Review

Molecular Genetics of Pediatric Soft Tissue Tumors

Clinical Application

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The application of molecular genetics to pediatric soft tissue tumors has grown tremendously over the last decade. It has resulted in the identification of novel genes that have provided us with an increased understanding of oncogenesis. Furthermore, these findings have identified diagnostic and potentially prognostic factors for patient management. Molecular diagnostic techniques, such as reverse transcription PCR (RT-PCR) and fluorescence *in situ* hybridization (FISH), have become important tools for evaluating pediatric soft tissue tumors. By detecting characteristic fusion genes, these techniques have greatly increased the diagnostic accuracy of histopathological classification. One of the exciting promises of the development of these molecular techniques is their ability to detect micrometastasis and minimal residual disease. Monitoring of minimal residual disease in pediatric soft tissue tumors by quantitative RT-PCR may provide important prognostic information. Furthermore, the potential development of targeted therapy based on the understanding of the molecular pathology of a specific soft tissue tumor may complement existing treatments and improve disease outcome. (J Mol Diagn 2003, 5:143–154)

The field of molecular genetics of pediatric soft tissue tumors has expanded enormously over the last decade. This has resulted in the identification of novel genes that have contributed to the understanding of oncogenesis. It is now known that most genetic abnormalities associated with pediatric soft tissue tumors are chromosomal translocations resulting in novel fusion proteins (Table 1). These fusion proteins often affect transcription factors resulting in a disruption of transcription regulation. This disruption may lead to activating inappropriate genes or inappropriately repressing some genes. These fusion

genes, which are fairly specific to the associated tumors, can also serve as targets for the molecular diagnosis of respective tumors and the development of targeted therapy. The knowledge obtained from these studies has further translated into diagnostic, prognostic, and therapeutic applications for patient management. A comprehensive summary of the molecular and cytogenetic lesions associated with pediatric soft tissue tumors is presented in Table 1.

This review is divided into three sections: the application of molecular techniques in clinical management, technical considerations for the commonly used molecular diagnostic techniques, and a brief review of the molecular genetics/pathogenesis of the most common and well-defined pediatric soft tissue tumors.

Application of Molecular Genetics in Clinical Management

Diagnostic Applications

For the purpose of clinical management, pediatric soft tissue tumors are broadly divided into: rhabdomyosarcomas and non-rhabdomyosarcomas (Ewing/peripheral primitive neuroectodermal tumor (PNET) and other sarcomas). Rhabdomyosarcomas are treated primarily with chemotherapy.^{1,2} The role of surgery is limited to initial biopsy, wide local excision (whenever clear margins are possible), and resection of residual disease. Radiotherapy in the form of external beam or brachytherapy is restricted to persistent or recurrent disease. The primary therapy for non-rhabdomyosarcomas is surgical resection, but adjuvant radiotherapy and chemotherapy are being used with increasing success.^{1,3} Another major difference between these two categories is involvement

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Table 1. Summary of Genetic/Molecular Lesions in Pediatric Soft Tissue Tumors

