1 to 19 years); 8 Ewing's sarcomas of bone (5 females, 3 males; mean age, 21.6 years; range, 17 to 31 years; 7 of them showing involvement of soft tissues), and 2 PNETs (1 male, 1 female, both 31 years old; 1 in a paraspinal location and the other in the femur).

Samples

Tumor and normal tissue samples were collected at the time of surgery and immediately frozen in liquid nitrogen. RNA extraction was performed by standard procedures.[14]

RT-PCR

RT-PCR detection of transcripts was performed by using 1 μg of total RNA as template and a GeneAmp RNA-PCR kit (Perkin-Elmer, Roche Molecular Systems, Branchburg, NJ) following the recommendations of the manufacturer. Reverse transcription included an incubation period of 45 minutes at 42°C, with random hexamers or transcript-specific primers, followed by a 5-minute period at 99°C to denature the enzyme. PCR was then performed with an initial denaturation step of 95°C for 2 minutes, followed by 35 cycles including a denaturation step at 94°C for 30 seconds, annealing at 65°C for 30 seconds, and extension at 72°C for 60 seconds, with 6 minutes of extension after the completion of the last cycle. Products were identified by 2% agarose gel electrophoresis and ethidium bromide staining.

Primers

Oligonucleotide primer pairs, used to amplify the chimeric transcripts of alveolar rhabdomyosarcoma, Ewing's sarcoma/PNET, and DSRCT have been described[4–6,11] and are listed in Table 1.

The quality of the extracted RNA was assessed by RT-PCR by using random hexamers and β-actin primers (Clontech, Palo Alto, CA). RNA samples pre-

Table 1. *Primers and Sequences Used in the RT-PCR Assays*

Designation	Gene/exon or intron	Sequence
WT1-10.2	*WT1*/exon 10	5′-GACACTGAACGGTCCCCGA-3′
WT1-10.1	*WT1*/exon 10	5′-GCCACCGACAGCTGAAGGGC-3′
EWS 22.3	*EWS*/exon 7	5′-TCCTACAGCCAAGCTCCAAGTC-3′
EWS-8.1	*EWS*/exon 8	5′-GCATGAGTGGCCCTGATAAC-3′
FLI-1 11.3	*FLI-1*/exon 11	5′-ACTCCCCGTTGGTCCCCTCC-3′
5′-*PAX3*	*PAX3*	5′-GCACTGTACACCAAAGCACG-3′
5′-*PAX7*	*PAX7*	5′-TTTGAGAGGACCCACTACCC-3′
3′-*FKHR*	*FKHR*	5′-AACTGTGATCCAGGGCTGTC-3′

pared from normal liver, kidney, and spleen were used as non-neoplastic controls in all procedures.

Cloning and Sequencing

Products of RT-PCR were isolated from 2% agarose gels with silicagel matrix (QIAGEN, Chatsworth, CA) and ligated into pUC18. Direct sequencing of purified recombinant plasmid DNA was done by a modified Sanger method[15] with the Circumvent kit (New England Biolabs, Beverly, MA),^{35}S-dATP (800 Ci/mmol/L, New England Nuclear, Boston, MA), and forward and reverse sequencing primers for pUC18 (Promega, Madison, WI) or exon-specific primers (Table 1). Products were evaluated on 6 to 8% polyacrylamide-urea sequencing gels. Sequences were analyzed with GeneWorks version 2.4 (Intelligenetics, Inc., Mountain View, CA).

Results

The *EWS-WT1* chimeric transcript was detected in 11 of 12 DSRCTs by RT-PCR using primers for *EWS* exon 7 (*EWS* 22.3) and *WT1* exon 10 (WT1 10.1). Ten of the eleven positive cases showed amplification of the expected 268- and 259-bp products corresponding to the two described splice variants of this portion of the *WT1* gene[11] (Figure 1, left *EWS-WT1* lane). Cloning and sequencing of PCR products in these cases showed in-frame fusions of *EWS* exon 7 to *WT1* exon 8 (Figure 2, typical chimeric transcript). Both splice variants (plus or minus 9 bp encoding lysine, threonine, serine (+KTS and −KTS)) were identified after sequencing multiple individual clones of the amplified chimeric transcript fusion fragments. No mutations in the segments close to the junction were seen. The full-length chimeric product in these cases included the first 7 exons of *EWS*, an effector domain with strong transactivating properties *in vitro*, and exons 8 to 10 of *WT1*, which encode the last three zinc fingers of the DNA-binding domain of *WT1* (Figure 2).

