

## Cellular and Molecular Characteristics of Established Childhood Soft-Tissue Sarcoma Cell Lines

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### Abstract

From 1984 to 2013, we established 29 childhood tumor cell lines, including 13 soft-tissue-sarcoma cell lines. Here, we provide an overview of these soft-tissue sarcoma cell lines, their origins, characteristics, and highlight their potential as valuable research tools for fundamental research and development of new treatments. The cell lines were established from three patients with rhabdomyosarcoma (RMS), five patients with Ewing sarcoma family tumors, four patients with rhabdoid tumors, and one patient with clear cell sarcoma of soft parts. In particular, we demonstrate the potential of the MRT cell lines in preclinical in vivo and in vitro studies to evaluate molecular target therapy using gefitinib and trastuzumab activated antibody-dependent cellular cytotoxicity. Moreover, the clear cell sarcoma cell line, MP-CCS-SY, was established from a metastatic tumor in the left Achilles tendon of a 17-year-old girl, which represents a rare cell line for this cancer, and will help to gain a better understanding of the molecular biology of this malignancy and serve as a useful tool for developing boron neutron capture therapy.

**Keywords:** Soft-tissue-sarcomas; Rhabdomyosarcoma; Ewing sarcoma family tumor; Malignant rhabdoid tumor; Clear cell sarcoma of soft parts; Clinical characteristics.

**Abbreviations:** RMS: rhabdomyosarcoma; PNET: peripheral primitive neuroectodermal tumor; ESFT: Ewing sarcoma family of tumor; MRT: Malignant rhabdoid tumor; CCS: clear cell sarcoma of soft parts

## Introduction

Soft-tissue sarcomas originate from immature embryonal mesoderm or mesenchymal tissues, and can therefore potentially develop from any anatomical site. Recent advances in progressive therapy have led to a high cure rate for soft-tissue sarcomas with combinations of chemotherapy, surgery, radiation therapy, and supportive therapy. In addition, chromosomal analysis and more recent advanced molecular biological techniques that can identify chromosomal aberrations have improved both the diagnosis and prognosis of the disease, along with insight into the underlying mechanism of tumor biology.

In the last 30 years, from 1984 to 2013, we have established 29 tumor cell lines from children, including 13 soft-tissue sarcoma cell lines at three Institutes (SCMS, KP and MP). Here, we established from children with [1] rhabdomyosarcomas (RMS), [2] Ewing sarcoma family tumors (EW), [3] malignant rhabdoid tumors (MRT), and [4] clear cell sarcoma of soft parts (CCS) (Table 1). The goal of this review is to further highlight the utility and potential of these soft-tissue sarcoma cell lines to improve understanding of basic and clinical aspects of pediatric oncology.

No	Cell line	Age/gen-der	Primary tumor	Stage	Culture Sample	Out- come	References
Rhadomyosarcoma							
1	SCMC-RM2	10y/F	Rt-abdominal wall	IV	Bone marrow	Dead	11
2	KP-RMS-DH	14y/M	Lt-mandibular cavity	IV	Bone marrow	Dead	Unpublished
3	KP-RMS-KH	14/F	Perinea	IV	Primary tumor	Dead	Unpublished
Ewing sarcoma family tumor							
1	KP-EW-YI	16y/M	Rt-pelvis	II	Primary tumor	Dead	17
2	KP-EW-MS	16y/F	Rt-breech	IV	Bone marrow	Dead	17
3	MP-ASKIN-SA	13y/M	Lt-chest	II	Primary tumor	Dead	18
4	KP-PNET-TO	15y/M	Lt-kidney	IV	Lt-iliac	Dead	Unpublished
5	KP-EW-AK	15y/M	Lt-scapula	IV	Primary tumor	Dead	Unpublished
Malignant rhabdoid tumor							
1	KP-MRT-NS	2m/F	Lt-kidney	II	Ascites	Dead	19, 20, 24
2	KP-MRT-RY	1m/M	Lt-kidney	I	Primary tumor	Dead	19, 20, 22, 23
3	MP-MRT-AN	3m/F	liver	IV	Peripheral blood	Dead	20, 21, 24
4	KP-MRT-YM	5m/M	Rt-chest wall	I	Primary tumor	Alive	20, 22, 23
Clear cell sarcoma of soft parts							
1	MP-CCS-SY	13y/F	Lt-Achillis	II	Lt-popliteall fossa	Dead	26, 28

Table 1: Our established soft-tissue-sarcoma cell lines from children

## Establishment of 13 soft-tissue sarcoma cell lines

The idea for establishing a cell line was born in 1983, when one of the authors (TS) was a clinical research fellow at Cell Culture Laboratory, Department of Molecular Biology, Roswell Park Memorial Institute (Buffalo, NY, USA), as establishment of tumor cell lines was considered to be an important mark of a researcher at that time. Here, we focus on the cell lines established for soft-tissue sarcomas with the ultimate goal to unravel novel diagnostic or therapeutic methods.