Type of tumor	Chromosomal abnormality	Gene involved or fusion gene	Prevalence	FISH	RT-PCR*	Prognosis [†]
Rhabdomyosarcoma						
Botryoid	NA	NA	NA	NA	NA	Good
Spindle cell	NA	NA	NA	NA	NA	Good
Embryonal	Gains of 2, 7, 8, 12, 13; losses of 1, 6, 9, 14, and 17 ⁵⁰	<i>IGF2, GOK, PTCH, TP53</i>	NA	NA	NA	Good
Alveolar	t(2;13)(q35;q14); t(1;13)(p36;q14)	<i>PAX3-FKHR</i> <i>PAX7-FKHR</i>	75% 10%	Ref.67	Ref.13	Poor [‡]
Undifferentiated	NA	NA	NA	NA	NA	Poor
Non-rhabdomyosarcoma-EWS family						
EWS/PNET	t(11;22)(q24;q12)	<i>EWS-FLI-1</i>	85–95%	Ref.108	Ref.109	Good with type I fusion transcripts [‡]
	t(21;22)(q22;q12)	<i>EWS-ERG</i>	5–10%			
	t(7;22)(p22;q12)	<i>EWS-ETV1</i>	<1%			
	t(17;22)(q21;q12)	<i>EWS-E1AF</i>	<1%			
	t(2;22)(q33;q12)	<i>EWS-FEV</i>	<1%			
	Inversion of 22q	<i>EWS-ZSG</i>	<1%			
DSRCT	t(11;22)(p13;q12)	<i>EWS-WT1</i>	>95%	NA	Ref.24	Poor
Clear cell sarcoma	t(12;22)(q13;q12)	<i>EWS-ATF1</i>	90%	Ref.110	Ref.37	Poor
Extraskeletal myxoid chondrosarcoma [§]	t(9;22)(q22–23;q11–12)	<i>EWS-TEC (CHN)</i>	75%	Ref.111	Ref.43, 96	Good
	t(9;15)(q22;q21)	<i>TCF12-TEC</i>	NA		Ref.43, 112	
	t(9;17)(q22;q11)	<i>TAF2N-TEC</i>	25%		Ref.113	
Extraskeletal mesenchymal chondrosarcoma	der(13;21)(q10;q10)	NA	NA	Ref.114	NA	Poor
Non-rhabdomyosarcoma-others						
Synovial sarcoma	t(X;18)(p11;q11)	<i>SYT-SSX1</i> <i>SYT-SSX2</i> <i>SYT-SSX4</i>	65% 35% rare	Ref.101 Ref.101	Ref.23 Ref.23 Ref.104	Poor [‡]
Congenital/infantile-fibrosarcoma	t(12;15)(p13;q25)	<i>ETV6-NTRK3</i>	80%	Ref.115	Ref.39	Good
Inflammatory myofibroblastic tumor	t(1;2)(q25;p23)	<i>TPM3-ALK</i>	NA	Ref.10	Ref.10	Good
	t(2;19)(p23;q13)	<i>TPM4-ALK</i>	NA	Ref.10	Ref.10	
	t(2;17)(p23;q23)	<i>CLTC-ALK</i>	NA	Ref.11	Ref.11	
	t(2;11;2)(p23;p15;q31)	<i>CARS-ALK</i>	NA	Ref.116	Ref.116	
Myxoid/round cell liposarcoma [§]	t(12;16)(q13;p11)	<i>TLS-CHOP</i>	>95%	Ref.109	Ref.117	Good
Tenosynovial giant cell tumor	t(12;22)(q13;q12)	<i>EWS-CHOP</i>	rare		Ref.97	
	t(1;2)(p11;q35–37)	NA	40%	Ref.118	NA	Good
	t(1;5)(p11;q22–31)		10%			
	t(1;11)(p11;q11–12)		10%			
	t(1;8)(p11;q21–22)		10%			
Lipoblastoma	Rearrangement of 8q12, polysomy 8	<i>PLAG1</i>	70% 20%	Ref.119,126	NA	Good
Malignant peripheral nerve sheath tumor	Complex changes	NA	NA	NA	NA	Poor
DFSP	Ring chromosome with sequences from chromosomes 17 and 22, t(17;22)(q22;q13)	<i>COL1A1-PDGFB</i>	>99%	Ref.121, 122	Ref.38, 123	Good
Desmoid tumor	+8, +20, Deletion (5)(q21–22)	NA NA	NA NA	Ref.24 NA	NA NA	Good
Alveolar soft part sarcoma	t(X;17)(p11;q25)	<i>ASPL-TFE3</i>	> 90%	NA	Ref.125	
Giant cell fibroblastoma (juvenile form of DFSP)	t(17;22)(q22;q13)	<i>COL1A1-PDGFB</i>	NA	Refs.122, 126	Refs.38, 123, 126	

DFSP, Dermatofibrosarcoma protuberans; NA, not applicable/not known.

[§]Rare in pediatric population.^{127, 128}

*The number shown is the corresponding number of references containing protocol for FISH or RT-PCR.

[†]Prognosis based on morphologic classification.

[‡]Fusion transcripts correlate with prognosis of patients, see text for detail.

of lymphatics. Rhabdomyosarcomas often involve the regional lymph nodes, indicating the importance of lymph node evaluation for staging. Non-rhabdomyosarcomas involve lymph nodes less commonly and the spread of these tumors is predominantly hematogenous.

As outlined above, the accurate diagnosis of pediatric soft tissue tumors is critical for clinical management. Accurate diagnosis requires integration of clinical findings (age, sites of involvement, pattern of disease spread, and radiographical characteristics), morphological evaluation, and ancillary tests including immunohistochemistry, cytogenetics, and molecular genetics.

Molecular diagnostic techniques, particularly RT-PCR and FISH, have become important tools to detect the characteristic fusion genes associated with pediatric soft tissue tumors (Table 1) since these molecular techniques require only a minimal amount of tissue. Recently, diagnostic procedure has been changed from open incisional biopsy to methods, such as core biopsy and fine-needle aspirate biopsy (FNAB), requiring smaller amounts of tissue without compromising the accuracy of diagnosis. Such methods are easily accepted by patients, may be performed in ambulatory clinics, and are less likely to have significant complications. The success of these methods (core biopsy and FNAB) relies heavily on the prudent use of the small amount of tissue obtained.

The contributions of molecular genetics have significantly improved the accuracy of diagnosis of pediatric soft tissue tumors.⁴⁻⁷ A practical diagnostic approach of integrating morphology, immunohistochemistry, and molecular genetics using small amounts of tissue is illustrated in Figure 1 with representative examples. The specimens are evaluated for adequacy during FNAB and/or core biopsy by immediate morphological interpretation of the cytology smear or frozen section. The initial differential diagnosis based on morphology evaluation is further refined using immunohistochemistry (Table 2). In the majority of cases, the above evaluation may be sufficient for diagnosis. In difficult cases such as monophasic synovial sarcoma with spindle cell pattern, molecular genetics can be used. Furthermore, whenever there is doubt due to either the atypical morphology or the unexpected immunohistochemistry findings, molecular genetic study may be performed for further evaluation. The exact nature of molecular targets to be tested will depend on the initial differential diagnosis based on morphology and immunohistochemistry as illustrated in Figure 1.

