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Use of a novel FISH assay on paraffin-embedded tissues as an adjunct to diagnosis of alveolar rhabdomyosarcoma

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A valuable diagnostic adjunct and important prognostic parameter in alveolar rhabdomyosarcoma (ARMS) is the identification of translocations t(2;13)(q35;q14) and t(1;13)(p36;q14), and the associated *PAX3-FKHR* and *PAX7-FKHR* fusion transcripts, respectively. Most RMS fusion gene type studies have been based on reverse transcriptase-polymerase chain reaction (RT-PCR) detection of the fusion transcript, a technique limited by RNA quality and failure of devised primer sets to detect unusual variants. As an alternative approach, we developed a fluorescence *in situ* hybridization (FISH) assay that can: (1) distinguish between the two most common ARMS-associated fusion genes; (2) identify potential unusual variant translocations; (3) assess histologic components in mixed alveolar/embryonal RMS; and (4) be performed on paraffinized tissue. FISH analyses of 75 specimens (40 ARMS, 16 ERMS, 8 mixed ARMS/ERMS, and 11 non-RMS tumors) using selected cosmid clone, bacterial, P1-derived, and yeast artificial chromosome probe sets were successful in all but two cases. Among specimens with informative results for both FISH and RT-PCR or standard karyotyping, *PAX/FKHR* classification results were concordant in 94.6% (53/56). The three discordant cases included one exhibiting a t(2;13) by FISH that was subsequently confirmed by repeat RT-PCR, a second showing a rearrangement of the *PAX3* locus only (consistent with the presence of a *PAX3* variant translocation), and a third revealing a t(2;13) by FISH that lacked this translocation cytogenetically. Both alveolar and embryonal components of the mixed ARMS/ERMS subtype were negative for *PAX3*, *PAX7*, and *FKHR* rearrangements, a surprising finding confirmed by RT-PCR and/or conventional karyotyping. These data demonstrate that FISH with newly designed probe sets is a reliable and highly specific method of detecting t(1;13) and t(2;13) in routinely processed tissue and may be useful in differentiating ARMS from other small round cell tumors. The findings also suggest that FISH may be a more sensitive assay than RT-PCR in some settings, capable of revealing variant translocations.
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Rhabdomyosarcoma (RMS) is the most common pediatric soft-tissue sarcoma related to the myogenic lineage. RMS can be divided into three primary histologic subtypes including embryonal RMS (ERMS), alveolar RMS (ARMS), and pleomorphic (anaplastic) RMS.[1,2] Anaplasia can be identified in any subtype of RMS.[2] ERMS encompasses the botryoid and spindle cell variants. ARMS may present as a solid pattern or mixed with both embryonal and alveolar features. These histologic subtypes are one of the most useful prognostic parameters in RMS.[1,3]

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The majority (80%) of ARMS are associated with specific chromosomal translocations occurring between chromosomes 2 and 13 or less commonly 1 and 13 leading to the fusion of either the transcription factor *PAX3* in the former case or the related transcription factor *PAX7* in the latter case to another transcription factor *FKHR*.[4,5] The remaining 20% of ARMS are translocation or fusion-negative forming a basically unexplored group, although two variant translocation associated fusion transcripts (*PAX3-AFX* and *PAX3-NCOA1*) have been described.[6,7]

Although the different *PAX-FKHR* fusion genes are readily identified by reverse transcriptase-polymerase chain reaction (RT-PCR), this technique may be limited by RNA quality and failure of devised primer sets to detect unusual variants. Previous FISH studies have been performed on a small series of RMS samples.[8,9] Therefore, we wished to develop a novel FISH assay that can distinguish between the two most common ARMS-associated fusion genes, identify potential unusual variant translocations, and provide reliable performance on formalin-fixed, paraffin-embedded tissue. We also assessed both alveolar and embryonal components of the mixed histologic subtype by FISH using paraffin-embedded tissue sections in which morphologic features could be identified.

Materials and methods

Histopathologic Assessment

The histopathologic classification of the RMS specimens was confirmed by the Intergroup Rhabdomyosarcoma Study Group (IRSG) Pathology Review Committee. Tumors were assessed in accordance with the International Classification of Rhabdomyosarcoma (ICR) criteria.[3] All of the mixed ARMS/ERMS cases had well-characterized alveolar and embryonal components (Figure 1); equivocal cases were omitted.

Tumor Samples

In total, 40 ARMS samples including 23 fusion-negative cases selected from the IRSG database, 16 ERMS samples, and eight mixed ARMS/ERMS samples were analyzed. In addition, 11 non-RMS tumors (three Ewing sarcomas, three neuroblastomas, two inflammatory myofibroblastic tumors, and three leiomyosarcomas) were included in this study. The histopathologic types of the samples studied are listed in Table 1.

