

# **ETV6 Rearrangements in Patients with Infantile Fibrosarcomas and Congenital Mesoblastic Nephromas by Fluorescence *In Situ* Hybridization**

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**Congenital mesoblastic nephroma (CMN) and infantile fibrosarcoma (IFS) are two pediatric tumors arising in the kidneys and soft tissues of infants, respectively. Recently, a t(12;15)(p13;q25) resulting in ETV6-NTRK3 gene fusion was detected in patients with IFS and in patients with the cellular type of CMN, suggesting a common pathogenetic pathway. We investigated the presence or absence of ETV6 rearrangements and numerical abnormalities of chromosome 11 by using fluorescence *in situ* hybridization on paraffin-embedded material from five cases of IFS, two of CMN, and one of mixed type (CMN and IFS) found in our files. In three cases of IFS, we found ETV6 gene rearrangement but a normal copy number of chromosome 11. One case each of IFS, the cellular type of CMN, and the mixed type (CMN and IFS) had both abnormalities. In a case of classic CMN, neither trisomy 11 nor gene rearrangement was found. It is possible that trisomy 11 is a later, nonessential event in the pathogenetic process or that this secondary aberration is associated with still-unrecognized clinical or biological characteristics. We confirmed that IFS and the cellular type of CMN are cytogenetically related and can occur synchronously in the same organ.**

**KEY WORDS:** *ETV6*, Fluorescence *in situ* hybridization, Infantile fibrosarcoma, Mesoblastic nephroma, Trisomy 11.

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Congenital mesoblastic nephroma (CMN) was described in 1967 by Bolande *et al.* (1) based on eight cases of the classic type. The cellular type of CMN, with lesions displaying necrosis, high cellularity, and atypia or mitosis, was reported later (2, 3). Trisomy 11 is the most consistent nonrandom karyotypic finding in the CMNs cytogenetically investigated so far (4), occurring almost exclusively in the cellular type of CMN and the mixed type of CMN and infantile fibrosarcoma (IFS; 5, 6). IFS was characterized by Chung and Enzinger (7) in their series of 53 cases, which showed patients with IFS to have a much better prognosis than adults with fibrosarcomas. A combination of numerical changes (chromosomal gains), including trisomy 11, initially were said to be characteristic cytogenetic findings in IFS (8, 9).

However, Knezevich *et al.* (10) reported a recurrent cryptic t(12, 15)(p13;q15) resulting in a fusion between the *ETV6* gene in the band 12p13 and the *NTRK3* gene in the band 15p15. Such cytogenetic features were not seen in any of the 18 control cases, which included cases of adult-type fibrosarcoma and infantile fibromatosis (10). It was subsequently established that both IFS and the cellular type of CMN can carry the same 12;15 translocation, suggesting a possible link between these two pediatric tumors (11, 12).

We retrospectively reviewed tumors coded as CMN or IFS in the Mayo Clinic surgical pathology files and in the consultation files of one of the authors (AGN). We also included a case in which morphologic features of both CMN and IFS were seen in transition-like areas. Molecular cytogenetic technique or fluorescence *in situ* hybridization (FISH) analysis was used to evaluate the presence or absence of *ETV6* gene rearrangements and the copy number of chromosome 11 in these cases without prior knowledge of the histopathologic diagnosis.

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## MATERIALS AND METHODS

### Selection of Cases

Eight cases were selected for study. After review of hematoxylin and eosin-stained slides, cases were classified as IFS (five cases), CMN classic type (one case), CMN cellular type (one case), or mixed type (IFS and CMN, one case). Clinical data are summarized in Table 1. One representative block from each case was sent for molecular cytogenetic analysis.

### Preparation of Nuclei From Paraffin Sections

Nuclei were extracted from paraffin-embedded 50- $\mu$ m-thick tissue sections as described by Kuchinka *et al.* (13). In brief, the sections were deparaffinized in 2  $\times$  5-minute baths in xylene and 2  $\times$  5-minute baths in ethanol. Digestion was performed by collagenase XI (Sigma, St. Louis, MO) for 2 hours, followed by 0.05% trypsin and EDTA (Life Technologies, Rockville, MD) for 30 minutes at 37° C. The cells were spread on positively charged slides, baked for 2 hours at 50° C, and treated with 30% sodium bisulfite (Pretreatment solution, Oncor, Gaithersburg, MD) for 10 minutes, followed by digestion in 250  $\mu$ g/mL proteinase K (Oncor) for 10 to 15 minutes at 45° C.

