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Establishment and Characterization of a Novel Human Clear-cell Sarcoma of Soft-tissue Cell Line, RSAR001, Derived from Pleural Effusion of a Patient with Pleural Dissemination

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Abstract. *Background/Aim: Clear cell sarcoma (CCS) of soft tissue is exceedingly rare and frequently exhibits aggressive behavior. Toward the goals of improving the aggressive course and poor prognosis of CCS, and establish new therapeutic methods, molecular genetic and biological characterizations of CCS are required. Materials and Methods: A new human CCS cell line (designated RSAR001) was established from the pleural effusion of a 44-year-old man with multiple lung metastases and pleural dissemination. The cell line and its xenograft were characterized including their morphology, immunohistochemistry, cytogenetic analysis, reverse transcription-polymerase chain reaction, direct sequencing analysis, and fluorescence in situ hybridization analysis. Results: The cell line has been maintained for over 12 months with more than 50 passages. RSAR001 cells exhibited a fascicular or diffuse growth pattern of short spindle- or oval-shaped cells with clear cytoplasm in heterotransplanted tumor, that was similar to the primary tumor. Immunophenotypically, RSAR001 cells in vitro and in vivo exhibited almost the same characteristics as the primary tumor. Cytogenetic analyses revealed a translocation, t(12;22)(q13;q12). Reverse transcription-polymerase chain reaction and direct sequencing analysis detected transcripts of the Ewing sarcoma breakpoint region 1–activating transcription factor 1 (EWSR1–ATF1) type 1 fusion gene. Fluorescence in situ hybridization using a break-apart probe for the EWSR1 gene on 22q12 showed a rearrangement. Conclusion: These findings indicate that the RSAR001 cell line harbors EWSR1–ATF1 type 1 chimeric fusion gene, which is specific to CCS. RSAR001 cells might be useful for investigating biological behaviors and developing new treatments such as molecular-targeting antitumor drugs or immunological drugs for CCS.*

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Key Words: Clear cell sarcoma of soft tissue, cell line, xenograft, *EWSR1–ATF1* type 1 chimeric fusion gene, fluorescence *in situ* hybridization.

Clear cell sarcoma (CCS) of soft tissue, also called malignant melanoma of soft parts, is an extremely rare soft-tissue sarcoma with uncertain differentiation that affects young adults between the ages of 20 and 40 years (1, 2). CCS most frequently occurs in the foot and ankle (approximately 40% of the reported cases) (1, 2). Although CCS usually grows slowly, it frequently exhibits aggressive behavior, resistance to multi-agent chemotherapy and radiation therapy, and fatal metastasis. The overall 5-, 10-, and 20-year survival rates are 67%, 33%, and 10%, respectively (1). Therefore, in order to improve the aggressive course and unfavorable prognosis of CCS over the long term, new treatments including molecular-targeting drugs or immunological drugs based on molecular, genetic, and immunobiological characterization of CCS are required.

To the best of our knowledge, only a few human CCS cell lines have been established (3-16). Molecular, genetic and biological characterizations of CCS using *in vitro* and *in vivo*

models are desired. To clarify the biological behavior of CCS, we established and characterized a new CCS cell line, RSAR001, derived from the pleural effusion of a patient with multiple lung metastases and pleural dissemination.

Materials and Methods

The patient's clinical history. A 20-year-old Japanese man with no remarkable past medical history was referred to our hospital after marginal resection for a primary soft-tissue tumor of the right ankle at his primary hospital. Since the resected tumor was pathologically diagnosed as synovial sarcoma, additional wide resection followed by six courses of chemotherapy with vincristine and high-dose methotrexate was performed at our hospital. Eighteen years after the primary surgical treatment, multiple lung metastases were found. Light microscopy of the biopsy specimens from the metastatic lung tumor showed diffuse proliferation of short spindle- or oval-shaped cells with melanin (Figure 1A). The tumor cells exhibited atypical nuclei, and mitotic figures were frequently seen. Immunohistochemically, tumor cells were positive for vimentin, S-100 protein (Figure 1B), human melanin black-45 (HMB-45) (Figure 1C), and melan-A (Figure 1D), but negative for AE1/AE3, alpha-smooth muscle actin (α-SMA), and desmin (Table I). The characteristics revealed by light microscopy and immunohistochemistry led us to change the diagnosis from synovial sarcoma to CCS. Notwithstanding the combined treatment with surgery, chemotherapy or proton therapy, metastatic lung tumors slowly grew larger. The patient died of his disease at the age of 44 years, 62 months after the appearance of lung metastases.

