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Molecular analyses of cell origin and detection of circulating tumor cells in the peripheral blood in alveolar soft part sarcoma

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Abstract Alveolar soft part sarcoma (ASPS) is a distinct, rare soft tissue tumor with an unknown histogenesis and a tendency for late widespread metastases to lung, bone, and brain. It is now clear that they are caused by a specific unbalanced translocation, der(17)t(X;17)(p11;q25), which results in the formation of an *ASPSCR1−TFE3* (alias *ASPL−TFE3*) fusion gene;. The rearrangement results in the expression of chimeric transcripts, which can be identified by means of reverse transcriptase−polymerase chain reaction (RT-PCR). We investigated the histogenesis of ASPS and attempted to detect circulating ASPS tumor cells in peripheral blood. The immunohistochemical and genetic details of four cases and one cell line of ASPS were examined. An immunohistochemical analysis and RT-PCR did not detect myogenic differentiation gene *MYOD1*. The sensitivity of nested RT-PCR for detection of circulating ASPS cells was assessed by demonstrating that the tumor cell−associated gene translocation could be detected in 50 tumor cells/2 mL of blood. Clinically, it was detectable in a peripheral blood sample (2 mL) of ASPS patient with distant metastases. The findings suggest that ASPS is not of skeletal muscle origin. ASPS tumor cells in the peripheral blood could be monitored by RT-PCR.

1. Introduction

Alveolar soft part sarcoma (ASPS), so named by Christopherson et al. [1], is a rare soft tissue sarcoma with a characteristic microscopic appearance. The histogenesis of ASPS has not yet been determined. A myogenic origin was favored initially [2−8], although there are conflicting data on the immunohistochemical demonstration of muscle-associated markers [9−11]. These disparities in the data derive pathological studies and molecular genetic studies, which are associated with various technical problems, such as the use of different processing methods of ASPS tissue, contamination of skeletal muscles in tumor samples, and the selection of immunohistochemical methods. Recently, ASPS tumors have been characterized by a specific unbalanced translocation, der(17)t(X;17)(p11;q25), which results in the formation of an *ASPSCR1−TFE3* fusion gene (alias *ASPL−TFE3*) [12].

Four fresh-frozen tumor samples of ASPS, one cell line, and a xenografted tumor in SCID mouse were collected and analyzed. In addition, reverse transcriptase−polymerase chain reaction (RT-PCR) for the *ASPSCR1−TFE3* was used to detect any circulating ASPS tumor cells in peripheral blood.

2. Materials and methods

2.1. Tumor samples

Patient characteristics and clinical features are given in Table 1. In all cases, fresh tissue samples were obtained from the tumor tissue specimens during surgery, and the tissue was then immediately frozen at −80°C for the following RNA extraction.

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Table 1
Clinical data for four patients with alveolar soft part sarcoma

Case	Sex	Age, yr	Location	Metastasis
1	M	12	thigh	lung
2	F	21	calf	lung, bone
3	F	22	buttock	lung, bone
4	F	75	foot	lung, bone

2.2. Immunohistochemistry

Each formalin-fixed and paraffin-embedded specimen (i.e., the resected specimens and xenograft of ASPS-KY cells) was stained with hematoxylin and eosin. The immunoperoxidase methodology with a simple stain MAX PRO system (Nichirei, Tokyo, Japan) was used. The immunohistochemical reagents used were as follows: desmin D33, myogenin F5D, MyoD1 5.8A, HHF35, EMA E29, vimentin V9, SMA 1A4, CD31 JC70A, NSE BBS/NC/VEH14, and F VIII and S-100 rabbit polyclonal from DAKO (Carpinteria, CA); CD34 QBEed-10 from Immunotech France (Marseille, France); and TFE3 rabbit polyclonal from Santa Cruz Biotechnology (Santa Cruz, CA).