Although different opinions exist regarding how to weigh the impact of molecular genetics testing results on diagnosing pediatric soft tissue tumors, most authorities concur that conventional morphological assessment, supplemented by ancillary techniques including molecular genetics, remains the standard diagnostic approach.⁴⁻⁷ This is because the specificity of fusion genes, although reasonably high, is not absolute. A good example is that *TPM3-ALK* and *CLTC-ALK* gene fusions have been recently identified in both inflammatory myofibroblastic tumors and anaplastic large cell lymphomas.⁸⁻¹¹ An appropriate guideline for handling discrepant histopathological findings and molecular genetics results has been recently suggested by Ladanyi et al⁶

Figure 1. Algorithm highlighting diagnostic contribution by molecular genetic study. Several examples are shown for illustrating purpose. For details please see text, Tables 1 and 2.

Briefly, if a gene fusion is absent in cases of tumor type typically having the gene fusion, the quality of molecular diagnostic methods should be further evaluated to avoid false-negative results. In contrast, if a given gene fusion is present by one molecular diagnostic test in cases of tumor type typically not having the gene fusion, other molecular diagnostic tests should be performed to exclude false-positive interpretation.

Prognostic Applications

Prognostic Significance of Fusion Proteins

Studies have suggested that the different fusion proteins in each specific soft tissue tumor may have prognostic significance. Anderson et al¹² recently reported the translocation, t(2;13)/*PAX3-FKHR*, to be an adverse prognostic factor for alveolar rhabdomyosarcoma. In contrast, t(1;13)/*PAX7-FKHR* was associated with a favorable prognosis and was more frequently observed in younger patients with relatively localized disease.¹² Sorensen et al¹³ reported that, among the patients with metastatic alveolar rhabdomyosarcoma, bone marrow involvement was significantly higher in *PAX3-FKHR*-positive patients. Furthermore, in patients presenting with metastatic disease, there was a striking difference in outcome between *PAX7-FKHR* and *PAX3-FKHR* patient groups (estimated 4-year overall survival rate of 75% for *PAX7-FKHR* versus 8% for *PAX3-FKHR*).¹³

Table 2. Differential Diagnosis of Pediatric Soft Tissue Tumors Based on Cyto/Histomorphology and Immunohistochemistry

Cyto/histomorphology	Soft tissue tumor	Diagnostic immunoprofile
Round cell	Rhabdomyosarcoma	MyoD1+
	PNET	CD 99 +
	DSRCT	WT1+, keratin+, EMA+, Desmin+
Spindle cell	Fibromatosis	Non-diagnostic
	Spindle cell lipoma	Non-diagnostic
	Schwannoma/neurofibroma	S-100 protein+
	Kaposi's sarcoma*	CD34+, CD31+
	Dermatofibrosarcoma protuberans	CD 34+
	Fibrosarcoma*	Non-diagnostic
	Leiomyosarcoma*	SMA +, Desmin +
	Malignant peripheral nerve sheath tumor	S-100 protein + (weak)-
	Malignant fibrous histiocytoma (some)*	Non-diagnostic
	Synovial sarcoma	Usually non-diagnostic (Keratin -/+, EMA -/+)
Epithelioid (polygonal) cell	Angiosarcoma (some)*	CD 31 +, CD 34 +, Factor VIII +
	Epithelioid hemangioma	CD 31 +, CD 34 +, S-100 protein+
	Granular cell tumor	Melanoma markers +
	Clear cell sarcoma	CD 34 +, CK +
	Epithelioid sarcoma	Non-diagnostic
	Alveolar soft part sarcoma	CD 31 +, CD 34 +, Factor VIII +
	Angiosarcoma (some)*	Non-diagnostic
Myxoid	Nodular fasciitis	Non-diagnostic
	Myxoma	Non-diagnostic
	Myxoid liposarcoma*	Non-diagnostic
	Myxoid chondrosarcoma*	Non-diagnostic
	Myxoid malignant fibrous histiocytoma	Non-diagnostic
Adipocytes	Lipoma	Non-diagnostic
	Lipoblastoma	Non-diagnostic
	Well differentiated liposarcoma*	Non-diagnostic

*Rare in pediatric population^{127, 128}

Similarly, recent studies have suggested that the most common type of *EWS-FLI1* fusion transcript, type 1 (*EWS* exon 7 fused to *FLI-1* exon 6), is associated with a favorable prognosis and appears to encode a functionally weaker transactivator, compared to other types of fusion transcripts.^{14,15} Among patients with localized disease, patients with a type 1 fusion product had a longer relapse-free survival than those with other variants of fusion transcripts.¹⁴ Finally, in localized synovial sarcoma, patients with the *SYT-SSX2* fusion variant had significantly longer metastasis-free survival than those with *SYT-SSX1* variant.¹⁶ Ladanyi et al¹⁷ recently reported that the patients with the *SYT-SSX2* fusion variant had better overall survival than those with *SYT-SSX1* variant.

