Congenital-Infantile Fibrosarcoma

A Clinicopathologic Study of 10 Cases and Molecular Detection of the \textit{ETV6-NTRK3} Fusion Transcripts Using Paraffin-Embedded Tissues

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\textbf{Key Words:} Congenital-infantile fibrosarcoma; \textit{ETV6-NTRK3} fusion transcripts; NTRK3; Reverse transcription–polymerase chain reaction; RT-PCR; Immunohistochemistry

\textbf{Abstract}

Congenital-infantile fibrosarcoma (CIFS) is a relatively indolent sarcoma that should be distinguished from more aggressive spindle cell sarcomas of childhood. CIFSs have been found to have a novel recurrent reciprocal translocation t(12;15)(p13;q25) resulting in the gene fusion \textit{ETV6-NTRK3} (ETS variant gene 6; neurotrophic tyrosine kinase receptor type 3). We studied immunohistochemical expression of NTRK3, and conducted a reverse transcription–polymerase chain reaction (RT-PCR) assay to detect the \textit{ETV6-NTRK3} fusion transcripts using archival formalin-fixed paraffin-embedded tissues from 10 CIFSs. Thirty-eight other spindle cell tumors were included as controls.

The \textit{ETV6-NTRK3} fusion transcripts were identified in 7 (70\%) of 10 CIFSs. Nucleotide sequence analysis showed that the fusion occurred between \textit{ETV6} exon 5 and \textit{NTRK3} exon 13. The 38 control tumors were negative for the fusion transcript. Immunohistochemically, CIFSs consistently expressed NTRK3. But the expression of NTRK3 also was observed in 22 of 38 control tumors. These results show the diagnostic usefulness of RT-PCR methods to detect \textit{ETV6-NTRK3} fusion transcripts in archival formalin-fixed paraffin-embedded tissue and the important role of NTRK3 in the development of CIFS, despite its being a protein of little importance in differential diagnosis.

Although most fibrosarcomas occur in adults, 25\% to 40\% occur in children during the first 5 years of life.\textsuperscript{1} Despite a similar histologic appearance, congenital-infantile fibrosarcoma (CIFS) is much less aggressive than adult-type fibrosarcoma.\textsuperscript{2,3} Its distinction from infantile fibromatosis may be extremely difficult owing to closely overlapping clinical and histologic features. Why the two should be distinguished has, in fact, been questioned.\textsuperscript{4,5} Moreover, distinction of CIFS from other spindle cell sarcomas of childhood, such as spindle cell rhabdomyosarcoma, hemangiopericytoma, monophasic fibrous synovial sarcoma, malignant peripheral nerve sheath tumor (MPNST), and leiomyosarcoma, is also problematic.

Recently, Knezevich et al\textsuperscript{6} found a novel gene fusion, \textit{ETV6-NTRK3} (ETS variant gene 6; neurotrophic tyrosine kinase receptor type 3), in CIFS resulting from a recurrent chromosomal rearrangement of t(12;15)(p13;q25). By using frozen tissues, the fusion gene was demonstrated in 4 cases of CIFS examined by Knezevich et al,\textsuperscript{6} 5 cases examined by Rubin et al,\textsuperscript{7} and 9 cases examined by Bourgeois et al.\textsuperscript{8} This gene fusion also has been identified in congenital mesoblastic nephroma, which shares some histologic features with CIFS and establishes a histogenetic link between the two lesions.\textsuperscript{7,9,10} The uniqueness of the \textit{ETV6-NTRK3} gene fusion is potentially a useful diagnostic tool, since it has not been detected in other soft tissue spindle cell lesions, including infantile fibromatosis and adult-type fibrosarcoma.\textsuperscript{6,8} \textit{ETV6-NTRK3} has been detected in paraffin-embedded tumor samples of CIFS in only 2 of 3 cases.\textsuperscript{8} Thus, we performed an immunohistochemical study for NTRK3 expression in CIFS and conducted a reverse transcription–polymerase chain reaction (RT-PCR) assay to assess the specificity of \textit{ETV6-NTRK3} fusion transcripts detected in archival formalin-fixed paraffin-embedded tissues of CIFS.
Materials and Methods

