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ORIGINAL ARTICLE

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Identification of syt-ssx fusion transcripts in both epithelial and spindle cell components of biphasic synovial sarcoma in small tissue samples isolated by membrane-based laser microdissection

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Abstract In order to confirm the presence of SYT-SSX fusion gene in epithelial and spindle cell components of synovial sarcoma, we performed a nested reverse transcriptase-polymerase chain reaction (RT-PCR) using microbeam microdissection of membrane-mounted native tissue (MOMeNT) technique applied on formalin-fixed, paraffin-embedded tumor specimens from two biphasic synovial sarcomas and a control tissue of adamantinoma. Small targeted portions of either an epithelial or spindle cell component of the tumor tissue were microdissected together with the supporter membrane, by using an ultraviolet (337-nm) pulsed laser microbeam coupled into a robot-stage microscope with infinity optics. The SYT-SSX fusion transcript was detected in epithelial and spindle cell components of both biphasic synovial sarcomas, but not in the control tissue. Southern blot analysis also confirmed that the detected messages were derived from the SYT-SSX fusion gene. In conclusion, the microbeam MOMeNT is a useful method for isolating selected small portions from tissue sections. The SYT-SSX fusion gene is present in both cellular components of biphasic synovial sarcoma and is involved in oncogenesis of the synovial sarcoma rather than in morphologic epithelial differentiation. Therefore, in spite of the variable proportions of each component, our results confirm that the synovial sarcoma is of monoclonal origin.

Keywords Biphasic synovial sarcoma · SYT-SSX fusion transcript · Microbeam microdissection of membrane-mounted native tissue (MOMeNT) · Cold photolysis

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Introduction

A specific chromosomal translocation, t(X;18)(p11.2, q11.2), has been demonstrated in synovial sarcoma [21], which results in the fusion of the SYT gene on chromosome 18 and SSX1 or SSX2 gene on chromosome X [5, 6]. The chimeric gene transcripts have been detected by reverse transcriptase-polymerase chain reaction (RT-PCR) in more than 90% of cases [6, 8, 9, 14]. Therefore, detection of this specific translocation by chromosome analysis and of SYT-SSX transcripts by RT-PCR are helpful in the diagnosis of synovial sarcoma [6, 8, 9, 20, 22, 24].

Recently, Hiraga et al. detected SYT-SSX fusion probe bound to the nuclei in both epithelial and spindle cell components of biphasic synovial sarcoma by in situ hybridization [10]. Birdsall et al. confirmed that the rearrangement was present in both cellular components using a two-color fluorescence in situ hybridization [1]. In the present study, to confirm and complement these findings, we performed a nested RT-PCR using microbeam microdissection of membrane-mounted native tissue (MOMeNT) technique applied on formalin-fixed, paraffin-embedded tumor specimens. Microbeam MOMeNT is a novel technique that utilizes membrane-based microdissection by using an ultraviolet laser microbeam, thus providing a flexible, easy-to-use, high-performance tool [2, 3]. Here, the feasibility of microbeam MOMeNT for RNA analysis is also demonstrated.

Materials and methods

Tumor samples

Formalin-fixed, paraffin-embedded samples of two histologically and immunohistochemically verified synovial sarcomas were obtained from the archives of the Department of Pathology, Fukuoka University. As a negative control, a paraffin-embedded tissue sample of adamantinoma of long bone (tibia) was used. All tumor samples were diagnosed according to internationally accepted cri-

Table 1 Clinicopathological and molecular features (*F* female, *M* male, *E* epithelial, *S* spindle, *RT-PCR* reverse transcriptase-polymerase chain reaction)

Case number	Sex	Age (years)	Site	Histology	Epithelial/spindle cell composed analyzed	RT-PCR	
						β-actin	SYT-SSX
1	F	60	Foot	Biphasic synovial sarcoma	E	+	+
					S	+	+
2	F	25	Thigh	Biphasic synovial sarcoma	E	+	+
					S	+	+
3	M	69	Tibia	Adamantinoma	E	+	–
					S	+	–

teria. The tissue sections were stained with hematoxylin and eosin and immunohistochemically labeled with cytokeratin (polyclonal, 1:500; DAKO JAPAN, Kyoto, Japan), epithelial membrane antigen (EMA, monoclonal, 1:50; DAKO), and vimentin (monoclonal, 1:50; DAKO) using a labeled streptavidin biotin and an alkaline phosphatase technique described earlier [11]. The clinical data are summarized in Table 1.