Cytogenetic Analysis

Standard culture and harvesting procedures were performed, as described previously.[10] Metaphase cells were banded with Giemsa trypsin, and the karyotypes were expressed according to the International System for Human Cytogenetic Nomenclature 1995.[11]

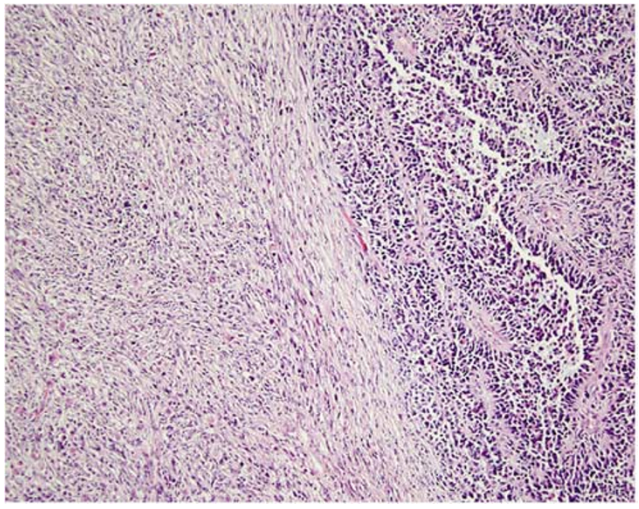

Figure 1 Mixed alveolar/ERMS (Case 57). An abrupt transition between embryonal (left) and alveolar (right) zones is seen.

RT-PCR Analysis

RT-PCR analysis for the presence of the *PAX3-FKHR* or *PAX7-FKHR* fusion transcript was performed as previously described.[12]

Probe Design and Development

Bacterial artificial chromosome (BAC), P1-derived artificial chromosome (PAC), yeast artificial chromosome (YAC) and cosmid clones for the *PAX3*, *PAX7*, and *FKHR* gene regions were identified utilizing the NCBI Map Viewer (http://www.ncbi.nlm.nih.gov/mapview), the Ensembl Genome Browser (http://www.ensembl.org), and the Whitehead Institute/MIT Center Genome Browser (http://www-genome.wi.mit.edu/cgi-bin/contig/yac_info). The probe mixtures used are listed in Table 2. Combinations of probe sets were fashioned to flank and span each gene.

Fluorescence *In Situ* Hybridization

Two-color FISH studies were performed on cytologic touch preparations ($n=25$), *in situ* sections ($n=2$), and formalin-fixed, paraffin-embedded tissue sections ($n=48$). Probes were directly labeled by nick translation with either Spectrum Green or Spectrum Orange-dUTP utilizing a modification of the manufacturer's protocol (Vysis, Downers Grove, IL, USA). An amount of 1 μg of DNA for each of three probes, 1.5 μg of DNA for each of two probes, or 3 μg of DNA for one probe was combined to total 3 μg of DNA per label. All nick translation reagents were then multi-

Table 1 A comparison between FISH and RT-PCR or cytogenetics from 64 tumor specimens

Case	*Histologic type*[a]	*Slide type*[b]	*Karyotype*[c]	*RT-PCR*[c]	*FISH*	*Correlation*
1	ARMS	TP	ND	Negative	Normal	Yes
2	ARMS	TP	ND	Negative	Normal	Yes
3	ARMS	TP	ND	Negative	*PAX3/FKHR*	No
4	ARMS	TP	ND	Negative	Normal	Yes
5	ARMS	TP	ND	Negative	Normal	Yes
6	ARMS	TP	ND	Negative	Normal	Yes
7	ARMS	TP	ND	Negative	Normal	Yes
8	ARMS	TP	ND	Negative	Normal	Yes
9	ARMS	TP	ND	Negative	Normal	Yes
10	ARMS	TP	46,XX[12]	Negative	Normal	Yes
11	ARMS	TP	ND	Negative	Normal	Yes
12	ARMS	TP	ND	Negative	Normal	Yes
13	ARMS	TP	46,XY[15]	Negative	*PAX3* variant	No
14	ARMS	TP	ND	Negative	Normal	Yes
15	ARMS	TP	ND	Negative	Normal	Yes
16	ARMS	TP	ND	Negative	Normal	Yes
17	ARMS	TP	ND	Negative	Normal	Yes
18	ARMS	TP	ND	Negative	Normal	Yes
19	ARMS (focal anaplasia)	TP	46,XY,-3,?add(12)(p12),+mar1, dmin,inc[12]/~94,idemx2, +mar3x2[8]	Negative	Normal	Yes
20	ARMS	TP	ND	Negative	Normal	Yes
21	ARMS	TP	48,XY,add(6)(p15),+11,+19[8]/46, XY[18]	Negative	Normal	Yes
22	ARMS	TP	ND	Negative	Normal	Yes
23	ARMS	TP	ND	Negative	Normal	Yes
24	ARMS	FFPE	ND	ND	Fail	Undecided
25	ARMS	FFPE	ND	Negative	Normal	Yes
26	ARMS	FFPE	ND	ND	*PAX3/FKHR*	Undecided
27	ARMS	FFPE	ND	ND	*PAX3/FKHR*	Undecided
28	ARMS	FFPE	ND	ND	*PAX3/FKHR*	Undecided
29	ARMS	FFPE	ND	*PAX3/FKHR*	*PAX3/FKHR*	Yes
30	ARMS	FFPE	ND	ND	*PAX3/FKHR*	Undecided
31	ARMS	FFPE	ND	Negative	Normal	Yes
32	ARMS	FFPE	ND	*PAX3/FKHR*	*PAX3/FKHR*	Yes
33	ARMS	FFPE	46,XY[19]	Negative	Normal	Yes
34	ARMS (solid variant)	FFPE	46,XY,del(6)(p21p25)[2]/46,XY[18]	Fail	*PAX3/FKHR*	No
35	ARMS (anaplasia)	FFPE	94,XXXX,+2,+2,t(2;13)(q35;q14)x2, 0-20dmin[10]/46,XX[2]	*PAX3/FKHR*	*PAX3/FKHR*	Yes
36	ARMS	FFPE	ND	*PAX7/FKHR*	*PAX7/FKHR*	Yes
37	ARMS	FFPE	ND	*PAX7/FKHR*	*PAX7/FKHR*	Yes
38	ARMS	FFPE	ND	*PAX7/FKHR*	*PAX7/FKHR*	Yes
39	ARMS	FFPE	ND	*PAX3/FKHR*	*PAX3/FKHR*	Yes
40	ARMS	FFPE	ND	*PAX3/FKHR*	*PAX3/FKHR*	Yes
41	ERMS	FFPE	ND	Negative	Normal	Yes
42	ERMS	FFPE	ND	ND	Normal	Undecided
43	ERMS	FFPE	ND	Negative	Normal	Yes
44	ERMS	FFPE	ND	Negative	Normal	Yes
45	ERMS	FFPE	ND	ND	Normal	Undecided