Cases of CIFS and other spindle cell lesions were retrieved from the files of the Department of Pathology and Oncology, School of Medicine, University of Occupational and Environmental Health, Kitakyushu, Japan; the Department of Pathology, Sahlgrenska University Hospital, Gothenburg University, Gothenburg, Sweden; the Department of Pathology, Tokyo University Hospital, Tokyo, Japan; and the Department of Pathology, Kanazawa Medical College Hospital, Kanazawa, Japan. The study included CIFS (10 cases), adult-type fibrosarcoma (7 cases), leiomyosarcoma (4 cases), a storiform-pleomorphic variant of malignant fibrous histiocytoma (4 cases), monophasic synovial sarcoma (4 cases), MPNST (3 cases), dermatofibrosarcoma protuberans (4 cases), schwannoma (4 cases), fibromatosis (4 cases), and nodular fasciitis (4 cases). H&E-stained sections of each case were reexamined, and representative paraffin blocks were selected for this analysis. Clinical data were reviewed, and follow-up information was obtained in all instances except 1 recent case.

Immunohistochemical Studies

A standard avidin-biotin complex method was used. Polyclonal rabbit anti-NTRK3 (also referred to as TrkC) immunoglobulin (798), recognizing an epitope corresponding to amino acids 798 to 812 of porcine TrkC p140 (1:100 dilution), was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Immunostaining were performed using the following panel of antibodies: CD34 (QBEND 10, Immunotech, Marseilles, France), alpha–smooth muscle actin (alpha-SMA) (1A4, Sigma, St Louis, MO), muscle-specific actin (HHF35, Enzo, New York, NY), desmin (D33, DAKO, Glostrup, Denmark), S-100 protein (DAKO), epithelial membrane antigen (DAKO), L26 (DAKO), CD45RO (clone CAM5.2, Becton Dickinson, San Jose, CA), cytokeratin clone AE1 and AE3 (AE1/AE3, Boehringer Mannheim, Indianapolis, IN), cellular adhesion molecule 5.2 (CAM5.2, Becton Dickinson), epithelial membrane antigen (DAKO), L26 (DAKO), CD45RO (clone UCHL-1, DAKO), CD3 (DAKO), CD4 (Novoceastra, Newcastle upon Tyne, England), and CD8 (DAKO).

For NTRK3 immunostaining, de waxed tissue sections were subjected to a microwave pretreatment (a 10-mmol/L concentration of sodium citrate buffer, pH 7.0, for 20 minutes at 600 W). Normal brain tissue was used as a positive control, and a negative control was obtained by exclusion of the primary antibody.

RNA Extraction

RNA was extracted from formalin-fixed paraffin-embedded tissues according to previously described methods. Briefly, tissue sections were cut from each representative paraffin block and deparaffinized with 2 changes of xylene and 3 washes with 100% ethanol. Tissue fragments were homogenized in 200 µL of lysis buffer (20 mmol/L tris(hydroxymethyl)aminomethane hydrochloride, pH 8.0; a 20-mmol/L concentration of EDTA; and 2% sodium dodecyl sulfate). They were then treated with 10 µL of proteinase K (100 mg/mL) and incubated at 55 C for 48 hours. Total RNA was extracted using Trizol reagent (Gibco BRL, Gaithersburg, MD), according to the manufacturer’s protocol and resuspended in 10 to 30 µL of water free of deoxyribonuclease (DNase) and ribonuclease (RNase). The RNA solution was treated with RNase I (Gibco BRL) for 15 minutes at 37 C.

RT-PCR and DNA Sequencing

Complementary DNA (cDNA) was synthesized using 10 µL of dissolved RNA, 1 µL of random primer (Gibco BRL), and 200 U of reverse transcriptase (Superscript II, Gibco BRL). Generated cDNA then was treated with 60 U of RNase H (Takara, Ohtsu, Japan) for 20 minutes at 37 C. To assess the integrity of RNA, parallel PCR for an internal positive control of the porphobilinogen deaminase (PBGD) gene was performed with the following primers: PBGD-S (5'-TGTCCTGTAACGCAATGCGGCTGCAAC-3') and PBGD-A (5'-TCAATGTTGCCACACACACTGTCCGTC-3'). These primers amplified a 127-base-pair (bp) fragment of the PBGD gene.