Microbeam microdissection of membrane-mounted native tissue

Microbeam microdissection of membrane-mounted native tissue (MOMeNT) allows a rapid, selective, and low-contamination procurement of tumor or other cells from histological sections by non-thermic, non-contact laser microdissection [2]. Tissue sections (4 µm thick) of formalin-fixed, paraffin-embedded tumor specimens were mounted on a specifically designed 1.5-µm-thick transparent supporter membrane. The membrane-covered glass slides were handled as ordinary glass slides, and hematoxylin and eosin staining was performed. Then, small targeted portions of epithelial or spindle cell component of the tumor tissue were microdissected together with the supporter membrane by an ultraviolet (337 nm) pulsed laser microbeam coupled into a robot-stage microscope with infinity optics (SL Microtest, Jena, Germany). The ultraviolet laser dissects by cold photolysis due to the high photon density of the microbeam rather than by local heating. The track of the laser microbeam can be preselected easily on a video screen, and the size and form of the dissectates can thus be adapted to the histological features of the section with a delineation accuracy in the micrometer range. The dissectates containing approximately 50–100 cells were removed on the tip of a 30-gauge needle and transferred into a PCR tube. These samples were utilized for RT-PCR and Southern blot analysis.

Reverse transcriptase-polymerase chain reaction

The microdissected tissue samples were homogenized and RNA was isolated according to the protocol described by Tsuji et al. [23], using a Trizol reagent (Life Technologies, Gaithersburg, Md.). Whole volume of total RNA was reverse transcribed using the T-Primed First-Strand kit: Ready-To-Go (Amersham Pharmacia Biotech, England). The resulting samples of complementary DNA (cDNA) were amplified by the nested PCR (RoboCycler). Two-microliter cDNA templates were initially amplified for 40 cycles with sytp3 (5′-GGATATAGACCA-ACACAGCC-3′) and ssxp2 (5′-CCAGATGCTTCTG[A/G]CACT-3′) primers. Then, aliquots of approximately 2 µl from the first PCR were reamplified for 30 cycles with sytp4 (5′-CAGCAGAGGCCTTATGGATA-3′) and ssxp1 (5′-C[G/T]TCCTCTGCTG-GCTTCT [T/C]G-3′) for the nested reaction [15]. Annealing temperature for all reactions was 55°C. The molecular size of the final PCR products was 55 bp for the nested PCR amplification of SYT-SSX. The PCR products were fractionated on 4.0% agarose gels and visualized by ethidium bromide staining and ultraviolet illumination. As positive controls for the integrity of cDNA in each case, a seminested PCR for ubiquitously expressed β-actin gene transcripts was performed with the following primers: act1 (5′-CCTTCTGACCCATGCCCACCA-3′), act2 (5′-TGATGATATCGCCGCGCTCGT-3′), and act3 (5′-ATGTGCA-AGGCCGGCTTCG-3′). This primer amplifies a 106-bp fragment of β-actin gene. As a negative control, an adamantinoma of long bone was analyzed by the same procedure. A positive control was a known case of previously detected chimeric product [11].

