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Establishment of an ASPL-TFE3 renal cell carcinoma cell line (S-TFE)

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Abbereviations: TFE3, transcription factor E3; RT-PCR, reverse-transcriptase polymerase chain reaction; FISH, fluorescence in situ hybridization; ASPS, alveolar soft part sarcoma

Xp11 translocation renal cell carcinoma is a rare disease diagnosed in children and adolescents in the advanced stage with an aggressive clinical course. Various gene fusions including the transcription factor E3 (TFE3) gene located on chromosome X cause the tumor. We established an Xp11 translocation renal cell carcinoma cell line from a renal tumor in a 18-y-old Japanese female and named it "S-TFE." The cell line and its xenograft demonstrated definite gene fusion including TFE3. They showed strong nuclear staining for TFE3 in immunohistochemistry, TFE3 gene rearrangement in dual-color, break-apart FISH analysis and ASPL-TFE3 type 1 fusion transcripts detected by RT-PCR and direct DNA sequencing. Although many renal cell carcinoma cell lines have been established and investigated, only a few cell lines are recognized as Xp11.2 translocation carcinoma. S-TFE will be useful to examine the characteristics and drug susceptibility of Xp11 translocation renal cell carcinoma.

Introduction

Xp11.2 translocation carcinoma has been recently recognized as a subtype of renal cell carcinoma primarily described in the 2004 World Health Organization classification of renal tumors.[1] Although this neoplasm is rare since the incidence is estimated to 1.5% of all renal tumors,[2] it is frequently observed in children and adolescents and was reported to account for 20–54% of renal tumors in children.[3,4] This neoplasm has a more aggressive clinical course than other subtypes of renal cell carcinoma;[5] however, little is known about the tumor characteristics.

Xp11.2 translocation carcinoma is caused by fusions between various genes and the transcription factor E3 (TFE3) gene located on the short arm of chromosome X. Several gene partners such as ASPL, PRCC, PSF, NONO and CLTC are reported to be reciprocal translocations.[6] ASPL-TFE3, t(X;17)(p11;q25) and PRCC-TFE3, t(X;1)(p11;q21) are the most common gene fusions. As a result of these translocations, the expression of TFE3 fusion pritein increases in the nuclei of tumor cells.

Morphologically, Xp11.2 translocation carcinoma resembles clear cell renal cell carcinoma, which typically presents as tan-yellow with various degrees of necrosis and hemorrhage. Although Xp11.2 translocation carcinoma usually has distinct microscopic findings such as papillary architecture composed of voluminous clear cells with psammoma bodies, this neoplasm often presents a nested or alveolar pattern with granular eosinophilic cells.[1] Therefore, on routine hematoxylin and eosin staining, these neoplasms may be misdiagnosed as conventional clear cell or papillary renal cell carcinoma in adult cases.[7] In general, the diagnosis of Xp11.2 translocation carcinoma can be confirmed by immunohistochemistry using antibodies against TFE3. The nuclear reactivity for TFE3 at low-power magnification under a microscope is specific to Xp11.2 translocation carcinomas.[8] In addition, molecular and cytogenetic methods such as reverse-transcriptase polymerase chain reaction (RT-PCR), karyotype analysis and fluorescence in situ hybridization (FISH) provide a reliable histological diagnosis.

In this study, we report the cytogenetic and biological characteristics of a renal cell carcinoma cell line with TFE3 gene fusion established from a young female with locally advanced renal cell carcinoma.

Results

Establishment of the "S-TFE" cell line and in vitro growth. For the first 6 mo from the primary culture, we observed a mixture of fibroblast-like cells and epithelial cells. In the subsequent culture, the proportion of the fibroblast-like cells became less prominent and the epithelial cells became dominant. The cells after passage 10 showed stable epithelial growth (**Fig. 1**). The cell line was named "S-TFE" with subcultures continued up to 80th passage. **Figure 2A** shows the growth curve of the cell line at 25th passage. The population doubling time obtained from the exponential phase of growth was 98.2 h.

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Tumorigenicity in nude mice. The xenograft tumors grew slowly but steadily. When the tumors reached a diameter of larger than 2.0 cm, the nude mice were euthanized. No metastases were macroscopically found in other organs. On the growth curve of the xenograft, the doubling time was estimated to be 24.2 d (**Fig. 2B**). The xenografts were frozen for preservation and fixed in 10% buffered formalin for cytogenetic and pathological examinations.

Immunohistochemical findings for primary and xenograft tumors. Macroscopically, the primary tumor resembled conventional clear cell renal cell carcinoma, having a tan-yellow color with hemorrhage. Histopathological examination revealed alveolar and nested architecture composed of abundant clear and eosinophilic cells with prominent nucleoli (**Fig. 3A**). There were few psammoma bodies or hyaline nodules. A close similarity in cellular morphology was observed in the xenograft (**Fig. 3B**), which had large nucleoli with pleomorphism. Immunohistochemical features of the primary tumor and xenograft were also similar, showing strong nuclear labeling for TFE3 (**Fig. 3C**). Immunoreactivities for AMACR and CD10 were diffusely positive; in contrast, these tumors underexpressed vimentin and epithelial immunohistochemical markers such as cytokeratin (**Table**).

Interphase FISH analysis. In the interphase FISH assay, there were several pairs of green and orange signals in each cell and 44% of primary tumor cells had split signals indicating TFE3 gene arrangement on the X chromosome (**Fig. 3D**). The same assay of xenografts showed a similar degree of split signals.