### Fluorescence *In Situ* Hybridization Analysis

FISH analysis was performed to assess the status of the *ETV6* gene using the yeast artificial chromosome clone 964c10, spanning the *ETV6* gene region in 12p13 (14). To increase the specificity of the analysis, 964c10 was hybridized to interphase nuclei together with another yeast artificial chromosome clone probe, 753f12, localized to the neighboring *KRAS2* region in 12p12 (Fig. 1A). The clones 964c10 and 753f12 were obtained from Fondation Jean Dausset (CEPH, Paris, France). Labeling was performed by random-priming octanucleotides (Life Technologies).

With use of these probes, normal interphase nuclei show four signals arranged as two pairs (Fig. 1B–C). Nuclei harboring a translocation involving *ETV6* show two signals for *KRAS2*, two

signals for *ETV6* adjacent to the *KRAS2* signals, and an additional *ETV6* signal. The centromeric alpha-satellite probes for chromosomes 11 (*D11Z1*) and 12 (*D12Z1*) were detected by biotinylated and digoxigenin-labeled alpha-satellite probes (Oncor).

Probes and extracted nuclei were denatured simultaneously on a HYBrite slide warmer (Vysis, Downers Grove, IL). This was followed by hybridization at 37° C overnight and stringency washing in 50% formamide/2 $\times$  SSC for 15 minutes and 2  $\times$  SSC for 8 minutes at 42° C. Biotinylated probes were detected by fluorescein isothiocyanate-avidin or Alexa594-streptavidin, and digoxigenin-labeled probes were detected by rhodamine or fluorescein isothiocyanate-conjugated anti-digoxigenin antibodies (Oncor). At least 50 cells with signals were analyzed for each hybridization. Nuclei from the karyotypically normal human fibroblast line GM498B (NIGMS Human Cell Repository, Camden, NJ) were used to determine normal hybridization patterns. Of 500 interphase cells hybridized with centromeric and single-copy probes, more than 90% showed only two signals per cell, and fewer than 5% showed three or more signals (Fig. 1D).

