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# INTERPHASE CYTOGENETIC ANALYSIS OF DISTINCT X-CHROMOSOMAL TRANSLOCATION BREAKPOINTS IN SYNOVIAL SARCOMA

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## SUMMARY

Synovial sarcomas show a specific translocation involving chromosomes X and 18, t(X;18)(p11.2;q11.2). Two distinct X-chromosomal breakpoints occur in different synovial sarcoma tumour samples. These breakpoints are located within two related genomic regions containing ornithine aminotransferase-like sequences, termed OATL1 and OATL2. Preliminary observations indicated the potential correlation of OATL1-associated breakpoints with biphasic tumours and OATL2-associated breakpoints with monophasic fibrous tumours. The present study uses interphase cytogenetics to investigate the nature of chromosomal aberrations in frozen synovial sarcoma tissue samples. Two-colour fluorescence *in situ* hybridization (FISH) was performed using probes specific for the centromeres of chromosome X or 18, along with yeast artificial chromosome probes corresponding to the distinct breakpoint regions on Xp. One monophasic epithelial and two monophasic fibrous synovial sarcomas showed an OATL2-associated breakpoint, while a biphasic tumour revealed a hybridization pattern indicating a breakpoint within the OATL1 region. These results confirm our previous suggestion of a relationship between alternative breakpoints in Xp11.2 and different histological phenotypes observed in synovial sarcomas. They also demonstrate the utility of the two-colour hybridization approach for the identification of chromosomal changes in interphase nuclei isolated from frozen tissues.

KEY WORDS—synovial sarcoma; immunohistochemistry; FISH; yeast artificial chromosome (YAC); interphase cytogenetics

## INTRODUCTION

Synovial sarcoma is a malignant soft tissue tumour that most frequently affects adolescents and young adults. The classical histological pattern of the tumour is biphasic, revealing two morphologically distinct cellular components, epithelial and spindle cells, that can be present in varying proportions. The equally common monophasic fibrous type is entirely composed of spindle cells, while monophasic epithelial synovial sarcoma is a rare histological variant clearly dominated by cells exhibiting epithelioid differentiation. There are also poorly differentiated forms of this neoplasm, in which the tumour cells have an indistinct morphology.[1]

Cytogenetic studies on synovial sarcomas have revealed a characteristic chromosomal translocation involving chromosomes X and 18, t(X;18)(p11·2;q11·2).[2] This chromosomal aberration is found in a high percentage of tumours, either alone or associated with further numerical and structural cytogenetic changes.[3,4] This striking correlation suggests that the t(X;18) translocation might represent a primary event in the development of this malignancy.

Molecular characterization of the cytogenetically described rearrangements has led to the identification of yeast artificial chromosomes (YACs)

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spanning the translocation breakpoint region on the X chromosome. Fluorescence *in situ* hybridization (FISH) on synovial sarcoma metaphase spreads and Southern analysis of a translocation chromosome-containing somatic cell hybrid led to the assignment of the breakpoint to the OATL1 region on Xp.[5,6] Another study localized the X-chromosomal break within the more proximal OATL2 cluster.[7] FISH analysis of additional tumour samples has confirmed the presence of two distinct breakpoints on the X chromosome in different tumours.[8,9] It has been suggested that a potential relationship exists between the two breakpoints and the different histological phenotypes of synovial sarcoma.[8]

In this study, we have used FISH to detect chromosomal translocations in interphase nuclei isolated from frozen synovial sarcoma tissue, instead of investigating metaphase spreads of tumour cells placed in culture. Our two-colour hybridization strategy employs a combination of centromere-specific probes and YAC probes specific for the alternative breakpoint regions on Xp. We compare these findings with the histological and immunohistochemical characterization of the tumours and thereby address the question of a potential correlation between the genetic events and the phenotype of the tumour.

## MATERIALS AND METHODS

### *Immunohistochemistry*

Immunohistochemistry was performed using a panel of monoclonal and polyclonal antibodies against different antigens, i.e., EMA, CEA, vimentin (V9), S-100, NSE, smooth muscle actin (HHF 35), UEA-1, collagen IV (all from Dakopatts) and keratin (KL1; Dianova). Five-micrometre sections were deparaffinized, rehydrated, and incubated with primary antibodies for 1 h at room temperature after blocking of non-specific binding. Antibody reactivity was visualized using a modified APAAP technique.[10]

### *Preparation of nuclei from frozen tissue*

Sections 50 μm in thickness cut from frozen tissue were minced with a scalpel on a microscope slide. The minced tissue was suspended in a small volume of phosphate-buffered saline (PBS) and transferred to a 15 ml tube. The tissue suspension was then pipetted repeatedly to release nuclei. After allowing the larger particles to settle, the supernatant, containing the nuclei, was fixed by adding ice cold 70 per cent ethanol. Nuclei were collected by centrifugation at 1200 rpm for 10 min. 50 μl of the nuclear suspension was spread on slides and dried at 65°C for 30 min.