Even more promising is the potential ability of gene expression profiles by cDNA microarray to detect different prognostic groups. In a pilot study, using cDNA microarray and cluster analysis, the gene expression profiles of different types of pediatric soft tissue tumors correctly discriminated patients who survived from those who did not, with *P* values less than 0.0001.¹⁸ Notably, none of the existing prognostic factors, including histological type and response to therapy, were as reliable for predicting the survival in these patients.¹⁸

Prognostic Significance of Detection and Quantitation of Minimal Disease

The sensitivity and specificity of the RT-PCR protocols have provided evidence for the existence of minimal disease which cannot be detected by physical/morphological/radiographical examination. Cumulative data has

demonstrated that most patients still harbor a significant tumor burden after resection. When patients with Ewing's sarcoma or PNET were studied using RT-PCR, while about 50% of patients with metastatic or relapsed disease had blood or marrow samples positive for the *EWS-FLI-1* transcripts; approximately 25% of patients without clinically metastatic disease did so as well.¹⁹ Similarly, using RT-PCR for detecting the *PAX3-FKHR* and *PAX7-FKHR* transcripts, Kelly et al²⁰ reported that some marrow samples, from patients with alveolar rhabdomyosarcoma, interpreted as negative by light microscopy were positive by RT-PCR. Recently, detection of fetal acetylcholine receptor by RT-PCR has shown to be a reliable marker for detecting minimal disease for both alveolar and embryonal rhabdomyosarcomas.²¹ Other RT-PCR methods to detect minimal disease of synovial sarcoma and desmoplastic small round cell tumors have also been described.²²⁻²⁴ However, the clinical significance of minimal disease remains to be evaluated.^{22,24}

It is anticipated that, similar to hematopoietic malignancies, monitoring of minimal residual disease by quantitative RT-PCR will, in the future, provide important prognostic information for the management of pediatric soft tissue tumors.^{25,26} In patients with chronic myelogenous leukemia, rising or persistently high levels of BCR-ABL (BCR-ABL:ABL ratio of >0.02% or >100 BCR-ABL transcripts/ μ g RNA), determined by quantitative RT-PCR, in two sequential specimens more than 4 months following stem cell transplantation, are predictive of overt clinical relapse.²⁶ Similarly, using real-time quantitative PCR to quantify the amount of t(14;18)(q32;q21) cells, we have recently observed that measurable tumor load $\geq$ 0.01%

after stem cell transplantation may predict subsequent relapse of follicular lymphoma (Chang C, Bredeson C, Juckett M, Logan B, Keever-Taylor CA, manuscript submitted). Furthermore, studies have shown that treating CML patients before overt clinical relapse of disease can improve survival of CML patients after stem cell transplantation.²⁷ Quantitation of minimal residual disease may lead to new therapeutic avenues to improve patient outcome in pediatric soft tissue tumors. Furthermore, quantitation of minimal residual disease has become clinically practical due to the recent emergence of devices for performing real-time RT-PCR.

Therapeutic Applications

Targeted therapy potentially can be developed based on the understanding of the molecular pathology of pediatric soft tissue tumors.^{18,28-34} For example, when the gene expression profiles of metastatic tumors were compared to that of non-metastatic and favorable tumors by cDNA microarray, several candidate genes that represent potential therapeutic targets were readily identified.¹⁸ These targets are analogous to the HER2/neu receptor in breast cancer and c-Kit in gastrointestinal stromal tumors. Allander et al³⁵ reported the identification of a new potential therapeutic target, ERBB2 (HER2/neu), for a subset of synovial sarcoma cases using cDNA microarray analysis.

Fusion proteins generated by chromosomal translocations are true tumor-specific antigens and are promising targets for immunotherapy.³¹ In a recent study, the induction of synovial sarcoma-specific cytotoxic T-lymphocytes from normal donor lymphocytes using *in vitro* stimulation with fusion peptide (derived from SYT-SSX fusion protein)-pulsed dendritic cells has been demonstrated. These cytotoxic T-lymphocytes have the ability to lyse human synovial sarcoma tumor cells expressing the fusion protein.³⁶ These findings suggest that a peptide derived from the fusion protein may work as a neoantigen and induce a tumor-specific immune response.

Other molecular targets currently under investigation include: antisense oligonucleotides to the fusion transcripts, signal transduction inhibitors, angiogenesis inhibitors, matrix metalloproteinase inhibitors, inhibitors of fusion protein, and differentiation inducers.^{29,30,32} These targeted approaches along with traditional therapeutic modalities will be valuable additions to the armamentarium of current methods for tumor management.

Technical Considerations of the Commonly Used Molecular Diagnostic Techniques

Although most of the characteristic chromosomal translocations in the soft tissue tumors can be detected by conventional cytogenetics, molecular diagnostic techniques have become an important complementary tool for detecting these fusion genes. Cytogenetics studies require fresh samples that may not always be available. Furthermore, cytogenetic studies are limited by growth failure of tumor cells, masking of tumor cells by over-

growth of karyotypically normal stromal cells, and long turnaround time (2 to 7 days).