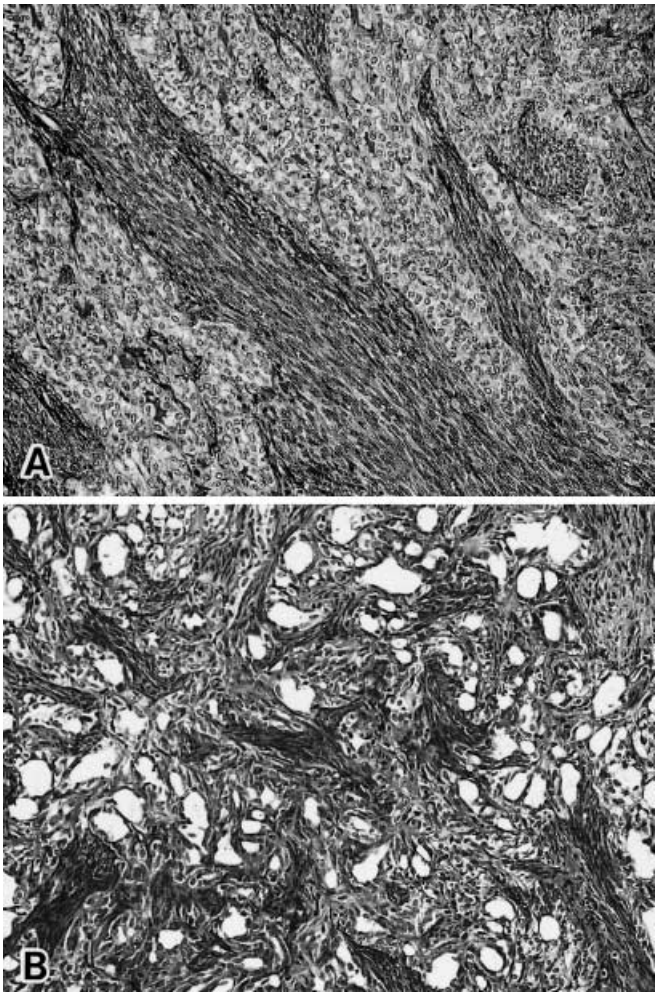


Fig. 1 Histologic features of synovial sarcoma. **A** Case 1. Typical biphasic synovial sarcoma with epithelial cells arranged into sheets separated by bundles of fibroblast-like spindle cells. **B** Case 2. Typical biphasic synovial sarcoma with epithelial cells forming gland-like structures admixed with fibroblast-like spindle cells. H&E, ×150

Southern blot analysis

The RT-PCR products were transferred onto nylon membranes (Oncor, Gaithersburg,Md.) for subsequent hybridization and de-