Establishment of the RSAR001 cell line. Tumor cells were collected from the pleural effusion obtained approximately 1 month prior to the patient's death. The tumor cells were collected by centrifugation and rinsed with Hanks' balanced salt solution with 1% penicillin-streptomycin mixed solution (Nacalai Tesque, Kyoto, Japan). Cells were cultured at an initial density of 1.0×10^6 viable cells/ml in RPMI-1640 medium (Fujifilm Wako Pure Chemical, Osaka, Japan) supplemented with 15% heat-inactivated fetal bovine serum (Biowest, Nuaillé, France). The cells were then inoculated into 100-mm plastic dishes (Greiner Bio-One, Kremsmünster, Austria) and incubated at 37˚C in a humidified atmosphere with 5% CO_2. The culture medium was changed twice per week. To harvest and transfer the cell line, the cells were treated with trypsin-EDTA solution (Lonza, Basel, Switzerland) after they reached a sub-confluent state. The cell line has been maintained for over 12 months under these culture conditions and was designated as RSAR001.

Morphological study. The growth and morphology of RSAR001 cells *in vitro* was observed by using an inverted microscope. Morphological characteristics were further determined by hematoxylin-eosin staining on paraffin-embedded sections.

Immunohistochemistry. An imunohistochemical analysis was performed on the primary tumor, RSAR001 cells at the 49th passage, and heterotransplanted tumors by the streptavidin-biotin complex method. The antibodies used in this study were against S-100 protein (polyclonal; Roche Diagnostics, Indianapolis, IN, USA), HMB45 (clone HMB45; Leica Biosystems, Wetzlar, Germany), melan-A (clone A103; Leica Biosystems), neuron-specific enolase (NSE) (clone 22C9; Leica Biosystems), AE1/AE3 (clones AE1/AE3/PCK26; Roche Diagnostics), desmin (clone DE-R-11, Leica, Biosystems), and α-SMA (clone 1A4; Dako, Glostrup, Denmark).

Table I. *Immunohistochemical reactivity of primary tumor and RSAR001 cells in vitro and from transplanted tumor.*

		RSAR001	
Antibody	Primary tumor	*In vitro*	*In vivo*
S-100 protein	3+	3+	3+
HMB-45	1+	4+	3+
Melan-A	2+	3+	3+
NSE	NT	3+	4+
AE1/AE3	–	–	–
Desmin	–	–	–
α-SMA	–	–	–

–, Negative; 1+, <10% positively stained cells; 2+, 10-50% positive cells; 3+, >50-90% positively stained cells; 4+, >90% positively stained cells. NT, not tested; HMB-45, human melanin black-45; NSE, neuron-specific enolase; EMA, epithelial membrane antigen; α-SMA, α-smooth muscle actin.

Cell population-doubling time. In order to determine the doubling time, RSAR001 cells were seeded at the 55th passage on six-well plastic plates (Greiner Bio-One) with fresh culture medium. Cell growth was measured by determination of the cell density using a CloneSelect™ Imager system (Molecular Devices, Sunnyvale, CA, USA) for 9 consecutive days.

Heterotransplantation. A NOG mouse (NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Sug}/ShiJic, male; 8 weeks old) was used for the heterotransplantation study. NOG mouse was developed by the Central Institute for Experimental Animals (Kanagawa, Japan) (17). The mouse was housed in a plastic cage in a pathogen-free state, at a temperature of 22±1˚C with 55±5% humidity, and a 12-h light/12-h dark cycle. The mouse was administered a single subcutaneous injection of 8.0×10^6 cells at the 43rd passage from cultured cells in the log phase of growth. The mouse was maintained in a pathogen-free environment and carefully observed after heterotransplantation.

Cytogenetics. The karyotype analysis of RSAR001 cells at the 54th passage was performed commercially by SRL (Tokyo) using the trypsin G-banding technique.

RNA extraction, cDNA synthesis, reverse transcription-polymerase chain reaction (RT-PCR) analysis, and direct sequencing analyses for mRNA expression of specific fusion gene. Total RNA from RSAR001 cells at the 54th passage was isolated using ISOGEN reagent (Fujifilm Wako Pure Chemical) according to the manufacturer's protocol. One microgram of RNA was reverse-transcribed to cDNA using a ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan) according to the manufacturer's protocol. Aliquots of cDNA (1.0 μl) were amplified using Takara Ex Taq HS polymerase (Takara Bio, Shiga, Japan) in a total volume of 50 μl.