G-band karyotype. Chromosomal analysis of both the primary culture and cell line at passage 29 did not show common clonal karyotype anomalies. The modal chromosomal number and the karyotype of the primary tumor were 37 and XX, add(1)(q21), –1, del(2)(q33), add(3)(q11), –3, –4, +5, +6, –7, add(8)(p21), +9, add(9)(p22) × 2, add(10)(p13), del(11)(q13), +i(12)(q10), –13, –14,–14, –15, –15, –16, –16, –18, add(19)(p13),–20, –20, –21, –22,–22, +4mar. Those of the cell line at passage 29 were 50–62, X, –X, add(1)(p22), +add(1)(p22), del(2)(q33), der(3;?6)(p10;p10), der(3;14)(p10;q10), –4, add(4)(p11), +5, +6, ?add(9)(p22), +11, i(12)(q10), –13, –13, add(15)(p11), –16, –17, –18, –19, +20, –21,–22, –22, +15-21mar, inc[cp8].

FISH mapping. In the cell line at passage 10, there were seven chromosomes with fluorescence signals (**Fig. 4A**). Three of them showed normal patterns with innate signals (green and aqua on a pair of X chromosomes, Cy3 and red on one chromosome 17, green and yellow arrowheads, respectively). On the other hand, two pairs of derived chromosomes were detected. A pair of longer chromosomes (white arrows) had adjacent Cy3 and aqua signals (centromeric site of ASPL and telomeric site of TFE3, **Fig. 4B**). Thus, the FISH mapping elucidated the hybridization signals of ASPL and TFE3, indicating gene fusions of ASPL-TFE3. In contrast, because another pair of shorter

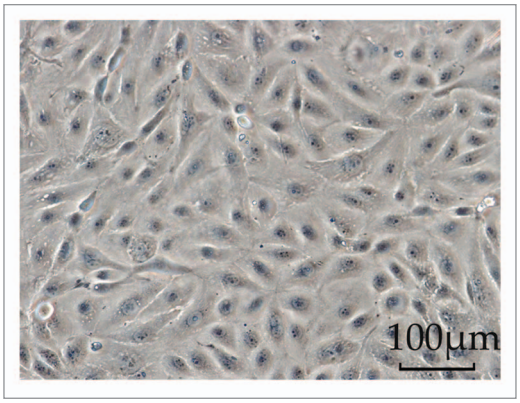

Figure 1. Morphological features of S-TFE in monolayer culture at passage 20. Tumor cells have a polygonal appearance (phase-contrast light micrograph, scale bar shows 100 μm).

derived chromosomes (red arrows) showed several signals having various intensities, it was unable to identify specific translocation patterns.

Multicolor FISH analysis. In multicolor FISH analysis of the cell line, one pair of chromosomes showed more complex structural abnormalities, including orange signals of TFE3 telomeric probes (white arrows, **Fig. 5A**). Although there were two complete X chromosomes with normal TFE3 green and orange signals (yellow arrowheads), two other X chromosomes showed only TFE3 centromeric green signals, lacking TFE3 telomeric orange signals (green arrowheads). Derived chromosomes containing TFE3 telomeric signals had centromeres of chromosome 9 and parts of chromosomes 3 and 10 (**Fig. 5B**). Although signals indicating chromosome 17 were not detected on multicolor FISH analysis, considering the results of FISH mapping and DNA sequences described later, it was highly likely that a very short region of chromosome 17 (ASPL) was located between the telomeric site of TFE3 and part of chromosome 10.

RT-PCR analysis. Although no transcript of PRCC-TFE3 were observed in the primary tumor tissue and cell line, fusion

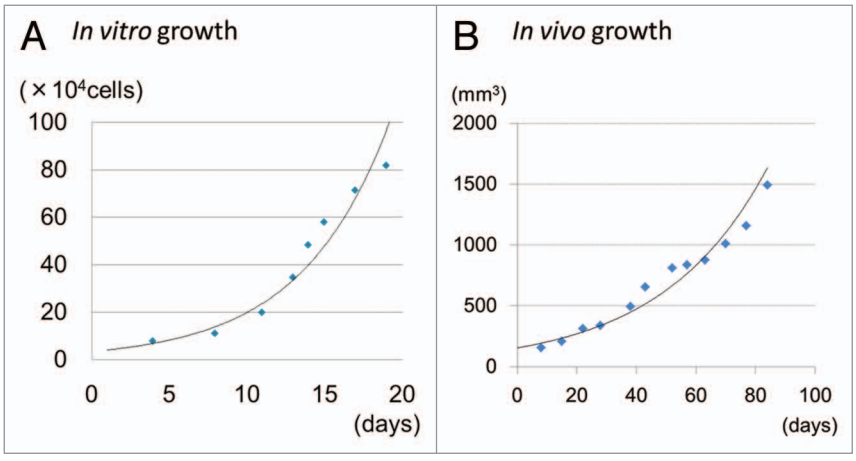

Figure 2. The growth curve of cell line S-TFE at passage 25 (**A**) and the xenograft (**B**). The population doubling times obtained from the exponential phase of growth were 98.2 h and 24.2 d in vitro and in vivo, respectively.