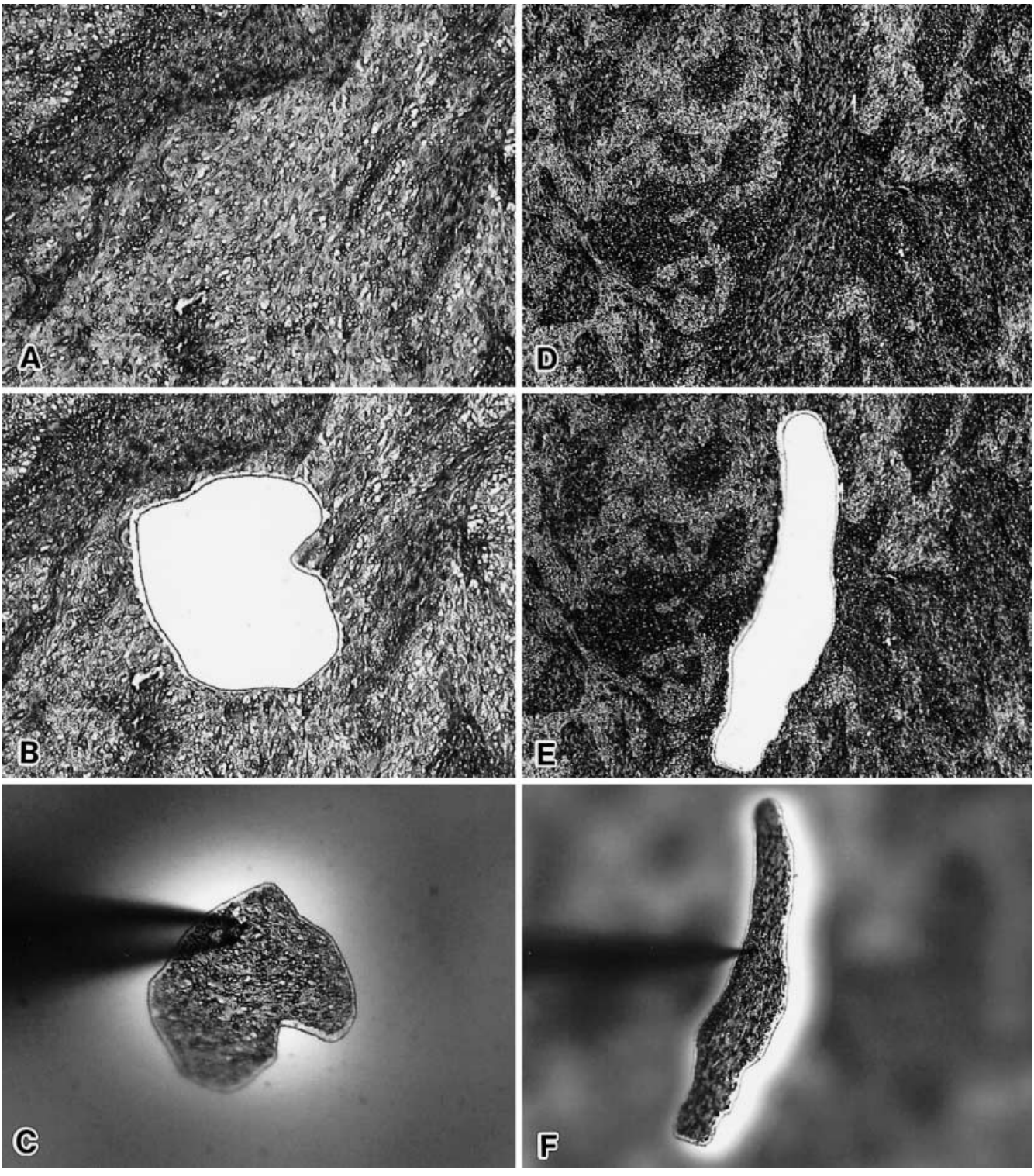


Fig. 2A–F Membrane-based laser microdissection (microbeam MOMeNT) of biphasic synovial sarcoma (Case 1). Hematoxylin and eosin stained 4 µm-formalin-fixed, paraffin-embedded sections are shown at original magnification ×260 (**A–C**), and ×130 (**D–F**). **A** Before microdissection of the epithelial cell component. **B** After removal of the dissectate. **C** Dissected sample from the epithelial cell component. **D** Before microdissection of the spindle cell component. **E** After removal of the dissectate. **F** Dissected sample from the spindle cell component

tection using ECL 3′-oligolabeling and detection systems (Amersham Life Science, UK). We used the oligonucleotide probe spanning the chimeric junction, SYT-SSX (5′-GGATATGACCAGATCATC-ATGCCCAAG-3′) [9].

Fluorescence in situ hybridization

In order to characterize the tumors in more detail, we employed fluorescence in situ hybridization (FISH). Fifty-micrometer thick

sections of paraffin-embedded tumor samples were disaggregated [13]. Isolated nuclei were spun onto silanized glass slides, fixated in 4% paraformaldehyde in PBS, and dried. Pretreatment consisted of 1 M sodium thiocyanate (NaSCN, Sigma, Munich, Germany) at 80°C for 3 min followed by digestion with 0.2% Proteinase K (Boehringer Mannheim, Germany) in PBS for up to 10 min. Two probes used in the analysis included the chromosome 18q11 region-specific probe (Human Band Specific Probes, Research Genetics, U.S.A. and Canada) and the chromosome Xp11.2–4 region-specific probe (Human Band Specific Probes, Research Genetics). Double-target in situ hybridizations were performed according to protocols described by Krams et al. [13]. Briefly, DNA probes were labeled with digoxigenin-dUTP or biotin-dUTP (both Boehringer Mannheim) with nick translation (Gibco BRL, Karlsruhe, Germany). Labeled probes were precipitated with human Cot-1 DNA (Gibco BRL) and sonicated salmon sperm DNA (Sigma).

The DNA pellet was dissolved in 10 µl hybridization mixture containing 5 µl deionized formamide, 1×SSC, and 10% dextran sulphate. The specimens were denatured in 70% foramide/2×SSC for 4 min at 75°C and dehydrated in cold ethanol. The hybridization mixture with the probe was denatured for 5 min at 75°C and pre-annealed for 10 min at 37°C. Hybridization, probe detection, and counterstaining were performed as described by Iwasaki et al. [11]. Hybridization signals were visualized by an in situ imaging system (Isis, Carl Zeiss Vision, Oberkochen, Germany).