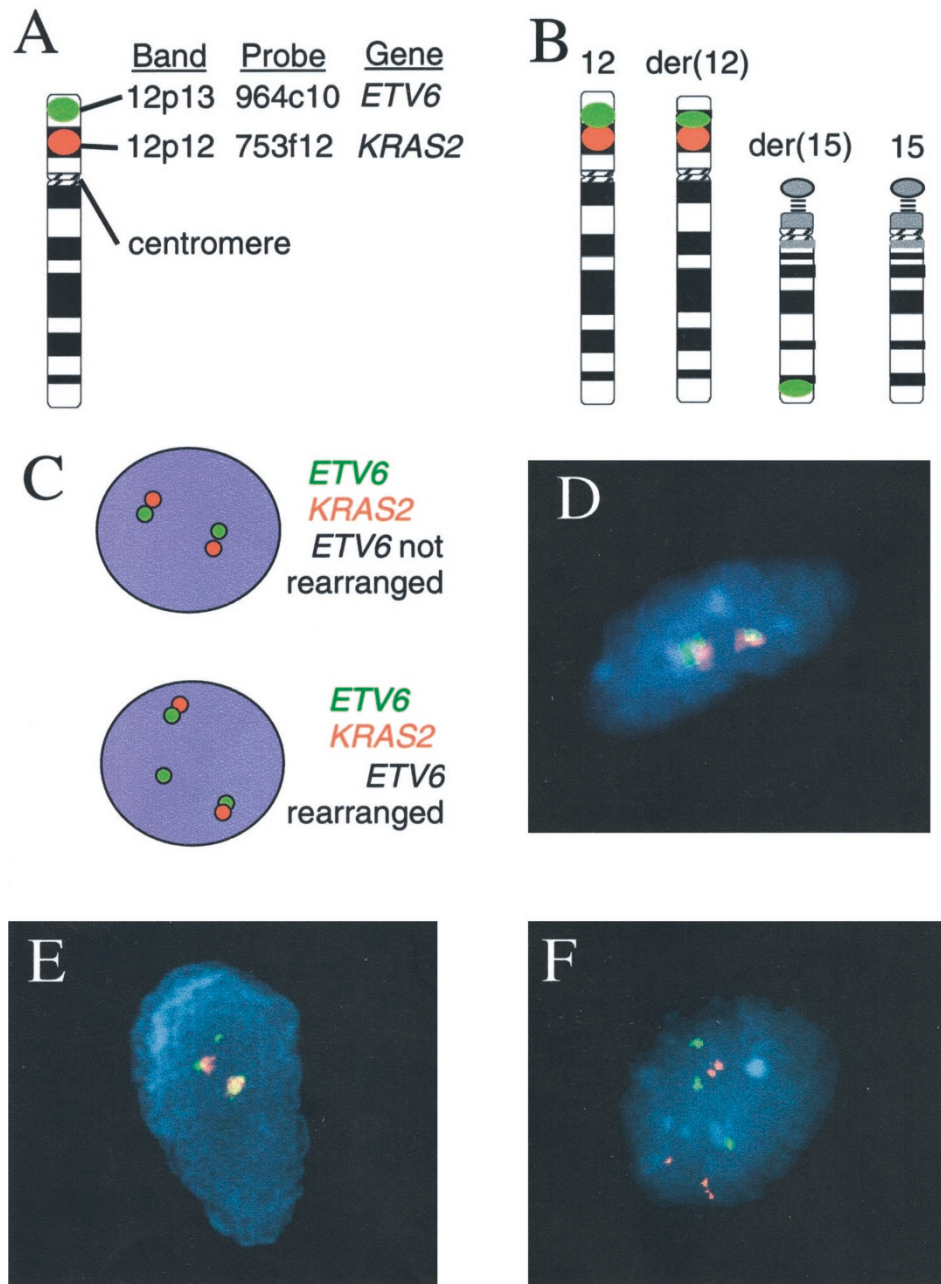
## RESULTS

The conditions studied occurred in four boys and three girls (records did not indicate the sex of the patient in Case 6). Mean age was 10 months (range, 4 months to 2 years; records did not indicate patient age in three cases). The five patients with IFS were affected in the extremities (three cases) and the head (two cases). The two patients with CMN were affected in the kidneys, as was the one patient with the mixed type, IFS/CMN (Table 1). The tumors in Cases 1, 2, 3, and 8 were composed of a hypercellular malignant spindle cell population arranged in a herringbone pattern, with numerous mitotic figures, consistent with the diagnosis of IFS. The tumor in Case 4 was composed of a multinodular hypocellular spindle cell population in a myxoid background, with moderately cellular areas and rare mitotic figures (Fig. 2). The neoplasm in Case 7 arose from the left kidney. On low-power view, two

**TABLE 1. Clinical, Histopathologic, and FISH Analysis Data on 8 Cases of IFS or CMN**

Case No.	Patient Age, Sex	Histopathologic Diagnosis	Tumor Location	Chromosome 11 Copy No.	<i>ETV6</i> Rearrangement
1	4 mo, M	IFS	Forearm	2	Present
2	Unknown, F	IFS	Lower leg	3	Present
3	6 mo, M	IFS	Thigh	2	Present
4	2 y, M	IFS	Postauricular	3,4	Absent
5	Unknown, M	CMN-Ce	Kidney	3	Present
6	Unknown, unknown	CMN-Cl	Kidney	2	Absent
7	4 mo, F	CMN/IFS	Kidney	3	Present
8	11 mo, F	IFS	Temporal	2	Present