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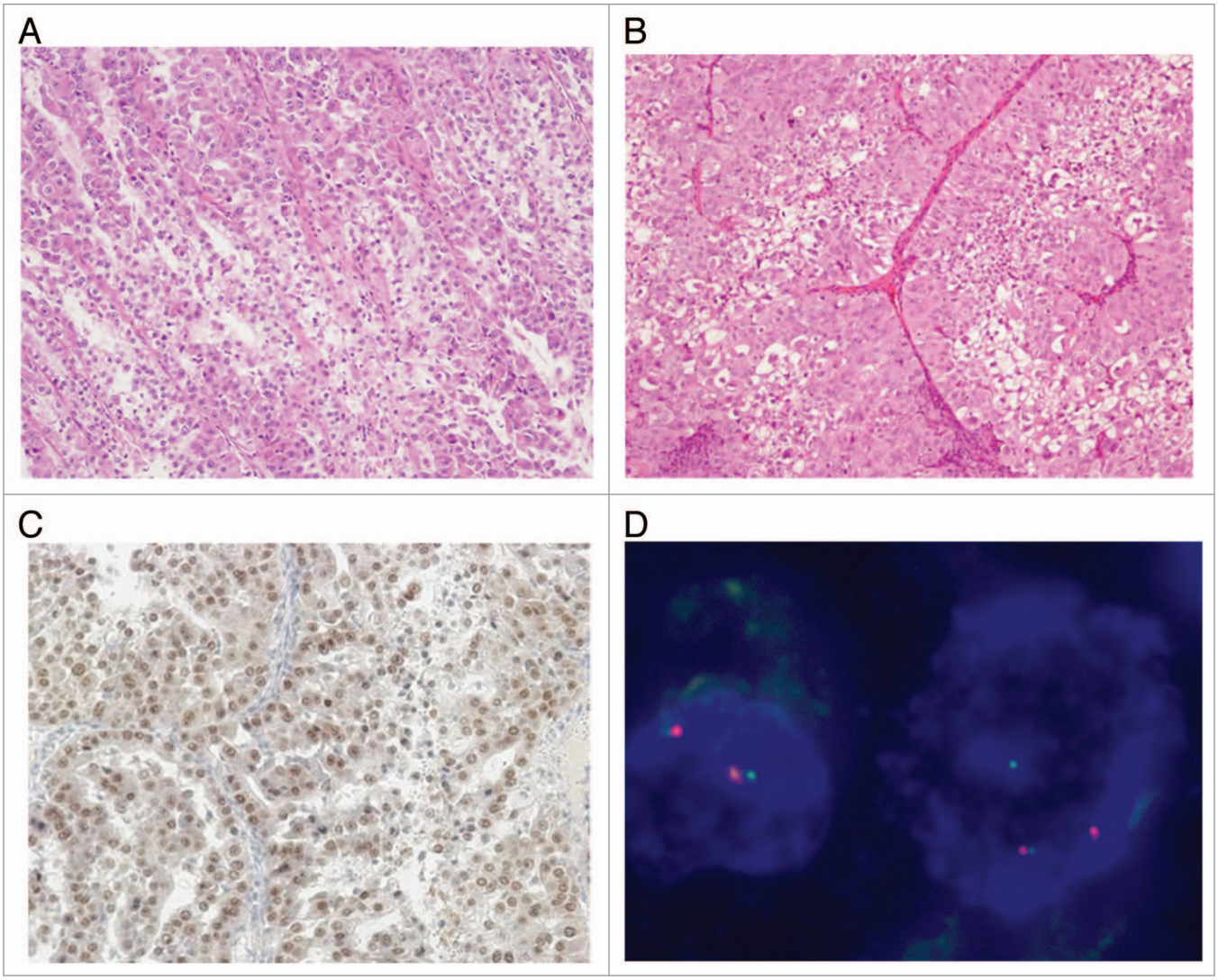

Figure 3. Microscopic features of the primary tumor and xenograft. (**A**) Histopathological features of the primary tumor (HE staining, magnification ×200). (**B**) Immunohistochemistry for TFE3 in the primary tumor shows strong nuclear staining (magnification ×200). (**C**) Interphase FISH analysis of the primary tumor with a dual-color, break-apart DNA probe. The TFE3 probes were labeled green at the centromeric site and orange at the telomeric site. Split signals are observed (arrows).

transcripts of ASPL-TFE3 were detected as a single band of approximately 200 bp in both the primary tumor and S-TFE cell line (**Fig. 6A**). These transcripts were shorter than those of the FU-UR-1 cell line with ASPL-TFE3 type 2 fusions.[9] No fusion transcripts were observed in ACHN or Caki-2.

DNA sequencing. PCR products using ASPL-TFE3 primer sets were purified for sequencing, and all nucleotide sequences and the sites of fusion were identified (**Fig. 6B**). The fusion transcripts of the primary tumor and S-TFE cell line had 195 bp, indicating ASPL-TFE3 type 1 fusion.

Discussion

In the present study, we established a new renal cell carcinoma cell line named S-TFE. Histologically, the primary tumor showed strong nuclear immunostaining for TFE3. FISH mapping and multicolor FISH analyses revealed more complicated TFE3 gene rearrangement than expected. Furthermore, the ASPL-TFE3 type 1 fusion gene was detected by RT-PCR and direct DNA sequencing.