For the detection of mRNA of Ewing sarcoma breakpoint region 1–activating transcription factor 1 (*EWSR1–ATF1*) fusion gene, PCR was performed as follows: denaturing at 94˚C for 10 min

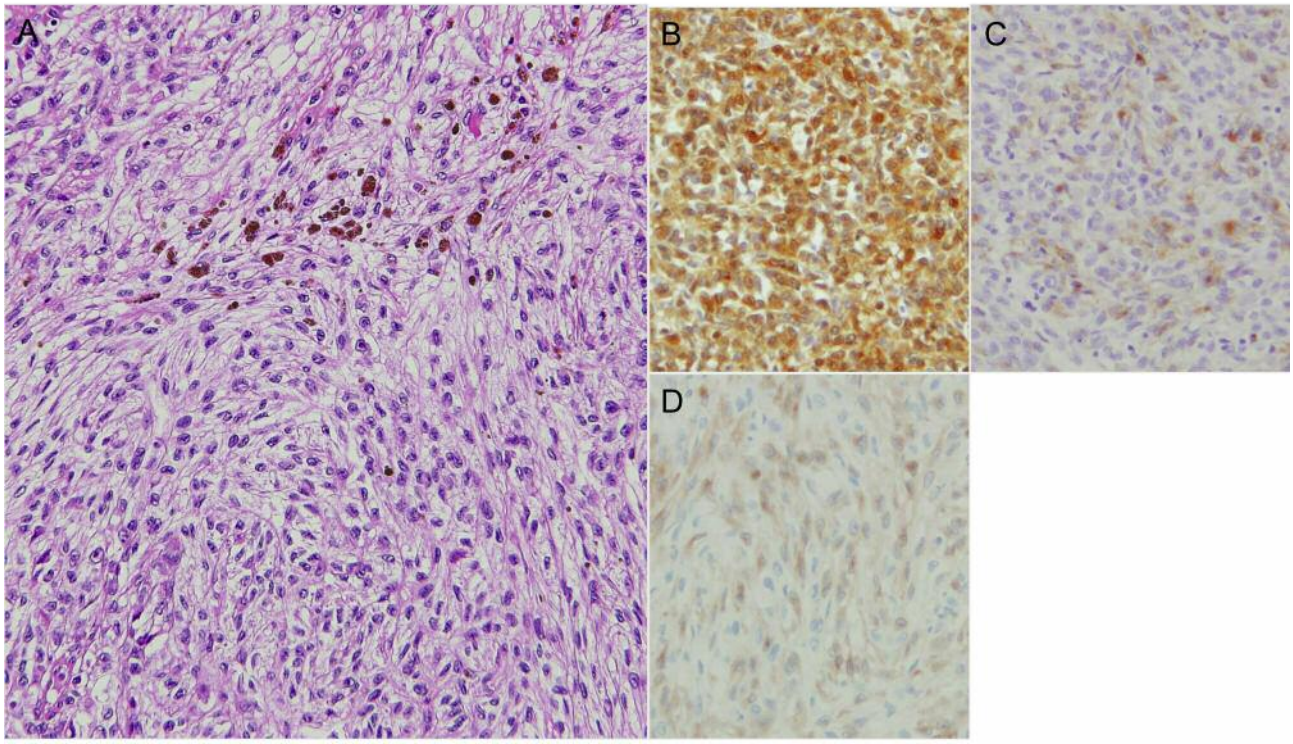

Figure 1. *Histology of the metastatic lung tumor. The tumor cells exhibited diffuse proliferation of short spindle- or oval-shaped cells with melanin expression (A) (hematoxylin and eosin, ×200). Immunohistochemically the tumor cells were positive for S-100 protein (B), human melanin black-45 (C), and melan-A (D) (immunoperoxidase stain, ×200).*

