Establishment and characterization of a renal cell carcinoma cell line (FU-UR-1) with the reciprocal ASPL-TFE3 fusion transcript

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Abstract. We have established a cultured cell line named FU-UR-1 from a large retroperitoneal tumor of a 24-year-old Japanese male patient who simultaneously had a small renal cell carcinoma (RCC). Cytogenetic analysis and fluorescence *in situ* hybridization of the retroperitoneal tumor, and the cell line established from this tumor demonstrated similar karyotypes including add(13)(p11).ish der(13)t(13;17)(p11;q11)t(X;17)(p11;q25)(wcpX+,wcp17+). FU-UR-1 had been propagated continuously for more than 70 passages, and the doubling time was 32 h. Successful heterotransplantation was performed by inoculation of the cultured FU-UR-1 cells into the subcutis of BALB/c nude mice. Reverse transcriptase-polymerase chain reaction (RT-PCR) and sequence analysis demonstrated reciprocal ASPL-TFE3 and TFE3-ASPL fusion transcripts in the retroperitoneal tumor, cultured FU-UR-1 cells and xenografted tumors. In addition, the pathological findings of these samples and the renal tumor resembled each other. These observations suggest that the FU-UR-1 cell line established from the retroperitoneal tumor is an RCC cell line. This well-examined cell line may become a useful system for studying the genetic and biologic characteristics of rare neoplasms with the reciprocal ASPL-TFE3 fusion transcript.

Introduction

Renal cell carcinoma (RCC) is the most common renal malignancy in adults, but it is rare in children and young adults, comprising only 2-6% of all renal tumors (1). The biological and clinical behavior as well as the histological and cytogenetical findings of RCC in children and young adults differ from those of adult RCC, and thus these two groups have been suggested to represent different disease entities (2-9).

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Pathologically, RCC in children and young adults mainly exhibits papillary histology, whereas RCC in adults frequently shows clear-cell, non-papillary features (3-5).

Cytogenetic studies have suggested that some populations of RCC in children and young adults may be characterized by aberration of chromosome Xp11.2 (6,7). In contrast, deletions or rearrangements of the short arm of chromosome 3 are by far the most common abnormalities in adult cases (7-9). Abnormalities of Xp11.2 in children and adults mainly exhibit t(X;1)(p11.2;q21), t(X;1)(p11.2;p34), and rarely t(X;17)(p11.2;q25.3) and some other variations (7). These translocations result in the fusion of PRCC on 1q21 (10), PSF on 1p34 (11), RCC17 or ASPL on 17q25 (12,13) and TFE3 located on Xp11.2 (14,15).

The t(X;17)(p11.2;q25.3) involving these genes has also been observed in human alveolar soft part sarcoma (ASPS) (16). ASPS is a rare malignant soft tissue neoplasm occurring mainly in children and young adults, and its origin is unknown (17). Recently, Sandberg and Bridge (18) proposed a relationship between RCC with the reciprocal ASPL-TFE3 fusion transcript and ASPS, and suggested that the expression of ASPL-TFE3 is balanced in RCC, whereas it seems to be unbalanced in ASPS.

We cultured a retroperitoneal metastatic tumor of RCC from a young adult to analyze the chromosomal aberrations. Since the cells subsequently grew into a permanent cell line, we named this cell line FU-UR-1 and investigated its morphologic, biologic and genetic characteristics.

Materials and methods

Source of the tumor cells. The original tumor tissue specimen was obtained from a surgically excised retroperitoneal tumor (11x7 cm in size; Fig. 1A) from a 24-year-old Japanese man. Since the tumor had involved the left renal artery and vein, the left kidney was resected simultaneously. A tiny renal tumor (0.5 cm in diameter) was found at the central portion of the kidney (Fig. 1B), and was considered to be an original lesion. Histopathologically, the retroperitoneal tumor was considered to be metastatic renal cell carcinoma.

After about a month, resection of the metastatic lesion from the lower lobe of the left lung was performed. The patient underwent interferon treatment, but it was ineffective. Multiple metastases occurred involving the lumbar spine, lungs and liver. He died 3 months later, but an autopsy was not performed.

