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Newly established clear cell sarcoma (malignant melanoma of soft parts) cell line expressing melanoma-associated Melan-A antigen and overexpressing *C-MYC* oncogene

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Abstract Clear cell sarcoma (CCS), malignant melanoma of soft parts, is a rare malignant tumor with a poor prognosis. In this study, a CCS cell line, designated MP-CCS-SY, was established from a metastatic tumor of a 17-year-old Japanese girl that originated in the left Achilles tendon. A small number of melanosomes were detected in the cytoplasm by electron microscopy. The melanosomes immunoreacted with two melanoma-associated antibodies, HMB45 and Melan-A. A Western blot demonstrated the existence of a Melan-A antigen in this cell line. Although a t(12;22)(q13;q12), which is characteristic of CCS, was not identified by a chromosomal analysis with conventional banding techniques, fluorescence in situ hybridization analysis with painting probes of chromosomes 12 and 22 revealed the insertion of a chromosome 12 fragment into one of the long arms of chromosome 22. The chimeric *EWS/ATF1* transcript was detected by the reverse transcriptase polymerase chain reaction. Extra copies and structural abnormalities of chromosome 8 were observed. Overexpression of c-myc mRNA was detected by Northern blot analysis and may have a role in malignant progression of CCS. The availability of this MP-CCS-SY cell line will help to understand the molecular biology of this malignancy and should be useful as a tool for developing an immunotherapy.

1. Introduction

Clear cell sarcoma (CCS), also known as malignant melanoma of soft parts, was originally described by Enzinger [1]. Clear cell sarcoma is a rare tumor that usually occurs in tendons and aponeuroses of extremities of young adults between 20 and 40 years old. Despite the slow clinical course, CCS tend to recur locally and eventually have a propensity to metastasize after a protracted clinical course. All patients suffering from metastasis finally die of progressive disease.

Histologically, CCS cells contain vesicular nuclei with prominent nucleoli and a clear cytoplasm. Clear cell sarcoma must be distinguished from other tumors with potential clear cell components, but it is difficult to do this without ancillary studies. One way of distinguishing these cells is through immunohistochemistry using a melanoma-associated antibody, HMB45. There is another melanoma-associated antibody, Melan-A, but it is unknown whether it reacts with this tumor. In melanoma, the HMB45 and Melan-A antigens have been utilized not only for diagnosis but for immunotherapy as tumor-associated antigens [2,3].

Cytogenetically, a reciprocal t(12;22)(q13;q12) has been reported as a characteristic chromosomal abnormality in CCS [4]. This chromosomal translocation results in the fusion of the N-terminal region of the Ewing sarcoma oncogene (*EWS*) and the C-terminal region of the gene encoding the activating transcriptional factor 1 (ATF1) [5]. This chimeric *EWS/ATF1* transcript serves as a valuable diagnostic marker for CCS. In addition to the t(12;22)(q13;q12), trisomy 8, which has been described in many tumor types, is commonly seen in CCS tumors and cell lines [6–8]. It is

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possible that trisomy 8 arises because of the selective advantage of cells with an increased dosage of particular genes located on this chromosome. One such gene may be *C-MYC*, which is located on the long arm of chromosome 8.

Established cell lines are potent tools for clarifying the biological and molecular characteristics of the malignancy, and can have clinical applications in terms of making more accurate diagnoses and developing therapeutic strategies. However, only five CCS cell lines are well-documented [9–13].

In this study, a new cell line was established from a metastatic tumor of the left femoral bone of a 17-year-old Japanese girl. The cell line was characterized and confirmed as CCS. This cell line expresses two melanoma-associated antigens, HMB45, and Melan-A, and should be a valuable tool for developing an immunotherapy. Furthermore, using this cell line, we found a possible association of c-myc overexpression with the extremely poor prognosis of CCS.

2. Materials and methods

2.1. Clinical history

A 13-year-old Japanese girl was referred to the Miyazaki Medical College in December 1994 with a tumor on her left tendon that had been growing for the last 8 months. Physical examination and computed tomography scan revealed a tumor, 8×4×2 cm in size, in her left Achilles tendon region (Fig. 1). No other tumor involvement including popliteal or inguinal lymph nodes was revealed. Biopsy of the tumor was performed in January 1995. Spindle, polygonal, and small round tumor cells contained round or oval nuclei with prominent nucleoli and eosinophilic to clear cytoplasm (Fig. 2). Many tumor cells showed positive immunoreaction for Melan-A, and more tumor cells were positive for HMB45. Electron microscopy revealed a small number of melanosomes in the cytoplasm of the tumor cells. From these results, the tumor was diagnosed as a CCS. The patient received four courses of chemotherapy consisting of cyclophosphamide, etoposide, pirarubicin, and cisplatin, and a wide resection of the primary tumor, followed by two courses of pirarubicin and ifosfamide. Furthermore, megatherapy consisting of etoposide, carboplatin, and melphalan with autologous peripheral blood stem cell transplantation was performed in November 1995. In spite of intensive therapy, the disease progressed slowly and the tumor metastasized to the left popliteal lymph nodes, right lung, and left femoral bone. The metastatic tumors were resected and combination chemotherapy with cisplatin, vincristine, pirarubicin, docetaxel, cyclophosphamide, and peplomycin was administered. Although a transient minor response was observed following this treatment, the patient finally expired due to her progressive disease in December 1999.