Tumor samples from patients for cell culture were obtained by biopsy, operation, or autopsy and were finely minced with a scalpel and cultured. Mononuclear cell fractions from the bone marrow or peripheral metastatic cells were prepared by Ficoll-Hypaque centrifugation. The cells were cultured in RPMI 1640 medium containing penicillin (100 U/ml), streptomycin (100 µg/ml), and 15% heated-inactivated fetal calf serum at 37°C in a 5% CO<sub>2</sub> atmosphere. The medium was replaced every 3–4 days. Cell lines were considered to be established when they were cultured for more than 60 passages over a 2-year period [1].

## RMS

### Incidence and pathological findings in RMS

RMS is derived from the immature embryological mesoderm or mesenchymal tissues during the formation of skeletal muscle cells, and is the most common soft-tissue sarcoma (50%), accounting for 5% of all childhood cancers. RMS can be classified into five groups: embryonal, botryoid, spindle, alveolar, and pleomorphic, as per the 1992 World Health Organization Classification of Soft Tissue Tumors [2]. Antibodies against skeletal muscle-specific proteins (e.g., desmin, alpha- and gamma-muscle actin, alpha-sarcomeric actin) are effective for the immunofluorescence-based diagnosis of RMS [3,4]

### Expression of *MyoD1* in RMS cell lines

Myoblast determination protein 1 (*MyoD1*) expression has been found to be limited to RMS, and is considered to be responsible for the lack of differentiation to mature skeletal muscle cells [5–7]. *MyoD1* was confirmed to be expressed in our RMS cell lines (RD, SCMC-RM2, KP-RMS-KH, KP-RMS-DH, SCMC-MM-1 series, and SMS-CTR series), and was not expressed in our neuroblastoma cells and other cell lines, including Wilms tumors and Ewing sarcoma cell lines. The positive expression of *MyoD1* mRNA specific to the RMS cell lines confirmed the diag-

nosis of RMS in these patients (Figure 1).

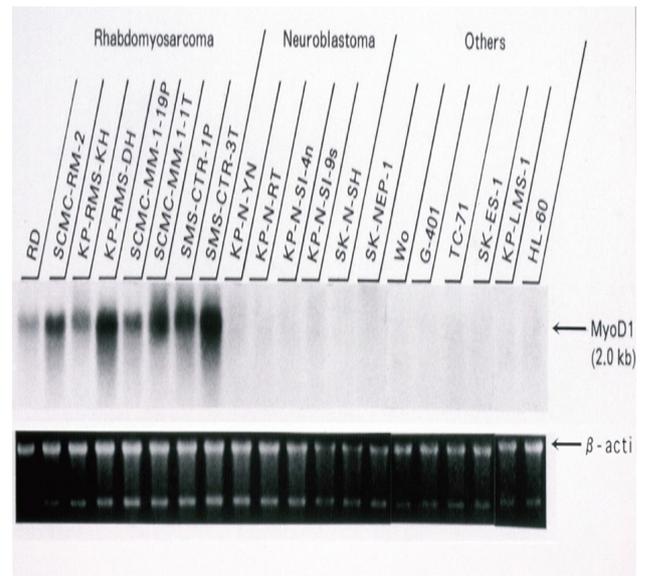


Figure. 1 Expression of *MyoD1* in rhabdomyosarcoma, neuroblastoma, and other tumor cell lines

### *PAX3/FKHR* chimera product in alveolar RMS cell lines

One of the molecular characteristics of alveolar RMS is the translocation  $t(2;13)(q35;q14)$ , which results in the *PAX3/FKHR* chimera product, whereas the translocation  $t(1;13)(p36;q14)$  results in the *PAX7/FKHR* chimera product. Indeed, the former translocation and *PAX3/FKHR* chimera were confirmed in our alveolar RMS cell lines (Figure 2), whereas the latter was not detected in any of the cell lines. Therefore, based on a reverse transcription-polymerase chain reaction (RT-PCR) results, alveolar RMS was confirmed in two of our cell lines (KP-RMS-DH and SCMC-RM2). Thus, using recent molecular biology techniques, we were able to confirm the presence of the *PAX3/FKHR* chimera gene to identify and diagnose alveolar RMS more specifically [8, 9].