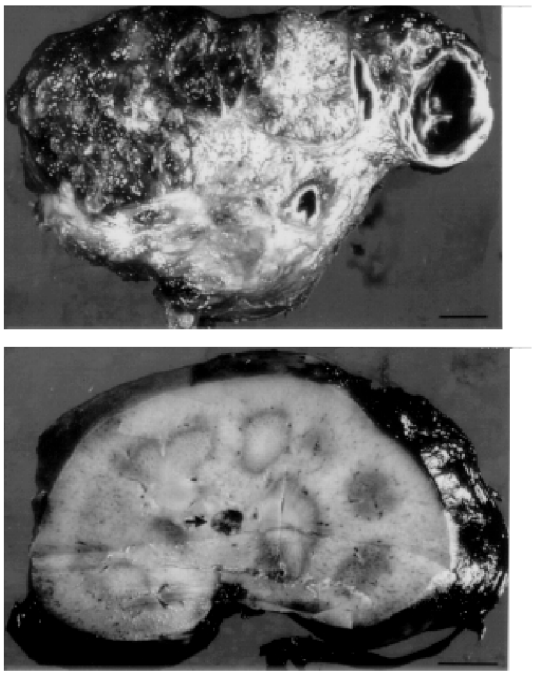

Figure 1. (A), Gross appearance of the surgically excised retroperitoneal tumor. (B), Cut section of the left kidney. A small tumor nodule (arrow) is present in the central portion of the kidney. Scale bar, 10 mm.

Cell culture. Immediately after the first surgery, minced fragments of the retroperitoneal tumor tissue were digested with 400 U/ml type II collagenase (Worthington Biochemical Corporation, Freehold, NJ, USA) in culture medium for 30 min at 37˚C. After the digestion, the tumor cells were washed, re-suspended in culture medium in plastic flasks (Falcon Primaria, Becton Dickinson), and incubated at 37˚C in a humidified atmosphere containing 5% CO_2 and 5% O_2. The culture medium was composed of a 1:1 mixture of DMEM and Ham's F12 (Gibco BRL, Paisley, UK) supplemented with 10-20% fetal calf serum (HyClone Laboratories, Logan, UT, USA) and kanamycin (100 mg/l). The medium was replaced twice a week. Four weeks later, the first subculture was performed using 0.02% EDTA and 0.1% trypsin.

Tumorigenicity in nude mice. Dispersed cells (passage 4, $1x10^7$ cells) suspended in PBS were inoculated in the dermis of congenital athymic nude mice (BALB/c nu/nu, Nippon Clea Japan Inc., Osaka, Japan). Mice carrying a transplanted tumor were euthanased 3 weeks after the inoculation. The xenografts were routinely processed for light microscopy and reverse transcriptase-polymerase chain reaction (RT-PCR).

Growth kinetics. At passage 51, a growth curve was estimated by inoculating $8x10^3$ cultured cells in 1 ml medium per well of 24-well culture plates (Corning, New York, USA). The number of viable cells was counted by dye exclusion (0.3% trypan blue in phosphate-buffered saline) every day.

Pathologic evaluations. For light microscopic studies, the retroperitoneal, renal, pulmonary and xenografts tumors were formalin-fixed and embedded in paraffin. Serial 3 μm thick sections were prepared on glass slides. Cultured cells grown on chamber slides (Lab-Tek, Miles Laboratories, Naperville, IL, USA) were washed in PBS and fixed in methanol for 5 min. These slides were stained with Giemsa and periodic acid-Schiff (PAS) with or without a diastase digestion test.

For immunocytochemistry of paraffin-embedded sections, antigen retrieval was performed for EMA, cytokeratins, desmin and vimentin using microwave pretreatment in a standard citrate buffer (0.01 M, pH 6.0), and for CD10 using steaming in the same solution. Cultured cells grown on chamber slides were fixed in cold acetone for 10 min. The samples were reacted with each of the primary antibodies (Table I) for 1 h at room temperature. The bound antibodies were visualized using a labeled streptavidin-biotin system and an alkaline phosphatase technique.

Cytogenetic analysis. Cells in primary cultures (7, 11 and 30 days after the beginning of the cultures) and from the cultured cell line (at passage 70) were used for G-band and fluorescence *in situ* hybridization (FISH) analyses. Metaphase preparations were made with a standard trypsin-Giemsa banding technique (19). Chromosomes were classified according to the 1995 International System for Human Cytogenetic Nomenclature (20).