2.2. Cell culture

A tumor sample for cell culture was obtained from the metastasis of the left distal portion of her femoral bone in May 1999. The tumor tissue was minced and suspended in a RPMI-1640 medium containing penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% heat-inactivated fetal calf serum. The cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. The medium was changed every 3 to 4 days. Upon reaching the confluent state, the monolayers were treated with trypsin and the dispersed cells were transferred into new culture flasks.

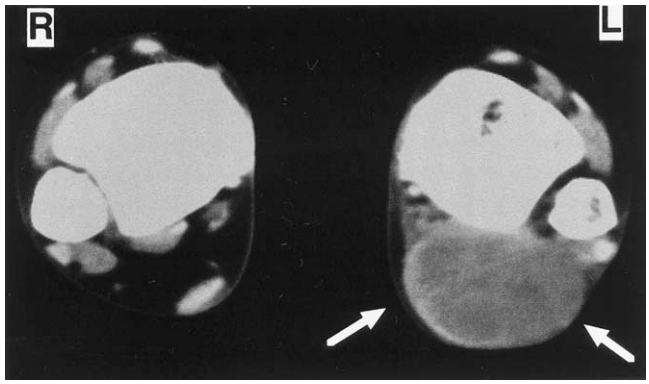

Fig. 1. Enhanced computed tomography scan of the right and left Achilles tendons (transaxial scan). A tumor (46×32 mm) with marginal enhancement on the left Achilles tendon was revealed.

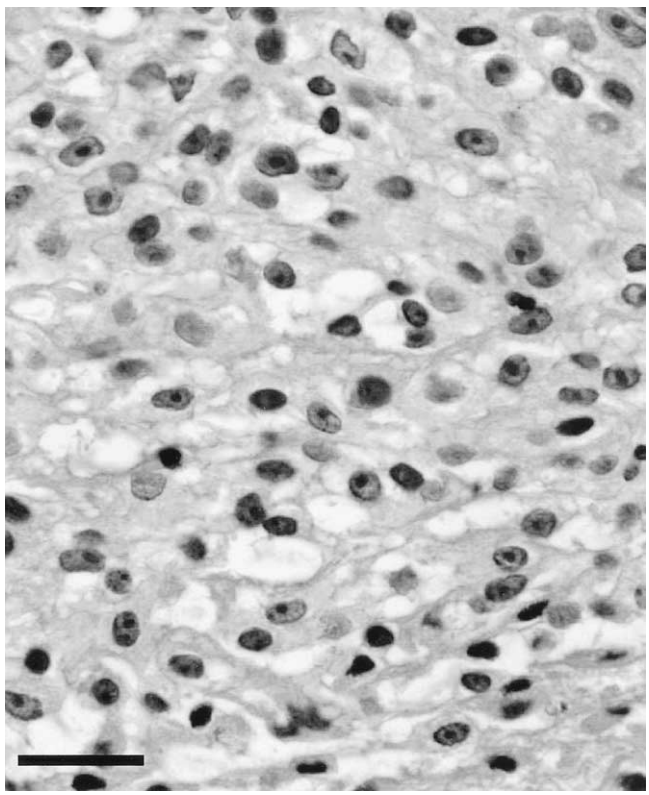

Fig. 2. Histological appearance of the primary tumor. Polygonal and spindle tumor cells proliferated in a solid pattern. These cells had vesicular nuclei and prominent nucleoli (×130). Scale bar: 50 μm.

2.3. Cell lines

One CCS cell line, SU-CCS-1 [9], one melanoma cell line, MeWo [14], and one promyelocytic leukemia cell line, HL60 [15], were cultured and used as controls.

2.4. Electron microscopy

The cultured cells were fixed with a combination fixative containing 4% neutralized formaldehyde and 1% glutaraldehyde for 1 hour at 4°C, and then postfixed in 2% osmium tetroxide for 1 hour at room temperature. The cells were dehydrated through a graded series of ethanol and embedded in Epon 812 using standard procedures. Ultrathin sections were stained with uranium acetate and lead citrate and examined with a JEM-200CX electron microscope (JEOL Ltd., Tokyo, Japan).

2.5. Immunohistochemistry

To test for the expression of melanoma-associated antigens in a tissue sample, two melanoma-associated monoclonal antibodies, HMB45 (Dako Japan, Kyoto, Japan) and Melan-A (Dako Japan) were used. Tissue was fixed in 10% formalin for 18 hours, embedded in paraffin, sliced into 4-μm–thick sections, and examined immunohistochemically with the standard streptavidin-biotin-immunoperoxidase staining method (Histofine SAB-PO kit, Nichirei, Tokyo, Japan). Preimmune mouse IgG was used as a negative control. The HMB45 antigen localizes primarily in premelanosomes [16], while the Melan-A antigen is believed to localize in pigmented melanosomes [17].

2.6. Indirect immunofluorescence for melanoma-associated antigens

Cells were seeded on coverslips and fixed with −20°C methanol. To investigate HMB45 binding, fixed cells were permeabilized by treatment with 0.5% Tween-20 in PBS. They were washed, stained with appropriately titered antibodies, and then observed with an immunofluorescence microscope as previously reported [18]. The melanoma cell line, MeWo, was used as a positive control. Negative controls were also used in all experiments.