### *Fluorescence* in situ *hybridization on interphase nuclei*

The following probes were used: pBamX5, specific for the X chromosome centromere; LI.84, specific for the chromosome 18 centromere; and two YAC clones, numbered 2 and 7, which correspond to the OATL1 and OATL2 clusters, respectively.

Hybridization and detection procedures were performed as previously described.[5,6] Probes were labelled by nick translation with either biotin-14-dATP or digoxigenin-11-dUTP. YAC probes were preannealed for 4 h at 37°C with a 50-fold excess of sonicated total human DNA, which was unnecessary for centromeric probes. All hybridizations were performed in 50 per cent (v/v) formamide, 10 per cent (w/v) dextran sulphate, 2 × SSC, 1 per cent (v/v) Tween-20, pH 7·0.

The slides were pretreated with porcine pepsin (Serva; 100 μg/ml in 0·01 M hydrochloric acid) at 37°C for 20 min, followed by fixation in 1 per cent (v/v) formaldehyde–PBS, pH 7·2, for 10 min at 4°C. After denaturation at 70°C for 2 min in 70 per cent formamide, 2 × SSC, pH 7·0, the slides were hybridized overnight at 37°C in a moist chamber with the indicated probes. Post-hybridization washes involved three changes of 50 per cent formamide, 2 × SSC at 42°C, 5 min each, followed by three washes, 5 min each, in 2 × SSC at 42°C.

Biotinylated probes were detected with FITC-conjugated avidin (1:500; Vector Laboratories). This signal was amplified by two rounds of incubation with rabbit-anti-FITC (1:250; DAKO) and FITC-conjugated mouse-anti-rabbit antibodies (1:100; Jackson Immunoresearch). Digoxigenin-labelled probes were visualized with rhodamine conjugated sheep-anti-digoxigenin antibodies (1:20; Boehringer Mannheim), followed by amplification with donkey-anti-sheep-Texas red antibodies (1:50; Jackson Immunoresearch). Finally, the slides were mounted in anti-fade medium (DABCO; Merck) containing diaminophenylindole (DAPI; Sigma) for nuclear counterstaining.

Using a Zeiss Axiophot epifluorescence microscope, at least 100 nuclei were evaluated per

Table I—Histological diagnosis, immunophenotype, and *in situ* hybridization data

| Case No. | Histological diagnosis | Immunophenotype | | | | | | | | | FISH |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | EMA | Keratin | CEA | VIM | S-100 | NSE | Actin | UEA-1 | Collagen IV | breakpoint region |
| 1 | MF | − | + | − | + | + | − | − | − | − | OATL2 |
| 2 | BI | + | + | − | + | + | − | − | − | + | OATL1 |
| 3 | ME | − | − | + | + | − | − | − | − | + | OATL2 |
| 4 | MF | − | − | − | + | − | − | − | − | − | OATL2 |

MF=Monophasic fibrous; BI=biphasic; ME=monophasic epithelial; +=tumour cells are positive; −=tumour cells are negative; EMA=epithelial membrane antigen; CEA=carcinoembryonic antigen; VIM=vimentin; NSE=neuron-specific enolase; OATL1, OATL2=X-chromosomal breakpoint within the ornithine aminotransferase-like clusters 1 and 2, respectively.

experiment. Separate digital images were recorded using a high-performance CH250/A cooled CCD camera coupled to a Macintosh IIci computer and analysed with the program BDS-image (Biological Detection Systems, Rockville U.S.A.).