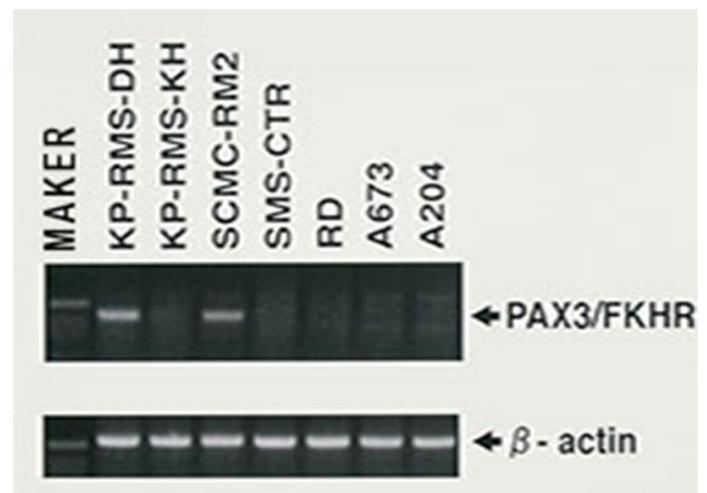


Figure. 2 *PAX3/FKHR* chimera gene in alveolar rhabdomyosarcoma cell lines

## MYCN amplification in RMS cell lines

In September 1985, a tumor on the right abdominal wall was observed in an 11-year-old female. A cell line designated SCMC-RM2 was then established from her bone marrow cells, and transferred to our department for further cellular and biological characterization.

Cytoplasmic protein analysis demonstrated a myogenic origin, with the expression of desmin (Figure 3B), myoglobin, alpha- and gamma-muscle actin (using the HHF35 monoclonal antibody) (Figure 3C), *MyoD1* mRNA (Figure 1), and alpha-sarcomeric actin [3,4].

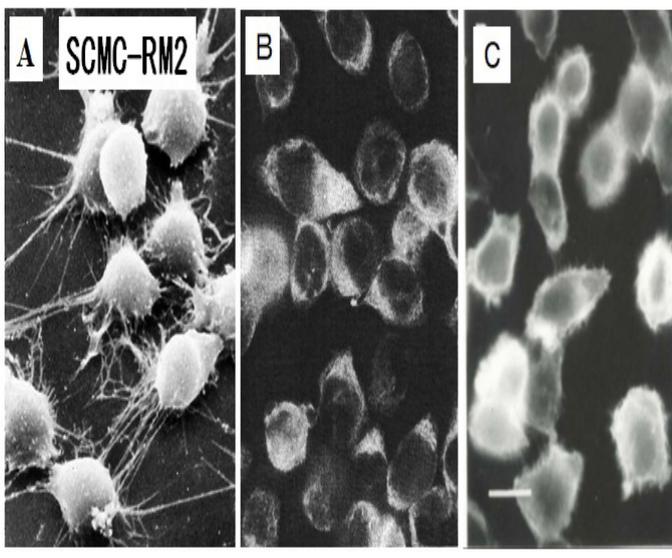


Figure 3 Cytoskeletal protein of SCMC-RM2 rhabdomyosarcoma cell line

According to Barr et al. [8], the *PAX3/FKHR* chimera product does not only indicate “an embryonal RMS cell line” as outlined in our previous study [11] but can also refer to alveolar RMS, as highlighted in the example described above (Figure 2).

Moreover, amplification of the N-Myc proto-oncogene *MYCN* has generally been considered to be related to neuroblastoma [10]; however, *MNYC* amplification and expression were observed in the SCMC-RM2 cell line with 8- and 7-fold elevation as detected by the HL60 cell line in Southern blotting (Figure 4A). Northern blots for detection of *MYCN* mRNA further revealed approximately 1/5 the *MYCN* expression in the SCMC-RM2 cell line as compared with the KP-N-RT neuroblastoma cell line (Figure 4B) [11]. This SCMC-RM2 cell line represented one of the first RMS cell lines showing *MNYC* amplification and overexpression. This finding has led to several studies examining the relationship between *MNYC* copy number and expression in RMS, along with potential associations with an adverse prognosis in the alveolar subtype [12–16].