2.7. Western blot analysis for expression of Melan-A antigen

Cultured cells were harvested from flasks by a rubber policeman, solubilized by sonication in RIPA lysis buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 1% SDS, 2 mM phenylmethylsulfonyl fluoride, 20 μM leupeptin) at 4°C, and centrifuged at 20,000×g for 10 minutes. The protein concentrations of the supernatants were measured with the Bio-Rad (Hercules, CA, USA) protein assay, and adjusted to the equal. Samples were boiled in sample buffer (2% SDS, 0.25% bromophenol blue) for 2 minutes and electrophoresed in an 8% SDS-polyacrylamide gel as previously reported [18]. After electrophoresis, the gel was transblotted onto a nitrocellulose membrane. The immunoblot was incubated with Melan-A antibody, and then analyzed using the ECL chemiluminescence system (Amersham, Arlington Heights, IL, USA).

2.8. Chromosome and fluorescence in situ hybridization (FISH) analyses

For cytogenetic studies, chromosomes were prepared by standard techniques and analyzed by both trypsin-Giemsa banding and Q-banding as previously described [19,20]. Karyotypes were determined according to the International System for Cytogenetic Nomenclature [19]. We also performed fluorescence in situ hybridization (FISH) analyses with whole chromosome painting probes (chromosomes 8, 10, 12, and 22) in order to define the rearrangements involving chromosomes 12, 22, and other unidentified chromosomes. Chromosome painting with DAPI-PI counterstaining was carried out according to the manufacturer's protocol.

2.9. Detection of chimeric transcript EWS/ATF1 by reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was extracted from the tissue sample and cultured cells using a TRIZOL RNA extraction Kit (GIBCO BRL, Rockville, MD, USA). Two other CCS cell lines, SU-CCS-1 [9] and KAO [13], were used as positive controls. The MeWo melanoma cell line, which is known to have no *EWS/ATF1* transcript, was used as a negative control [21]. Reverse transcription of 1μg of total RNA was carried out using a reaction mixture of a first-strand cDNA synthesis kit according to the manufacturer's instructions (Takara, Kyoto, Japan). Polymerase chain reaction (PCR) amplification was performed in a 50-μl reaction volume containing 10 mM Tris-HCL (pH 8.3), 50 mM KCL, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 μM of forward and reverse primers, and 2.5 U of Taq DNA polymerase (Takara). The sense and antisense primers for the fusion gene were 5′-CCCACTAGTTACCCAC CCCA-3′ and 5′-AAAACTCCACTAGGAAATCCATTT-3′, respectively [5]. Thermal cycling was performed with an initial denaturation at 94°C for 2 minutes, 35 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, and extension at 72°C for 1 minute. The PCR products were electrophoresed in 2% agarose gels in the presence of 0.5 μg/ml of ethidium bromide and visualized by UV irradiation. The β-actin gene was also amplified as a control to demonstrate the integrity of the cDNA after PCR.

2.10. C-MYC gene amplification and expression

High molecular-weight DNA and total RNA were isolated by standard methods. Ten micrograms of DNA digested with restriction enzyme *Eco*RI (Boehringer Mannheim, Mannheim, Germany) was electrophoresed through a 0.8% agarose gel and transferred to a nylon membrane by Southern blotting as previously described [22]. Twenty micrograms of total RNA was electrophoresed through a 1.5% formaldehyde-agarose gel and transferred to a nylon membrane by Northern blotting as previously described [22].

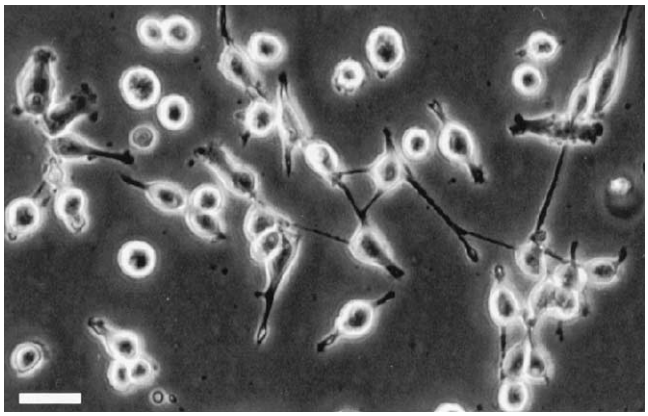

Fig. 3. Phase-contrast microphotograph of the MP-CCS-SY cell line. Small round cells and polygonal, spindle-shaped cells with slender processes are shown (×200). Scale bar: 10 μm.

The membranes were hybridized with a ^{32}P-labeled 660-bp DNA fragment corresponding to the second exon of the *C-MYC* gene. As an internal control, the β-actin gene was also used. The relative intensities of the hybridization signals were quantified by scanning the autoradiograms with a densitometer. The human promyelocytic leukemia cell line HL60, which amplifies and overexpresses c-myc, was used as a positive control [23,24]. Peripheral lymphocytes of the patient were used as a negative control.

3. Results

3.1. Establishment of the MP-CCS-SY cell line

Tumor cells obtained from a metastasis of the left distal femoral bone grew in the form of an adherent monolayer. Two types of cells were obtained, small round cells, and polygonal, spindle cells with slender processes (Fig. 3). The cells have been maintained for more than 60 passages over an 18-month period. This new cell line was named MP-CCS-SY. The doubling time of the cultured cells is 36 hours.

3.2. Electron microscopy of the MP-CCS-SY cell line

The ultrastructure of the MP-CCS-SY cells showed abundant cytoplasm and large irregular nuclei with prominent nucleoli. Many round to oval mitochondria were present. Glycogen particles were observed in most of the cells with a patchy distribution in the cytoplasm. The cells also contained dispersed melanosomes in various stages of development (Fig. 4).