Table 1 Continued

Case	*Histologic type*[a]	*Slide type*[b]	*Karyotype*[c]	*RT-PCR*[c]	*FISH*	*Correlation*
46	ERMS	FFPE	ND	Negative	Fail	Undecided
47	ERMS	FFPE	ND	ND	Normal	Undecided
48	ERMS	FFPE	ND	Negative	Normal	Yes
49	ERMS	FFPE	ND	ND	Normal	Undecided
50	ERMS	FFPE	48–51,X,−X,del(3)(q13.33q24),+der(5)t(5;8)(q35.1;q13.3),del(6)(q14q21),+8,+12,+17,+20,+21,der(22)t(1;22)(q21;q13),+r[cp14]/46,XX[43]	Fail	Normal	Yes
51	ERMS	FFPE	55,XY,+del(6)(q22q26),+8,+12,+13,+13,+18,+19,+19,+21[17]/56,idem,+17[3]/46,XY[1]	Fail	Normal	Yes
52	ERMS	FFPE	53,X,−Y,+8,+8,+8,+13,+13,+19,+20,+22[9]/54,idem,+2[1]/106,idemx2[2]/46,XY[8]	Negative	Normal	Yes
53	ERMS (anaplasia)	FFPE	68–70,X,−X,del(X)(q21),add(1)(p36.3),del(1)(q21),−4,+der(5)t(2;5)(p11.2;q33),−6,t(8;20)(p11.2;q13.3),der(9)t(1;9)(q21;q12),−10,del(11)(q12),add(12)(q13),+13,+del(13)(q22q33),−15,−16,i(17)(q10),+19,+der(19)t(19;21)(p13.2;q11.2),+der(19)del(19)(p13.2)t(11;19)(?;q13.2),+21,−22,+0−1mar,5–25dmin[20]	Negative	Normal	Yes
54	ERMS	*In situ*	46,XX[16]	ND	Normal	Undecided
55	ERMS	FFPE	ND	Negative	Normal	Yes
56	ERMS	FFPE	ND	Negative	Normal	Yes
57	Mixed ARMS/ERMS	FFPE	ND	ND	Normal	Undecided
58	Mixed ARMS/ERMS	FFPE	Fail	ND	Normal	Undecided
59	Mixed ARMS/ERMS	FFPE	ND	Negative	Normal	Yes
60	Mixed ARMS/ERMS	FFPE	ND	Negative	Normal	Yes
61	Mixed ARMS/ERMS	FFPE	ND	Negative	Normal	Yes
62	Mixed ARMS/ERMS	FFPE	ND	Negative	Normal	Yes
63	Mixed ARMS/ERMS	*In situ*	56,XX,+add(1)(p11),+add(1)(p11),+2,+7,+7,+18,+18,+20,+20,+mar[7]/57,idem,+del(1)(q21)[3]/46,XX[1]	ND	Normal	Yes
64	Mixed ARMS/ERMS	TP	65,XY,−X,−1,−4,−5,−6,+8,+8,−9,add(12)(q22),−15,−16,−17,−18,add(19)(p13.3),+21,−22,+2r,+mar1,+mar2,1dmin[18]/46,XY[2]	ND	Normal	Yes

[a]ARMS, alveolar rhabdomyosarcoma; ERMS, embryonal rhabdomyosarcoma.

[b]TP, touch preparation; FFPE, formalin fixed, paraffin-embedded tissue.

[c]ND, not detected.

Table 2 Optimized rhabdomyosarcoma FISH probe sets

Probe set	*Clone*[a]	*Location*	*Label*
RP11-89L15	BAC	Proximal portion of *FKHR* locus	Spectrum orange
RP11-181D10	BAC	Distal portion of *FKHR* locus	Spectrum green
RP11-89L15/RP11-181D10	BAC cocktail	Spans *FKHR* locus	Spectrum green
RP11-71J24	BAC	Proximal to *PAX3* locus	Spectrum orange
RP11-384O8	BAC	Distal portion of *PAX3* locus	Spectrum green
923A10	YAC	Spans *PAX3* locus	Spectrum orange
RP1-93P18	PAC	Proximal to *PAX7* locus	Spectrum green
RP1-8B22[b]	PAC	Distal to *PAX7* locus	Spectrum orange
P-Cos-HPax7[b]	Cosmid	Distal portion of *PAX7* locus	Spectrum orange
RP3-394P21/RP1-93P18	PAC cocktail	Spans *PAX7* locus	Spectrum orange

[a]BAC, bacterial artificial chromosome; YAC, yeast artificial chromosome; PAC, P1-derived artificial chromosome.
[b]These two probes are combined and labeled as a cocktail to examine the distal flanking portion of the *PAX7* locus.

plied by the total μg of DNA used in the cocktail. Amounts ranging from 200 to 600 ng of each probe were hybridized to the target DNA and blocked approximately 15 times with a combination of Human Cot-1 DNA (Invitrogen, Carlsbad, CA, USA) and human placental DNA.