In clinical laboratories, the most common molecular diagnostic techniques used to detect these fusion genes are RT-PCR (qualitative and/or quantitative) and FISH, although other methods can be applied as well (see below). Molecular studies require a minimum of 100 mg of representative viable tumor tissue frozen (at -80°C in OCT embedding compound, Sakura Tissue Tec, Torrance, CA, or snap-frozen in liquid nitrogen) and stored in a -80°C freezer. Technologies such as comparative genomic hybridization (CGH), spectral karyotyping (SKY), multicolor fluorescence *in situ* hybridization (M-FISH), and cDNA microarray remain predominantly research tools to investigate the oncogenesis of these tumors.

Southern Blot

Southern blot is the oldest nucleic acid-based method and is used extensively in research settings. It is a simple but powerful basic tool for molecular studies. Using a probe targeting a specific oncogene, this method is particularly useful in detecting a translocation when the partners of a translocation of a specific gene are multiple or unknown. Furthermore, this method can quantitatively determine the number of copies of a gene when compared with an appropriate control. However, this method suffers from the disadvantages of a turnaround time of 4 to 5 days. It further requires a relatively large quantity (micrograms) of fresh DNA. Thus, this method cannot be applied when only a small amount of tissue sample is available (such as in needle core biopsies) or when freshly frozen samples are not stored. Ultimately, these factors discourage its application for diagnostic purposes.

RT-PCR-Based Methods

Most characteristic translocations reported in pediatric soft tissue tumors have genomic breakpoints located more or less randomly within large introns. This precludes using DNA to detect these translocations by PCR, although DNA is easier to handle and more readily retrieved from paraffin-embedded tissue than RNA. Nevertheless, the breakpoints of these translocations are clustered enough so that primers can be designed to detect most of the translocations using RT-PCR (Table 1).

The RT-PCR-based methods have become the most practical method for detecting fusion transcripts. The advantage of RT-PCR-based methods is that only a small amount of tissue is required. However, preservation of good quality RNA is essential. Thus, appropriate handling of tissue samples is critical. For RT-PCR, tissue can be snap-frozen in liquid nitrogen or be immediately immersed in OCT embedding compound and then snap-frozen with dry ice or liquid nitrogen. Both methods preserve adequate quality RNA for RT-PCR (conventional and real-time quantitative RT-PCR); however, embedding tissue in OCT compound is more readily combined with microdissection technique. The recently available RNA,

RNAlater (Ambion RNA Diagnostics, Austin, TX), provides an alternative way to preserve RNA when immediate freezing is not possible. The quality of RNA preserved with RNAlater is comparable to that preserved with the above two methods (personal experience). Although RNA extracted from paraffin-embedded tissue has been used for RT-PCR to detect fusion transcripts,^{37–43} the diagnostic yield with this approach may be inferior.⁷ A recent multi-center study revealed significantly decreased diagnostic yield of IgH gene rearrangement when paraffin-embedded tissue was used.⁴⁴ Fresh-frozen tissue, preferably containing a minimum of 100 mg (approximately 5 × 5 × 5 mm) of viable tumor, remains the most favorable testing material.

The major disadvantage of RT-PCR is the need for scrupulous laboratory practices to avoid contamination of locations and equipment with PCR products. In our laboratory, we have successfully used uracil-N-glycosylase (UNG) to reduce contamination problems without compromising the efficiency of real-time PCR. Furthermore, vigorous negative controls including “no RNA (water)” and “no reverse transcriptase” controls should be included in the RT-PCR assays. The former is to evaluate reagent contamination and the latter is to address the possibility of contamination of the RNA samples themselves.

In addition to detection, quantitative RT-PCR can also be used for the quantitation of fusion transcripts. The conventional quantitative RT-PCR techniques by either competitive or limiting dilution methods are too labor intensive for clinical practice. The recently developed real-time quantitative RT-PCR technology using fluorescence resonance energy transfer (FRET) is easy to perform with quick turnaround time.^{45–49} Assay time, including RNA extraction, is about 6 hours, compared with 2 days for standard RT-PCR methods. The assay time can be further shortened to 4 hours if an instrument capable of rapid cycling, such as LightCycler (Roche Diagnostics, Indianapolis, IN), is used.⁴⁵ It is likely that this technology will become the mainstream method for detecting the fusion transcripts and quantitating their levels.^{46–49}

Recently, Peter et al⁴⁹ reported the development of a multiplex real-time RT-PCR which enabled the detection with identical PCR conditions of the different fusions specifically observed in Ewing tumors, alveolar rhabdomyosarcoma, synovial sarcoma, small round cell desmoplastic tumors, extraskeletal myxoid chondrosarcoma, malignant melanoma of soft parts, and congenital fibrosarcoma. The multiplex reaction can reduce cost and turnaround time significantly. Furthermore, this type of technology constitutes an important step toward the complete automation of detecting cancer-specific gene fusions.

Fluorescence in Situ Hybridization

Fluorescence *in situ* hybridization (FISH) offers several advantages over Southern blot and PCR-based methods. FISH has the ability to examine individual cells. Thus, it can potentially distinguish tumor cells from normal cells and detect heterogeneity within tumor cells. However,

this may be a difficult task at times. Interphase FISH can be applied to cytology smears and histological sections from formalin-fixed, paraffin-embedded tissue.⁶ However, FISH is more time consuming and labor intensive than RT-PCR. Surprisingly, despite the importance of detecting fusion genes in the diagnosis of pediatric soft tissue tumor, there are no commercially available FISH probes for the vast majority of these genes. The applications of FISH in various soft tissue tumors as reported in the literature are summarized in Table 1.