In a single case of DSRCT (referred to as variant case), RT-PCR for *EWS-WT1* carried out with primers for *EWS* exon 7 (*EWS* 22.3) and *WT1* exon 10 (*WT1* 10.1) yielded 442 bp (−KTS) and 451 bp (+KTS) DNA fragments (Figure 1, left *EWS-WT1* lane). Cloning and sequencing of these fragments confirmed a different chimeric transcript junction. Alignment of the sequence with wild-type *EWS* and *WT1* showed fusion of *EWS* exon 8 to *WT1* exon 8. Therefore the chimeric transcript in this variant case differed from the typical chimeric product by including *EWS* exon 8. In addition, a 4-bp deletion at the 5′ end of *WT1* exon 8 and a 6-bp insertion in the junction between *EWS* and *WT1* were present, resulting in an in-frame fusion of the two genes (Figure 2, variant chimeric transcript). A very faint 270-bp DNA band was also detected with *EWS* exon 7 (*EWS* 22.3) and *WT1* exon 10 (*WT1* 10.1) primers. It may be that alternate splicing produces a minor component of *EWS-WT1* transcripts without *EWS* exon 8 as a naturally occurring alternatively spliced form of wild-type *EWS* lacking exons 8 and 9 has been detected. This requires clarification by cloning and sequencing this minor product. RT-PCR with primers complementary to *EWS* exon 8 and *WT1* exon 9 detected chimeric transcripts only in this case and not in any other DSRCT (data not shown).

EWS-WT1 chimeric transcripts were not detected in any of the Ewing's sarcomas/PNET, Wilms' tumors, and rhabdomyosarcomas (Figure 1). Results are summarized in Table 2.

RNA from 7 of 13 alveolar rhabdomyosarcomas directed amplification of a 436-bp fragment by RT-PCR using primers for *PAX3* and *FKHR* (Table 1). In addition, 2 other alveolar rhabdomyosarcomas showed amplification of a 695-bp fragment with primers for *PAX7-FKHR*. These are the expected sizes of PCR products from the *PAX3-FKHR* and *PAX7-FKHR* chimeric transcripts.[5,6] All 9 cases had been classified histologically as alveolar type. Cloning and sequencing of these PCR products confirmed the *PAX3-FKHR* or *PAX7-FKHR* gene fusions. None of the nine rhabdomyosarcomas classified as the embryonal type showed amplification of the *PAX3-FKHR* or *PAX7-FKHR* products. RNA from DSRCTs, Ewing's sarcomas, Wilms' tumors, and PNETs did not yield *PAX3-FKHR* or *PAX7-FKHR* RT-PCR products.

All eight Ewing's sarcomas and the two PNETs demonstrated the presence of the *EWS-FLI-1* chimeric transcript by RT-PCR (Figure 1). All other tumors, including DSRCT, were negative for *EWS-FLI-1* chimeric transcript.

Discussion

This study demonstrates that RT-PCR detection of *EWS-WT1* chimeric transcripts represents a very sensitive and specific molecular marker for DSRCT, being that these transcripts were not present in any of 49 other tumors considered in its differential diagnosis. The detection of chimeric transcripts has been shown to be very useful in the diagnosis of Ewing's sarcoma/PNET[3] and alveolar rhabdomyosarcoma.[13]