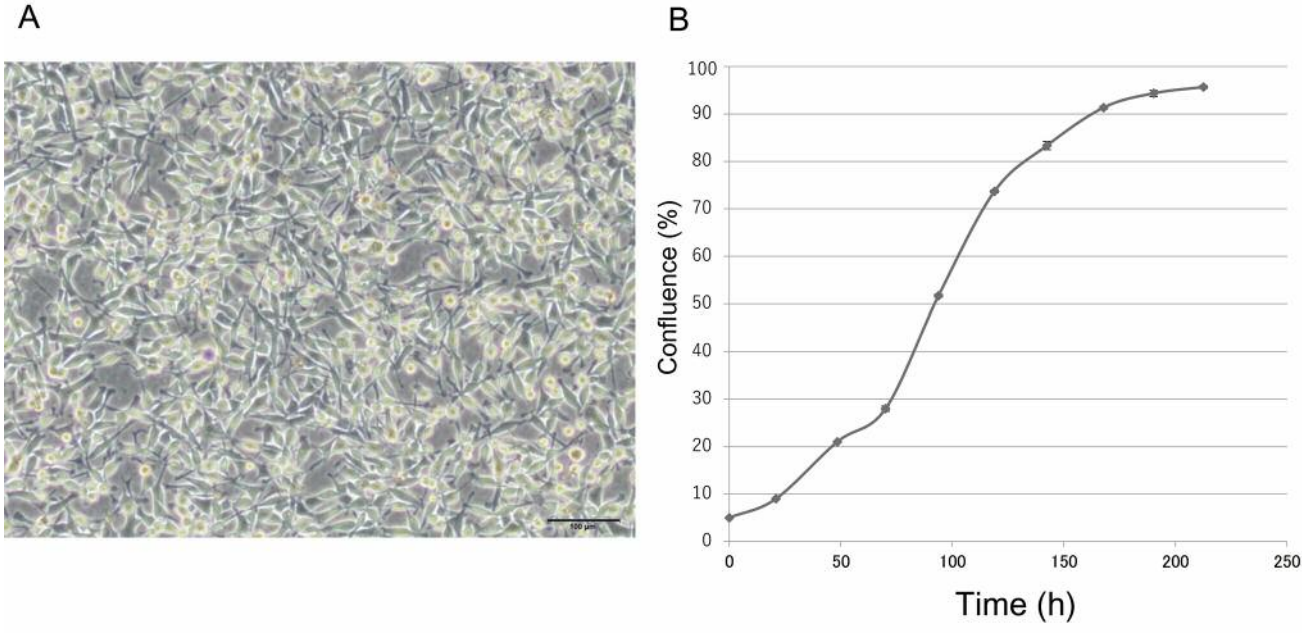

Figure 2. *A: Inverted microscopy of RSAR001 cells in vitro (43rd passage). Adherent cultured cells were found to be atypical, small, polygonal or short spindle-shaped without contact inhibition (bar=100 μm). B: The doubling time of RSAR001 cell line was approximately 35.0 h.*

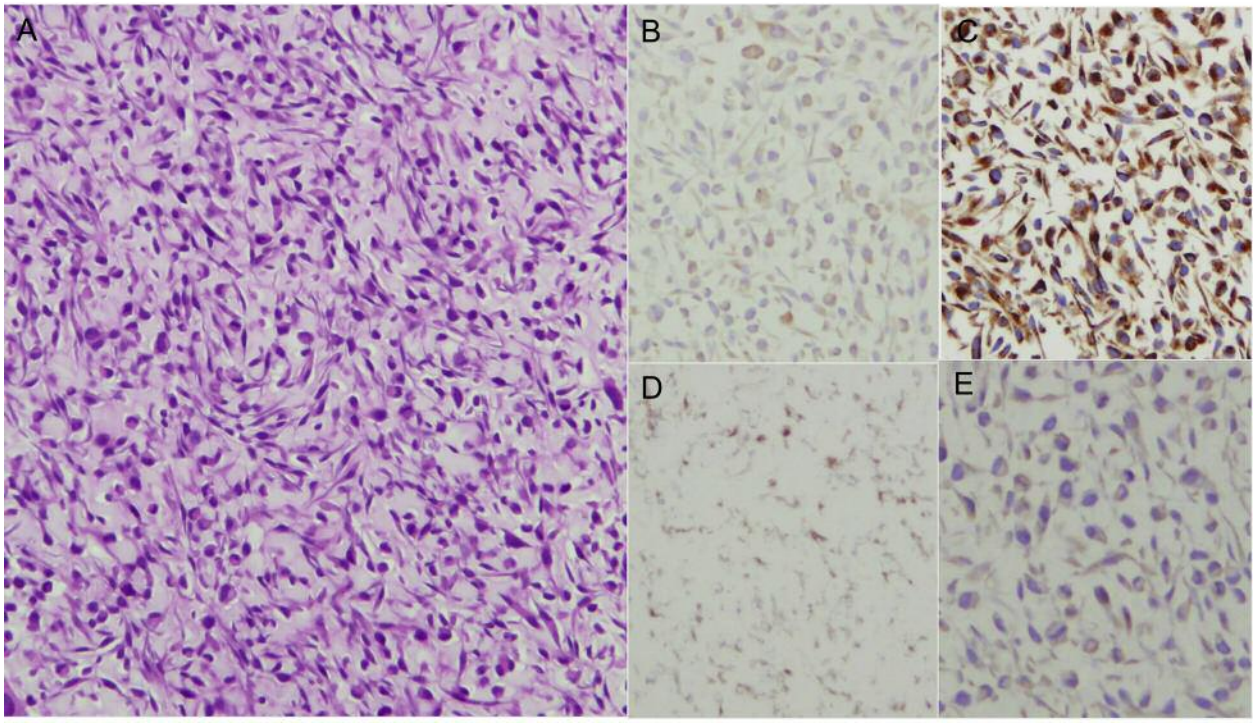

Figure 3. *Microscopically, RSAR001 cells comprised small, polygonal or short spindle-shaped cells (A) (hematoxylin and eosin, ×200). RSAR001 cells were immunohistochemically positive for S-100 protein (B), human melanin black-45 (C), melan-A (D) and neuron-specific enolase (E) (immunoperoxidase stain, ×200).*