The procedure for FISH was based on published methods (21). For three-colored chromosome painting, chromosome 13, 17 and X specific painting probes (chromosome 13: Cotasome13 Total Chromosome Probe, Oncor, Gaithersburg, MD, USA; chromosome 17: Chromosome Painting System, Cambio17B, Cambridge, MA, USA; chromosome X: CotasomeX Total Chromosome Probe, Oncor, and Chromosome Painting System, were used. To detect chromosome X, we used a 1:1 mixture of CotasomeX and CambioXB.

Visualization of hybridization was performed according to the manufacturer's protocol. Chromosome 13 (Cotasome13) was detected with rhodamine-labeled anti-digoxigenin (red) whereas chromosome 17 (Cambio17B) was detected with avidin-fluorescein isothiocyanate (green). Chromosome X (CotasomeX and CambioXB) was visualized with both reagents (yellow). The slides were counterstained with DAPI (4'-6'-diamino-2-phenyl-indole, Sigma Chemical Co., St. Louis, MO, USA) and visualized using an *in situ* imaging system (Isis, Carl Zeiss Vision, Oberkochen, Germany).

RT-PCR analysis. Total RNA was isolated from fresh frozen tissue of the retroperitoneal tumor, xenografts and FU-UR-1 cells (at passage 60) using a standard organic extraction method (TRIzol, Life Technologies, Gaithersburg, MD, USA). Samples were treated with DNase I (Gibco BRL) and reverse-transcribed using Superscript II reverse transcriptase (Gibco BRL), according to the manufacturer's conditions. PCR amplification of the complementary DNA product was performed as described in the literature (13,22). As a positive control for the integrity of the mRNA isolated from each sample, PCR for the ubiquitously expressed glyceraldehyde-3-phosphate dehydrogenase (G3PDH) gene was performed (23). All amplification products were stained with ethidium bromide and analyzed in 3% agarose gels.

Table I. Immunostaining results and antibody descriptions.

Antibodies	Renal tumor	Retroperitoneal tumor	Pulmonary tumor	Xenograft tumor	Cultured tumor	Dilution cells	Vendor
EMA	+f	+f	-	±	-	1:100	Dako
Cytokeratin AE1/AE3	+f	-	-	-	-	1:50	Dako
Cytokeratin 7	+f	-	-	-	-	1:50	Dako
CAM5.2	+f	±	-	±	+	1:1	Becton Dickinson
HMB45	-	-	-	-	-	1:50	Dako
S-100	-	-	-	-	-	1:1	Histofine
Desmin	-	-	-	-	-	1:100	Dako
MyoD1	-	-	-	-	-	1:50	Dako
HHF35	-	-	-	-	-	1:50	Enzo
CD10	+f	+f	+f	+f	+[a]	1:10	Novocastra
Vimentin	+f	+f	-	+f	+	1:100	Dako

[a]Diffuse and strong. -, Negative; +, positive; +f, focally positive; ±, faint and diffuse. EMA, epithelial membrane antigen. Dako Japan, Kyoto, Japan. Novocastra, Newcastle, UK. Histofine, Nichirei, Tokyo, Japan. Becton Dickinson, Mountain View, CA, USA. Enzo, New York, USA.

Cloning of RT-PCR products. RT-PCR products were subcloned using a TA Cloning Kit (Invitrogen, San Diego, CA, USA) following the manufacturer's instructions, and sequenced by the dideoxy method using a BigDye™ Terminator kit (ABI, Foster City, CA, USA) and an ABI 377 DNA sequencer.

Results

Establishment of the FU-UR-1 cell line. The primary culture exhibited polygonal tumor cells of various sizes admixed with normal fibroblasts. We maintained the cultures without subculturing until the tumor cells grew to confluence. The fibroblasts gradually disappeared so that confluent tumor cell layers were obtained within 3 months of the primary culture. The population doubling time was 32 h at passage 51. The cell line was named FU-UR-1 and has been in continuous culture up to passage 98 (Fig. 2).