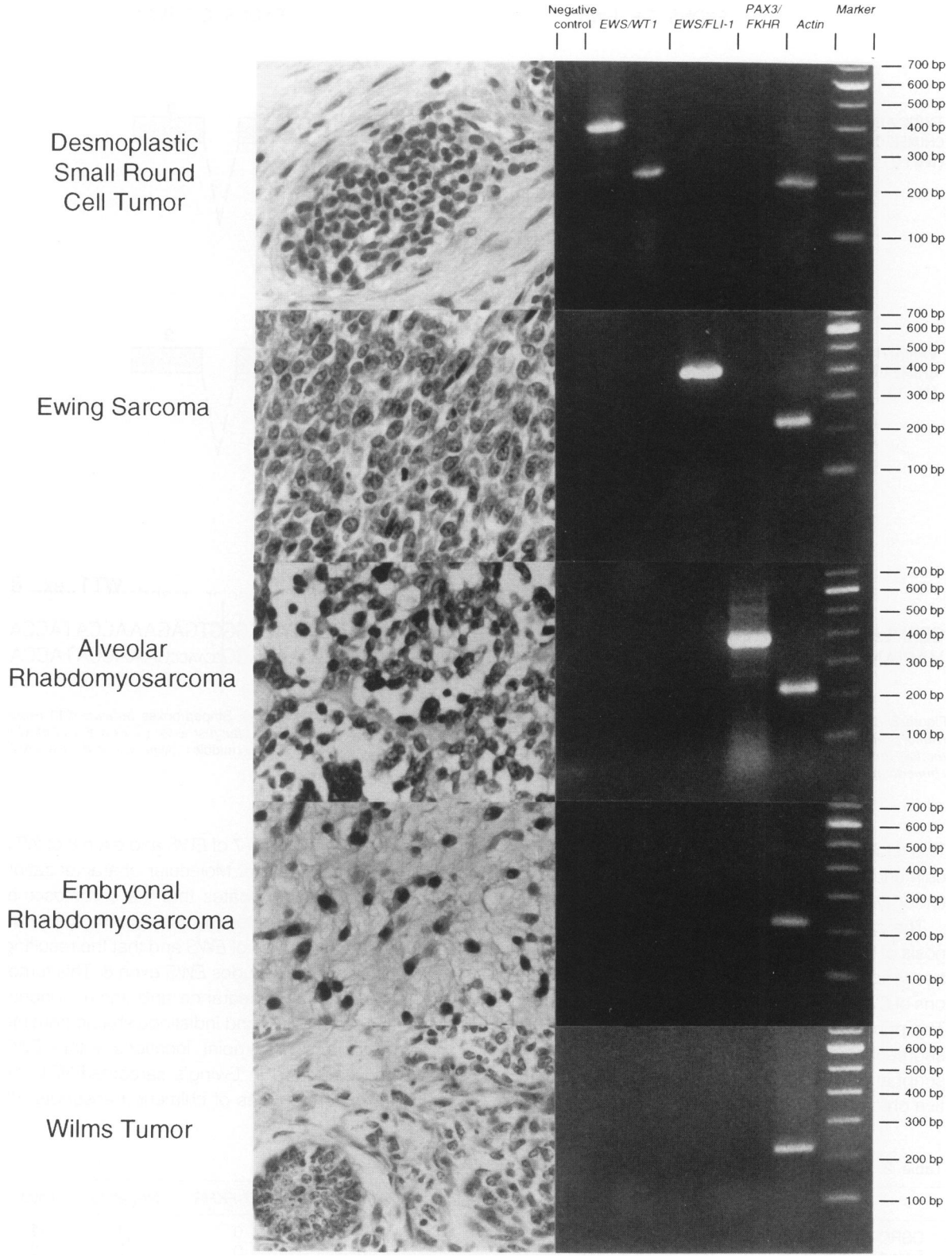

Figure 1. *Representative photomicrograph (H&E, ×400) and RT-PCR results for each particular tumor type. RT-PCR was performed with a panel of three different pairs of primers* (rows). *Results with two cases of DSRCT are shown; a case carrying a typical chimeric transcript displays a 268-bp band* (right EWS-WT1 *lane, top*), *whereas the size of the band in the case with a variant chimeric transcript is 451 bp* (left EWS-WT1 *lane, top*). *A small DNA band in the negative control lane of alveolar rhabdomyosarcoma is presumably a primer-dimer artifact. The negative control RT-PCR is performed without RNA template.*