followed by 35 cycles of amplification (95˚C for 10 s, 60˚C for 20 s, and 72˚C for 20 s) and a 10-min extension at 72˚C in a thermal cycler (TProfessional TRIO Thermocycler; Biometra, Göttingen, Germany). The sequencing primers were as follows: *EW*Sex7-F2: TATAGCCAACAGAGCAGCAGC, and *ATF1*R2: CTGTAAGG CTCCATTTGGGGC (18). These primers were obtained from Sigma-Aldrich Japan (Hokkaido, Japan). The PCR reaction products were electrophoresed through 2% agarose gels containing 0.2 mg/ml ethidium bromide. The PCR product was cut and purified with a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). Products were directory sequenced with the use of a Big-Dye Terminator v3.1 Cycle sequencing kit (Applied Biosystems, Carlsbad, CA, USA) following the manufacturer's instructions. The oligonucleotide sequences of EWSR1–ATF1 fusion gene were then analyzed using a sequencer and the sequences compared with the germline sequences recorded in the GenBank database.

Fluorescence in situ hybridization (FISH) analysis. The FISH analysis for EWSR1 gene rearrangement was performed commercially on RSAR001 cells at the 54th passage by SRL.

Ethics statement. This study was approved by the Ethical Review Committee of Fukushima Medical University (no. 1765). Written informed consent for study inclusion was obtained from the patient and his younger brother. The animal experiments in the present study were approved by the Institutional Animal Care and Use Committee of Fukushima Medical University (no. 25028).

Results

Establishment of the RSAR001 cell line. We established a new human CCS cell line (RSAR001) from the pleural effusion of a patient with multiple metastases of CCS (Figure 2A). The doubling time of this cell line was approximately 35.0 h (Figure 2B). The cell line has been stably maintained for more than 50 passages over a period of more than 12 months. Cultured cells comprised small, polygonal or short-spindle-shaped cells (Figure 3A). Loss of contact inhibition was observed. Immunohistochemically, cultured cells were positive for S-100 protein (Figure 3B), HMB45 (Figure 3C), melan-A (Figure 3D) and NSE (Figure 3E), but negative for AE1/AE3, desmin, and α-SMA (Table I).

Heterotransplantation. The RSAR001 cell line was successfully heterotransplanted into a NOG mouse. The NOG mouse was given a single subcutaneous injection of RSAR001 cells; a tumor with a diameter of approximately 7 mm developed 13 weeks after inoculation. The cut surface of the xenografted tumor was solid and white. Under light microscopy, the heterotransplanted tumor of the NOG mouse showed a morphological pattern similar to that of the primary tumor (metastatic lung tumor) (Figure 4A). The heterotransplanted