To detect the ETV6-NTRK3 fusion transcripts in formalin-fixed paraffin-embedded tissue samples, we designed a pair of primers that specifically amplify a relatively small 111-bp region flanking the published fusion point of the ETV6-NTRK3 gene transcript (Oligo Primer Analysis Software, Molecular Biology Insight, Cascade, CO). The ETV6 forward primer was 5'-GCTTACATGAAACACACACACCATCAGG-3', and the NTRK3 reverse primer was 5'-GAAGTCTGCTACAGAGAGG-3'. PCR was performed under the following conditions: 94 C for 10 minutes followed by 45 cycles at 94 C for 50 seconds, 60 C for 30 seconds, 72 C for 1 minute, and a final extension at 72 C for 10 minutes. In each PCR procedure, a no-reverse transcription control, a no-cDNA template control, and a known fusion transcript–positive case of congenital mesoblastic nephroma (positive control) were included. The positive PCR products were cloned into a pCR2.1 vector using a TA cloning kit (Invitrogen, San Diego, CA) and sequenced by an automated sequencing system (ALFexpress DNA sequencer; Pharmacia Biotech, Uppsala, Sweden).

Results

Clinical Summary

Clinical data for the 10 patients with CIFS are summarized in Table 1. There were 7 male and 3 female infants.
Table 1
Clinical Data for 10 Cases of Congenital-Infantile Fibrosarcoma

<table>
<thead>
<tr>
<th>Case No./Sex/Age (mo)</th>
<th>Tumor Location</th>
<th>Tumor Size (cm)</th>
<th>Follow-up</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/F/2</td>
<td>Lower leg (L)</td>
<td>2.5 - 3.5</td>
<td>No evidence of disease, 29 y</td>
</tr>
<tr>
<td>2/M/1</td>
<td>Thigh (R)</td>
<td>6 - 3 - 2</td>
<td>No evidence of disease, 28 y</td>
</tr>
<tr>
<td>3/F/3</td>
<td>Jaw angle (L)</td>
<td>NA</td>
<td>No evidence of disease, 31 y</td>
</tr>
<tr>
<td>4/F/3</td>
<td>Wrist (R)</td>
<td>2 - 2</td>
<td>No evidence of disease, 32 y</td>
</tr>
<tr>
<td>5/M/3</td>
<td>Lower leg</td>
<td>NA</td>
<td>No evidence of disease, 22 y</td>
</tr>
<tr>
<td>6/M/3</td>
<td>NA</td>
<td>5.5 - 3.6 - 3.2</td>
<td>Recent case</td>
</tr>
<tr>
<td>7/M/1</td>
<td>Lower leg (L)</td>
<td>4 - 2 - 1</td>
<td>No evidence of disease, 22 y</td>
</tr>
<tr>
<td>8/M/4</td>
<td>Lower leg (L)</td>
<td>10 - 8</td>
<td>No evidence of disease, 28 y</td>
</tr>
<tr>
<td>9/M/3</td>
<td>Thigh (L)</td>
<td>2.8 - 1</td>
<td>No evidence of disease, 15 y</td>
</tr>
<tr>
<td>10/M/7</td>
<td>Ankle (L)</td>
<td>3.6</td>
<td>NA, not available.</td>
</tr>
</tbody>
</table>

Clinical Data for 10 Cases of Congenital-Infantile Fibrosarcoma

- Ranging from 1 to 7 months of age at diagnosis. Four tumors occurred in the lower leg, 2 in the thigh, 1 on the head, 1 in the wrist, and 1 in the ankle. The main symptom was a mass or swelling of the soft tissues. All lesions were locally excised with adequate margins. All patients were alive and well without local recurrence or metastasis after a follow-up of 14 to 32 years.