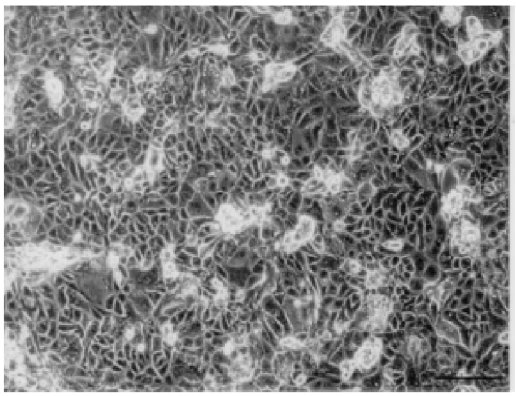

Figure 2. Phase-contrast light micrograph of the FU-UR-1 cell line at passage 40. The cultured cells show various sizes of polygonal cytoplasm in the culture flask. Scale bar, 100 μm.

Tumorigenicity in nude mice. The FU-UR-1 xenografts grew rapidly, within 3 weeks after inoculation of the cultured cells. Round nodules developed and reached approximately 2.5x1.5 cm in size at the sites of injection. The mice were sacrificed humanely, and no metastatic lesions were disclosed at autopsy.

Histopathologic findings. Histopathologic examination of the retroperitoneal tumor showed a proliferation of atypical polygonal cells with abundant clear or eosinophilic cytoplasm forming both papillary and alveolar structures, with necrotic foci and inflammatory infiltration (Fig. 3A). The tiny renal tumor mainly exhibited a papillary growth pattern with eosinophilic and clear cells, and the periphery showed an alveolar pattern composed of nests of clear cells (Fig. 3B). Both the retroperitoneal and renal tumors showed some calcified psammoma bodies.

The metastatic pulmonary tumor showed nodular proliferation of clear cells forming occasional glandular structures (Fig. 3C). The xenograft tissue showed atypical epithelial cells with clear or eosinophilic cytoplasms forming nests and alveolar structures. The cells showed more atypia than those of the retroperitoneal or renal tumors (Fig. 3D). The cultured FU-UR-1 cells had various sizes of polygonal cytoplasm, and hyperchromatic nuclei.

All samples possessed PAS-positive glycogen granules in the cytoplasm, which were removed by a diastase predigestion. In addition, a small amount of PAS-positive, diastase-resistant cytoplasmic granules were found in the retroperitoneal tumor cells (Fig. 4).

Immunohistochemical findings. The immunohistochemical profiles are summarized in Table I. In all samples the tumor