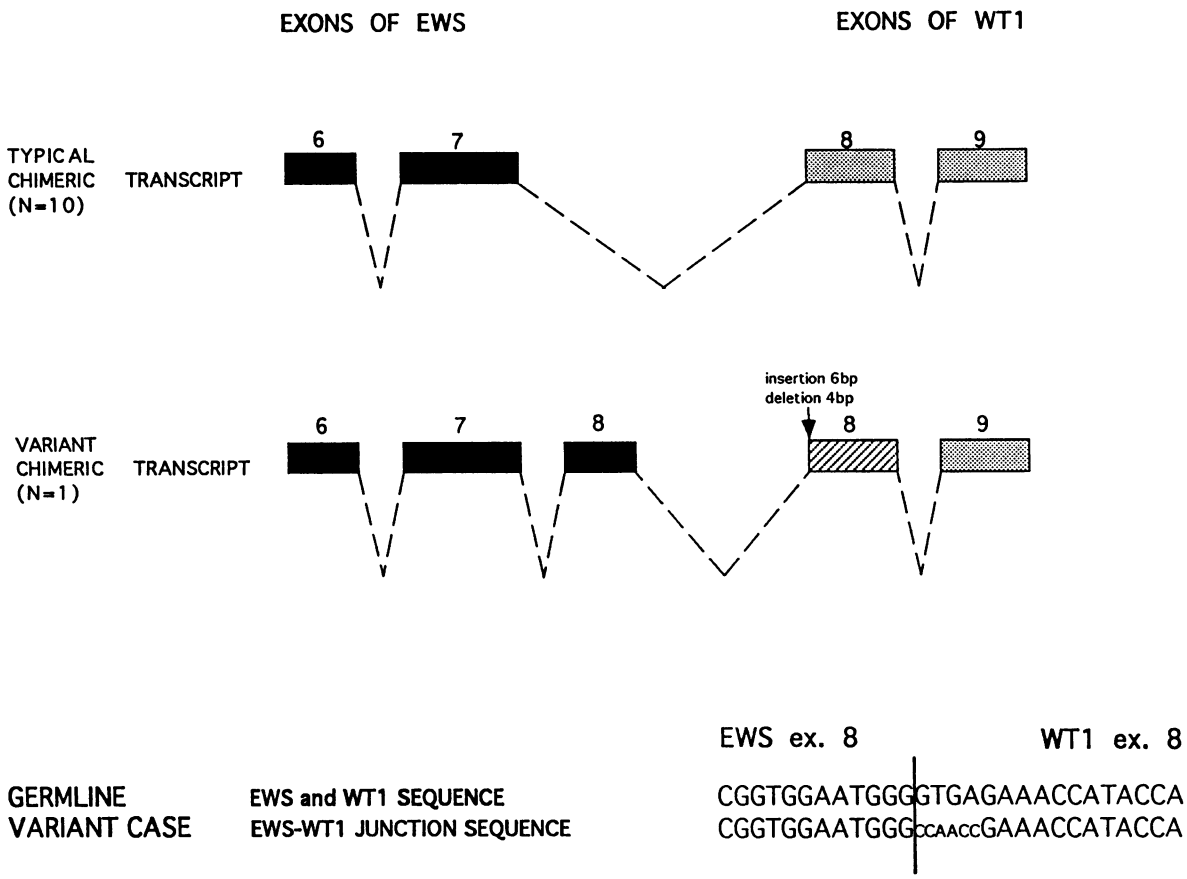

Figure 2. *Diagram of the junction region of* EWS-WT1 *chimeric transcripts.* Black boxes *indicate* EWS *exons.* Striped boxes *indicate* WT1 *exons. Numbers above boxes indicate the exon number of each gene. The typical chimeric transcript is composed of the first seven exons of* EWS *linked to the last three exons of* WT1 (top), *except in one case (variant case) in which* EWS *exon 8 is also present* (middle). *Sequence of the* EWS-WT1 *chimeric transcript junction for the variant case is shown at the bottom.*

This study, with a panel of primers for individual chimeric RNA transcripts, delineated sharply each tumor type associated with that transcript.

The specificity of RT-PCR in the molecular diagnosis of DSRCT relies on the structure of the chimeric transcript, which is composed of the first seven exons of *EWS* joined to the last three exons of *WT1*.[11,12] The primers we used direct amplification of a short segment close to the *EWS-WT1* junction in this transcript and corresponds to the product of the derivative chromosome 22. In the present study the fusion occurred between exon 7 of *EWS* and exon 8 of *WT1* in all cases except one. Molecular characterization of the latter tumor indicates that the chromosomal breakpoint is likely located in, or is distal to, the intron between exons 8 and 9 of *EWS* and that the resulting chimeric transcript includes *EWS* exon 8. This tumor had a histological appearance and immunophenotype typical of DSRCT and indistinguishable from the others. Different breakpoint locations within *EWS* have been reported in Ewing's sarcoma/PNET, resulting in different types of chimeric transcripts.[3,16]

Table 2. *Detection of Chimeric Transcripts in Small Round Cell Tumors*

Diagnosis	*EWS-WT1*	*EWS-FLI-1*	*PAX3-FKHR*	*PAX7-FKHR*	Negative	Total
DSRCT	11	0	0	0	1	12
Ewing's sarcoma	0	8	0	0	0	8
PNET	0	2	0	0	0	2
Wilms' tumor	0	0	0	0	17	17
Alveolar rhabdomyosarcoma	0	0	7	2	4	13
Non-alveolar rhabdomyosarcoma	0	0	0	0	9	9
Total	11	10	7	2	31	61

However, this is the only known example of DSRCT with a chimeric transcript that includes *EWS* exon 8. Fusion of exon 8 of *EWS* to any exon of *FLI-1* is out of frame and extremely infrequent in Ewing's sarcoma/PNET.[17] Likewise, fusion of *EWS* exon 8 to *WT1* exon 8 would result in an out-of-frame product. However, in the case described here, there is a small deletion of *WT1* and insertion of six nucleotides at the junction of the chimeric transcript resulting in an in-frame product. The genomic breakpoint region from this case is currently being cloned and sequenced to further define this variant gene fusion. The prognostic and biological significance of this variant translocation in DSRCT, if any, remains unknown.