Prior to hybridization, the touch preparations and *in situ* slides were pretreated in 2 × saline sodium citrate (SSC) at 72°C for 2 min and in pepsin solution (20 μl 10% pepsin in 50 ml of 0.1 N hydrochloric acid (HCl)) at 37°C for 3 min, washed in 1 × phosphate-buffered saline (PBS) at room temperature for 5 min, fixed in 1% formaldehyde at 4°C for 5 min, and again washed in 1 × PBS at room temperature for 5 min. The slides were then dehydrated in an ethanol series (70, 85, and 100%) at room temperature for 2 min each and air-dried. The cells and probes were codenatured at 75°C for 1 min and incubated at 37°C overnight using the HYBrite™ denaturation/hybridization system (Vysis). Formalin-fixed, paraffin-embedded tissue sections were cut at a thickness of 4 μm. Tissue sections were deparaffinized in Hemo-De three times at room temperature for 10 min each, followed by dehydration in 100% ethanol twice for 5 min each, and air-dried. Tissue sections were then pretreated in 0.2 N HCl at room temperature for 20 min and in 1 M sodium thiocyanate at 80°C for 30 min, rinsed in distilled water at room temperature for 1 min, followed by washing in 2 × SSC twice for 5 min each, digested in pepsin solution (2.5 mg/ml in 0.9% sodium chloride, pH 2.0) at 37°C for 10 min, and finally washed in 2 × SSC twice at room temperature for 5 min each. The cells and probes were codenatured at 74°C for 6 min and incubated at 37°C overnight using the HYBrite™ denaturation/hybridization system.

Posthybridization washing was performed in 0.4 × SSC/0.3% NP-40 at 72°C for 2–3 min, followed by 2 × SSC/0.1% NP-40 at room temperature for 1–2 min. The slides were air-dried in the dark and counterstained with 4′,6-diamidino-2-phenylindole (DAPI II; Vysis).

Before reviewing the FISH assay, we confirmed the appropriate areas of both alveolar and embryonal components of the mixed histologic subtype using a parallel hematoxylin and eosin (H&E) stained section. Hybridization signals were assessed in 200 interphase nuclei with strong, well-delineated signals and distinct nuclear borders by two different individuals. An interphase cell specimen was interpreted as abnormal if spanning probe signals for the *PAX3* or *PAX7* genes fused with the spanning probe signal for the *FKHR* gene, or if a split of flanking probe signals was detected in more than 10% of the cells evaluated (more than two standard deviations above the average false-positive rate). Positive controls included one t(1;13) positive (CW9019) and two t(2;13) positive (RH28 and RH30) ARMS cell lines. Negative controls included normal peripheral blood lymphocytes, cytologic touch preparations and paraffin-embedded tissue sections of pathologically unremarkable skeletal muscle, and one translocation negative ERMS cell line RD. Images were acquired by use of the CytoVision Image Analysis System (Applied Imaging, Santa Clara, CA, USA).

Results

In the present study, cytologic touch preparations, *in situ* sections, or formalin-fixed, paraffin-embedded tissue sections from 75 tumor specimens were evaluated using the FISH technique. The cytogenetic, RT-PCR and FISH findings of the 64 RMS specimens are summarized in Table 1. The 11 non-RMS tumors were normal by FISH. FISH studies were successfully performed on all but two RMS cases. Prolonged exposure to formalin may be responsible for the two failed hybridizations despite repeated efforts.

Among all specimens with informative results for both FISH and RT-PCR or conventional cytogenetic analysis, *PAX/FKHR* classification results (ie positive or negative) were concordant in

94.6% (53/56). When normal metaphase cells are obtained, they are assumed to arise from normal stromal cells, and conventional cytogenetic studies are therefore considered uninformative. There were two discordant cases between FISH and RT-PCR and one discordant case between FISH and conventional cytogenetic analysis (Table 1). One discordant case exhibited rearrangements and fusion of the *PAX3* and *FKHR* gene loci by FISH (Figure 2), but had been initially reported as negative by RT-PCR from the IRSG database. Repeat RT-PCR analysis confirmed the presence of a *PAX3-FKHR* fusion transcript. A second discordant case exhibited splitting of the *PAX3* breakpoint flanking probe set and amplification of the probe signal located just distal to the *PAX3* breakpoint (Figure 3a). However, FISH analysis with the *FKHR* breakpoint flanking probe set revealed normal results; the signals remain juxtaposed to each other (Figure 3b). FISH analysis with the *PAX3* and *FKHR* breakpoint spanning probes showed amplification of the *PAX3* spanning probe, but no fusion with or amplification of the *FKHR* spanning probe (Figure 3c). These findings suggest the presence of a possible *PAX3* variant translocation. In the third discordant case, *PAX3* and *FKHR* rearrangements and *PAX3-FKHR* fusion were detected by FISH (Figure 4a), but cytogenetic analysis showed no clear evidence of the t(2;13) translocation (Figure 4b). Unfortunately, we were unable to evaluate this case by RT-PCR because the quality of RNA was suboptimal.