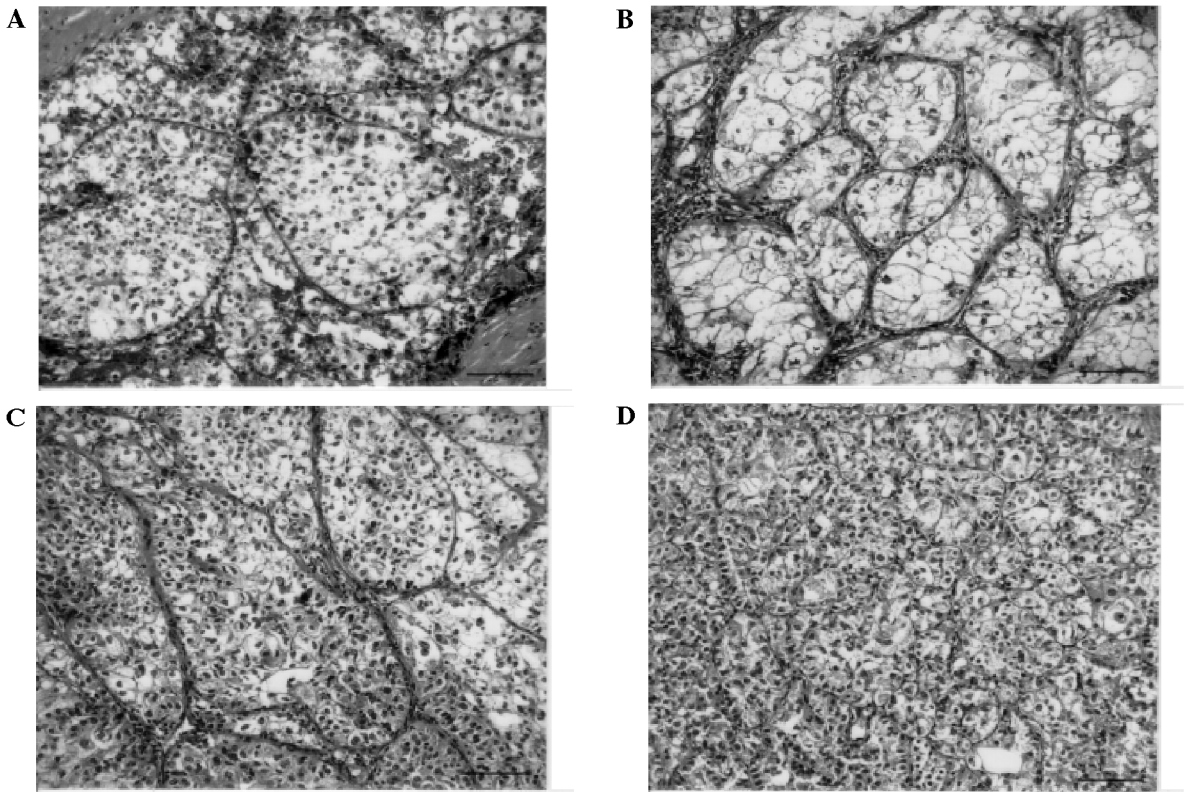

Figure 3. (A), Histologic features of the retroperitoneal tumor showing distinct alveolar growth patterns (H&E). (B), Renal tumor showing nested structures consist of clear cells (H&E). (C), Metastatic pulmonary tumor showing an alveolar growth pattern with clear cells (H&E). (D), Xenograft tumor resected from a nude mouse showing solid nested structures (H&E). Scale bars, 100 μm.

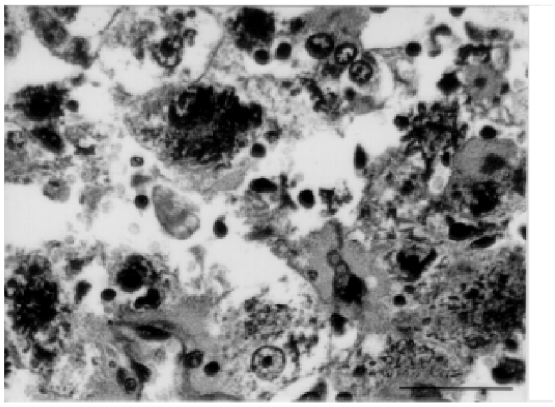

Figure 4. Retroperitoneal tumor shows diastase-resistant periodic acid-schiff granules in the cytoplasm (PAS). Scale bar, 50 μm.

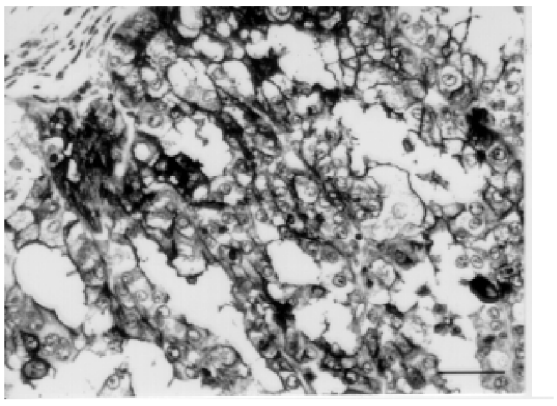

Figure 5. Immunohistochemical expression of CD10 in a xenograft tumor showing strong surface membrane staining. Scale bar, 50 μm.

cells showed positive reactions for CD10 (Fig. 5) and vimentin, but were uniformly negative for S-100 protein, HMB45, MyoD1, HHF35 or desmin reactions. The reactions for EMA, cytokeratin7, CAM5.2 and cytokeratin AE1/AE3 varied for each sample.

Cytogenetic analysis. Sixty-five adequate metaphases were obtained from the retroperitoneal specimen that had been cultured for 30 days. Other harvests did not contain any analyzable metaphases. The metaphases showed hypodiploid to pseudodiploid karyotypes with complex abnormalities. The modal chromosomal number was 45. The G-banded karyotype showed: 45,XY,del(X)(p11.2),del(11)(q23), add(13)(p11),+add(13)(p11),add(16)(p11),-17,-18[cp17]/ 44,idem,- 4[3],-8[3] [cp14]]/43,idem,-Y[6],-4[4],-21[6][cp15] (Fig. 6A).