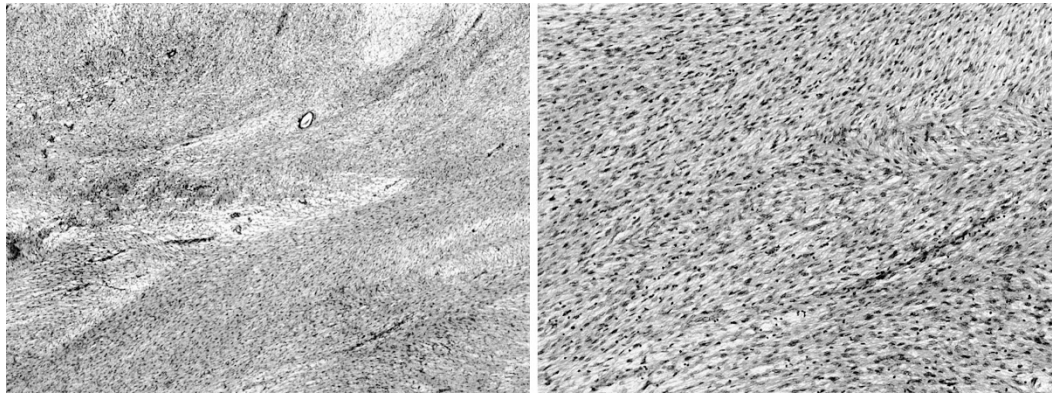
Ce, cellular type; Cl, classic type.



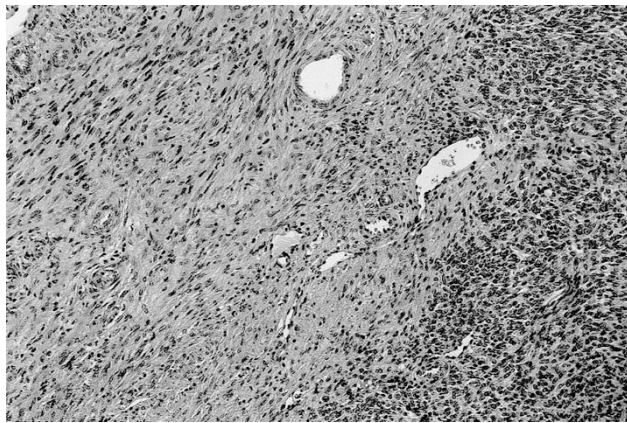
**FIGURE 1.** **A**, ideogram of a G-banded chromosome 12 with positions of the *ETV6* and *KRAS2* probes. **B**, schematic description of hybridization pattern resulting from a 12;15 translocation involving the *ETV6* gene region: the *ETV6* signal (**green**) is split between the derivative (*der*) chromosomes 12 and 15, whereas the *KRAS2* signal remains on the *der*(12). **C**, schematic interphase pattern in nuclei without and with rearrangement of the *ETV6* region. **D**, interphase nucleus with a normal hybridization pattern; in other words, two pairs of colocalized *ETV6* (**green**) and *KRAS2* (**red**) signals; blending of red and green results in **yellow** fluorescence. **E**, nucleus from Case 3, showing two copies of the *KRAS2* region (**red**) and three signals for the *ETV6* region; the smaller green signal is located some distance from the red-green-yellow signals, indicating that it is translocated to another chromosome. **F**, nucleus from Case 7, showing one intact and one split signal for *ETV6* (**red**) and three signals for the centromere of chromosome 11 (**green**).

distinct areas were seen. One area was composed of hyperchromatic, malignant-looking spindle cells arranged haphazardly in a fascicular pattern with a high mitotic index. The other area was composed of a bland-looking spindle cell population with an absence of mitotic figures, consistent with CMN (Figs. 3 and 4). The tumors in Cases 5 and 6 had morphologic features consistent with the cellular type and the classic type of CMN, respectively.