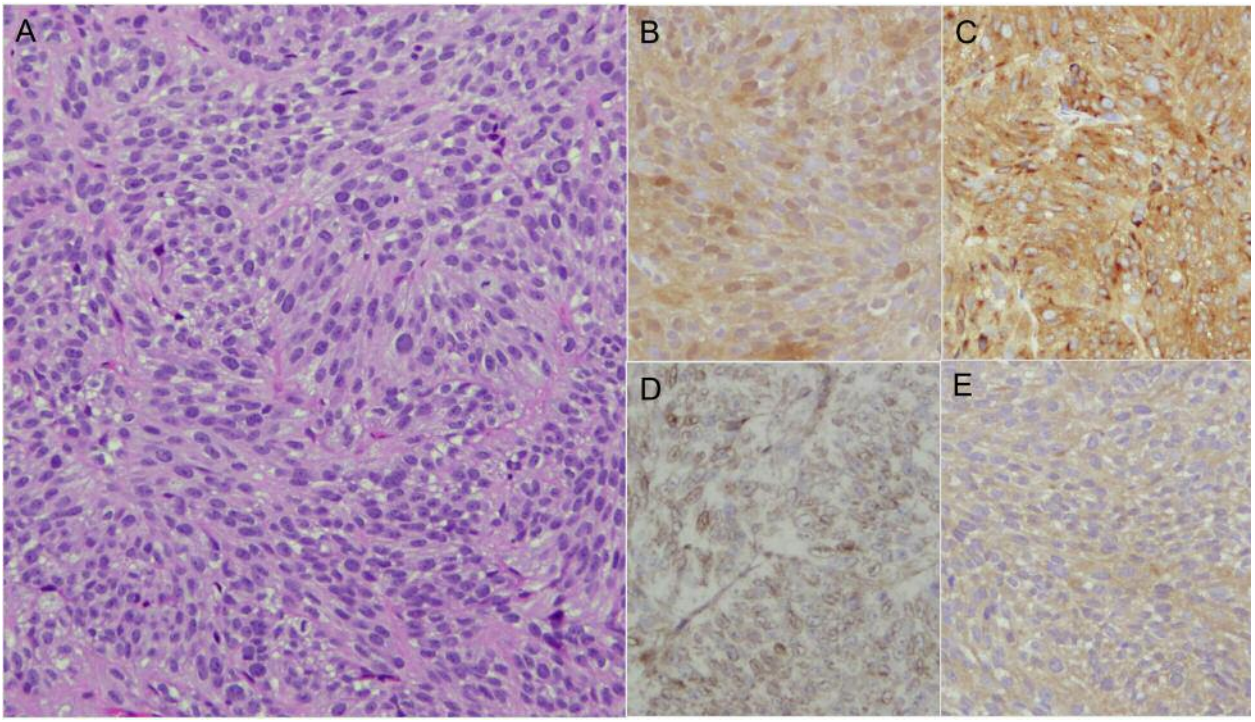

Figure 4. *On microscopic findings of heterotransplanted tumor in the NOG mouse, RSAR001 cells from the NOG mouse exhibited a morphological pattern that was similar to that of the primary tumor (A) (H&E, ×200). RSAR001 cells displayed positive immunohistochemical reaction for S-100 protein (B), human melanin black-45 (C), melan-A (D) and neuron-specific enolase (E) (immunoperoxidase stain, ×200).*

tumor cells exhibited almost the same immunophenotype as that of the primary tumor cells (Figure 4B-E) (Table I).

Cytogenetics. The karyotype analysis of G-banded chromosomes for RSAR001 cells revealed the following composite karyotype: 61<2n> (modal number: 61), XY, +der(1;9)(q10;p10)×2, t(1;2)(p31;p23)×2, +add(3)(p11), ?t(3;17)(p21;q23)×2, +der(7)t(7;8)(p11.2;q11.2)×2, +8, +i(8)(q10), add(9)(p11)×2, t(12;22)(q13;q12), +14, +15, +16, +17, +19, der(22)t(12;22), +mar1, +mar2, +mar3 (Figure 5).

RT-PCR and direct sequencing analyses for specific fusion gene mRNA expression. EWSR1–ATF1 type 1 chimeric fusion gene transcript mRNA, a specific gene for CCS, was detected in the RSAR001 cells (Figure 6A and B).

FISH analysis for split signal of EWSR1 gene. In the RSAR001 cell line, the FISH analysis using a break-apart probe specific to the *EWSR1* gene on 22q12 showed a rearrangement in 100 of 100 nuclei assessed (Figure 6C).

Discussion

We established the RSAR001 cell line from the pleural effusion of a patient with a CCS. RSAR001 cells exhibited typical morphological characteristics of CCS in a heterotransplanted tumor, with diffuse proliferation of short spindle- or oval-shaped cells. The immunohistochemical findings of the RSAR001 cell line both *in vitro* and *in vivo* also showed a pattern that was similar to that of the primary tumor cells. Moreover, several genetic findings showed that the RSAR001 cell line harbored *EWSR1–ATF1* type 1 chimeric fusion gene, which is specific to CCS and is the most common type of *EWSR1–ATF1* chimeric fusion gene (1, 2).

To the best of our knowledge, 15 human CCS cell lines have been reported to date (Table II) (3-16). Among these CCS cell lines, nine harbor *EWSR1–ATF1* type 1 fusion gene (3, 6, 8-10, 13, 14, 16), two harbor type 2 fusion gene (11,15), and one harbors type 3 fusion gene (12). Heterotransplantation into immunodeficient model mice was reported for only three of the previously identified nine cell lines with *EWSR1–ATF1* type 1 fusion gene (3,8,14). The value of RSAR001, the 16th human CCS cell line with *EWSR1–ATF1* type 1, is its utility for both *in vitro* and *in vivo* experiments as well as its rarity.