Pathologic Findings

- Tumor size (known in 7 cases) ranged from 2 to 10 cm in the greatest dimension. The cut surface was firm, fleshy, and gray-white. Focal cystic change was seen in 1 tumor (case 9); hemorrhage and necrosis were not observed.

- Microscopically, most tumors were cellular and composed of uniform oval to spindle-shaped cells with hyperchromatic nuclei separated by variable amounts of collagen. One case, however, had a small number of large, bizarre tumor cells (case 9). All tumors were composed of elongated cells arranged in fascicles. In 3 lesions, a herringbone pattern similar to that of adult-type fibrosarcoma was seen Image 1. Three tumors displayed a prominent hemangiopericytoma-like vascular pattern Image 2. In case 2, the tumor was less structured and consisted mainly of more immature, small, round to oval cells with minimal amounts of collagen. Eight of 10 cases had patchy to diffuse chronic inflammatory cell infiltrates composed predominantly of lymphocytes. The number of mitoses per 10 high-power fields (400) was 1 to 4 in 6 cases, 5 to 9 in 2 cases, and 10 or more in 2 cases. Diffuse infiltration of neighboring fat or skeletal muscle was observed in all cases. Tumor necrosis or hemorrhage was not observed microscopically in any case.

Immunohistochemical Results

- Immunohistochemical staining results are summarized in Table 2. All 10 CIFSs had membranous and cytoplasmic staining for NTRK3 Image 3. In 8 cases, more than 50% of the tumor cells were positive for NTRK3. NTRK3 also was found in leiomyosarcoma (4/4), nodular fasciitis (4/4), fibromatosis (4/4), MPNST (3/3), schwannoma (3/4), adult-type fibrosarcoma (2/7), and malignant fibrous histiocytoma (2/4).

- Two of 10 CIFSs were positive for CAM5.2; 1 tumor showed patchy (case 4) and 1 showed diffuse staining (case 6). Diffuse S-100 protein immunostaining was seen in 1 tumor (case 7); this case also was focally positive for alpha-SMA and HHF35. Focal reactivity for desmin was seen in 1 case (case 9). None of the CIFSs was positive for CD34, AE1/AE3, or epithelial membrane antigen.

- Except for case 9, 7 of 8 CIFSs with chronic inflammatory infiltrates were available for analysis of the immunophenotype of these inflammatory cells. The majority of the cells were T cells (positive for CD45RO and CD3), while a small number of them were B cells (positive for L26). CD8+ T cells were obviously much more in quantity than CD4+ cells. We found no difference in the cellular phenotype among these 7 cases.

RT-PCR and Sequencing

- The results of RT-PCR are summarized in Figure 1. The PBGD gene transcripts were amplified consistently in all 10 CIFSs. By using the ETV6 forward primer and the NTRK3 reverse primer, 111-bp fragments were identified in 7 of 10 CIFSs, including the case with a small number of large, bizarre tumor cells, 3 tumors displaying a hemangiopericytoma-like vascular pattern, and 2 of 3 tumors with a herringbone pattern Image 4. In the 8 tumors with inflammatory cell infiltrates, 1 tumor (case 10) was negative for the fusion transcripts, while the others were positive. Nucleotide sequencing of these PCR products demonstrated that ETV6 exon 5 was fused consistently to NTRK3 exon 13 Image 2. There was no apparent relationship among ETV6-NTRK3 fusion transcript detection, the immunohistochemical staining results, and specific microscopic features.
The clinicopathologic features of our cases are similar to several published series of CIFS. However, the reported rates of local recurrence and distant metastasis are quite variable; in contrast, there were none in our series.

The differential diagnosis of pediatric soft tissue spindle cell lesions is indeed difficult. Infantile fibromatosis, especially a cellular variant designated aggressive fibromatosis, is probably the most difficult one to diagnose. The degree of cellularity, exhibited as alternating cellular and more collagenous areas; foci of residual fat or muscle; and an infiltrative growth pattern are all features of aggressive fibromatosis that overlap with CIFS, making it difficult to distinguish between the lesions. However, the distinction is not so critical owing to their similar biologic behaviors.