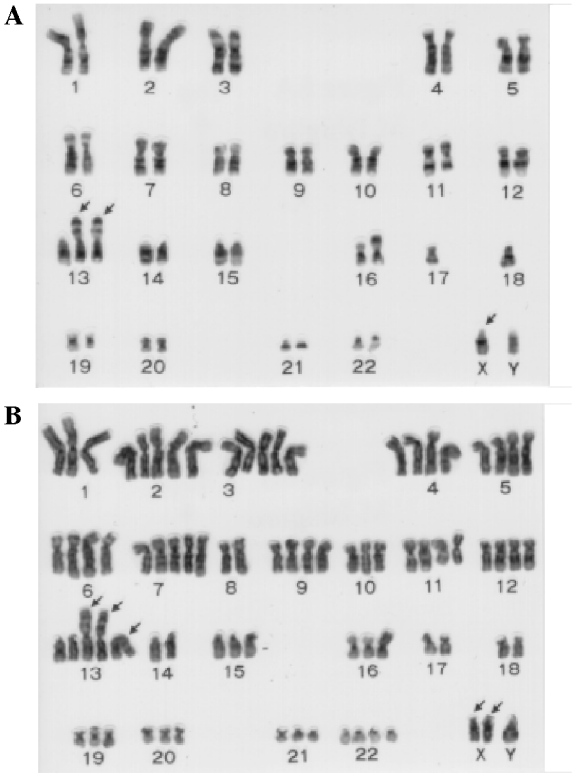

Figure 6. (A), Representative G-banded karyotype of the retroperitoneal specimen. (B), Representative G-banded karyotype of the cell line at passage 70. Both samples exhibit the same structual chromosome aberrations: del(X)(p11.2),del(11)(q23),add(13)(p11),add(16)(p11) (arrows).

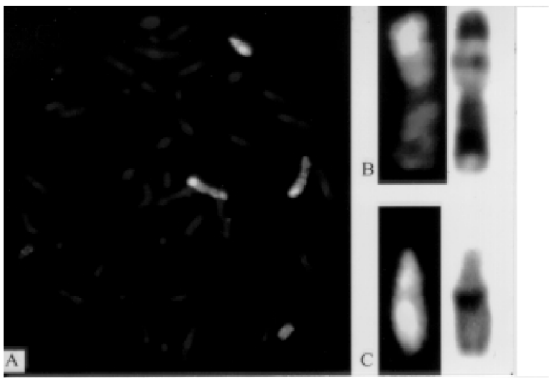

Figure 7. (A), Representative images from the FISH analysis are shown. FISH with a probe for chromosome 13 (red), chromosome 7 (green) and chromosome X (yellow) showing chromosomes. (B), Partial karyotype showing the add(13). By G-band and FISH analysis, add(13) was identified as der(13)(p11)t(13;17)(p11;q11). (C), Partial karyotype showing the del(X) (p11.2).

The FU-UR-1 cell line taken at passage 70 showed a triploid clone. The karyotype had the following description: 74-83,XXY,del(X)(p11.2),+2[4],+3[5],+4[6],+5[6],+6,+7[3],+add(7)[6],-8[3],del(11)(q23),+del(11)(q23),+12[6],add(13)(p11),+add(13)(p11)x2,-14[5],add(16)(p11),+add(16)(p11)[3],-17,-18[cp7] (Fig. 6B). Marked cell-to-cell variability was seen.

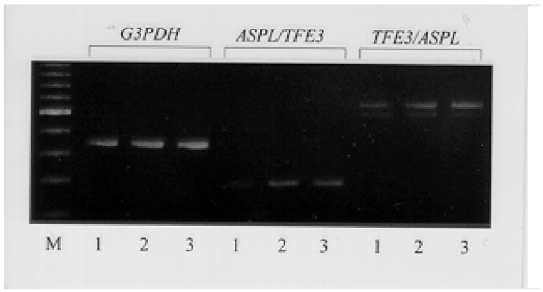

Figure 8. Detection of ASPL/TFE3, AFE3/ASPL, G3PDH fusion transcripts by RT-PCR. Lane 1, retroperitoneal tumor; lane 2, renal tumor; lane 3, cell line.