Comparative Genomic Hybridization

Comparative genomic hybridization (CGH) is particularly useful for the detection of yet unidentified genomic regions that are amplified or deleted in a particular tumor.^{50–57} For example, studies using this technology have generated significant clues in the oncogenesis of rhabdomyosarcoma,^{50,51} clear cell sarcoma,⁵⁸ synovial sarcoma,⁵⁵ and Ewing tumors.⁵³ The major limitation of CGH is that it cannot detect low copy number amplifications, small deletions, and balanced rearrangements such as inversions or translocations. This method requires imaging hardware and software that is present in most commercially available systems.

Spectral Karyotyping (SKY)

Using spectral karyotyping (SKY), a universal chromosome painting technique, all of the chromosomes can be labeled at once, providing a comprehensive FISH screening test for detecting cytogenetic changes.⁵⁹ This technology recognizes extremely complex or subtle gene rearrangements. This technology is based on metaphase analysis and can only be performed on viable tissue. A related technology, multi-fluorophore fluorescence *in situ* hybridization (M-FISH), has also been developed.^{60,61} Both methods are limited by the inability to detect intrachromosomal anomalies, abnormalities involving the p-arms of acrocentrics, and areas rich in highly repetitive DNA.⁶¹

cDNA Microarray

Preliminary works have shown promise for classifying soft-tissue tumors based on gene expression profiles using cDNA microarray, which have the capability of simultaneously examining the expression of more than 12,000 genes.^{18,62–65} For example, Khan et al⁶⁵ have recently shown that alveolar rhabdomyosarcoma cells show a characteristic pattern of gene expression, which allows this group of tumor to be identified by cDNA microarray analysis. Triche¹⁸ reported preliminary results indicating that simple hierarchical clustering analysis of gene expression profiles by cDNA microarray categorized all but two of 23 “small round cell tumors” of childhood. With more sensitive clustering methods for the analysis of gene expression profiles, subcategories of tumors (such as embryonal *versus* alveolar rhabdomyosarcoma) and even subsets of alveolar RMS (such as

with a *PAX3/FKHR*, *PAX7/FKHR*, or without any translocation) could be readily distinguished.¹⁸ Additionally, this technology has been explored for predicting prognosis of pediatric soft tissue tumors and for developing targeted therapy (see above). The power of this technology can be further enhanced with laser microdissection technology to select a pure tumor population. Further studies are required to determine the clinical role of the cDNA microarray technology.

Brief Review of Molecular Genetics/ Pathogenesis of the Most Common and Well-Defined Pediatric Soft Tissue Tumors

Rhabdomyosarcoma

Rhabdomyosarcoma is the most common type of pediatric soft tissue sarcoma, comprising about 50% of cases. Its subtypes vary according to histological appearance, clinical presentation, and outcome. The major morphological subtypes are alveolar (20% of cases) and embryonal (65% of cases). Survival may vary from greater than 95% (embryonal subtype) to less than 25% (alveolar subtype).⁶⁶ The contributions of molecular genetics have significantly improved the accuracy of diagnosis within subtypes and enhanced the understanding of oncogenesis.

Alveolar Rhabdomyosarcoma

A characteristic translocation, t(2;13)(q35;q14), is seen in about 75% of cases.^{67–70} With this translocation, the *PAX3* gene on chromosome 2 is fused with the *FKHR* (also known as *FOX10A* or *ALV*) gene on chromosome 13, resulting in a chimeric transcription factor derived from the 5' end of the *PAX3* gene and the 3' end of *FKHR* gene (exons 2 and 3).⁷⁰ Both portions encode DNA-binding domains. A less frequently associated translocation, t(1;13)(p36;q14), is observed in 10% of cases. With this translocation the *PAX7* gene on chromosome 1 is involved. Recently, Barr et al⁷¹ reported several genetically distinct subsets of alveolar rhabdomyosarcoma (ARMS) cases without characteristic *PAX3-FKHR* or *PAX7-FKHR* transcripts. These subsets include cases with low expression or atypical presentation of standard fusions, variant fusions with other genes, and true fusion-negative cases.

The *PAX3* and *PAX7* genes belong to a family of *PAX* genes that encode transcription factors important in normal embryonic development.⁷² Both of these genes encode DNA-binding domains, referred to as "paired box" and "homeobox" domains. *PAX3* and *PAX7* are highly homologous, suggesting similar functions. Both genes are expressed in developing myotomes, which may explain the involvement of these genes in rhabdomyosarcoma. The *FKHR* gene is a member of a different family of genes that also encode transcription factors involved in the developing embryo.