2.3. Cell culture, cytogenetic analysis, and spectral karyotyping

The ASPS-KY cells used in this study were obtained from Yokohama City University and were passaged [13] and cultured in RPMI-1640 medium (GIBCO BRL, Gaithersburg, MD) supplemented with 10% fetal calf serum (M. A. Bioproducts, Walkersville, MD) and 200 μg/mL of kanamycin sulfate (Meiji Seika, Tokyo, Japan). The cultures were maintained at 37 °C in a humidified incubator with a constant flow of 5% CO_2 in air. The medium was renewed every 5−7 days (Fig. 1). Cytogenetic analysis was performed using the standard trypsin−Giemsa banding technique. Additional slides were made for spectral karyotyping (SKY) analysis according to the manufacturer's protocol (Applied Spectral Imaging [ASI], Migdal Ha'Emek, Israel). The SKY probe was a mixture of whole-chromosome paint probes for each chromosome, combinatorially labeled with five fluorochromes. Briefly, the probe was denatured, preannealed with Cot-1 DNA for 1 hour, hybridized with the separately denatured chromosomes for 48 hours, washed, and detected according to the ASI protocol.

2.4. Tumor implantation in SCID mice

ASPS-KY cells (5×10^6), obtained from the cultures after 50 passages, were suspended in RPMI 1640 and injected subcutaneously into the backs of six 2-week-old female athymic SCID mice (CB-17/Icr scid; Jcl CLEA Japan, Osaka, Japan).

2.5. RT-PCR

Total RNA was extracted from the tumors and cultured ASPS-KY cells using the guanidium thiocyanate−phenol−chloroform extraction method [14]. RNA (1.0 μg) was converted into cDNA by reverse transcription using Moloney murine leukemia virus (MMLV) reverse transcriptase (GIBCO-BRL) and the oligo(dT)15 primers (Promega, Madison, WI). The oligonucleotide primers have been previously reported [12], and inner primers for *ASPL* and *TFE3* were designed in this laboratory (Table 2).

Blood samples (2 mL) were collected on EDTA at room temperature from case 1 and from a disease-free volunteer. Ten thousand ASPS-KY cells were added to the disease-free volunteer blood and a serial dilution of the tumor cells was made. Each dilution sample contained 10,000, 1,000, 100, 50, 10, or 0 ASPS-KY cells per 2 mL blood. They were diluted in 8 mL of ice-cold red blood cell lysis buffer (155 mmol/L NH_4Cl, 10 mmol/L $KHCO_3$, 1 mmol/L EDTA, pH 7.4 [15]) and the total RNA was extracted. The RNA (1.0 μg) was converted into cDNA by reverse transcription using MMLV reverse transcriptase (GIBCO-BRL) and oligo(dT)15 primers (Promega, Madison, WI). Nested PCR was used to amplify the *ASPSCR1−TFE3* fusion transcripts, as previously described [16]. The amplified products were separated by electrophoresis on a 1.5% agarose gel containing ethidium bromide and photographed under ultraviolet light.

3. Results

3.1. Immunohistochemical study of four patients

Nuclear staining of TFE3 was found in all specimens. Desmin was partly positive in two cases, but myogenin, MyoD1 and HHF35 were negative in all patients. Vimentin

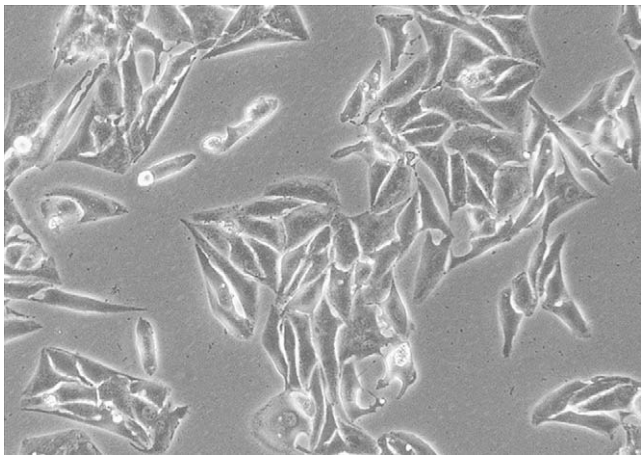

Fig. 1. Phase-contrast micrograph (200 ×) of alveolar soft part sarcoma cell line ASPS-KY. The cultured cells were composed of spindle-shaped mononuclear forms.