3.3. Indirect immunofluorescence for melanoma-associated antigens

The MP-CCS-SY cell line reacted with two antibodies (HMB45 and Melan-A), which produced granular and cytoplasmic staining patterns (Fig. 5). The staining patterns were similar to those observed in the melanoma cell line MeWo.

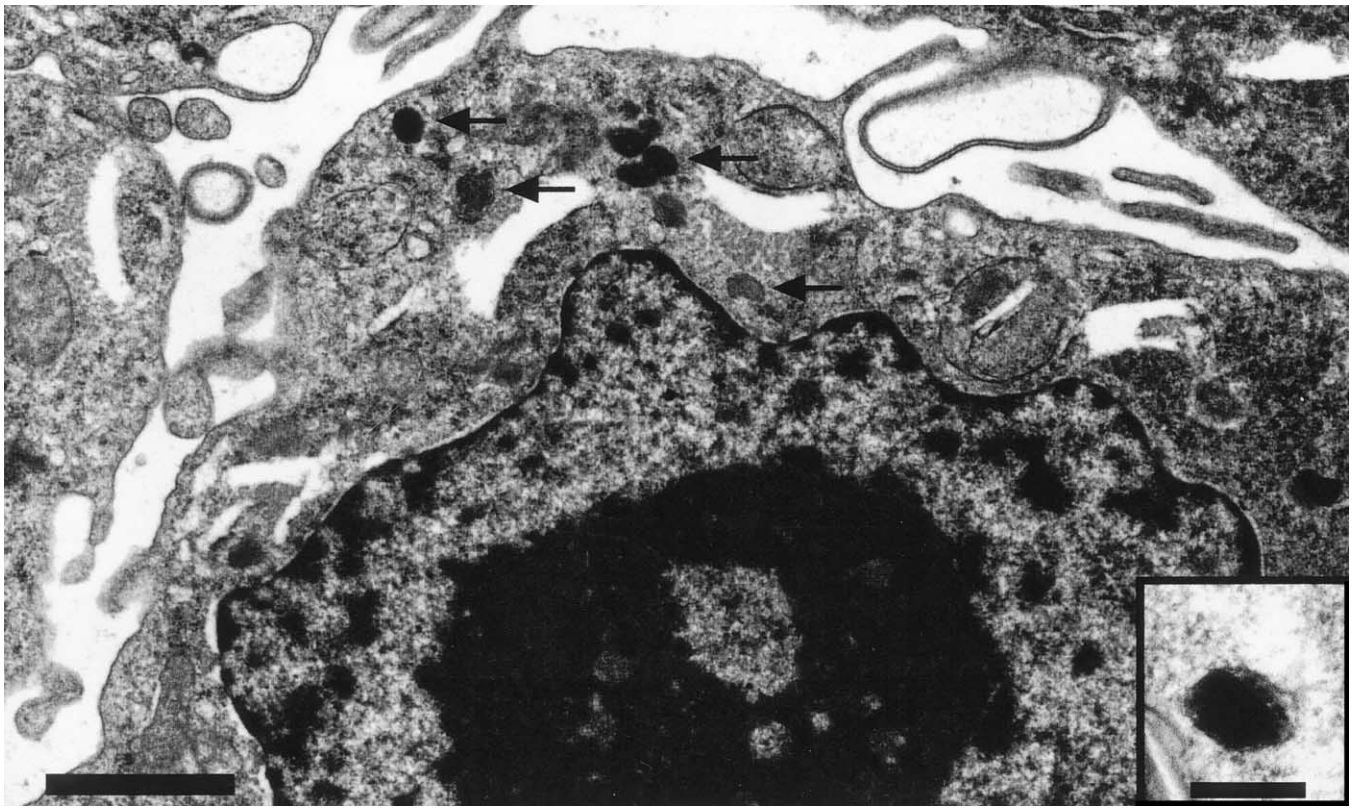

Fig. 4. Electron microphotograph of the MP-CCS-SY cell line. The cytoplasm contained melanosomes (arrows) in various developmental stages (×8000). Scale bar: 1 μm. Inset: As shown here, mature melanosomes were seen in this cell (×25,000). Scale bar: 200 nm.