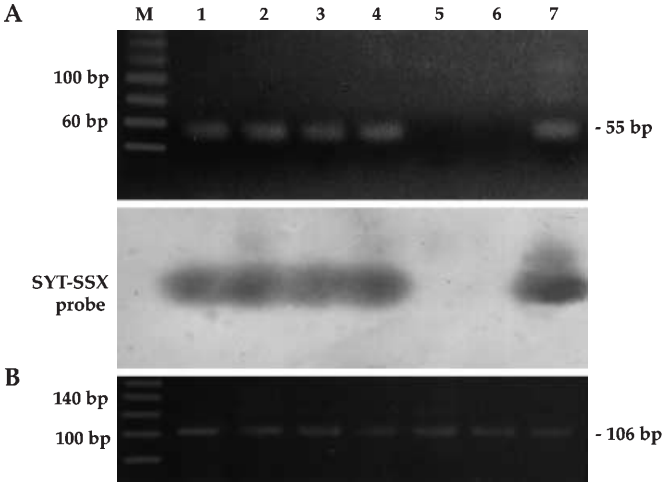


Fig. 3 RT-PCR amplification of (**A**, top) 55 bp SYT-SSX fusion gene transcripts and (**B**) 106 bp β-actin gene transcripts from formalin-fixed, paraffin-embedded microdissected samples. The specificity of the products was confirmed by transfer and hybridization with an oligonucleotide probe spanning the chimeric function, SYT-SSX (**A,** bottom). *M*, molecular weight marker. *Lane 1*, epithelial cell component from Case 1; *lane 2*, spindle cell component from Case 1; *lane 3*, epithelial cell component from Case 2; *lane 4*, spindle cell component from Case 2; *lane 5*, epithelial cell component from Case 3 (negative control); *lane 6*, spindle cell component from Case 3 (negative control); *lane 7*, positive control of synovial sarcoma

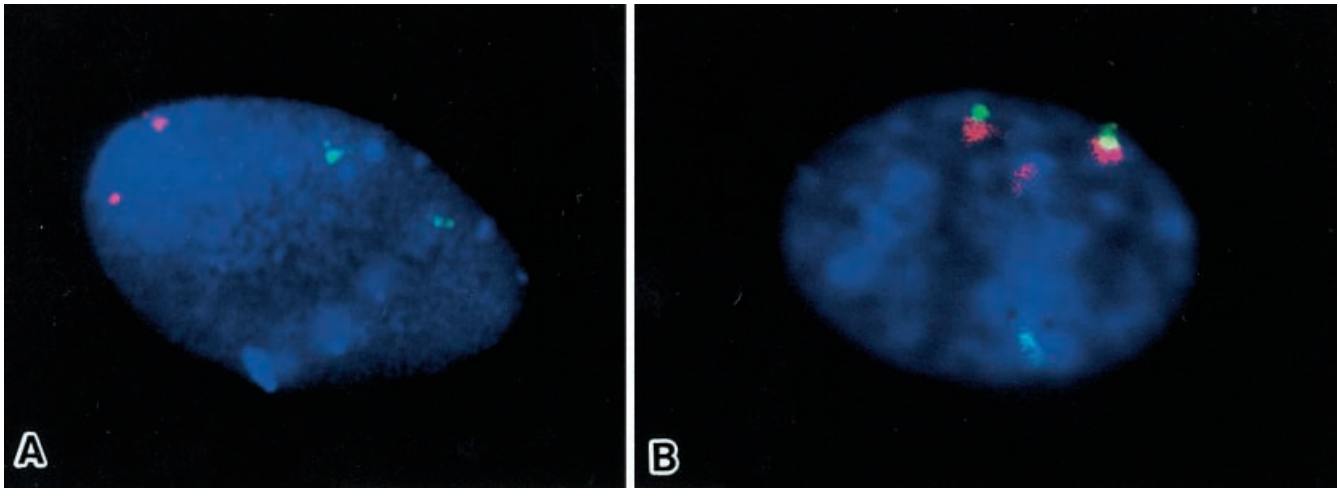
Fig. 4 Fluorescence in situ hybridization (FISH) analysis on isolated nuclei from a case of biphasic synovial sarcoma, Case 1, with human band specific probes for chromosome 18q11 (*green*) and chromosome Xp11.2–4 (*red*). **A** One normal nucleus showing two chromosome 18q11 region and two chromosome Xq11.2–4 region-specific probe signals that are separate from each other. **B** One tumor nucleus showing two areas with closely adjoining signals of chromosomes 18q11 and Xq11.2–4, corresponding to derivative chromosome 18 and derivative chromosome X. These findings confirmed the presence of the translocation t(X;18)

Results

Histologically, the two synovial sarcomas consisted of epithelial cells, resembling those of carcinoma arranged into pseudoglandular structures or sheets, and fibroblast-like spindle cells (Fig. 1). Immunohistochemically, epithelial cell components were positive for cytokeratin and EMA, while spindle cell components were positive for vimentin.

The precision for cutting out irregular lesions and specifically ablating undesired cells was probably the outstanding feature of microbeam MOMeNT (Fig. 2). In each sample, the test lesion was dissected accurately and immediately by the laser microbeam, followed by transfer of the dissectates to the PCR tube. The transfer process was enhanced by electrostatic attraction between the polyester supporter membrane and the transfer needle and PCR tube, respectively. The size of most dissectates was 0.5–1.0 mm and each of the obtained samples contained about 50–100 tumor cells.