Table 2
Primers for reverse transcriptase−polymerase chain reaction

	Forward	Reverse
ASPSCR1−TFE3[a]	5′-AAAGAAGTCCAAGTCGGGCCA-3′	5′-CGTTTGATGTTGGGCAGCTCA-3′
inner	5′-CCAAGTCGGGCCAGGA-3′	5′-CCACGCCTTGACTACTGT-3′
desmin	5′-ACACCTGCGAGATTGACG-3′	5′-TCAATCTCCTGCTCC-3′
myogenin	5′-GTGTAAGAGGAAGTC-3′	5′-CATGGATGAGGAAGGGGAT-3′
MyoD1	5′-AGCACTACAGCGGCGACT-3′	5′-GCGACTCAGAAGGCACGTC-3′

[a] Fusion gene alias *ASPL−TFE3*.

was positive for all cases, and NSE was partly positive in three cases. All other reagents were negative in all cases.

3.2. Cytogenetic findings of ASPS-KY

According to a cytogenetic analysis of five samples, a section of the X chromosome was added to chromosome 17 (Fig. 2). The tumor karyotype was identified as 44,XX, −4, −10,ider(17)(q10)t(X;17)(p11;q25).

3.3. Tumor formation in SCID mouse

ASPS-KY cells were successfully formed transplanted tumors in all three SCID mice examined. Small nodules were palpable at 2 weeks after inoculation, and the tumors eventually grew to 2 cm in diameter. The tumors were removed and immersed in 4% paraformaldehyde in 0.1 mol/L phosphate buffer for 5−7 days. Transplanted ASPS-KY cell tumors were characterized by uniform, organoid nests of polygonal tumor cells, separated by a fibrovascular pattern. Although fine periodic acid-Schiff−positive granules were found in some tumor cells, no crystal-like structures were found in the tumor. Immunohistochemically, the transplanted tumor cells possessed the characteristics of ASPS, including positive staining for vimentin, NSE, and TFE3 (Figs. 3, 4).

3.4. RT-PCR

The genetic analysis from four patients and the ASPS-KY cells showed that they were all positive for *ASPSCR1−TFE3*. RT-PCR confirmed the presence of the disease-specific fusion product in the RNA, correlating to the diagnosis in all cases. Desmin was positive in two cases and in the ASPS-KY cells. In case 2, myogenin was positive but MyoD1 could not be detected (Table 3).

The RT-PCR protocol designed for detection of *ASPSCR1−TFE3* in the peripheral blood was determined using a dilution series of ASPS-KY cells. The sensitivity level was 50 free tumor cells/2 mL peripheral blood (approximate nucleated cell content, 1×10^7) (Fig. 5). When we applied one round of RT-PCR, the sensitivity level was 1,000 free tumor cells/2 mL peripheral blood. All peripheral blood samples from 10 healthy volunteers were negative for *ASPSCR1−TFE3*. The identification of tumor cells from peripheral blood of the patient who had metastasis at presentation was confirmed (Fig. 6).

4. Discussion

Although desmin expression has been reported in ~50% of cases of ASPS, desmin expression is not limited to myogenic tumors and is observed in a wide variety of mesenchymal tumors [17]. There have been inconsistent