Inflammatory myofibroblastic tumor/inflammatory fibrosarcoma (IMT), which also affects infants and young children, may arise in the histologic differential diagnosis if a prominent chronic inflammatory infiltrate is present. However, the clinical features of IMT differ from CIFS. Recent identification of the TPM-ALK fusion gene and expression of the ALK gene in a subset suggest that IMT has a distinctive genetic background.

The CIFSs with considerable vascularity also should be distinguished from infantile hemangiopericytoma and infantile myofibromatosis. Infantile hemangiopericytoma usually is located superficially and has a distinct lobular arrangement with more regularly distributed vessels, whereas infantile myofibromatosis displays a typical zonal appearance with distinct myofibroblastic differentiation.

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Immunohistochemical Results for 10 Cases of Congenital-Infantile Fibrosarcomas</th>
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<tbody>
<tr>
<td>Case No.</td>
<td>NTRK3</td>
</tr>
<tr>
<td>1</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>++</td>
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<td>3</td>
<td>+</td>
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<tr>
<td>9</td>
<td>++</td>
</tr>
<tr>
<td>10</td>
<td>++</td>
</tr>
</tbody>
</table>

AE1/AE3, cytokeratin clone AE1 and AE3, Boehringer Mannheim, Indianapolis, IN; alpha-SMA, alpha-smooth muscle actin, 1A4, Sigma, St Louis, MO; CAM5.2, cellular adhesion molecule 5.2, Becton Dickinson, San Jose, CA; EMA, epithelial membrane antigen, DAKO, Glostrup, Denmark; HHF35, muscle-specific actin, Enzo, New York, NY; NTRK3, neurotrophic tyrosine kinase receptor type 3, Santa Cruz Biotechnology, Santa Cruz, CA; –, negative; +, less than 50% immunoreactive cells; ++, 50% or more immunoreactive cells. Desmin, D33, DAKO; S-100, DAKO; CD34, QBEND 10, Immunotech, Marsilles, France; desmin, D33, DAKO; S-100, DAKO.
Immunohistochemically, 2 of our cases expressed low-molecular-weight cytokeratin (CAM5.2); 1 was positive for S-100 protein, alpha-SMA, and HHF35, and 1 was focally positive for desmin. Coffin et al identified actin-positive cells in 30% of CIFS cases and desmin in 19%. The actin-positivity seems to represent myofibroblastic differentiation based on corroborating ultrastructural studies.

The expression of cytokeratin and S-100 protein in CIFS has been noted in some cases. This immunophenotypic diversity may cause problems in differentiating CIFS from MPNST, spindle cell rhabdomyosarcoma, leiomyosarcoma, and synovial sarcoma in infants and young children. However, a serpentine or wavy nuclear contour, nuclear palisading, and distinctly alternating hypercellular and hypocellular zones characteristic of MPNST were not observed in our cases. Spindle cell rhabdomyosarcoma has a predilection for the paratesticular area and demonstrates cross-striations and invariably strong desmin expression.

Pediatric leiomyosarcomas are extremely rare; most of these are reported as low-grade and display typical smooth muscle features, such as interwoven fascicles of spindle cells with cigar-shaped nuclei and well-delineated eosinophilic, fibrillar cytoplasm. Uncommonly, synovial sarcoma may occur in infancy. We found 3 such cases, 2 of which were misdiagnosed previously as CIFS. They had plumper nuclei, focal whorled cell arrangements, and many mast cells. These cases strongly expressed cytokeratin, and the SYT-SSX fusion transcript was identified.

In our 7 CIFSs with inflammatory cell infiltrates, we found that T lymphocytes were more prominent than B lymphocytes, and most of the T cells were CD8+ cytotoxic or suppressor T cells. We did not find a relationship between RT-PCR results and immunophenotypes of the infiltrating inflammatory cells. But the number of our cases was small, and further studies of a large number of cases are necessary.