In contrast to the alveolar histologic subtype, all cases of ERMS and non-RMS tumors were negative for *PAX3*, *PAX7*, and *FKHR* gene loci rearrangements. Moreover, both alveolar and embryonal

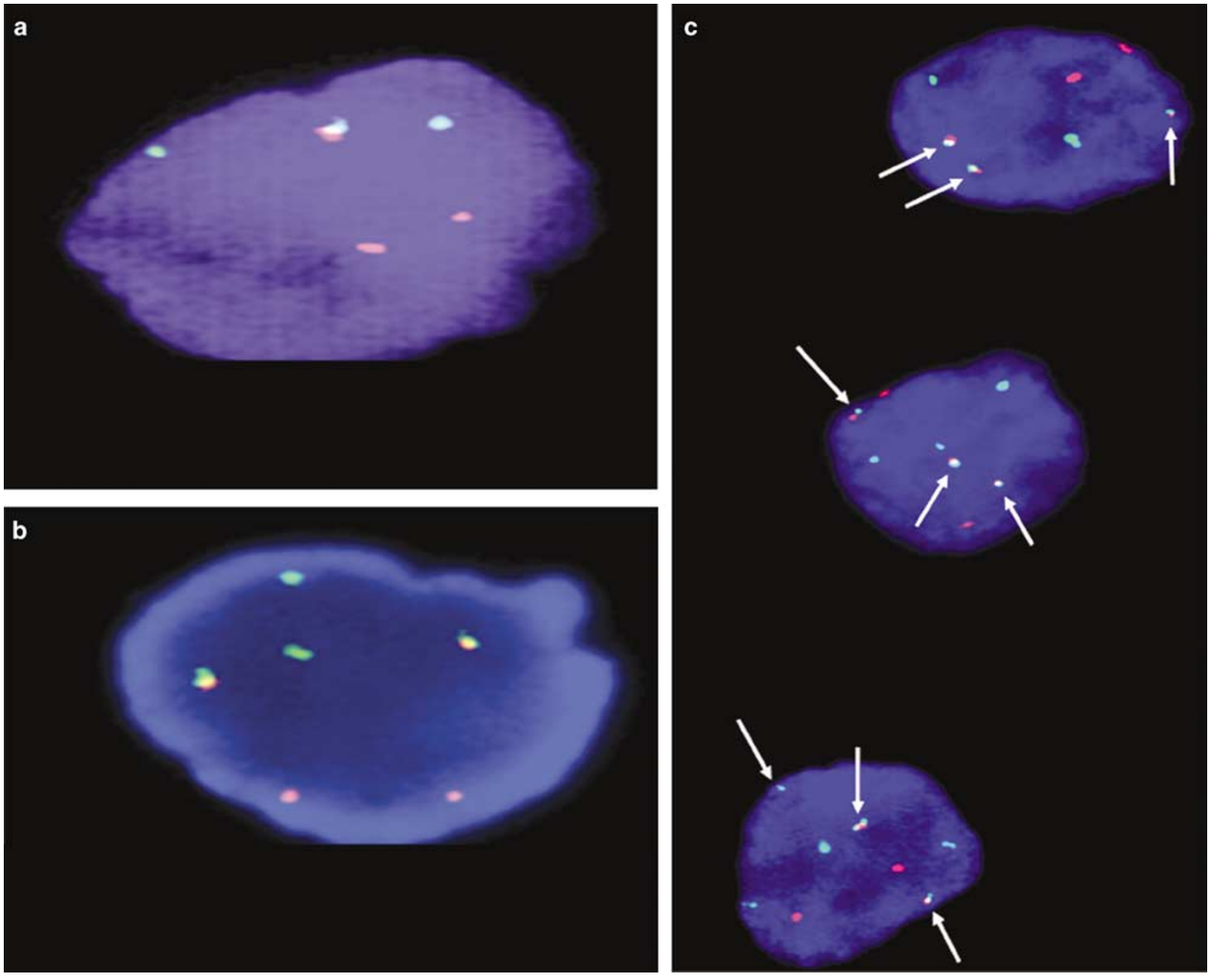

Figure 2 FISH analyses performed on cytologic touch preparations of Case 3 (ARMS). (**a**) FISH analysis with the *FKHR* breakpoint flanking probe set (proximal portion, orange; distal portion, green) shows splitting of the orange and green signals, indicating disruption of *FKHR*. (**b**) FISH analysis with the *PAX3* breakpoint flanking probe set (proximal portion, orange; distal portion, green) shows splitting of the orange and green signals, indicating disruption of *PAX3*. (**c**) FISH analysis with the *PAX3* (orange) and *FKHR* (green) spanning probes shows juxtaposed green and orange or overlapping yellow-white signals (arrows), confirming the presence of a *PAX3-FKHR* fusion.