The FU-UR-1 cell line had the same abnormalities as the retroperitoneal tumor, including: del(X)(p11.2),del(11)(q23), add(13)(p11),add(16)(p11),-17,-18. New abnormalities which were not seen in the retroperitoneal tumor could be defined, including add(7) and +12.

The FISH analysis demonstrated that the aberrant chromosome 13 consisted of chromosomes 13, X and 17 (Fig. 7). The main clone of the retroperitoneal specimen was established as follows: 45,XY,del(X)(p11.2),del(11)(q23), add(13)(p11)ish der(13)t(13;17)(p11;q11)t(X;17)(p11;q25)(wcpX+,wcp17+), +add(13)(p11)ish der(13)t(13;17) (p11;q11)t(X;17)(p11;q25) (wcpX+,wcp17+),add(16)(p11),-17,-18.

RT-PCR reaction. The results of the RT-PCR are shown in Fig. 8. The cells of the FU-UR-1 cell line, retroperitoneal tumor, and xenografts demonstrated PCR products of the same size. The ASPL-TFE3 fusion transcript was detected as a single band of 300 bp. Cloning and sequencing of this PCR product showed in-frame fusion of ASPL to TFE3 exon 3 (Fig. 9A). The TFE-ASPL fusion transcripts showed 631 bp and 697 bp products. Both products were also identified after cloning and sequencing individual clones. As a result, both splice products showed in-frame fusion of TFE3 exon 2 to ASPL, and the 631 bp product lacked 65 bp from the TFE3 sequence (Fig. 9B). The junction sequence of the TFE3 gene was between exons 2 and 3. The G3PDH transcripts from each extract revealed a single band of approximately 450 bp.

Discussion

In the present study, we have established a renal cell carcinoma cell line, FU-UR-1, from a large metastatic tumor in the retroperitoneum, which exhibits reciprocal ASPL-TFE3 and TFE3-ASPL fusion transcripts, characteristic of RCC in children and young adults.

In 1993, we reported this same case of a retroperitoneal metastatic renal cell carcinoma with del (X)(p11) in a young adult (24), and subsequently established the FU-UR-1 cell line from this tumor as described here.

Histologically, the retroperitoneal, renal, pulmonary and xenograft tumors exhibited an alveolar pattern similar to that of alveolar soft part sarcoma (ASPS). Furthermore, histochemical staining demonstrated PAS-positive diastase-resistant

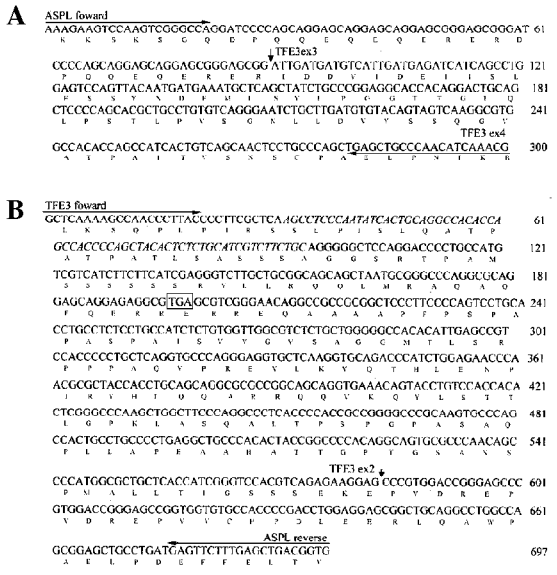

Figure 9. (A), Neucleotide sequence of the ASPL/TFE3 fusion gene transcript obtained by RT-PCR and the predicted amino acid sequence. The site of fusion point is indicated by the arrow head. ASPL and TEF3 specific primers are underlined with directional arrow. (B), Neucleotide sequence of the TFE3/ASPL fusion gene transcript obtained by RT-PCR and the predicted amino acid sequence. The site of fusion point is indicated by the arrowhead. TFE3 and ASPL specific primers are underlined with directional arrow. The absence in ASPL of a 65-nucleotide segment is shown in italics and the terminal codon is shown in the square.

granules (possible precursors of the crystals) in the retroperitoneal tumor. These granules are similar to those formed in ASPS (25). Therefore, we cannot exclude the possibility that the retroperitoneal tumor was ASPS.