Both the predicted 55-bp product of the SYT-SSX fusion transcript and the transcript of β-actin gene were identified in all four microdissected samples from each



of epithelial and spindle cell component of the two biphasic synovial sarcomas (Fig. 3 A, top panel and B). The specificity of the RT-PCR product was confirmed by hybridization with the SYT-SSX oligonucleotide (Fig. 3A, bottom panel), which spans the common junction point of both chimeric mRNAs. The adamantinoma of long bone showed no PCR product of the SYT-SSX fusion transcript, although the transcript of the β-actin gene could be detected (Fig. 3A, top panel and B). The results of analyses of synovial sarcomas and control are summarized in Table 1.

Interphase FISH on isolated nuclei demonstrated two areas with closely adjoining signals of chromosome 18q11 and chromosome Xq11.2–4, corresponding to derivative chromosome 18 and derivative chromosome X, in both samples. Figure 4 shows the FISH analysis of case 1, revealing one normal nucleus and one tumor nucleus.

Discussion

Nonrandom occurrence of t(X;18)(p11.2,q11.2) has been consistently found in synovial sarcoma. Clark et al. suggested that the formation of SYT-SSX fusion transcript represents the key event in tumor development [5]. Therefore, the SYT-SSX fusion transcript is considered as a highly sensitive diagnostic marker for synovial sarcoma and we previously reported a primary prostatic synovial sarcoma diagnosed by cytogenetic analysis [11].

The histogenesis of synovial sarcoma is unclear, although most investigators currently regard the cell of origin to be a primitive mesenchymal precursor stem cell, unrelated to synovial lining cell [4, 17,19]. On the other hand, other investigators have suggested that the histogenesis of biphasic synovial sarcoma involves epithelial differentiation of mesenchymal cells [7, 16,18].

Reporting the results of in situ hybridization analysis of a single case of biphasic synovial sarcoma using an antisense probe, Hiraga et al. suggested that SYT-SSX fusion gene was expressed in both cellular components [10]. Birdsall et al. used a two-color fluorescence in situ hybridization and demonstrated the presence of a specific genetic rearrangement in both cellular components of biphasic synovial sarcoma [1]. More recently, Kasai et al. reported that SYT-SSX fusion transcripts were detected in both cellular areas of biphasic synovial sarcoma using laser capture microdissection and RT-DOP-PCR-PCR [12]. In addition, they tried ordinary PCR and nested PCR, but failed to detect SYT-SSX fusion transcripts and porphobilinogen deaminase gene transcripts in the microdissected samples. We, on the other hand, were able to detect the SYT-SSX fusion gene transcripts and the β-actin gene transcripts in microdissected samples by the nested RT-PCR. The photographs in the former study (Kasai et al.) illustrated only dissection of the spindle cell component, while the separation of the epithelial cell component was unclear. In the present study, we clearly demonstrated the isolation of both epithelial and spindle cell components using the microbeam MOMeNT technique. The reliability of our results was further supported by data gained by means of FISH analysis.

Using the FISH technique on interphase nuclei, we could confirm the specific translocation t(X;18) in both biphasic synovial sarcomas.

Microbeam MOMeNT is a reliable method for the detection of tumor-specific alterations when using PCR assays and allows a rapid, selective, and low-contamination procurement of tumor cells from membrane-mounted tissue sections. Using this method, it is possible to accurately microdissect the tissue using a computer pointing device (e.g., mouse) and the procedure can be clearly seen on the monitor screen. Böhm et al. suggested that microbeam MOMeNT provides the flexibility and accuracy that may be critical in many irregular or infiltrated neoplastic lesions or in the procurement of specific cell clusters in development biology [2,3]. In the present study, we have demonstrated that efficient analysis of RNA from small tissue samples is feasible using the above procedure. To our knowledge, this is the first report of RNA analysis using formalin-fixed paraffin-embedded samples isolated by microbeam MOMeNT.

In conclusion, the microbeam MOMeNT is a useful method for isolating selected small portions from tissue sections. Our findings of the presence of the SYT-SSX fusion gene in both cellular components of biphasic synovial sarcoma confirmed that the synovial sarcoma is of monoclonal origin.

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