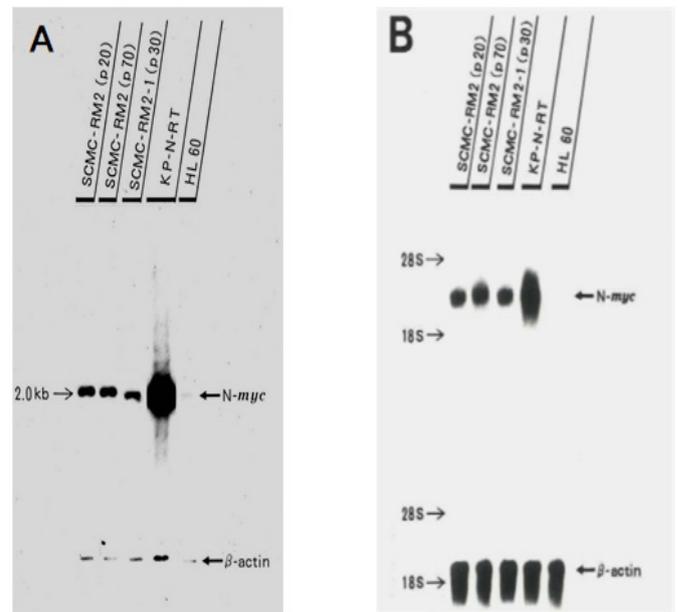


Figure 4 *MYCN*-amplification (a) and expression (b) in SCMC-RM2 rhabdomyosarcoma cell line

## Ewing sarcoma family tumor

Classical Ewing sarcoma of the bone, extraskeletal Ewing sarcoma (ES), Askin tumor of the thoracic wall, and peripheral primitive neuroectodermal tumor (PNET), also known as peripheral neuroepithelioma, are small, blue, round tumors that are highly aggressive, and poorly differentiated with unknown histogenesis. Such tumors are classified as part of the Ewing sarcoma family of tumors (ESFT) based on molecular evidence demonstrating shared immunological and genetic traits.

### *EWS/FLI1* chimeric gene in established EW sarcoma cell lines

The *t* (11;22) (*q24;q12*) and *t* (21;22) (*q22;q12*) chromosomal translocations are specific to the ESFT, including ES, PNET, and Askin tumors [17].

We previously reported two types of *EWS/ERG* chimeric genes in two ES cell lines in addition to six types of *EWS/FLI-1* chimeric genes in 10 ES cell lines, including KP-EW-YI and KP-EW-MS [17].

However, we did not find a clear association between the type of chimeric mRNA and clinical features such as sex, age, primary site, and histopathology of the patients. All of the chimeric mRNAs were generated from in-frame junctions and are considered to encode fusion proteins that may be involved in the molecular mechanism underlying the progression of ESFT [17].

## C-Myc and focal adhesion kinase (FAK) genes in the MRT cell lines

Askin tumor is a malignant small, round-cell tumor that originates from the thoraco pulmonary region and is a member of the ESFT. The MP-ASKIN-SA cell line was established from a tumor identified in the left-posterior pulmonary cavity of a 13-year-old boy (Figure 5, Table 1). The MP-ASKIN-SA cell line was identified by the presence of *EWS/ERG* fusion mRNA with RT-PCR (Figure 6A). Moreover, high expression of C-Myc (which is associated with enhanced cell growth and proliferation; (Figure 6B) and overexpression of the FAK gene (associated with focal adhesion formation and cell migration) were detected in these cells, which appear to play a role in the poor prognosis of patients with ESFT [18].

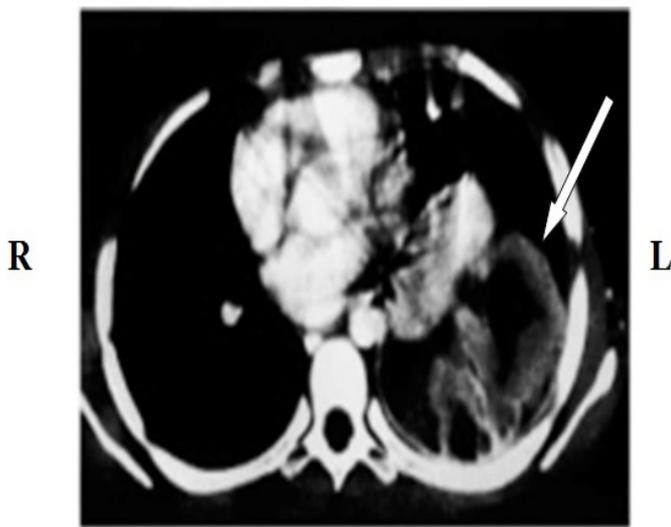


Figure. 5 Establishment of the MP-ASKIN-SA cell line from the left pulmonary thoracopulmonary tumor from a 13 yr-old boy