Identification of specific genetic alterations in certain malignancies such as lymphoma and sarcoma is helpful not only for diagnosis but also for better treatment. TFE3 gene fusions have been implicated in alveolar soft part sarcoma (ASPS) in addition to Xp11.2 translocation renal cell carcinoma. ASPS is a rare tumor mostly arising in the extremities or the head and neck regions of adolescents or young adults. Both neoplasms have gene fusions between X (TFE3) and chromosome 17 (ASPL). The other gene fusion partners confirmed in Xp11 translocation carcinoma are PRCC, PSF, NONO and CLTC, situated on chromosomes 1q21, 1p34, Xq12 and 17q23, respectively.[6] TFE3 is a member of the microphthalmia transcription factor (MiTF) family, which

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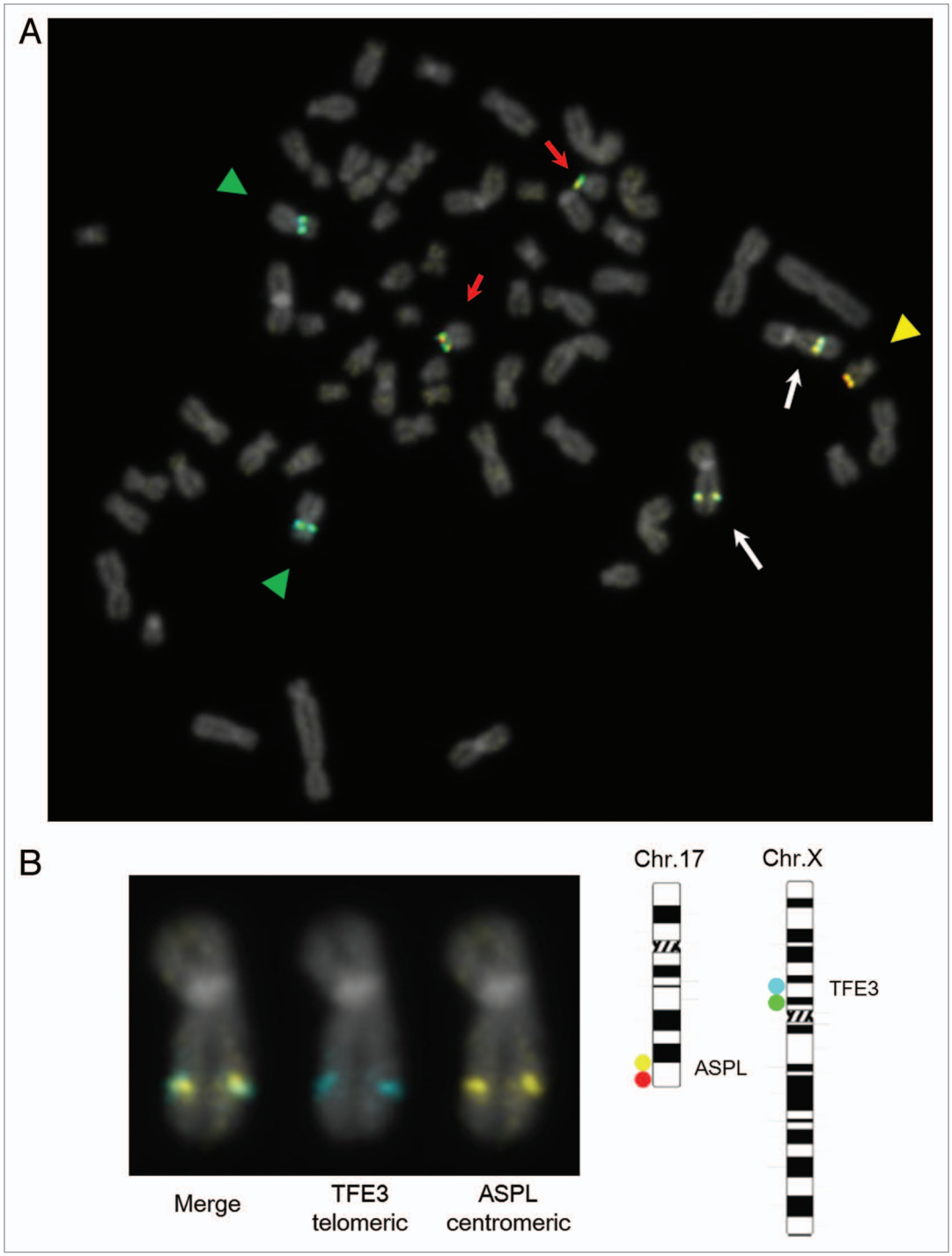

Figure 4. FISH mapping. (**A**) FISH analysis of S-TFE cell line using 4-color signals; There were seven chromosomes with fluorescence signals. Two pairs of derived chromosomes were detected (white arrows and red arrows). The other three chromosomes had normal patterns with innate green and aqua signals on chromosome X (green arrowheads) and Cy3 (yellow) and red signals on chromosome 17 (yellow arrowheads). (**B**) Fusion pattern of derived chromosome. Of these derived chromosomes, the longer ones [white arrows in (**A**)] had adjacent signals of the telomeric site of TFE3 (aqua) and centromeric site of ASPL (Cy3, yellow signals). The merge image shows the gene fusion signals of ASPL and TFE3. Because there was another pair of derived chromosomes [(red arrows in (**A**)] having various degrees of 4-color signals, specific translocation patterns were not determined.

includes transcription factor EB (TFEB), transcription factor EC (TFEC) and MiTF. These members code for the basic helix-loop-helix leucine-zipper (BHLH-LZ) transcription factor that binds DNA as homodimers or heterodimers.[10] They have important roles in appropriate development and survival of retinal pigment epithelial cells, osteoclasts and melanocytes. Although another subtype of translocation renal cell carcinoma showing t(6; 11) (p21; q12) involving TFEB has been reported,[11] it shares clinical,