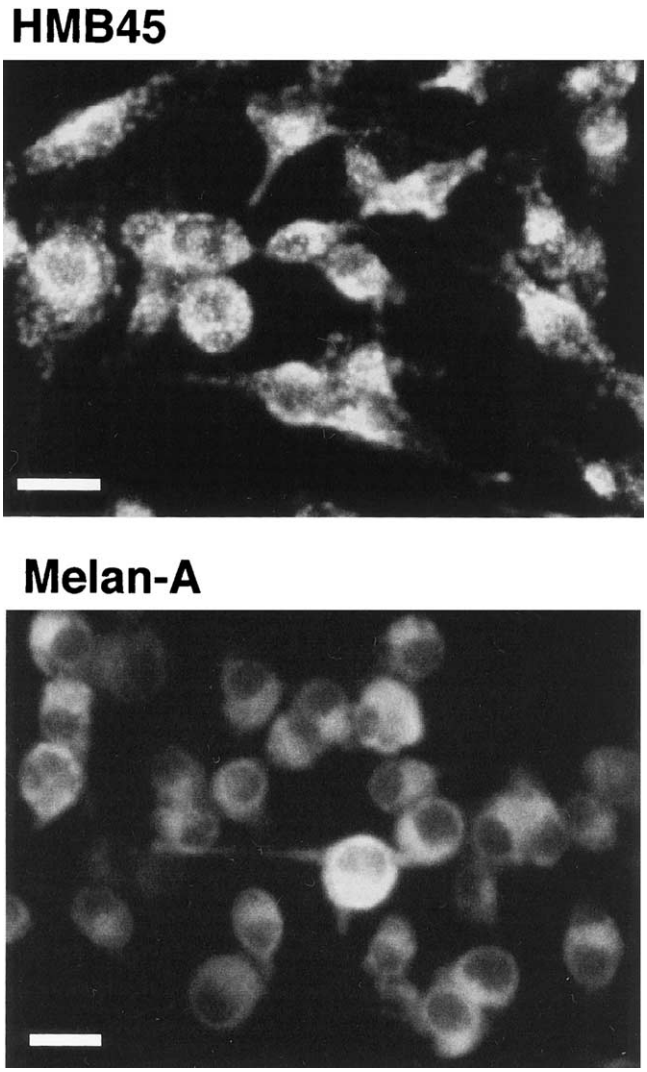

Fig. 5. Indirect immunofluorescence of the MP-CCS-SY cell line with HMB45 and Melan-A antibodies. Most MP-CCS-SY cells reacted with both antibodies, demonstrating granular and cytoplasmic staining patterns (×400). Scale bar: 10 μm.

One of the established CCS cell lines, SU-CCS-1, had a positive reaction to HMB45, but a negative reaction to Melan-A (data not shown).

3.4. Western blot analysis of Melan-A protein expression

A 20-kDa melanoma-associated Melan-A antigen was found in the MP-CCS-SY cells and, as expected, in the MeWo cells, but not in SU-CCS-1 cells (Fig. 6). These results are consistent with the indirect immunofluorescence data.

3.5. Chromosome and FISH analyses

A chromosome analysis was performed after four times transfer in vitro. A total of 32 metaphase cells from MP-CCS-SY cells were examined by both the G- and Q-banding methods. The modal chromosome number was 50 and structural abnormalities involving chromosomes 1, 2, 7, 8, 9, 10, 12, 13, 15, and 17 were clonally observed in almost all the metaphases analyzed. In particular, an unbalanced translocation between chromosomes 1 and 2 resulted in loss of one of the short arms of chromosome 1 and partial trisomy of a long arm of chromosome 2. Trisomy of chromosome 7, partial trisomy for a long arm of chromosome 1, and partial tetrasomy of chromosome 8 were also identified as characteristic numerical changes. An additional fragment, which may have been due to a translocation, was observed on a short arm of chromosomes 7, 13, and 15. A reciprocal transloca-

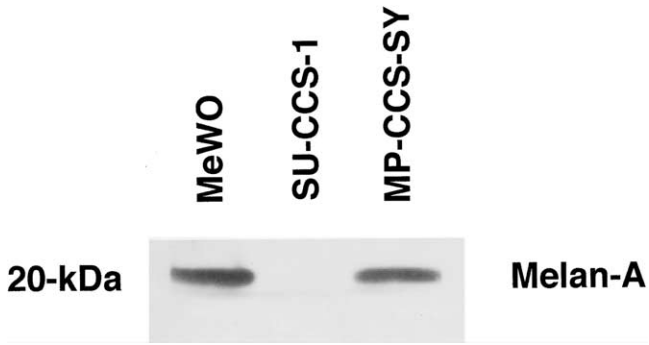

Fig. 6. Western blot analysis of the MP-CCS-SY cell line using the Melan-A antibody. An immunoreactive band (20-kDa) was detected in the MP-CCS-SY cells. The MeWo cell line was used as a positive control.

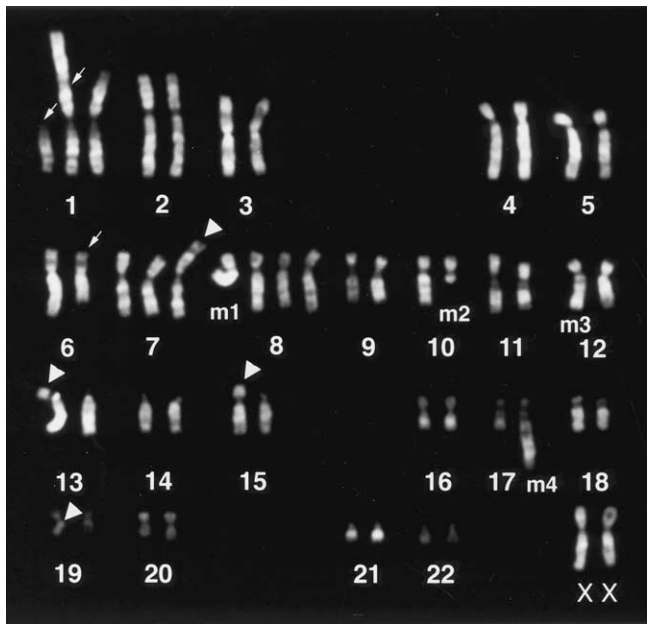

Fig. 7. A representative Q-banded karyotype of the MP-CCS-SY cell line: 50,XX,+del(1)(p11),der(1)t(1;2)(p34;q13),del(6)(q23),+add(7)(p22),+8, +der(8)t(8;10)(q22;?),del(9)(p22),der(9)t(9;12)(p22;),del(10)(q21),der(12) t(10;12)(q15;?),add(13)(p11),add(15)(p11),der(17)t(10;12;17)(?;?;q25),der(19) t(19;?)(q13.3;?). Arrows indicate chromosomal abnormalities whose origin was determined, and arrowheads indicate structural changes with unknown segments. The chromosomes with structural changes, designated m1, m2, m3, and m4, were defined by fluorescence in situ hybridization analyses. This metaphase cell also showed additional structural abnormalities in chromosomes 6 and 19.