## RESULTS

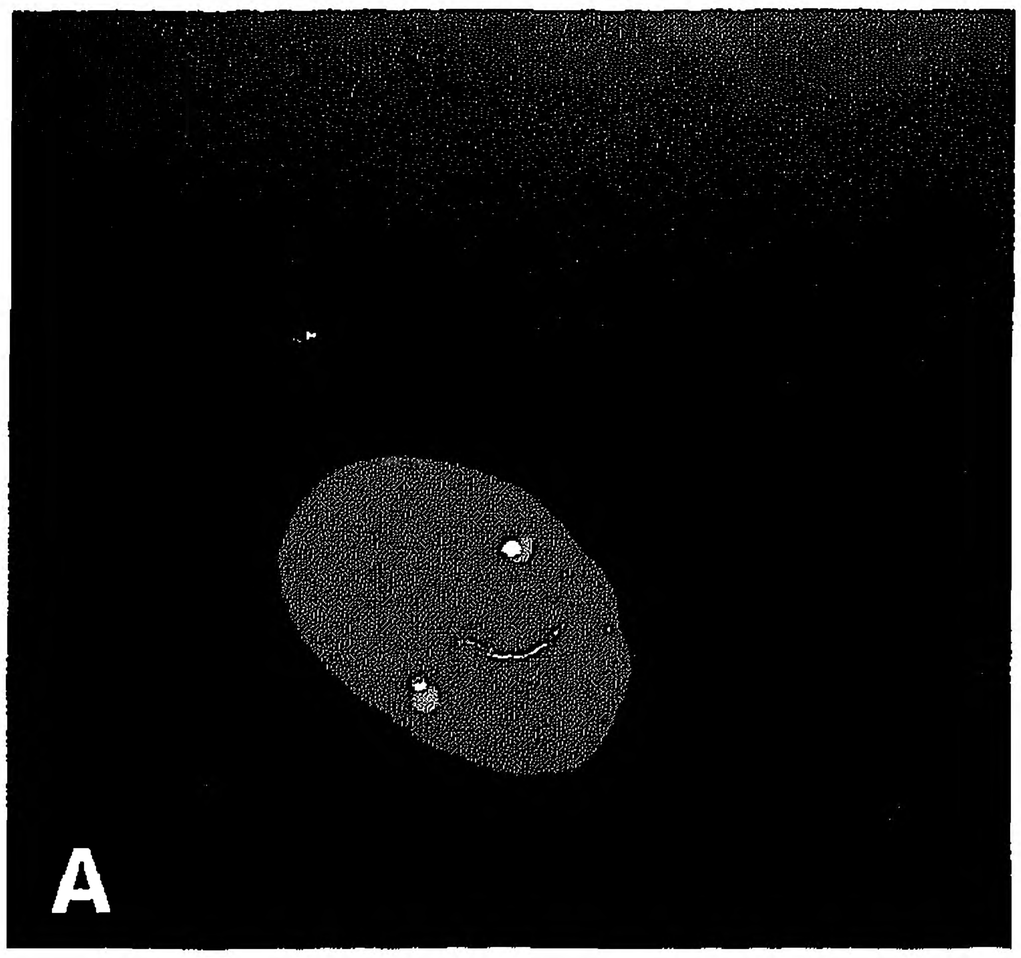
Histological examination of four separate cases of synovial sarcoma revealed two examples of the monophasic fibrous type, one monophasic epithelial type, and one biphasic tumour. Immunohistochemically, three of the four cases showed positive staining for one or two epithelial markers (i.e., keratin, EMA, or CEA) and all tumours stained positively for vimentin (Table I and data not shown).

FISH was performed using combinations of differently labelled probes. One was a centromeric probe specific for either chromosome X or 18, detected in red, and the other was either YAC 2 (OATL1) or YAC 7 (OATL2), detected in green. Simultaneous hybridization of the centromere X probe and either one of the YAC probes to normal interphase nuclei yielded discrete, closely spaced signals, consistent with their physical linkage (Fig. 1A). In contrast, the combination of the centromere 18 probe and the YAC probes indicated well-separated hybridization domains, showing a random distribution within the nuclei. A few cells exhibited alterations in this pattern, such as loss of signals or doublet signals. These are likely to result from overlapping hybridization domains or to reflect nuclei in a phase of the cell cycle when chromosomal condensation and replication are occurring.

Hybridization of the centromere X probe (in red) and the YAC 2 probe (in green) to tumour cells of cases 1, 3, and 4 revealed a close proximity for one pair of red and green signals, while the other signals were located at random in the nucleus (Fig. 1B). Using a combination of centromere 18 and YAC 2 probes, a consistent juxtaposition was observed for one red and one green signal (Fig. 1C). These findings indicated a chromosomal break occurring between the X centromere and the OATL1 cluster, with subsequent translocation of the Xp regions recognized by YAC 2 to chromosome 18. The use of YAC 7, corresponding to the more proximal OATL2 cluster, confirmed and refined these observations. Hybridization of YAC 7 resulted in three signals of unequal size, indicating that the region encompassed by this YAC was split by a translocation event (Fig. 1D). Moreover, one out of the three YAC 7 hybridization domains was consistently located near one centromere 18 signal (Fig. 1E). Taken together, these observations indicate a translocation involving chromosomes X and 18 in cases 1, 3, and 4 and localize the chromosomal break to the OATL2 cluster on Xp.