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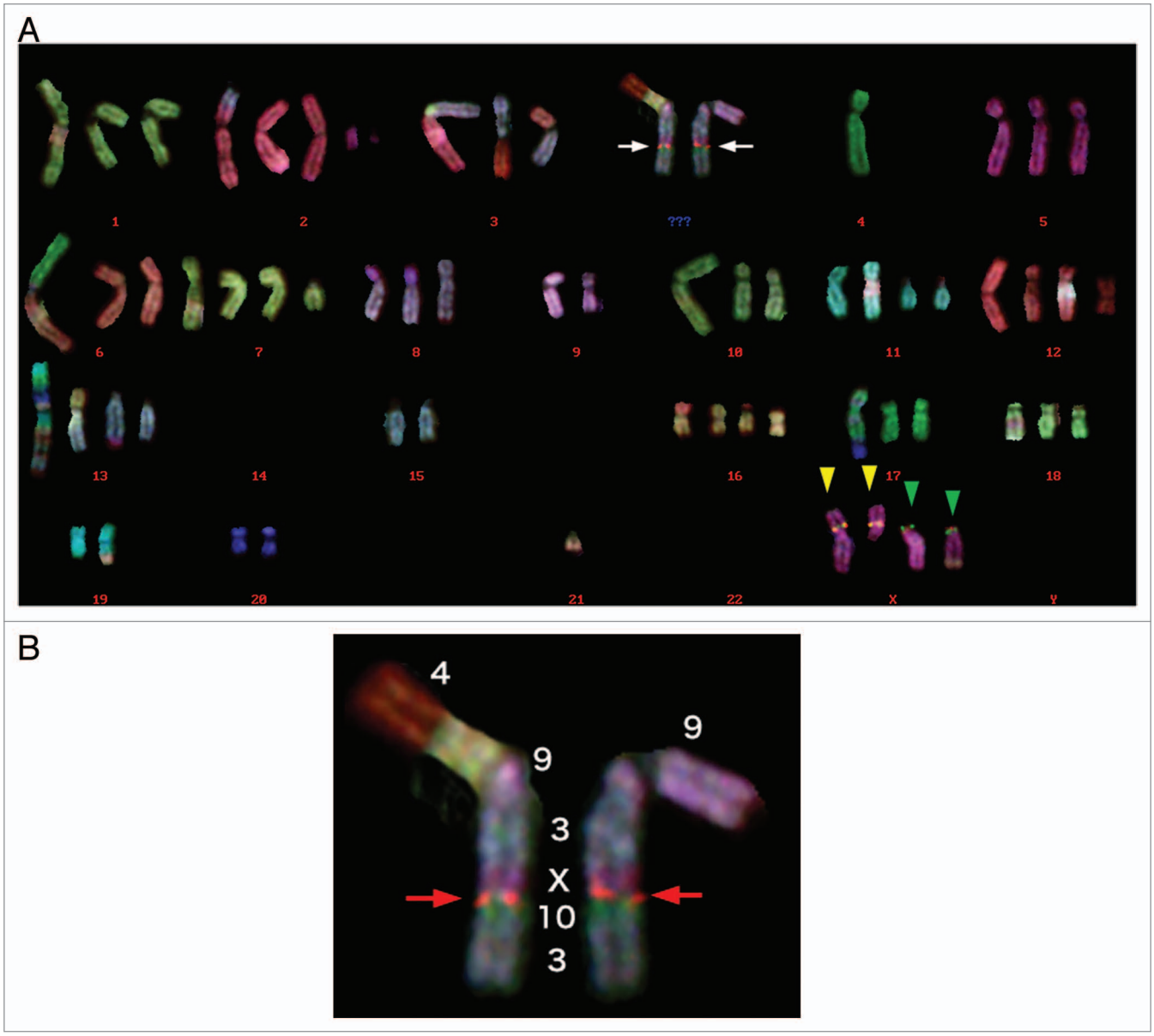

Figure 5. Multicolor FISH analysis of S-TFE cell line. (**A**) Multicolor FISH analysis was performed using the Multicolor FISH-Human probe set and a dual-color, break-apart TFE3 probe. There was a pair of chromosomes with a complex structural abnormality containing TFE3 telomeric orange signals (white arrows). Although two X chromosomes show normal TFE3 signals (yellow arrowheads), the other two X chromosomes show only TFE3 centromeric green signals, lacking TFE3 telomeric orange signals (green arrowheads). (**B**) Derived chromosomes containing TFE3 telomeric orange signals (red arrows) had centromeres of chromosome 9 and parts of chromosome 3 and 10.

pathological and molecular features with Xp11.2 translocation renal cell carcinoma. Argani et al.[12] have proposed regrouping these neoplasms as "MiTF/TFE translocation carcinomas."