The fusion proteins encoded by the *PAX3/FKHR* and *PAX7/FKHR* transcripts are similar. The DNA-binding do-

main of *PAX3* and *PAX7* are retained in the fusion proteins.⁷³ The *FKHR* portion of the fusion protein, although it has lost its DNA-binding domain, contributes an activation domain that is lost from the native *PAX3* or *PAX7* proteins as a result of the translocation. Thus, the activation domain of the *FKHR* protein may lead to aberrant levels of expression of the target genes of *PAX3* or *PAX7*. This aberrant gene expression may affect the control of growth, apoptosis, differentiation, and motility of myogenic precursor cells.⁷³ In a recent study, the expression levels of *Itih2A*, *Fath*, *FLT1*, *TGFA*, *BVES*, and *EN2* genes correlated with the levels of *PAX3-FKHR* expression in rhabdomyosarcoma cell lines. These genes may be part of the *PAX3-FKHR* regulatory pathway during tumor formation.⁷⁴

Embryonal Rhabdomyosarcoma

"Tumor-specific" genetic changes have not been reported in embryonal rhabdomyosarcoma. However, consistent/recurrent cytogenetics changes (+2, +7, +8, +11, +12, etc) have been observed. This pattern of changes may be helpful in classifying the lesion, although it is not definitive or tumor specific. Recently, using comparative genomic hybridization (CGH), Bridge et al⁵⁰ reported gains of chromosomes or chromosomal regions [2 (50% of cases), 7 (42%), 8 (67%), 11 (42%), 12 (58%), 20 (33%) and 13q21] and losses of 1p35–36.3 (42%), 6 (33%), 9q22 (33%), 14q21–32 (25%), and 17 (25%) as the most common genetic abnormalities in embryonal rhabdomyosarcoma. Loss of heterozygosity at chromosome 11p15 leading to overexpression of *IGF2* (insulin growth factor 2) through loss of imprinting or paternal disomy has been reported in some cases.⁷⁵ Chromosome fragment transfer studies have further suggested the presence of other unknown tumor suppressor gene(s) in chromosome 11p15.⁶⁹ Most recently, Bridge et al⁵¹ reported that genomic amplification was detected, by CGH, in 23% of embryonal rhabdomyosarcoma cases with anaplastic features. One amplicon, corresponding to the locus of the insulin-like growth factor type I receptor (*IGF1R*) gene located at 15q25–26, may play a role in the development or progression of this subset of rhabdomyosarcomas. Finally, p53 mutation may have a role in the development of rhabdomyosarcoma.⁵⁰ Rhabdomyosarcoma is the most common sarcoma observed in Li-Fraumeni syndrome. The majority of patients with this syndrome demonstrate a germ-line mutation in p53.

Ewing's Sarcoma and Peripheral Primitive Neuroectodermal Tumor

The shared genetic anomalies have confirmed a common histopathogenesis between these histopathologically similar tumors. Both tumors are associated with a reciprocal translocation involving chromosome 22 at q12.^{76–78} In about 90% of cases, the other involved chromosome is 11 at q24.⁷⁹ This translocation fuses the *FLI-1* gene (on chromosome 11) with the *EWS* gene (on chromosome 22), resulting in a chimeric protein derived from the 5'

end of *EWS* and 3' end of *FLI-1*. The breakpoints are variable with at least 10 different transcripts of different sizes, ranging from 300 to 700 bps, detectable by RT-PCR.⁸⁰ The most frequent fusion transcripts join exon 7 of *EWS* to either exon 6 or exon 5 of *FLI-1*. They are designated as type 1 and type 2 fusion transcripts, respectively.⁸¹ The type 1 fusion transcript is associated with a favorable prognosis and appears to encode a functionally weaker transactivator as compared to other fusion types.^{15,81}

The normal function of the *EWS* gene remains to be defined, although it is ubiquitously expressed. Ewing's sarcoma (EWS) is believed to function as an adaptor between the RNA polymerase II transcription complex and RNA splicing factors.^{77,82} The *FLI-1* gene is a member of the *ETS* family of proto-oncogenes and contains a DNA-binding domain and functions as a transcription activator. The fusion protein retains the polymerase II-interacting domain but loses the C-terminal domain of *EWS* necessary for normal interaction with splicing factors. Studies have shown that the fusion protein inhibits YB-1 mediated splicing of pre-mRNA and that the ability to inhibit pre-mRNA splicing coincides with the transforming ability of this fusion protein *in vitro*.^{77,83,84} Furthermore, the fusion protein acts as a transcriptional activator or repressor of a set of target genes identified by microarray analysis.⁸⁵ These findings suggest that the fusion protein plays a critical role in oncogenesis. Finally, studies have shown that the p53 pathway and the basic fibroblast growth factors (bFGF) pathway may play an important role in modulating fusion protein oncogenicity.^{77, 86} P53 alteration appears to define a small clinical subset of patients with EWS/PNET with a markedly poor outcome.⁸⁷

To date, at least four additional but less common translocations have been reported in Ewing's sarcoma and PNET (Table 1). All of them maintain a common theme of fusing a portion of the *EWS* gene to another gene similar to the *FLI-1* gene.⁷⁶ All these genes belong to the *ETS* family of transcription factors. Among these translocations, t(21;22)(q22;q12) is the most common and is seen in up to 5% of cases. Some variants with more complex translocations involving more than two chromosomes have been reported, but the *EWS* gene seems to be a consistent feature.⁸⁸ Recently, a novel fusion gene, *EWS/ZSG*, caused by inversion of 22q has been reported.⁸⁹ However, the definite interpretation of this tumor appears to be controversial since this tumor has overlapping histological features of desmoplastic small round-cell tumor (DSCRT) and PNET.