Most cases of CCS exhibit slowly growing but aggressive behavior. Because they show resistance to multi-agent chemotherapy and radiation therapy, a wide resection with an adequate margin is the only established treatment for CCS at this time (19). Thus, distant metastases, which often appear long after surgery for the primary tumor, may be fatal if they cannot

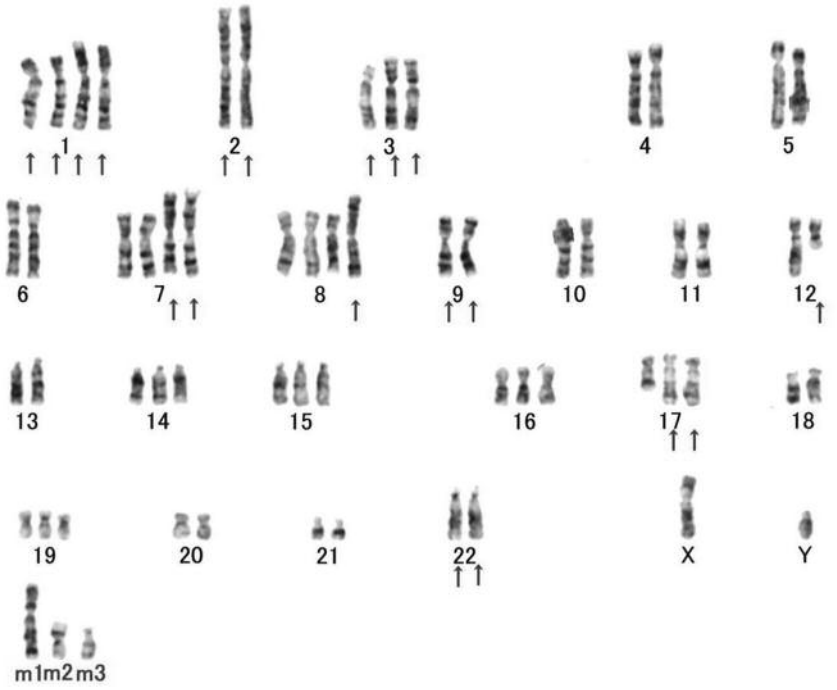

Figure 5. *Representative G-banded karyotype of the RSAR001 cell line at the 54th passage, exhibiting the following abnormal complement: 61<2n> (modal number: 61), XY, +der(1;9)(q10;p10)×2, t(1;2)(p31;p23)×2, +add(3)(p11), ?t(3;17)(p21;q23)×2, +der(7)t(7;8)(p11.2;q11.2)×2, +8, +i(8)(q10), add(9)(p11)×2, t(12;22)(q13;q12), +14, +15, +16, +17, +19, der(22)t(12;22), +mar1, +mar2, +mar3. Arrows indicate the abnormal chromosomes.*

Table II. *Previously established clear-cell sarcoma of soft tissue cell lines.*

No.	Cell line (Ref.)	Patient's age, years/gender	Tumor site	*EWSR1–ATF1*	Heterotransplantation
1	SU-CCS1 (3)	16/F	Heel	Type 1	Yes
2	HS-MM (4)	39/M	Knee	NA	Yes
3	NCS-1 (5)	38/M	Foot	NA	Yes
4	DTC1 (6)	NA	Chest wall	Type 1	NA
5	MST-1 (7)	14/F	Knee	NA	Yes
6	Kao (8)	9/F	Thigh	Type 1	Yes
7	MP-CCS-SY (9)	17/F	Ankle	Type 1	NA
8	GG-62 (10)	25/F	Lower leg	Type 1	NA
9	UM-CCS-1 (11)	60/F	Thigh	Type 2	Yes (*in vivo* only)
10	KAS (12)	25/M	Lymph node metastasis	Type 3	Yes
11	MST2 (13)	60/M	Knee	Type 1	NA
12	MST3 (13)	34/M	Groin	Type 1	NA
13	CCS292 (14)	NA	NA	Type 1	Yes
14	Hewga-CCS (15)	34/F	Toe	Type 2	Yes
15	Senju-CCS (16)	NA	NA	Type 1	NA
16	RSAR001 (present study)	43/M	Pleural effusion	Type 1	Yes

F: Female; M: male; EWSR1– ATF1: Ewing sarcoma breakpoint region 1-activating transcription factor 1; NA, data not available.

be resected surgically. This contributes to the unfavorable 10-year survival rate of CCS compared to the 5-year survival rate.

Recent progression in treatments including molecular targeted therapy and immunotherapy for several malignancies, especially for malignant melanoma (20) which has many similarities to CCS (21-25), has therefore attracted considerable attention for CCS. Although a number of reports indicate the possible efficacy of new therapeutic agents against CCS (23-29), most are case reports of single cases or a very small number of cases. Therefore, establishing the evidence of efficacy of these agents against CCS, both in basic research and clinically are required. The RSAR001 cell line can be expected to be useful for investigating the efficacy of antitumor agents against CCS both *in vitro* and *in vivo*.