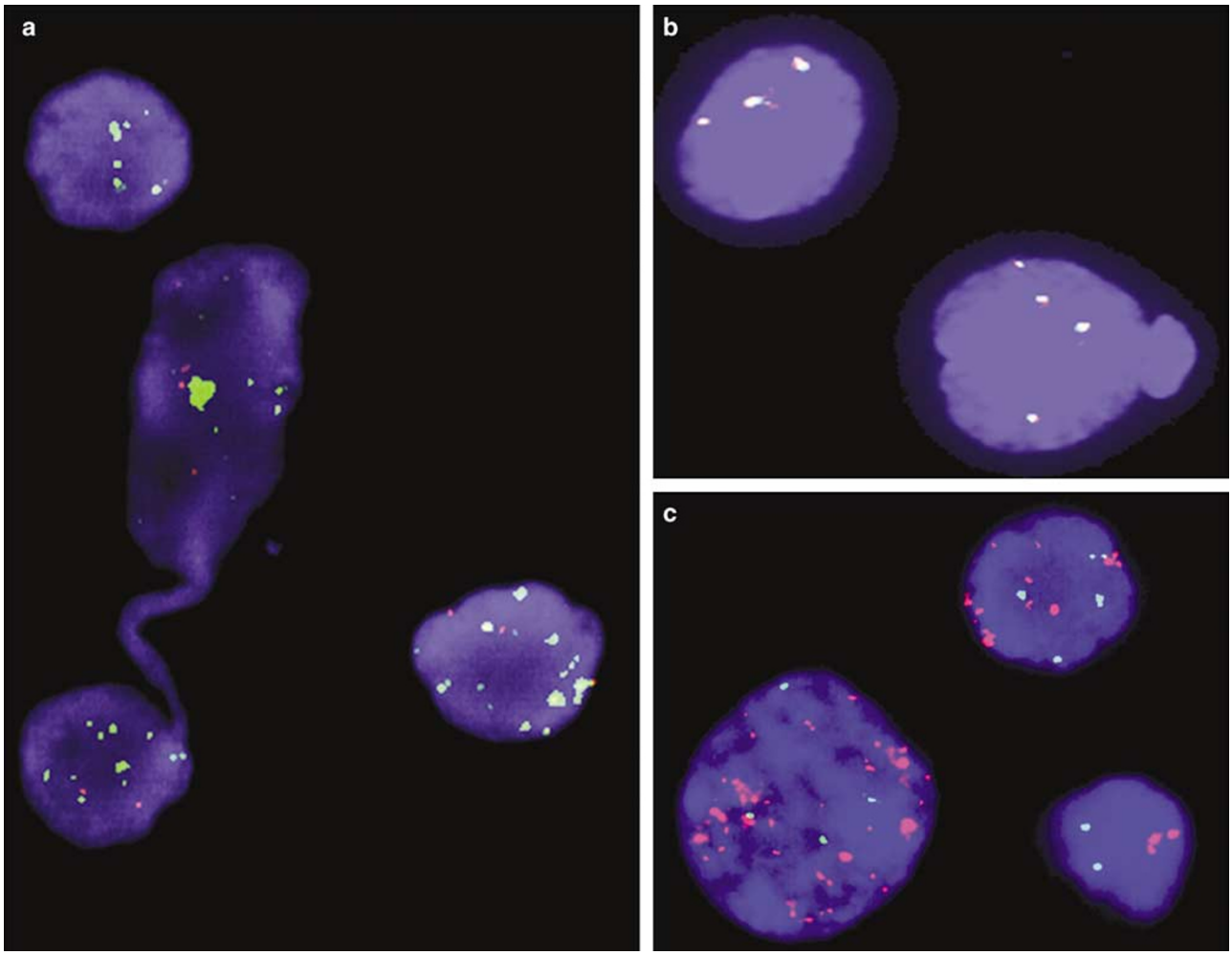

Figure 3 FISH analyses performed on cytologic touch preparations of Case 13 (ARMS). (**a**) FISH analysis with the *PAX3* breakpoint flanking probe set (proximal portion, orange; distal portion, green) reveals splitting of the orange and green signals and amplification of the green signal (located just distal to the *PAX3* breakpoint), indicating disruption and amplification of *PAX3*. (**b**) FISH analysis with the *FKHR* breakpoint flanking probe set (proximal portion, orange; distal portion, green) reveals normal results; the orange and green signals remain juxtaposed to each other. (**c**) FISH analysis with the *PAX3* (orange) and *FKHR* (green) spanning probes reveals amplification of the orange signal, but no fusion with or amplification of the green signal. These findings suggest the presence of a possible *PAX3* variant translocation. (A single normal cell is present in lower right-hand corner.)

components of the mixed ARMS/ERMS were negative for *PAX* and *FKHR* rearrangements (Figure 5), a finding confirmed by RT-PCR or conventional karyotyping.

Discussion

The diagnosis of pediatric small round cell tumors can be difficult because of overlapping histologic and/or immunohistochemical features. Moreover, the increasing use of minimal biopsy makes the diagnosis of these tumors more challenging. The ability to identify tumor-specific chromosomal translocations and associated fusion gene transcripts using interphase FISH and/or RT-PCR on paraffin-embedded material can be extremely useful in such cases.[13–23]

In the present study, we describe the largest series of the detection of RMS fusion gene type by FISH to date. Previous FISH studies have been applied to relatively few ARMS cases exhibiting a t(2;13) translocation.[8,9] Moreover, most of these have been performed on touch preparations of fresh or snap-frozen tissue, or on metaphase spreads and/or interphase nuclei from tumor cells grown in short-term culture. However, fresh or frozen tissue for analysis is not always available, and the most widely available tumor tissue is formalin-fixed and paraffin-embedded. Here, we have developed an optimized two-color FISH assay, which is applicable to formalin-fixed, paraffin-embedded tissue and allows a more extensive study of archival material from patients for whom there are detailed data of treatment and outcome.