In 2002, Sandberg and Bridge (18) reported on some comments regarding the diagnostic aspects of ASPS and renal tumors with t(X;17). Renal tumors with t(X;17) demonstrated both reciprocal ASPL-TFE3 and TFE3-ASPL fusion transcripts, as identified by RT-PCR (13), whereas in ASPS only the ASPL-TFE3 fusion transcript has been reported (22).

In our case, RT-PCR detected the ASPL-TFE3 (301 bp) fusion transcript and 2 sizes of the TFE3-ASPL (697 bp, 632 bp) fusion transcript in the retroperitoneal tumor tissue, the cell line and the xenograft tissue. These fusion transcripts were reciprocal. One of the TFE3-ASPL fusion transcripts (632 bp) lacked a 65-nucleotide segment of the TFE3 gene, which leads to premature termination within the portion encoded by the TFE3 gene. This in-frame TFE3-ASPL fusion transcript (632 bp) would probably not encode a functional protein.

Cytogenetic analyses of RCC in children and young adults have described them as bearing a translocation involving Xp11.2, irrespective of the sex or histologic type (7,26,27). However, in our case, the karyotype exhibited deletion of the short arm of chromosome X. In order to decide whether chromosome X was a deletion or a translocation, we performed a FISH analysis by chromosome painting of the cell line and the cultured retroperitoneal tumor. As a result, we concluded that the short arm of chromosome X represents a del(X)(p11.2) whereas add(13) is a der(13)(p11)t(13;17)(p11;q11)t(X;17)(p11;q25).

In view of the results of the RT-PCR, aberrant chromosome X may contain a fragment of chromosome 17q25. However, we could not find any chromosome 17 material on chromosome X by G-band and FISH analysis. This discrepancy may possibly be due to the fragment of chromosome 17 being too small for detection by G-band and FISH with whole painting probes.

The retroperitoneal, renal, pulmonary and xenograft tumors in our case share histopathologic features with ASPS. Although the histopathologic appearances of RCC in children and young adults are variable, ASPS often bears a striking resemblance to RCC with rearrangement at Xp11.2 (25,27,28).

We detected PAS-positive diastase-resistant cytoplasmic granules in the retroperitoneal tumor. These granules are a hallmark of ASPS (25) but, as these granules have also been reported in some RCC (22), this observation may not be specific for ASPS.

In our immunohistochemical studies, the lack of staining for S-100 protein, HMB45, desmin, MyoD1, and HHF35 within all samples excluded clear cell sarcoma and myogenic sarcomas. Furthermore, CD10 was positive in all our tumor samples, and the antibody has been shown to stain most clear cell and papillary renal cell carcinomas (28-30).

RCC in children and young adults, and ASPS are often non-immunoreactive for EMA and cytokeratin (25,28). In our case, there were some differences in the expression of cytokeratins, EMA and vimentin in each material. These controversial results may be due to the different fixation conditions.

The histogenesis of ASPS and its relationship to RCC with reciprocal fusion transcripts (ASPL-TFE3) still remain unclear. Three cases of RCC demonstrated only the ASPL-TFE3 fusion transcript (22).

The present samples, especially the retroperitoneal tumor, partly resembled ASPS in the histopathological features, and moreover, the renal tumor was very small for the original tumor. Thus, we cannot absolutely exclude the possibility that the present retroperitoneal tumor was ASPS. In the near future, RCC with t(X;17) and ASPS with unbalanced t(X;17) may possibly be classified into the same category.

Since RCC in children and young adults and ASPS are extremely rare, histopathologic, cytogenetic and molecular genetic data are limited. Culture systems have many advantages for examination of the genetic and biological characteristics of tumors. Therefore, this newly established FU-UR-1 cell line may be utilized as an appropriate tool for further investigations into the nature of RCC and the relationship between ASPS and RCC with reciprocal ASLP-TFE3. Furthermore, its ability to produce tumors in nude mice will facilitate comparative *in vitro* and *in vivo* studies not only for pathogenesis but also for modulation of the chemosensitivity in this type of tumor.

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