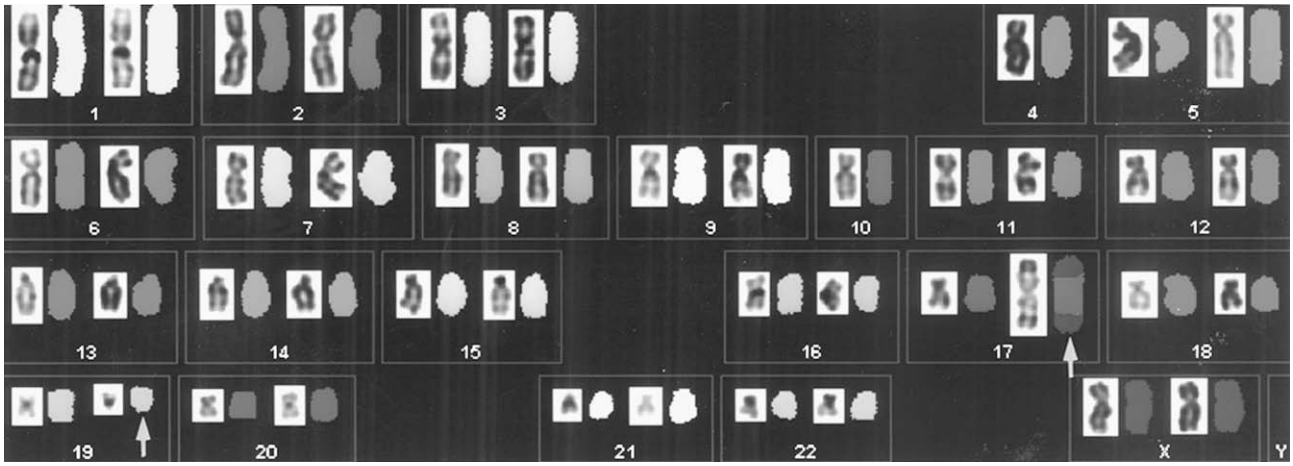

Fig. 2. Representative spectral karyotyping karyogram of the ASPS-KY cell line. The tumor karyotype was finally identified as 44,XX,−4,−10, ider(17)(q10)t(X;17)(p11;q25).

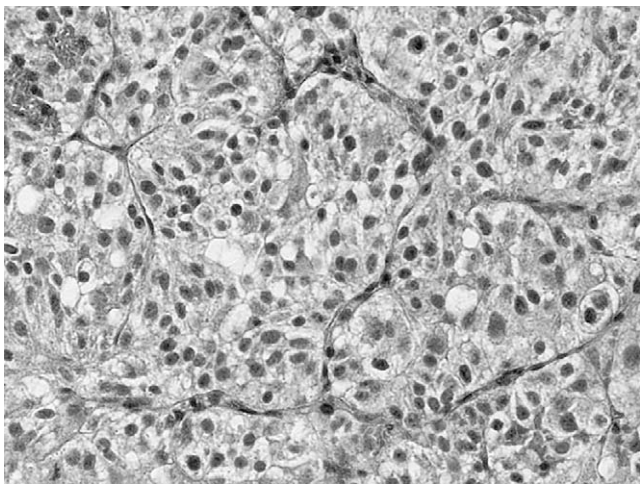

Fig. 3. Hematoxylin and eosin-stained section of the tumor implantation in SCID mouse shows the appearance of alveolar soft part sarcoma, composed of undefined cell borders, arranged in cohesive nests (200 ×).

reports of the detection of nuclear MyoD1 in ASPS and many reports have shown positive cytoplasmic staining of MyoD1. This finding has been suggested to be due cross-reactivity with an undetermined cytoplasmic antigen. Subsequent studies have usually yielded negative results [9–11,18–22]. Nakano et al. [6] reported that expression of myogenic mRNA is found in some cases of ASPS. However, the positive expression of myogenic mRNA including MyoD1 and myogenin, may represent a contamination of skeletal muscle cells in ASPS tumor tissue.

To our knowledge, there have been no previous established ASPS cell lines apart from ASPS-KY. Histological characteristics of ASPS-KY were previously reported in the Japanese literature [13], and the present article is the first English-language report characterizing the ASPS-KY cell line, including molecular analyses. According to both cytogenetic and immunohistochemistry findings from the transplanted SCID mouse and cell culture, ASPS-KY cells possessed the typical characteristics of ASPS. ASPS-KY cells, which have a specific fusion gene (*ASPSCR1–TFE3*), expressed mRNA of desmin, but not myogenin or MyoD1. The results of RT-PCR in ASPS-KY cells that had no contaminating tissue indicate that ASPS is not of skeletal muscle origin.

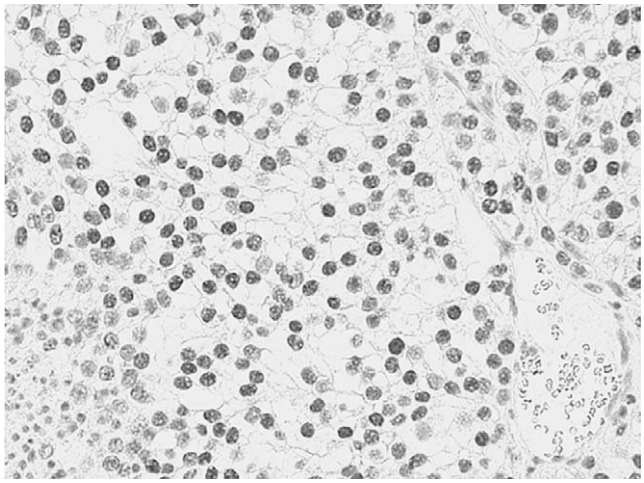

Fig. 4. Immunohistochemical demonstration of nuclear *TFE3* expression of xenografted tumor in SCID mouse (200 ×).