Xp11.2 translocation carcinoma has distinctive pathological findings with mixed papillary and nested/alveolar architecture composed of cells with clear and/or eosinophilic voluminous cytoplasm, and occasionally there are hyaline nodules and psammoma bodies.[1] However, since Xp11.2 translocation carcinomas are morphologically heterogeneous and sometimes difficult to distinguish from other types of renal cell carcinoma, many cases may be misdiagnosed as other subtypes of renal cell carcinoma such as the clear cell or papillary type. In addition, a patient presenting with multilocular cystic renal cell carcinoma-like features was reported.[13] The most definitive immunohistochemical finding of Xp11.2 translocation carcinoma is strong nuclear staining for TFE3 protein, which is absent in normal tissue and other subtypes of renal cell carcinoma. This antibody recognizes the C-terminal portion of the TFE3 protein.[8] Native TFE3 is ubiquitously expressed in normal tissue but its expression cannot be detected by TFE3 immunostaining. Although Argani et al. previously reported that TFE3 immunostaining was a superior diagnostic tool with high specificity and sensitivity,[8] it is also noted that the immunohistochemistry may have problems with its difficulty to assess TFE3 nuclear immunoreactivity. This may

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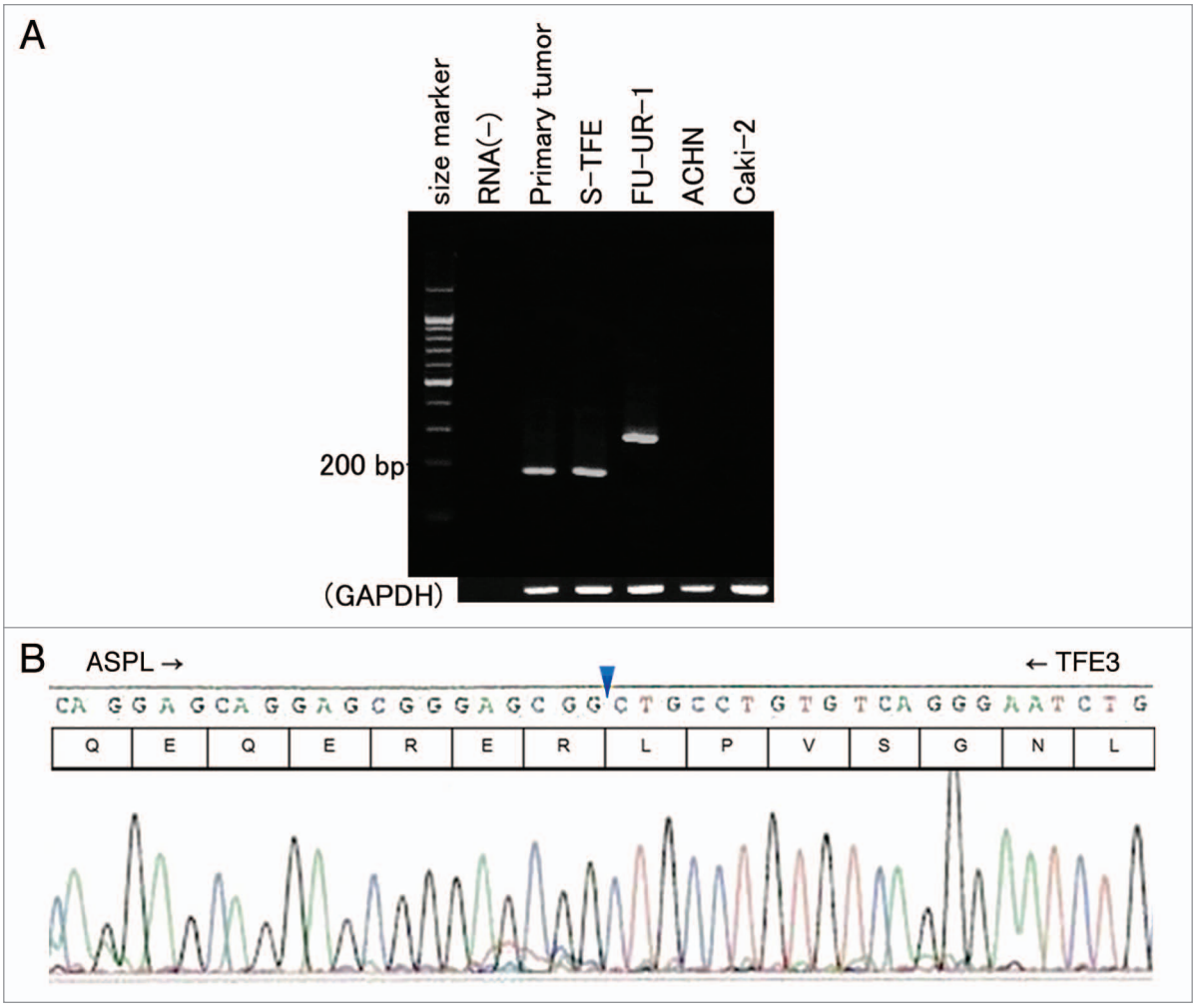

Figure 6. RT-PCR and DNA sequences. (**A**) Detection of the ASPL-TFE3 fusion transcript by RT-PCR. Approximately 200 bp chimeric transcripts were observed in the primary tumor and S-TFE cell line. FU-UR-1, a cell line already demonstrated to have ASPL-TFE3 type 2 fusions. ACHN and Caki-2, used as negative controls, are frequently used cell lines without TFE3 gene fusions. (**B**) A part of the nucleotide sequence from the ASPL-TFE3 fusion transcripts; arrowhead indicates the fusion point between ASPL and TFE3.

be because the sensitivity and specificity for detection of TFE3 gene fusions depend on the methodology of TFE3 immunohistochemistry.[14] Thus, additional examinations are mandatory for more precise diagnosis.