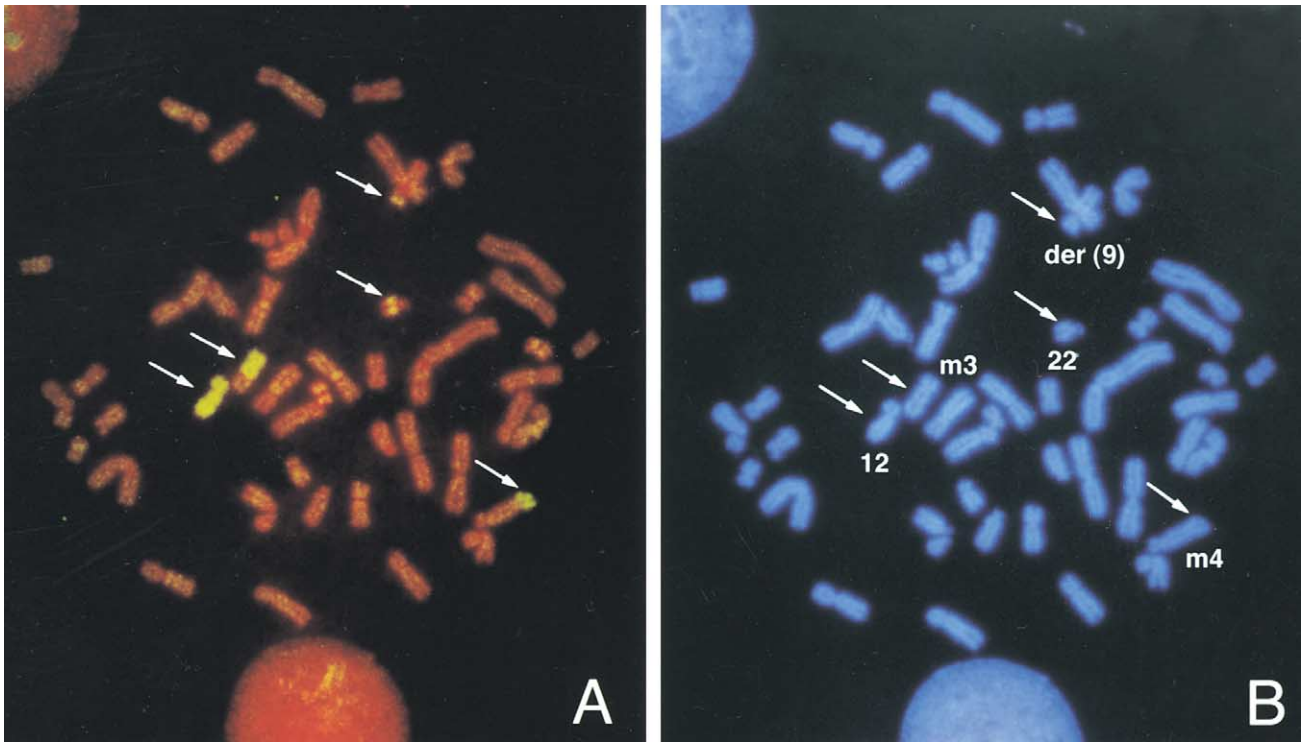

Fig. 8. (A) Fluorescence in situ hybridization of MP-CCS-SY cell line with a whole chromosome 12 painting probe. Arrows indicate signals of segments from chromosome 12. (B) DAPI-banded chromosomes of the same metaphase indicated in (A).

tion between chromosomes 12 and 22, which is a chromosome change that occurs specifically in CCS, and which leads to fusion of the *EWS* and *ATF-1* genes, was not detected in the chromosomes with the banding methods. Therefore, using the FISH technique with whole chromosome painting probes of chromosomes 12 and 22, we identified the insertion of the chromosome 12 fragment into one of the long arms of chromosome 22 (22q12∼q13). The signals from the chromosome 12 painting probe were also detected in the terminal region of a rearranged short arm of chromosome 9, in the extra region of one of the long arms of chromosome 17 (m4) and on one of the unidentified chromosomes (m3) except for a normal chromosome 12 (Fig. 7). No rearrangement was identified in FISH with the chromosome 22 painting probe. In order to define the origin of the rearranged chromosomes, the cells were subjected to FISH with painting probes for chromosomes 8 and 10. We detected the signals of chromosome 10 at the terminal region on the long arm of derivative chromosome 8 (m1). An additional region on one of the long arms of chromosome 17 consisted of fragments of chromosomes 10 and 12 (m3). Furthermore, the smallest marker derived from chromosome 10 (m2) and the other marker consists of regions of chromosomes 8 and 12 (m3). The additional segments on one of the short arms of chromosomes 7, 13, and 15 have not been determined yet. As a result, the representative karyotype including the results of FISH analyses is 50,XX,+del(1)(p11),der(1)t(1;2)(p34;q13),+add(7)(p22),+8,+der(8)t(8;10)(q22;?),del(9)(p22),der(9)t(9;12)(p22;?),del(10)(q21),der(12)t(10;12)(q15;?),add(13)(p11),add(15)(p11),der(17)t(10;12;17)(?;?;q25). Some mitotic cells also showed nonclonal structural abnormalities (Fig. 8).