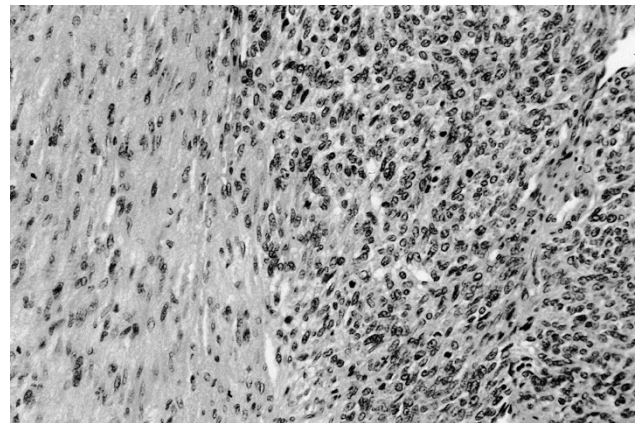
FISH analysis was performed on interphase nuclei from all eight cases (Table 1). An increased proportion of cells exhibited three signals positive for *ETV6* probe (964c10) per nucleus in Cases 1, 2, 3, 5, 7, and 8 (44%, 47%, 36%, 27%, 13%, and 26% of nuclei, respectively). In these cells, dual-color FISH with the *ETV6* and *KRAS2* probes showed two colocalized *ETV6*-*KRAS2* signals and an additional *ETV6* signal (Fig. 1E). One of the three *ETV6* signals



**FIGURE 2.** (Case 4). Photomicrograph of a bona fide IFS with mild to low cellular areas. *Left*, low-power view showing areas with moderate and high cellularity consistent with the diagnosis of IFS. (Hematoxylin and eosin stain; original magnification 4 $\times$ .) *Right*, higher power view of cellular areas with fascicles of spindle cells. (Hematoxylin and eosin stain; original magnification 20 $\times$ .)



**FIGURE 3.** (Case 7). A moderately cellular neoplasm consistent with a congenital mesoblastic nephroma (*left two-thirds of slide*) and a malignant spindle cell neoplasm consistent with infantile fibrosarcoma (*right third*). (Hematoxylin and eosin stain; original magnification 10 $\times$ .)



**FIGURE 4.** Higher-power view of portion of field shown in Figure 3. Compare the cell population of the congenital mesoblastic nephroma (*left side*) with the more atypical cell population and higher mitotic index of the infantile fibrosarcoma (*right side*). (Hematoxylin and eosin stain; original magnification 20 $\times$ .)

usually exhibited weaker fluorescence than the other 2, indicating an asymmetric split. Three signals for chromosome 11 were observed in Cases 2, 5, and 7 (63%, 32%, and 17% of cells, respectively; Fig. 1F). In Case 4, both the chromosome 11 centromeric probe and the *ETV6* probe showed increased frequencies of cells with three (22% and 26%) and four (15% and 13%) signals. An additional hybridization with a centromeric probe for chromosome 12 also showed three or four signals in a substantial percentage (20% and 36%) of cells, indicating Polyploidy or Polysomies 11 and 12 in this case. Case 6 had a normal hybridization pattern for *ETV6* and centromere 11.

## DISCUSSION

Previous authors have suggested that IFS and the cellular and mixed type of CMN are histogenetically linked (11, 12), and a t(12, 15)(p13;q25) resulting in

*ETV6-NTRK3* gene fusion has been observed in both entities.

In this study, FISH analysis demonstrated that rearrangements of the *ETV6* region occurred in four of the five cases of IFS and in two of the three cases of CMN. A combination of *ETV6* rearrangement and trisomy 11 occurred in one case each of IFS (Case 2), the cellular type of CMN (Case 5), and the mixed type, IFS and CMN (Case 7), but not in the classic type of CMN (Case 6). These findings are consistent with previously reported findings in cases of CMN and IFS (4–6, 8–12, 15–17).

In Case 4, two abnormal populations of cells carrying trisomy and tetrasomy for both chromosomes 11 and 12 were found in the absence of *ETV6* rearrangement. This may reflect the presence of cells with additional copies of these two chromosomes or with near-triploid and near-tetraploid modal numbers. It is interesting to note that this tumor had a lower-grade morphology than the tumors in the other cases of IFS. Previous studies have shown

that multiplication of the chromosome complement, tetraploidy in particular, may be a very early stage in tumor evolution that is followed by more specific chromosomal changes, usually occurring in a near-diploid cell population (18, 19). FISH analysis may thus have detected an earlier stage in the development of IFS.