In contrast, hybridization of YAC 7 to tumour cells of case 2 revealed no detectable alterations in the normal hybridization pattern, using either the centromere X or the centromere 18 probe. However, the more distal YAC 2 probe showed three signals in experiments with both centromeric probes (Fig. 1F). This is consistent with a translocation between chromosomes X and 18 occurring within the OATL1 region.

Two cases (2 and 4) showed a significant proportion of nuclei exhibiting a hybridization pattern identical to that of normal nuclei. Correspondingly, a high frequency of non-tumour cells was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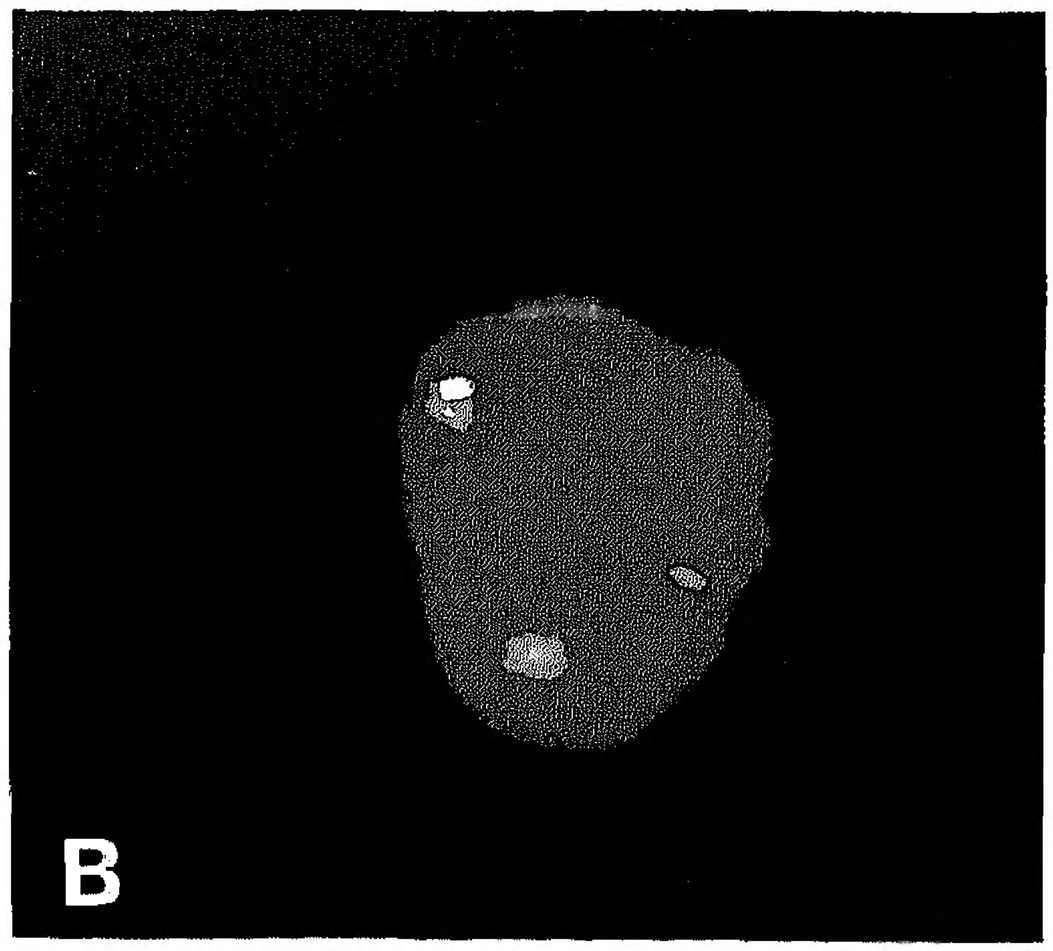