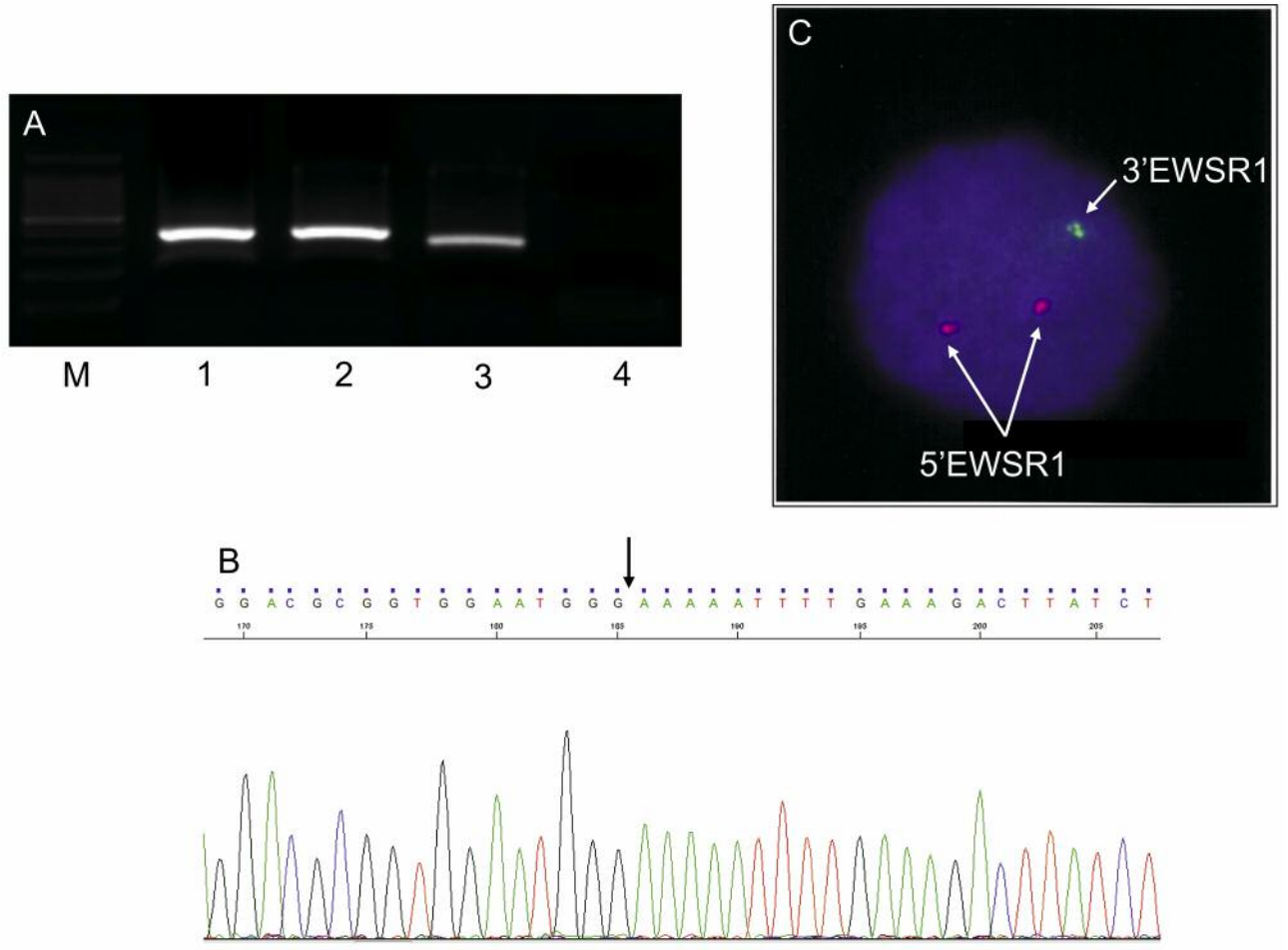

Figure 6. *A: Reverse transcription-polymerase chain reaction amplification of Ewing sarcoma breakpoint region 1–activating transcription factor 1 (EWSR1–ATF1) chimeric fusion gene transcripts from RSAR001 cells. M, Marker (100-bp DNA ladder); lane 1, RSAR001; lane 2, EWSR1–ATF1 type-1 positive control; lane 3, EWSR1–ATF1 type-2 positive control; lane 4, negative control. B: Direct sequencing analysis showed a junction between EWSR1 exon 8 and ATF1 exon 4 (confirming EWSR1–ATF1 type 1). C: Fluorescence in-situ hybridization analysis showed the split signal of the EWSR1 gene in 100/100 cells (arrows).*

Conclusion

The RSAR001 cell line established in the present study will prove useful for investigating biological behaviors and developing new therapies against CCS.

Conflicts of Interest

The Authors state that they have no conflicts of interest to declare in regard to this study.

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