Desmoplastic Small Round-Cell Tumor

DSRCT, originally described as intra-abdominal DSRCT, is a polyphenotypic tumor with a tendency for widespread peritoneal involvement. DSRCT occurs predominantly in young males and has a distinctive histological appearance. Recently, the spectrum of DSRCT has been expanded to include other histological variants and other sites including the ovary, pleura, and even the posterior cranial fossa.^{6,90}

Figure 2. The phenotype of the tumor may vary according to the fusion partners of *EWS* gene.

In DSRCT, the *EWS* gene on chromosome 22 is fused with the *WT1* gene (Wilms tumor suppressor gene) on chromosome 11.⁹¹ The resulting chimeric protein is derived from the 5' end of the *EWS* gene and the 3' end of the *WT1* gene. As in Ewing's sarcoma, the fusion gene in DSRCT includes up to exon 7 and rarely exon 8, 9, or 10 of *EWS*.⁹² The fusion includes exons 8–10 of *WT1* that encode most (but not all) of the portion of the DNA-binding domain. The *WT1* gene is normally expressed in kidney during the time of its transition from a mesenchymal phenotype to an epithelial phenotype.^{93,94} This gene is also expressed in mesothelium, which may lead to involvement of mesothelium in DSRCT and the polyphenotypic nature of DSRCT.^{93,94} In DSRCT, the retained portion of *WT1* behaves as a transcriptional activator, acting on some of the target genes of the normal *WT1* protein.⁹³ For example, it has been shown that expression of *EWS-WT1* induces the expression of endogenous platelet-derived growth factor-A (*PDGFA*), whose promoter contains many potential *WT1*-binding sites.⁹⁵ The induction of *PDGFA*, a potent fibroblast growth factor, may contribute to the characteristic reactive fibrosis associated with this unique tumor.⁹⁵

In addition to Ewing's sarcoma/PNET and DSRCT, the fusion of *EWS* gene is observed in clear-cell sarcoma,³⁷ extraskelatal myxoid chondrosarcoma,^{43,96} and myxoid/round cell liposarcoma,⁹⁷ though the counterpart genes in all these tumors are different (Figure 2). This suggests

that the fusion partner may determine the tumor phenotype. The fusion partner's DNA-binding properties may determine which target genes are activated, thus giving rise to a specific tumor phenotype.^{98,99}

Synovial Sarcoma

A characteristic translocation t(X;18)(p11;q11), has been identified in up to 95% of cases of synovial sarcoma.^{66,100} In the vast majority of cases, this translocation leads to a fusion transcript of the 5' portion of the *SYT* gene on chromosome 18 with the 3' portion of one of two genes, *SSX1* or *SSX2*, on chromosome X.^{23,40,101} Both monophasic (purely mesenchymal morphology) and biphasic (with both epithelial and mesenchymal features) variants of synovial sarcoma are associated with this translocation. Most synovial sarcomas with *SYT/SSX1* show biphasic morphology; in contrast, most synovial sarcomas with *SYT/SSX2* show monophasic morphology.⁴⁰ Recently, fusion transcript variants of a novel fusion gene *SYT/SSX4* have been reported.¹⁰²⁻¹⁰⁴

The function of these fusion proteins remains to be defined. In the *SYT/SSX* fusion transcript, the potential transcriptional activator domain of *SYT* gene replaces the potential transcriptional repressor domain of the *SSX* genes. Hypothetically, this could lead to aberrant expression of *SSX* target genes. In one recent study, *SYT-SSX* was shown to stabilize cyclin D1 and was critical for cyclin D1 expression in synovial sarcoma cells.¹⁰⁵ *SYT/SSX*-dependent expression of cyclin D1 may be a significant event in the development and progression of synovial sarcoma.¹⁰⁵ Additionally, Nagai¹⁰⁶ recently reported that the N-terminal 181 amino acids of *SYT-SSX1* were essential for the transformation of cultured fibroblasts and were shown to mediate binding to the chromatin remodeling factor hBMR/hSNF α . For further comprehensive review of molecular genetics of synovial sarcoma, the reader is referred to a recent article by Sandberg and Bridge.¹⁰⁷

Conclusion

Molecular diagnostic techniques to detect characteristic fusion genes have proved to be of significant importance as a tool for evaluating pediatric soft tissue tumors. The detection of these fusion genes has strengthened existing concepts of oncogenesis and greatly enhanced diagnostic accuracy. One of the exciting promises of the molecular techniques is the ability to detect fusion genes in the setting of micrometastasis and minimal residual disease. In the future, the challenge for the medical community is to establish the prognostic significance of micrometastasis and minimal residual disease. To surmount this challenge, it is important to standardize the RT-PCR methods used to detect and quantitate the characteristic fusion transcripts within the molecular pathology community. Furthermore, the potential development of targeted therapy based on the understanding of the molecular pathology of specific soft tissue tumor may further com-

plement the existing treatments to improve the disease outcome.

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