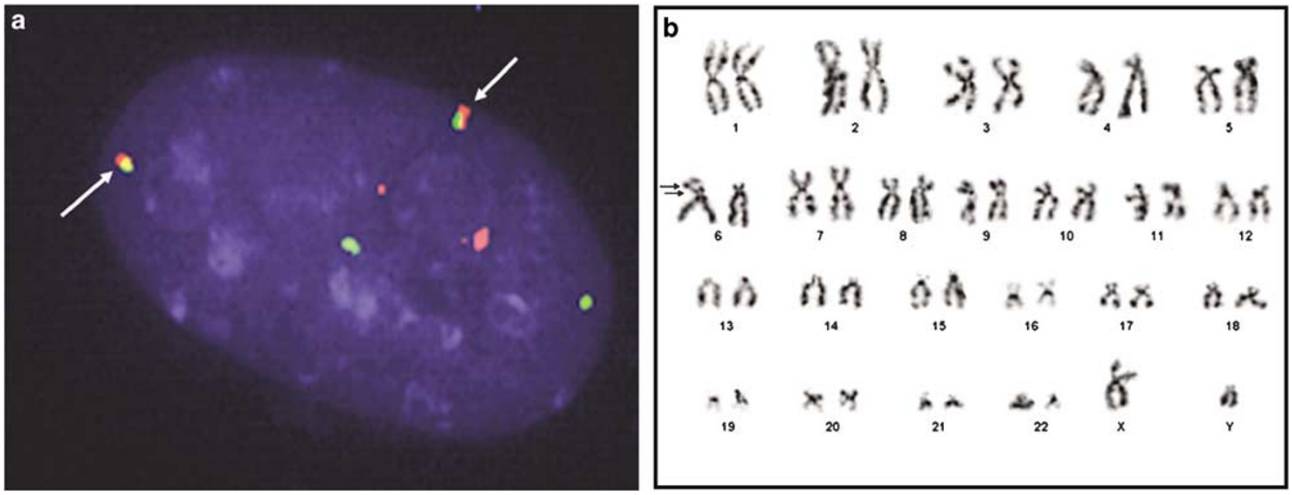

Figure 4 FISH and G-banding analyses of Case 34 (solid variant ARMS). (**a**) FISH analysis with the *PAX3* (orange) and *FKHR* (green) spanning probes reveals *PAX3-FKHR* fusion signals (arrows), indicating the presence of a t(2;13) translocation. (**b**) Representative karyotype showing the following abnormal complement: 46,XY,del(6)(p21p25). Arrows indicate the involved breakpoints on the normal chromosome 6 homologue on the left. Cytogenetic studies did not reveal the characteristic translocation involving chromosomes 2 and 13.