Table 3
Expression of mRNA of four tumor samples and ASPL-KY cells

	Case 1	Case 2	Case 3	Case 4	ASPS-KY cell line
ASPSCR1–TFE3[a]	type 1	type 1	type 2	type 2	type 1
desmin	−	+	−	+	+
myogenin	−	+	−	+	−
MyoD1	—	−	−	−	—

[a] Fusion gene alias *ASPL–TFE3*.

The ASPSCR1–TFE3 fusion protein localizes to the nucleus and can function as an aberrant transcription factor. Nuclear immunoreactivity of TFE3 is highly specific and sensitive for ASPS among sarcomas (sensitivity, 97.5%; specificity, 99.6%; Argani et al. [23]). In the present study, diffuse nuclear immunoreactivity for TFE3 could be observed in all four tumor samples, the ASPS-KY cells, and the SCID mouse. The detection of specific chromosomal translocations of sarcomas in peripheral blood by RT-PCR has been reported in Ewing sarcoma– primitive neuroectodermal tumor (EWS–FLI1, EWS–ERG) [24,25]; myxoid liposarcoma (FUS–CHOP, EWS–CHOP) [26]; alveolar rhabdomyosarcoma (PAX3FKHR) [27]; and synovial sarcoma (SYT–SSX) [28].

Because *ASPSCR1–TFE3* was detected in ASPS-KY cells, the sensitivity of RT-PCR for this fusion gene was used to find circulating tumor cells in the peripheral blood (2 mL) in patients with ASPS. The nested RT-PCR could detect 50 cells in 2 mL peripheral blood. In peripheral blood from 10 healthy volunteers, no transcription of *ASPSCR1–TFE3* was detected. A specific transcript was found in the peripheral blood of one patient with metastatic ASPS. In malignant diseases with specific translocations in which the genes involved have been cloned, RT-PCR is a sensitive, reliable and rapid assay for diagnosis and accurate staging of the disease. Furthermore, when applied to peripheral blood, it can be used for monitoring the response

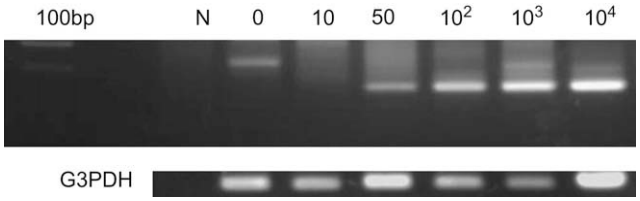

Fig. 5. Sensitivity of nested polymerase chain reaction for *ASPSCR1–TFE3* (alias *ASPL–TFE3*) fusion gene in ASPS-KY, as cells per 2 mL blood. Lane N: no cells in 2 mL blood of volunteer.

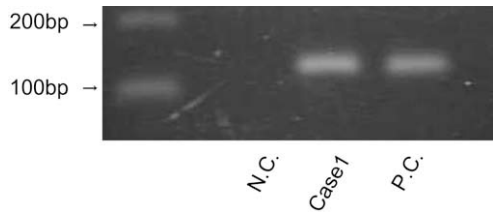

Fig. 6. Nested PCR detection of circulating ASPS cells in case 1. P.C., positive control (isolated from the cell line ASPS-KY); N.C., negative control (water).

to treatment, to detect minimal residual disease, and for the early diagnosis of metastasis in patients with ASPS.

The dissemination of tumor cells in peripheral blood may be common in ASPS. The tumor is richly vascular, causing pulsation or a distinctly audible bruit in some instances [29]. A massive hemorrhage often occurs during an open biopsy. The detection of micrometastasis in peripheral blood using RT-PCR may be a reliable and convenient assay to diagnose ASPS. Therefore, a nested PCR assay of the peripheral blood could provide very important information for the diagnosis and management of ASPS. Studies of patients with various tumor stages and treatments may reveal the clinical efficacy of monitoring tumor cells in peripheral blood. Further studies should try to elucidate the therapeutic and prognostic implications of micrometastases detected by RT-PCR.

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