3.6. Detection of chimeric transcript EWS/ATF1 by RT-PCR

EWS/ATF1 chimeric transcripts were detected in the metastatic tumor tissue obtained from the femoral bone of the patient and the MP-CCS-SY cell line (Fig. 9). Sequencing of the amplified DNA revealed the same in-frame junction as previously described [5]. In contrast, no PCR product was detected in the melanoma cell line MeWo.

3.7. C-MYC gene amplification and expression

Southern blot analysis revealed that the *C-MYC* gene was not amplified in the metastatic tumor tissue of the femoral bone and MP-CCS-SY cells. However, c-myc mRNA was definitively overexpressed in the metastatic tumor tissue, the MP-CCS-SY cell line, and the HL60 cell line, but not in the patient's peripheral blood cells (Fig. 10).

4. Discussion

The MP-CCS-SY cell line was established from the bone metastatic tissue of a 17-year-old girl, who presented with relapsed CCS. The cells were identified as CCS by their microscopic and electron microscopic appearance, their reaction to melanoma-associated HMB45 antibody by indi-

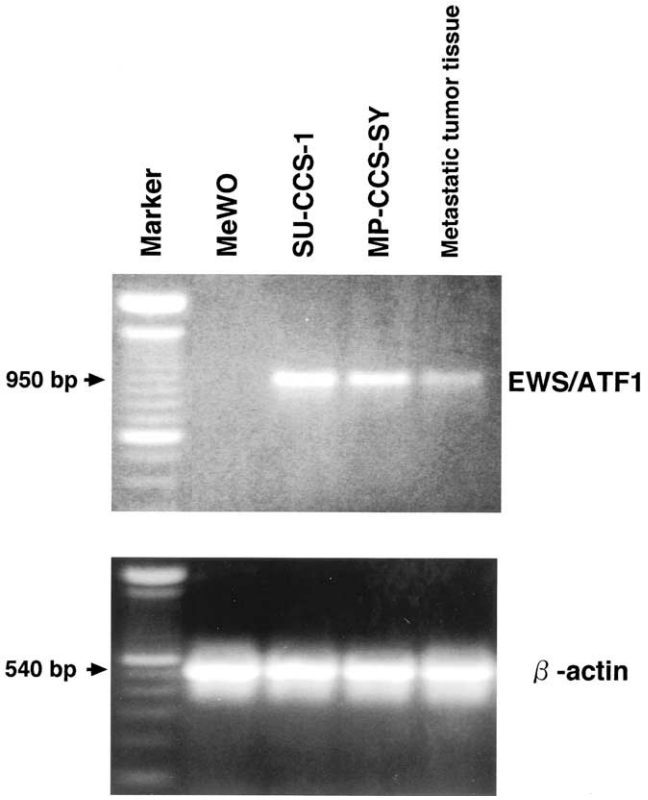

Fig. 9. Detection of the EWS/ATF-1 transcript in the MP-CCS-SY cell line by RT-PCR. The MP-CCS-SY cells had an EWS/ATF-1 transcript of approximately 950 bp. The metastatic tumor from which this cell line was established had the chimeric transcript. The SU-CCS-1 cell line was used as a positive control. The amplification of β-actin was observed in all samples confirming the RNA quality.

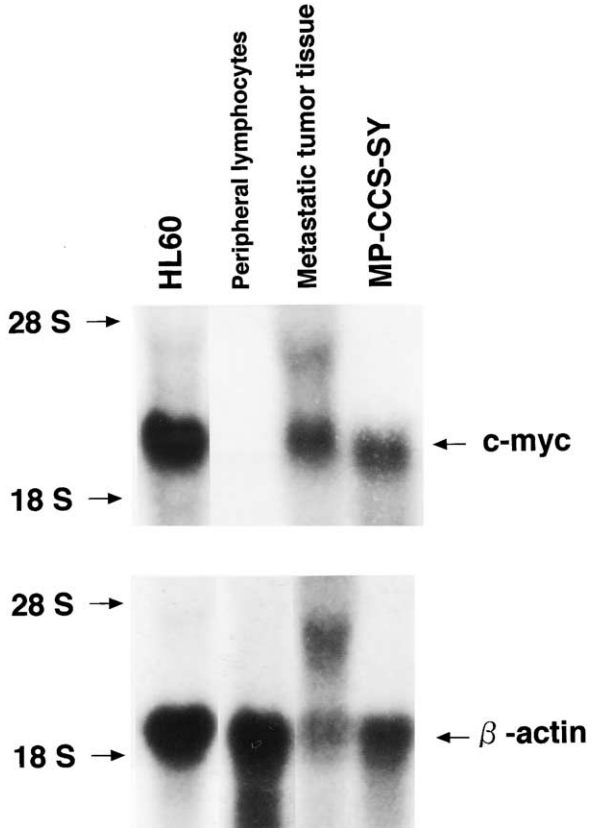

Fig. 10. c-myc expression. c-myc mRNA of the metastatic tumor and the MP-CCS-SY cell line was greatly overexpressed, as compared with the negative control. The β-actin gene was used for an internal control.

rect immunofluorescence, and the existence of the chimeric EWS/ATF-1 transcript. This is the sixth well-documented CCS cell line.