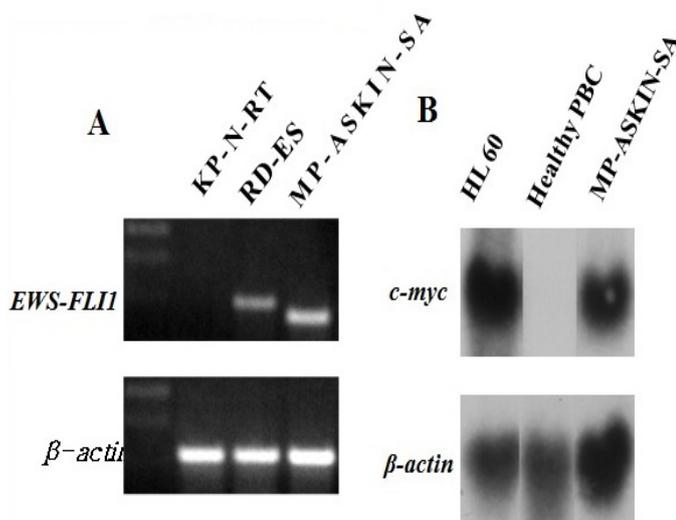


Figure. 6 *EWS/FLI1* chimera gene (a) and *c-myc* expression (b) in the MP-ASKIN-SA cell line

## MRT cell lines

### MRT

MRT was first described as a variant of Wilms' tumor of the kidney in 1978. MRTs are a rare and highly malignant cancer type, which have also been reported outside of the kidney, including in the liver, soft tissue, and central nervous system. Several cases of primary intracranial MRT have been reported since its recognition as a separate entity in 1978. The term rhabdoid was used to describe these cases because of the similarity in appearance to RMS under the light microscope. Regardless of the location, all rhabdoid tumors are highly aggressive, have a poor prognosis, and tend to occur in children less than two years of age.

To date, there are less than 10 MRT cell lines available. Four of these were established in our departments, and thus the cellular and genetic characterization of these cell lines will be useful for new diagnosis methods and novel therapeutic development.

### Establishment of four MRT cell lines

#### KP-MRT-NS cell line

The KP-MRT-NS cell line was established from a 2-month-old infant who presented with abdominal distension and macrohematuria in October 1991. Clinical examination revealed a left upper abdominal mass arising from the primary tumor from the left kidney (Table 1). Morphological observations of the KP-MRT-NS cell line by electron microscopy showed spindle cells or flat cells (Figure 7A), and a para nuclear whorl of intermediate filaments, which is a common characteristic of MRT cells (Figure 7B). The phenotype of the KP-MRT-NS cell line [19] was similar to that of neuroblastoma cells, indicating a neural crest origin of MRT similar to neuroblastoma [4] (Figure 8 and 9).

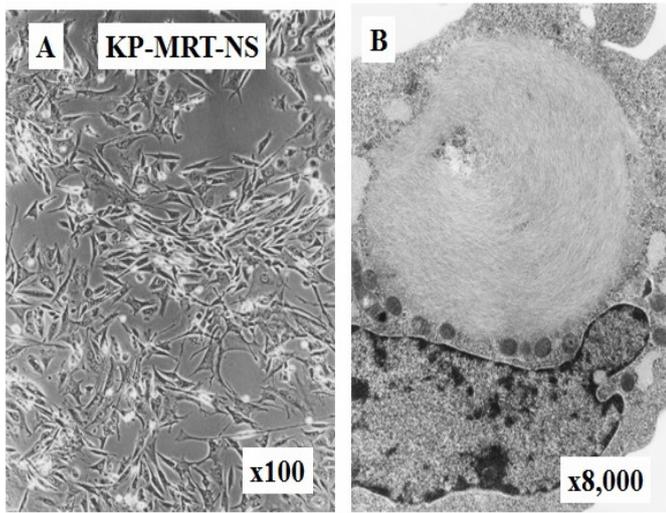


Figure. 7 Inverted microscopic (a) and electron microscopic (b) analysis of the KP-MRT-NS cell line