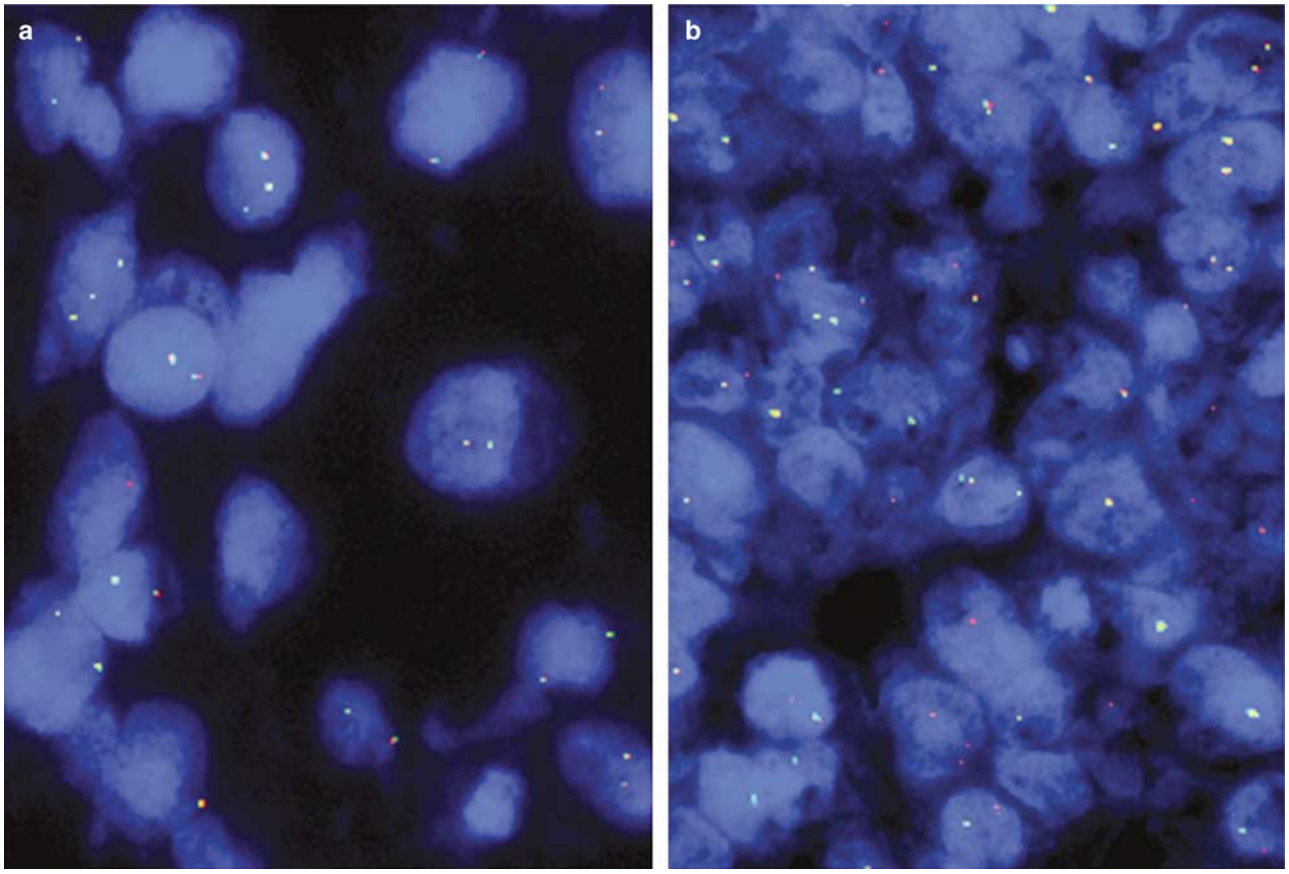

Figure 5 FISH analysis performed on paraffin-embedded tissue sections of Case 57 (mixed ARMS/ERMS) using the *FKHR* breakpoint flanking probe set (proximal portion, orange; distal portion, green). The orange and green signals remain fused in both embryonal (**a**) and alveolar (**b**) components of the mixed histologic subtype, indicating no rearrangement of *FKHR*.

The newly developed probes that we have described localize to the breakpoints on chromosomes 1, 2, and 13 and can distinguish between the t(2;13) and t(1;13) translocations. The distinction between these translocations in ARMS is important, because patients with t(2;13) have a more adverse

outcome than patients with t(1;13) despite indistinguishable histologic features.[5] On the other hand, previous FISH studies have used only cosmid probes to detect the disruption of *FKHR* gene locus at 13q14.[8,9] In the current study, we used BAC clones covering the entire *FKHR* gene in a single hybridization experiment. In contrast to the cosmid probes, the signals generated by the BAC probes are larger and brighter. This is particularly important for working with paraffin-embedded tissue in the routine clinical diagnostic setting and revealing chromosomal rearrangements in cases with complex variant translocations.

Although there was a high correlation between the results of FISH and those of RT-PCR or conventional cytogenetic analysis, three discordant cases were identified. One case (Case 3), while positive for the presence of the t(2;13) translocation by FISH, did not show a *PAX3-FKHR* fusion transcript by the initial RT-PCR. Repeat RT-PCR identified the presence of *PAX3-FKHR* fusion transcript. This finding indicates that these approaches are complementary.

An additional advantage of FISH is its ability to identify unusual variant or cryptic translocations. Two *PAX3* variant translocations have been previously described: *PAX3-AFX* and *PAX3-NCOA1*.[6,7] Our FISH studies suggest that one of the discordant cases, Case 13, also represents a *PAX3* variant translocation. FISH analysis of this case revealed rearrangement of the *PAX3* gene with no fusion of signals occurring between the *PAX3* and *FKHR* gene loci. We are currently performing rapid amplification of cDNA ends experiments in an effort to further characterize this rearrangement.

In the third discordant case (Case 34), the t(2;13) or t(1;13) translocations or derivative chromosomes 1, 2, or 13 originating from them were not observed on conventional cytogenetic analysis, but FISH analysis revealed the presence of the t(2;13) translocation. Similarly, several cases of ARMS have previously been reported that failed to demonstrate tumor-specific chromosomal translocations on cytogenetic analysis, but were shown to express *PAX-FKHR* fusion transcripts by RT-PCR and/or FISH.[24–26] Possible explanations for these findings are the difficulty of identifying the small derivative chromosome 13 or the presence of a cryptic translocation. FISH and RT-PCR analyses are valuable techniques that should be performed to detect the presence of tumor-specific chromosomal translocations or associated fusion gene transcripts even when cytogenetic analysis is successful.

Some RMS cases exhibit a mixture of alveolar and embryonal patterns, and these have been called mixed alveolar/ERMS. According to the ICR criteria, tumors with any alveolar features are classified as ARMS.[3] In our FISH analysis, both the alveolar and embryonal components of the mixed histologic subtype were negative for *PAX3*, *PAX7*, and *FKHR* rearrangements. These surprising results were confirmed by RT-PCR or conventional karyotyping in all cases that sufficient material was available for analysis. To the contrary, Biegel *et al*[27] reported a case of mixed ARMS/ERMS with a t(1;13)(p36;q14) translocation. The reason for this discrepancy is unclear. Of note, in two cases of mixed ARMS/ERMS, *N-myc* amplification was present only in the alveolar component.[28] Additional studies are required to determine the genetic characteristics of this histologic subtype.

In summary, these findings: (1) demonstrate that FISH analysis with these newly designed probe sets is a reliable and highly specific method of detecting t(1;13) and t(2;13) in routinely processed tissue (including formalin-fixed, paraffin-embedded tissue) and may be useful in differentiating ARMS from other small round cell tumors; (2) suggest that FISH may be a more sensitive assay than RT-PCR in some settings (capable of revealing variant translocations); and show that both the alveolar and embryonal components of the mixed ARMS/ERMS are negative for *PAX3*, *PAX7*, and *FKHR* gene loci rearrangements.

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