DSRCT is a tumor with unique clinical, morphological, and immunohistochemical features.[9,18] The clinicopathological spectrum of this tumor is, however, wider than initially reported.[18,19] There are three reported cases of DSRCT in a pleural location[20,21] and one in the posterior cranial fossa.[22] Some cases of DSRCT express MIC-2, a marker detected in most Ewing's sarcomas, although there are some differences in the subcellular distribution of this antigen between the two tumor types (W. L. Gerald, unpublished observation). Some cases of DSRCT have unusual histological features such as little desmoplasia,[23] prominent tubular or gland-like formation,[18] spindle cell areas, pseudorosette formation (W. L. Gerald, unpublished observations), or foci of larger anaplastic cells.[9] For this reason a specific assay, like the detection of the *EWS-WT1* chimeric transcript by RT-PCR, may prove to be useful for accurate classification and diagnosis of this member of the small round cell tumor family.

We were not able to detect the *EWS-WT1* chimeric transcript in one case. This may be a result of sampling error, as the tumor contained rare foci of tumor cell nests; however, the possibility of variant translocations or lack of *EWS-WT1* fusion in this case should also be considered. Studies to assess the sensitivity of the test are in progress, although it is expected to be similar to that of RT-PCR for *EWS-FLI-1*, which is approximately one tumor cell in 10^4-10^5.[3] Because of its high sensitivity, one of the potential utilities of RT-PCR to *EWS-WT1* is to detect the presence of tumor cells in peripheral blood or ascitic fluid, thus allowing a more accurate staging and more sensitive monitoring for residual or recurrent disease in patient follow-up.

DSRCT is the third tumor type associated with translocation of the *EWS* gene. The others are Ewing's sarcoma/PNET[3] and clear cell sarcoma of soft parts,[24] but only DSRCT is associated with a consistent translocation of *WT1*.[11] All chimeric proteins encoded by gene fusions with *EWS* have a similar structure, with a transactivator domain in the amino-terminal region and a DNA-binding domain in the carboxyl-terminal region. In each case the histological tumor classification correlates with a different translocation partner of *EWS*. In DSRCT, its chimeric protein is predicted to modulate transactivation at *WT1*-responsive genes. Interestingly, the locations reported so far for DSRCT are related to the serosal lining of body cavities, a structure that, like the urogenital system, has a high transient fetal expression of the *WT1* gene.[25]

The specificity of molecular assays, such as those used in this study, may be helpful in the differential diagnosis of small round cell tumors. During the preparation of this manuscript, a study supporting the utility of RT-PCR for the diagnosis of pediatric small round cell tumors was published.[26] One important potential use is in the differential diagnosis between DSRCT and Ewing's sarcoma/PNET as the latter are positive for *EWS-FLI-1* or *EWS-ERG* and consistently negative for *EWS-WT1*. The detection of the t(2;13) translocation (*PAX3-FKHR* transcript) and t(1;13) (*PAX7-FKHR* transcript) were very specific for alveolar rhabdomyosarcoma in our series. The sensitivity of this test was high, although 4 of 13 alveolar rhabdomyosarcomas were negative. Because certain gene rearrangements have proven to be very specific, it will be interesting to apply these analyses to some other subsets of developmental tumors like extra-osseous Ewing's sarcomas,[27] extra-renal rhabdoid tumors,[28] polyphenotypic non-desmoplastic small cell tumors,[21] mesenchymal chondrosarcomas, and small cell osteosarcomas.

Molecular analysis of small round cell tumors of childhood has been very informative concerning tumor classification. Tumors formerly viewed as distinct entities such as PNET and Ewing's sarcoma have been shown to have similar genetic alterations and probably belong to the same family.[26,29–31] Alveolar and embryonal rhabdomyosarcomas are two different clinicopathological entities associated with different genetic changes and share only skeletal muscle differentiation.[13] Molecular analysis, in conjunction with morphology and immunohistochemical data, will help to better define biologically homogeneous clinicopathological entities and provide a useful diagnostic tool for these aggressive developmental tumors.

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