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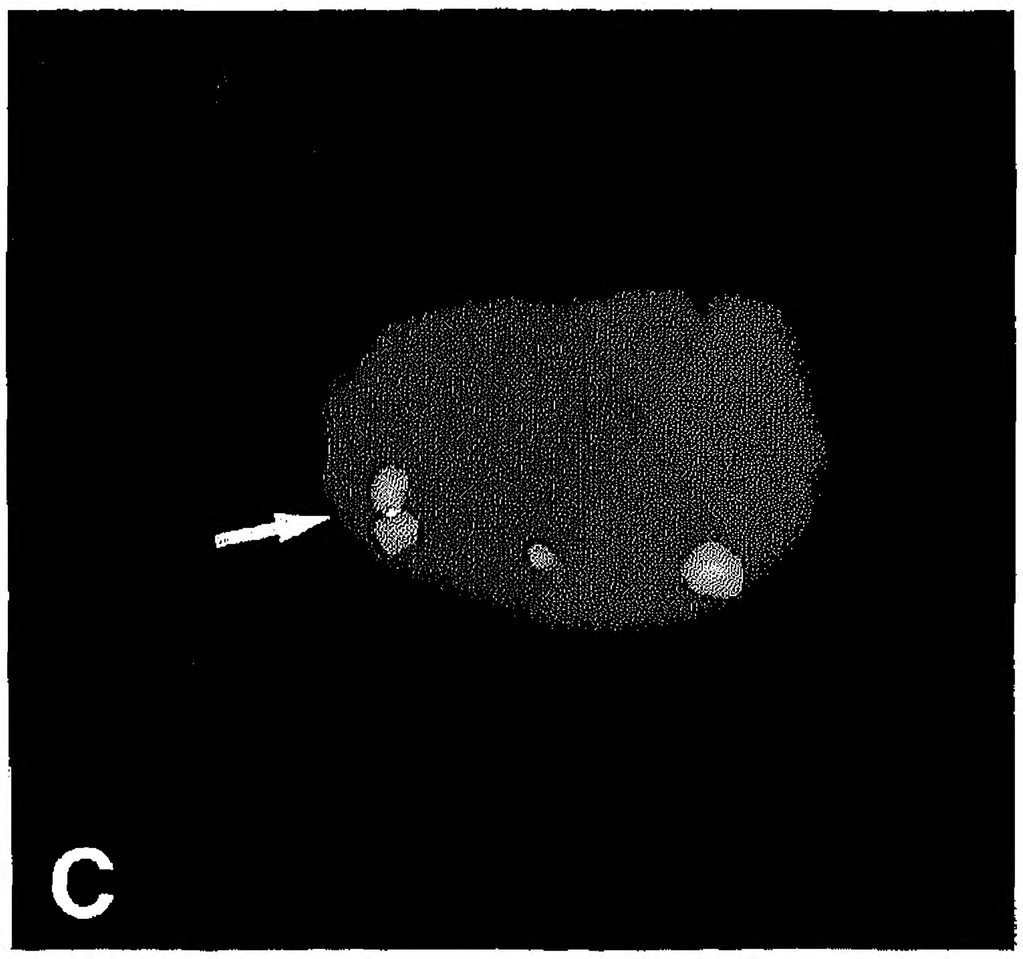
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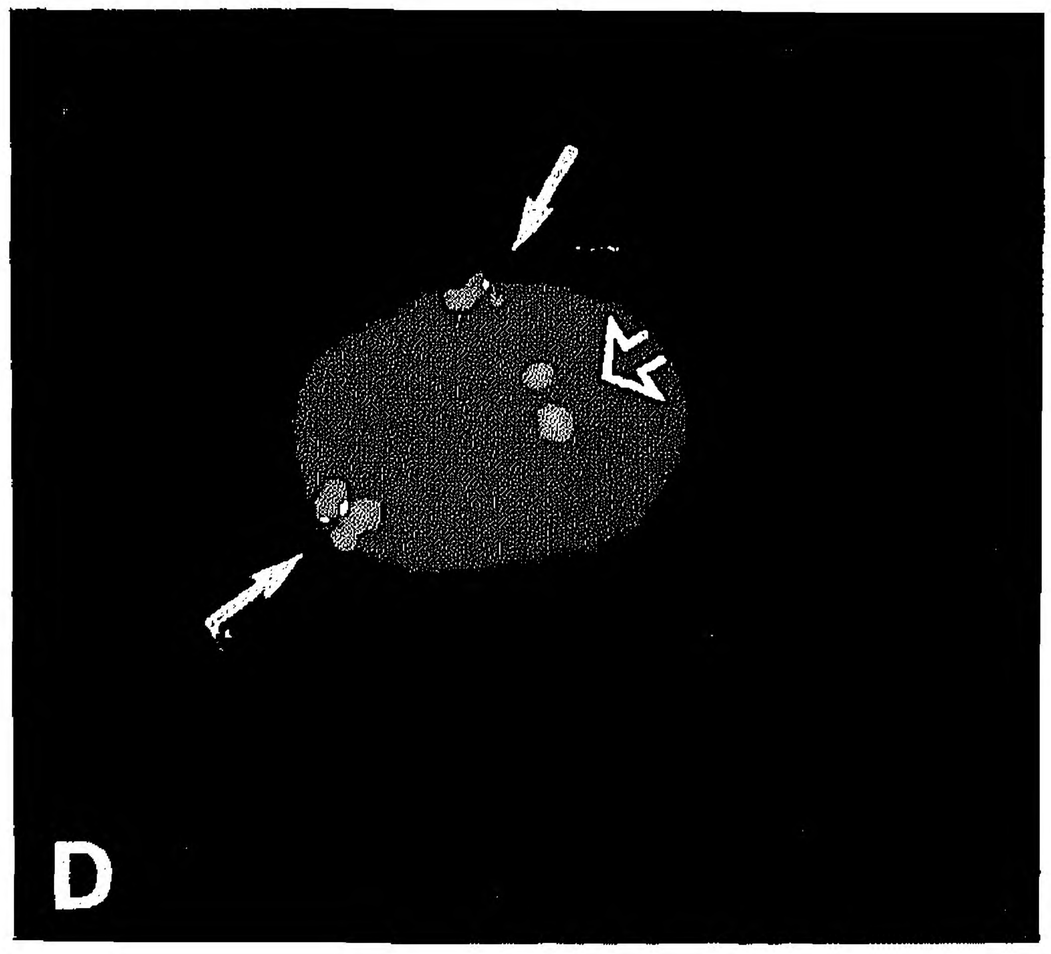
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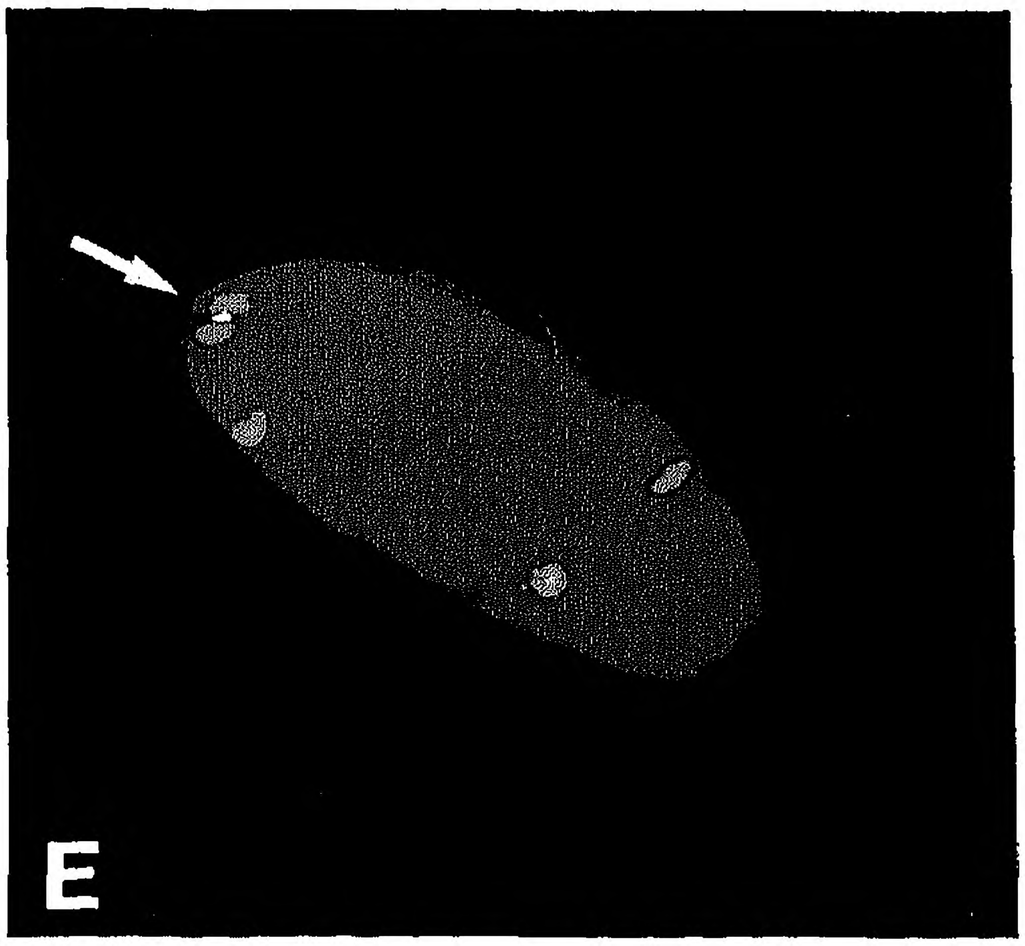
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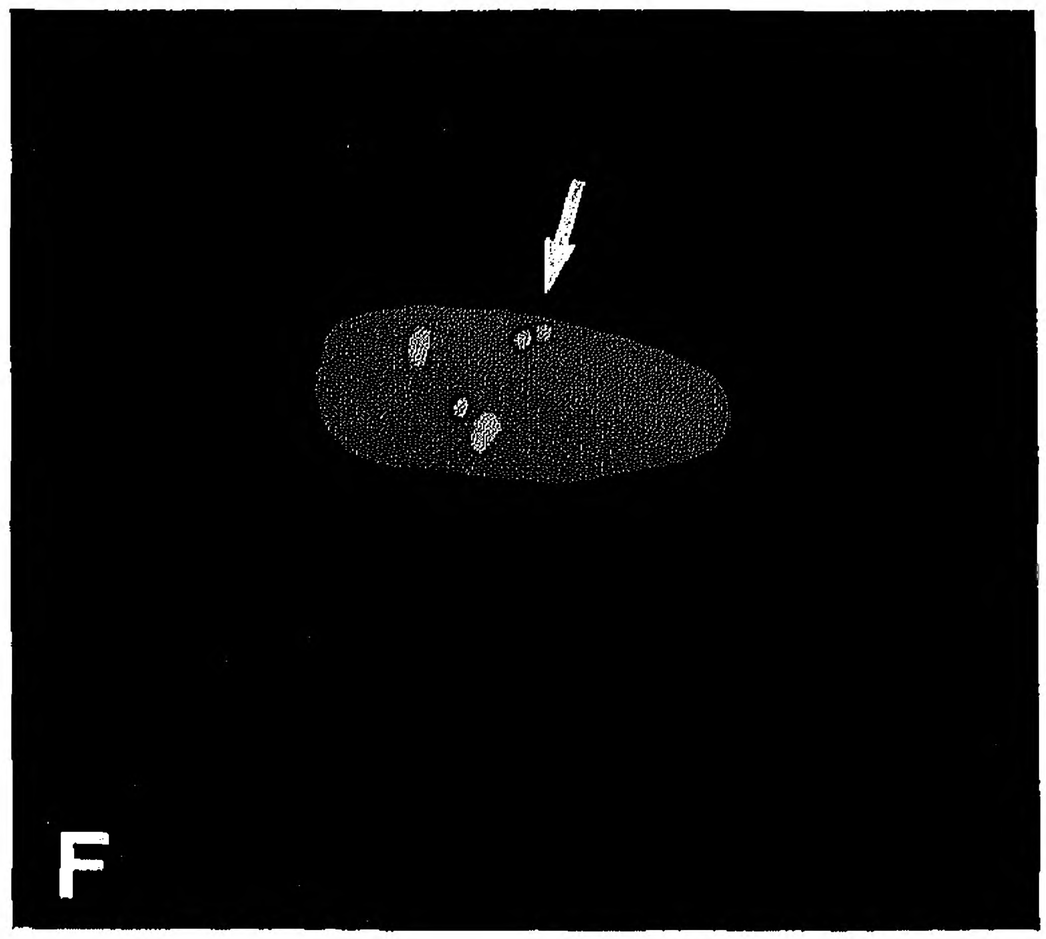
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observed in representative histological sections. These cases were therefore evaluated by correlating the percentage of tumour cells on the haematoxylin and eosin-stained sections with the percentage of cells presenting an abnormal signal pattern.