Some studies reported other immunohistochemical patterns of Xp11.2 translocation carcinomas.[5,15] These patterns included lack of staining with cytokeratin antibodies (CK7, AE1/AE3, EMA and Cam5.2) and strong expression of both CD10 and AMACR. Expression of vimentin is rare and focal. These findings may also be useful for distinguishing between Xp11.2 translocation carcinomas and other subtypes of renal cell carcinoma, especially the clear cell and papillary types. As noted above, TFE3 is a member of the MiTF family. "MiTF" is a transcription factor that regulates the development and survival of melanocytes and retinal pigment epithelium. It was recently reported that some Xp11.2 translocation carcinoma cases represented the immunoreactivity of melanosome markers such as HMB45 and Melan A. However, neither the primary tumor nor xenograft showed expression of melanocyte markers such as HMB45 and MiTF.

Although we performed routine G-banding karyotyping, it was impossible to identify the translocation and partner genes of TFE3. Chromosomal aneuploidy is the state of cells in which there are numerical aberrations of chromosomes compared with the normal condition, and it is considered to be a common phenomenon in many epithelial cancers, often caused by chromosomal instability. It is not known exactly why G-band karyotypes of the primary tumor, in addition to the present cell line, were more complicated than those of previously reported cases. Once cell lines are cultured for long periods of time, secondary mutations that were not identified in earlier passages and/or clonal evolution of cells that existed as very small populations in earlier passages can become a major problem.[16] There is a possibility that the numerical changes seen in the G-band karyotype could reflect artifacts of in vitro culture and that the underlying t(x;17) translocation could be masked by secondary mutations.

Multicolor FISH analysis showed more complicated chromosomal rearrangements than expected, but it also failed to detect apparent reciprocal translocations between chromosome X and

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17. On the other hand, interphase FISH analysis revealed that the primary tumor and xenograft had translocations involving TFE3; moreover, FISH mapping and DNA sequencing identified ASPL-TFE3 type 1 gene fusion. Some patients have been diagnosed as having translocation renal cell carcinoma by karyotyping. While these patients had simple reciprocal translocations written as t(X;17)(p11.2;q25), others may have unpredictable complex chromosomal rearrangements like our patient. Thus, it may be impossible to identify all chromosomal translocations only by commonly used karyotyping.

Ladanyi et al.[17] detected two types of ASPL-TFE3 fusion transcripts by RT-PCR in ASPS patients, type 1 (fused to TFE3 exon 4) and type 2 (fused to TFE3 exon 3). Although RT-PCR assays could be reliable diagnostic tools for ASPS and ASPL-TFE3 translocation renal cell carcinoma, frozen tissue is not always available for most real cell carcinomas in the routine clinical setting. Interphase FISH assay for formalin-fixed and paraffin-embedded tissue is helpful to detect TFE3 gene arrangement.

Xp11 translocation carcinoma is a rare tumor in children and adolescents usually diagnosed in the advanced stage with an aggressive clinical course. Its clinical and molecular characteristics remain to be fully elucidated, and it is unclear whether treatments using cytokines or molecular targeting drugs have a beneficial effect on improving its prognosis. In vivo and in vitro studies using this S-TFE cell line will be useful for examination of the tumor characteristics and drug susceptibility of Xp11 translocation carcinoma.

Materials and Methods

Origin of the tumor cells. Tumor cells were obtained from an 18-y-old Japanese female patient with a left renal tumor. She had suffered from an abdominal mass for several months. CT revealed a huge renal mass (90 mm in diameter) and lymph node involvement. Radical nephrectomy and lymph node dissection were performed without neoadjuvant treatments. This primary tumor was pathologically diagnosed as renal carcinoma associated with Xp11 translocations and pT3apN2. Six months after nephrectomy, multiple lung and liver metastases occurred. Molecular targeting agents such as sunitinib and radiation therapy were given and she had responded partially to these treatment. However they gradually became less effective, were given and she was alive with disease at the latest follow-up 29 mo after nephrectomy.

Cell culture methods and determination of in vitro growth. The tumor tissue was minced into small pieces, placed on a plastic dish with culture medium under sterile conditions and incubated at 37°C in a humidified atmosphere of 5% CO_2. The culture medium, RPMI 1640 (Invitrogen), was supplemented with 10% heat-activated fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen). Half of the culture medium was renewed every 3–4 d, and when the cells became subconfluent they were harvested from the bottom of the dish using 0.05% trypsin in EDTA solution. The cells were implanted again in a new culture flask.

The cells reaching subconfluence at the 25th passage were used for determination of in vitro growth. The cells (1×10^5/well) were seeded to 6-well plates. They were then incubated and their numbers were counted every 2–3 d up to 18 d. Average numbers of cells from triplicate wells were determined at given time points.

Tumorigenicity in nude mice. Cell suspension adjusted to 2×10^7/0.2 ml PBS was subcutaneously inoculated into the backs of 6-week-old athymic nude mice (BALB/c nu/nu, Hokudo Co Ltd.) to establish xenografts. After tumor formation was observed, the diameter was measured with a micrometer caliper, and tumor volume was calculated using the formula: tumor volume = $(\text{width})^2 \times (\text{length}) \times \pi/6$ (mm^3). The animal experiment was performed in accordance with a protocol that was approved by the Ethics Committee of Sapporo Medical University School of Medicine.