Diagnosis of CCS by only conventional morphological studies is difficult. Detection of melanosomes by electron microscopy, which supports the histogenesis of CCS form the neural crest, is useful for an accurate diagnosis. Melanosomes in various developmental stages are found in CCS. Mature melanosomes were observed in the MP-CCS-SY cells (Fig. 4), but not in another CCS cell line, SU-CCS-1 [9]. The appearance of melanosomes may reflect the maturation stage of different CCS.

The HMB45 antigen is a transmembrane protein that localizes in premelanosomes. A positive immunoreactivity with HMB45 is helpful in distinguishing CCS from other tumors, such as synovial sarcoma, malignant peripheral nerve sheath tumor, metastatic round cell carcinoma, and others. The presence of this antigen was determined by indirect immunofluorescence in the MP-CCS-SY cells (Fig. 5). Another melanoma-associated antigen, Melan-A antigen, is also a transmembrane protein, which is believed to localize in pigmented melanosomes. The presence of Melan-A antigen, which has never been studied in CCS, was demonstrated in the MP-CCS-SY cells by both indirect immunofluorescence (Fig. 5) and Western blot analysis (Fig. 6). The different reactivities of the MP-CCS-SY and SU-CCS-1 cells to Melan-A antibody may be due to the distinct differentiation stages of the melanosomes. In cutaneous malignant melanoma, the expression of melanoma-associated antigens (i.e., immunoreactivity with HMB45 and Melan-A) was reported to be lost with tumor progression [25]. Further studies are needed to determine whether the different expressions of these melanoma-associated antigens in CCS are related to the prognosis.

These melanoma-associated antigens are recognized by cytotoxic T lymphocytes [26,27] and are currently being investigated for their potential for immunotherapy against melanoma. Rosenberg et al. [2] reported that vaccination with a modified analogue of an HLA-A2-binding peptide of HMB45 induced tumor regression in 42% of patients with metastatic melanoma. In the same way, these melanoma-associated antigens may have applications in the immunotherapy of CCS.

The differential diagnosis of CCS only by morphological and immunohistochemical studies is still difficult, especially in patients with metastatic malignant melanoma from an unknown primary site. In these cases, genetic studies can lead to an accurate diagnosis. In a previous review [28], the 12;22 translocation was identified in 60–75% of CCS cases. However, none of the cutaneous malignant melanomas revealed

this chromosomal abnormality [8,28]. These results suggest that the cases with this translocation may be diagnosed as CCS and distinguished from cutaneous malignant melanoma. This translocation generates an *EWS/ATF-1* chimeric transcript, which is comprised of the N-terminal domain of the *EWS* gene and the C-terminal domain of the *ATF-1* gene [5]. In the present study, the 12;22 translocation was not identified in the MP-CCS-SY cells by conventional banding techniques. However, the FISH analyses with whole chromosome painting probes revealed the insertion of a chromosome 12 fragment into one of the long arms of chromosome 22 (22q12∼q13). The CCS cases without the 12;22 translocation may have submicroscopic changes such as the insertion confirmed in our present case. Therefore, it will be necessary to confirm the chimeric transcript and submicroscopic chromosome abnormality using molecular cytogenetic techniques. In the present study, one possible explanation for the results of the FISH analyses is that the insertion resulted in the production of the *EWS* and *ATF-1* chimeric transcript.

In fact, using RT-PCR, the chimeric transcript was detected in the MP-CCS-SY cells, indicating that detection of this chimeric gene is a more sensitive diagnostic method (Fig. 9). The resulting chimeric protein probably functions as a constitutive transcriptional activator and is likely to initiate CCS tumorigenesis [29,30]. However, the genetic aberrations related to malignant progression of CCS have not yet been elucidated.

Of the karyotypic changes observed in the MP-CCS-SY cell line, the numerical abnormality of chromosome 8 has been detected in more than one half of CCS cases [6–8]. Extra copies of chromosome 8 have been frequently reported in hematological disorders, such as chronic myeloid leukemia, acute myeloid leukemia, and myelodysplastic syndrome [31,32]. Extra copies of chromosome 8 have also been frequently observed in various malignant solid tumors, such as Ewing sarcoma, rhabdomyosarcoma, and breast cancer [33–35]. It is possible that these additional copies of the chromosomes increase the dosage of particular genes located on this chromosome.

One candidate gene is *C-MYC*, which is located on the long arm of chromosome 8. This is because *C-MYC* has been clearly associated with malignant progression in chronic myeloid leukemia [36], breast cancer [37], and Ewing sarcoma [38]. We didn't observe amplification of the *C-MYC* gene in the MP-CCS-SY cells by Southern blot analysis (data not shown), but we did detect a high level of c-myc mRNA by Northern blot analysis. This could be due to an increase of copy number, rearrangement of a long arm of chromosome 8, or influence by up-regulator of the *C-MYC* gene, such as T-cell factor-4 gene [39]. Since the c-myc mRNA was also overexpressed in the metastatic tumor of the patient's femoral bone, the possibility that this occurred during in vitro culture can be excluded. The overexpression of c-myc in CCS cells has not been previously reported. There may be a strong association between c-myc overexpression and malignant progression in CCS.

Thus, the MP-CCS-SY cell line can be useful for clarifying the biology of CCS, and as a tool for developing therapeutic strategies including immunotherapy of this rare and usually fatal malignancy.

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