In the present study, we identified the fusion transcripts in 7 (70%) of 10 cases of CIFS, all of which were studied by RT-PCR using archival, formalin-fixed, paraffin-embedded tissues. Nucleotide sequence analyses of the positive PCR products revealed that exon 5 of the ETV6 gene was fused consistently with exon 13 of the NTRK3 gene, as reported previously. No fusion transcripts were demonstrated in 38 other spindle cell lesions, including 7 adult-type fibrosarcomas and 4 fibromatoses, although the PBGD
housekeeping gene was amplified in these cases. The detection of ETV6-NTRK3 fusion transcripts in most CIFSs indicates that this RT-PCR is a diagnostically useful technique when analyzing formalin-fixed paraffin-embedded tissues. This view is in line with that of a recent study, although only 3 paraffin-embedded CIFSs were examined.

Although 127-bp fragments of the PBGD gene were amplified in all 10 CIFSs, 3 were negative for ETV6-NTRK3 fusion gene transcript. Failure to detect the fusion transcript in these cases probably was not due to the RNA degradation, since the amplified fragment of the fusion transcript (111 bp) is smaller than that of the PBGD gene transcript (127 bp). Similar to the findings of Argani et al, our primers recognized only part of the fusion transcript covering the previously described fusion point; they could not amplify variant fusion transcripts containing unknown fusion points.

NTRK3 is a member of the NTRK family of receptor tyrosine kinase that binds with neurotropine-3 and mediates nervous system growth and development. Its expression has been found primarily to be restricted to neuronal cells. A recent study has shown that NTRKs also are expressed in human nonneuronal tissues, including epithelium and smooth muscle of the colon, as well as skeletal muscle, but not normal fibroblasts. Chromosome translocations resulting in fusion of ETV6 (which is expressed in a wide variety of mammalian tissues) and NTRK3 seem to induce the expression of a fusion protein that contains an intracellular tyrosine kinase domain of NTRK3. A recent study demonstrated that the ETV6-NTRK3 chimeric protein is an oncprotein with potent transforming activity in NIH3T3 cells. Dimerization of the chimeric protein (mediated by the helix-loop-helix domain of ETV6) has been suggested to cause activation of the protein tyrosine kinase domain in NTRK3, thus producing an oncogenic effect by dysregulation of NTRK3 signal-transduction pathways.

It is possible that other mechanisms, such as point mutations, also are involved in activation of the NTRK3 gene and the development of CIFS. Since the tyrosine kinase receptor gene can be a target for point mutations associated with tumors or other diseases, we suspect that the NTRK3 gene might be activated by point mutations in the fusion gene–negative tumors. However, we could not identify any point mutations in the main portion of the intracellular tyrosine kinase domain (exons 13-18) of NTRK3 using PCR–single-strand conformation polymorphism analysis (unpublished data). Further studies are necessary to elucidate other unknown mechanisms of NTRK3 overexpression in fusion-negative tumors.

In the present study, we also examined the immunohistochemical expression of NTRK3 in CIFS to corroborate its oncogenetic function in this tumor. In contrast with the results of Bourgeois et al, who found only low and inconsistent expression of NTRK3 in 5 CIFSs, all 10 CIFSs in the present study expressed NTRK3, including the 3 fusion transcript-negative cases. The antibody used recognizes a carboxyl terminal portion of NTRK3; hence, the NTRK3-positive immunohistochemical staining results suggest aberrant expression of NTRK3 or NTRK3-related molecules, most of which seem to be the result of the ETV6-NTRK3 fusion gene. Thus, unscheduled expression of NTRK3 or an NTRK3-derived protein seems to be important in the development of CIFS. Since the immunohistochemical expression of NTRK3 also was observed in other spindle cell tumors and tumor-like lesions (mentioned in the “Results” section), we think it is not helpful to the differential diagnosis.

CIFS is a distinctive spindle cell tumor of newborns and infants with a favorable prognosis. It may be difficult to distinguish from other pediatric spindle cell sarcomas and sarcomatous lesions owing to histologic and immunophenotypic variations. The detection of ETV6-NTRK3 fusion transcripts in most CIFSs using RT-PCR methods with archival, formalin-fixed, paraffin-embedded tissues therefore is a useful diagnostic adjunct. Aberrant expression of NTRK3 owing to gene rearrangement or unknown genetic alteration seems to be an important oncogenetic factor in CIFS.
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References