## DISCUSSION

The classical biphasic type of synovial sarcoma has a distinctive morphology and thus rarely poses a problem diagnostically. Cases that do not exhibit distinct epithelial and spindle cell components, on the other hand, require more careful investigation. In particular, monophasic fibrous tumours and the poorly differentiated forms must be distinguished from a variety of morphologically similar soft tissue tumours, including fibrosarcoma, malignant schwannoma, haemangiopericytoma, and leiomyosarcoma. Together with advances in the immunohistochemical characterization,[11,12] the ability to identify the characteristic t(X;18) translocation could significantly facilitate the diagnosis, as well as enhancing its reliability.

Interphase FISH can overcome the limitations of conventional cytogenetic analysis in many instances. This can be useful for the cytogenetic characterization of solid tumours that are difficult to culture *in vitro*. FISH on interphase nuclei has proven to be a powerful tool for the detection of numerical aberrations in malignancies and in the context of pre-and post-natal diagnosis.[13,14] However, few reports present its application for the identification of structural changes.[15,16]

In the present study, interphase cytogenetics have been used to investigate chromosomal translocations in synovial sarcoma. Four different sets of experiments were performed on all tumours using probes specific for centromere X or 18, along with YAC probes spanning the two breakpoint regions on Xp. Advantage was taken of the fact that in synovial sarcoma the breakpoints are located adjacent to the X and 18 centromeres. This results in the YAC probes localizing in close proximity to centromere in normal cells and to centromere 18 in cells carrying a reciprocal t(X;18) translocation. This approach provided simultaneous information, on a single cell basis, about the chromosome copy number, the occurrence of additional, abnormal signals, and the physical linkage (or non-linkage) of the target sequences. Furthermore, it permitted a precise characterization of structural aberrations in interphase cells, more so than that possible with chromosome painting probes. It should be possible to apply this approach to solid tumours on a routine basis in cases where a specific translocation is associated with a tumour, provided that appropriate probes are available.

In tumours from four female patients, we have observed results consistent with the presence of one normal and one rearranged X chromosome. In the same cases, X signals appeared in close proximity to the 18 centromere-specific probe. This agrees with a t(X;18) translocation having occurred in the tumour samples. In three examples, one monophasic epithelial and two monophasic fibrous synovial sarcomas, it was possible to assign the breakpoint to the OATL2 region, while in the other case, a biphasic tumour, the hybridization pattern indicated an OATL1 breakpoint.

Thus, our findings corroborate the demonstration of two distinct X-chromosomal breakpoints in the t(X;18) translocation characteristic of synovial sarcoma and are relevant to the question of whether distinct chromosomal breakpoints can be correlated with different tumour phenotypes, as recently proposed.[8] With one exception,[9] biphasic tumours correspond to a breakpoint within the OATL1 cluster, whereas the majority of monophasic fibrous tumours show a breakpoint mapping to the OATL2 region.[8,9] Our results, while representing only a limited number of cases, support this working hypothesis.

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Fig. 1—Fluorescence *in situ* hybridization on interphase nuclei. Centromeric probes specific for chromosome X or 18, along with either YAC 2 or YAC 7, are shown. The latter correspond to the OATL1 and OATL2 regions on Xp11·2, respectively. The centromere-specific probes are detected in red and the YAC probes in green. (A) Normal interphase nucleus (female) revealing close proximity of the OATL1-specific YAC 2 probe to the chromosome X centromere. (B) Case 3 (female): centromere X probe in combination with YAC 2. One of the YAC signals is separated from one of the centromere X signals. (C) Case 1 (female): centromere 18 probe and YAC 2, with juxtaposition of one YAC 2 and one centromere 18 signal (arrow). (D) Case 3: centromere X probe in combination with YAC 7. Two YAC 7 signals are in close proximity to the chromosome X centromeres (arrows), whereas a third YAC 7 signal (which appears to be split due to computer processing; open arrow) shows no linkage to the centromeric signals. (E) Case 1: centromere 18 probe and YAC 7. Three YAC 7 signals are detectable, one of which is juxtaposed with one centromere 18 signal (arrow). (F) Case 2 (female): centromere 18 probe in combination with YAC 2. One of the three YAC 2 signals is located close to one centromere 18 signal (arrow)

Little is known about how synovial sarcoma originates or about the factors responsible for the striking morphological variety of the tumour. The interesting question is whether the pathways leading to epithelial- or mesenchymal-like differentiation are determined by epigenetic, environment factors, or whether they are related to distinct events at the chromosomal level. The full characterization of the breakpoints and the evaluation of additional tumour material will help to answer these questions and to provide further insight into the pathogenesis of synovial sarcoma.

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