Immunohistochemistry. Both the primary tumor and xenograft were fixed with 10% buffered formalin and embedded in paraffin. The paraffin blocks were sliced at 4 μm and mounted onto gelatin coated-slides. Hematoxylin-eosin staining and TFE3 immunostaining were performed. A polyclonal antibody to TFE3 was purchased from Santa Cruz Biotechnology (sc-5958). Heat-induced epitope retrieval for immunostaining of TFE3 was performed using microwave pretreatment in a standard citrate buffer (0.01 M, pH 6.0) for 5 min. The TFE3 primary antibody was manually diluted to 1:1,000 in phosphate-buffered saline, and the sections were incubated at room temperature for 60 min. The immunoreactivity was verified by reference to previously confirmed positive and negative control tissue sections. The immunoreactivity to the TFE3 antibody was scored as 0 or 1+ (negative), 2+ (moderately positive) or 3+ (strongly positive) as described by Argani et al.[8] Immunostaining for AMACR (α-methylacyl-CoA racemase), CD10, cytokeratin (AE1/AE3), CK7, vimentin and HMB45 was also performed (**Table 1**).

Interphase FISH analysis. The primary tumor and xenograft were examined by interphase FISH analysis of formalin-fixed and paraffin-embedded tissues according to the methods described by Noguchi et al.[18] A custom-made TFE3 dual-color, break-apart rearrangement probe was created using bacterial artificial chromosome clones (centromeric [RP11-57A11 + RP11-211H18] and telomeric [RP11-343N17 + RP11-735G22], Chromosome Science Labo Inc.). The TFE3 probes were labeled with SpectrumGreen-dUTP at the centromeric site and SpectrumOrange-dUTP at the telomeric site. In this analysis, green and orange signals are found a certain distance away from each other if there is chromosomal rearrangement. If 10% or more of tumor cells show split signals in the nuclei, the tumor is considered to have TFE3 gene rearrangement.

G-banding karyotype. Chromosomal analyses of the primary culture (cultured for 7 d) and cell line (at 29th passage) were performed using G-banding.

FISH mapping and multicolor FISH analysis. FISH mapping with 4 colored probes was performed for the cell line at the 10th passage. For FISH mapping, in addition to the TFE3 probes described above, dual-color break-apart DNA probes for ASPL were used (centromeric [RP11-634L10] and teromeric

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Table 1. Conditions and results of immunohistochemistory for primary and xenograft tumors

Antibodies		Clone	Dilution	Vendor	Primary	Xenograft
TFE3	poly	gout	1:1,000	Santa Cruz	(3+)	(3+)
AMACR	poly	rabbit	1:30	Diagnostic Biosystem	(3+)	(3+)
CD10	mono	56C6	1:50	Novocastra	(3+)	(2+)
Cytokeratin	mono	AE1/AE3	prediluted	DAKO	(1+), focal	(1+), focal
CK7	mono	OV-TL 12/30	prediluted	DAKO	(–)	(–)
Vimentin	mono	V9	prediluted	DAKO	(–)	(–)
Melanosome	mono	HMB-45	1:100	DAKO	(–)	(–)
MiTF	mono	D-5	1:100	DAKO	(–)	(–)

[RP11-1033I6]). The TFE3 probes were labeled green at the centromeric site and aqua at the telomeric site, and the ASPL probes were labeled Cy3 (yellow) at the centromeric site and red at the telomeric site. Multicolor FISH analysis for the cell line at passage 10 was performed using human chromosome-specific paints according to the manufacturer's instructions (Multicolor FISH-Human, Cambio). These FISH images were captured and merged with the CW4000 FISH application program of Leica Microsystems Imaging Solutions using a DMRA2 microscope (Leica Microsystems).

The FISH mapping was performed and multicolor FISH images were obtained as described by Tamura et al.[19] On the assumption of gene fusions including TFE3, the dual-color break-apart TFE3 probes described above were hybridized after multicolor FISH analysis, and the images of TFE3 signals were merged with the multicolor FISH images on the same metaphase spreads.

RT-PCR analysis. The expression of fusion genes including TFE3 was determined by RT-PCR. Total RNA from both fresh frozen primary tumor tissue and the cell line at 10th passage were purified via a standard extraction method using an RNeasy Mini Kit (Qiagen). The total RNA was converted into cDNA using SuperScript III reverse transcriptase (Invitrogen) according to the manufacturer's instructions. To detect the ASPL-TFE3 and PRCC-TFE3 fusion transcripts, primer sequences described in the previous literature were used:[20,21] forward primer from ASPL, AAA GAA GTC CAA GTC GGG CCA; TFE3 exon 4 reverse primer, CGT TTG ATG TTG GGC AGC TCA; forward primer from PRCC, CAC TGA GCT GGT CAT CAC; TFE3 exon 2 reverse primer, AGT GTG GTG GAC AGG TAC TG. As negative controls, renal cell carcinoma cell lines ACHN and Caki-2 were used (American Tissue Culture Collection). As a reference for the ASPL-TFE3 fusion pattern, RT-PCR was performed for cell line FU-UR-1, in which ASPL-TFE3 type 2 fusion has been confirmed.[9] PCR amplification was performed using AmpliTaq Gold 360 Master Mix (Applied Biosystems).

DNA sequencing. Agarose gels were cut out and PCR products were extracted using a QIAquick Gel Extraction Kit (Qiagen). The fusion transcripts were verified by direct DNA sequencing with the dideoxy method of the BigDye Terminator v3.1 Cycle Sequencing Kit and ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). The results of DNA sequencing were compared with the relevant sequences in the NCBI BLAST database (www.ncbi.nlm.nih.gov/BLAST/).

Disclosure of Potential Conflicts of Interest

No potential conflict of interest was disclosed.

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