Studies have distinguished CMN from Wilms tumor on the basis of dissimilar molecular profiles (20, 21). In 1980, Snyder *et al.* (22) distinguished the classic type of CMN from its cellular variant. Based on the results of a clinicopathologic study, these authors considered the cellular type of CMN to be a precursor of sarcoma. The finding of an *ETV6-NTRK3* fusion gene in only the cellular type of CMN supports the notion that the classic and the cellular types of CMN are genetically distinct entities. Besides CMN/IFS, *ETV6* rearrangements have been found in several lymphoid and myeloid hematologic neoplasms. In these conditions, as in CMN/IFS, *ETV6* is frequently fused to genes coding for tyrosine-kinase receptors, such as, *PDGFRB*, *JAK2*, and *ABL1* (23). However, trisomy 11, which often is concurrent with the 12;15 translocation in CMN/IFS, rarely is found in leukemias with *ETV6* rearrangements, suggesting that trisomy 11 is a typical rearrangement for solid tumors with the *ETV6-NTRK3* fusion. In the present study, trisomy 11 was found in only three of the six cases with *ETV6* rearrangement. Therefore, gain of chromosome 11 may be a later event in tumor progression. On the other hand, this secondary aberration may be associated with still-unrecognized clinical or biological characteristics. Like other authors, we believe that the cellular type of CMN is the renal counterpart of IFS (11, 12).

Our results also indicate that interphase FISH may be highly useful for detecting genetic abnormalities in archived materials. Recently, several reports have indicated that reverse transcription-polymerase chain reaction (RT-PCR) analysis is more sensitive in detecting *ETV6-NTRK3* gene fusion than conventional cytogenetic and immunohistochemical analysis (15–17). The present study demonstrates that interphase FISH analysis may be a comparably sensitive method for detecting *ETV6* rearrangements in CMN/IFS. Compared with RT-PCR analysis, the FISH technique allows analysis of very small sample volumes, even if they consist of a few single nuclei. Because FISH may be used to detect rearrangements of a single gene, by disruption of the fluorescence pattern, it also may detect *ETV6* rearrangements involving partners other than *NTRK3*. Such variant rearrangements would be missed by an *ETV6-NTRK3*-specific RT-PCR approach. Further analysis of cases exhibiting *ETV6* rearrangements detected by FISH in the absence of *ETV6-NTRK3* fusions detected by RT-PCR could

lead to the identification of other oncogenic fusion products in CMN/IFS.

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## Book Review

**Nickoloff BJ, editor: *Melanoma Techniques and Protocols: Molecular Diagnosis, Treatment, and Monitoring*, 366 pp, Totowa, NJ, Humana Press, 2001 (\$125.00).**

This book, in accordance with the editor's intent, is a cutting-edge summary of the most important advances regarding melanoma with particular emphasis on the biologic, immunologic, pathologic, and genomic aspects of the disease. Despite the heavy focus on molecular principles, the book is designed with practical application in mind and provides information that is clear and useful to readers who are not melanoma experts. The numerous contributing authors are from around the globe and were selected based on their forward-thinking perspectives as well as their close links to the clinical practice of medicine.

The book is divided into four major sections pertaining to melanoma: biology, diagnosis, treatment, and monitoring of patients with the disease. Laboratory techniques (*e.g.*, isolation of tumor suppressor genes, induction of melanocytic lesions) are described in step-wise detail throughout the book. Virtually all imaginable

topics pertaining to melanoma, from etiology to immunocytochemistry to gene therapy, are discussed in this book. Each topic is clearly and abundantly referenced in journal format, particularly useful for readers who wish to learn more about specific details. The book is divided into 20 chapters, and, although the index is scanty, each chapter is subdivided with bold, descriptive titles that facilitate easy recognition of topics of interest. Many useful tables appear throughout the book and provide readers with important "at-a-glance" summaries, lists, and comparisons.

This book is comprehensive yet succinct and will serve as a valuable reference text for laboratory personnel, researchers, and physicians (particularly pathologists, oncologists, and surgeons who frequently encounter melanoma). The book presents what is currently known about melanoma while also providing readers with a look toward the future of the disease.

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