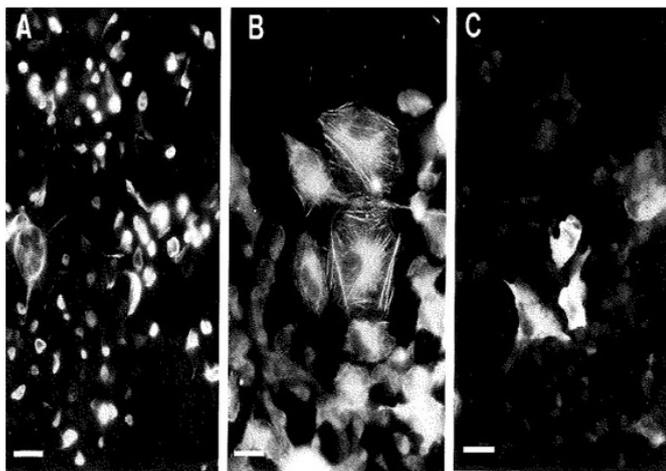


Figure. 8 Indirect immunofluorescence with (a) neurofilament, (b) alpha-smooth-muscle actin, and (c) SM1 isoforms of the smooth-muscle-myosin heavy chain in the KP-MRT-NS cell line

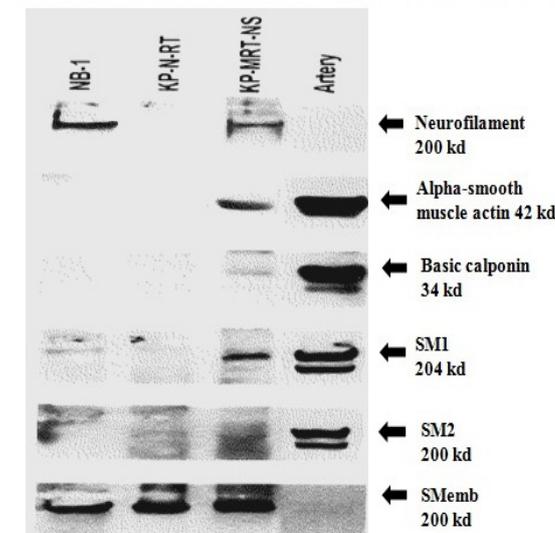


Figure 9. Western blot analysis of cytoplasmic proteins of KP-MRT-NS cell line with neurofilament, alpha-smooth-muscle, basic calponin and SM1 isoforms.

**KP-MRT-RY cell line**

The KP-MRT-RY cell line was established from a 1-month-old infant who presented with abdominal distension (Table 1, Figure 10). The left renal tumor was resected, and the KP-MRT-RY cell line was cultured and characterized as described previously [20].

**MP-MRT-AN cell line**

The MP-MRT-AN cell line was cultured from liver biopsy specimens (Figure 11). Immunohistochemical assays detected the expression of vimentin and cytokeratin. RT-PCR assays revealed that this cell line did not express smooth muscle myosin heavy chain isoforms or MyoD1 [21].

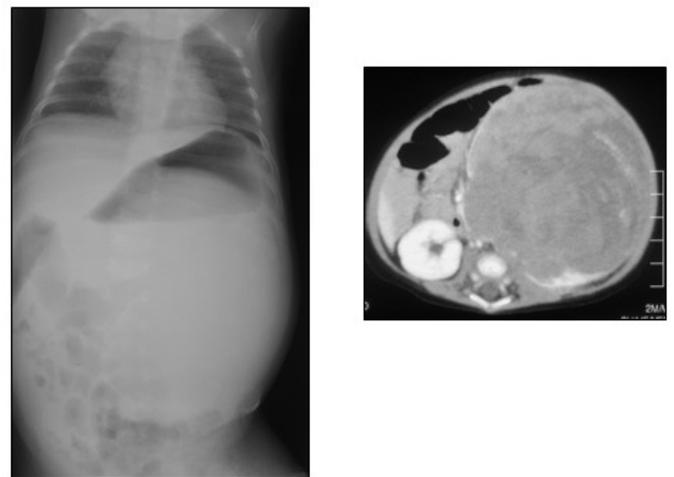


Figure. 10 Chest XP and abdominal computed tomographic imaging of an established KP-MRT-RY cell line

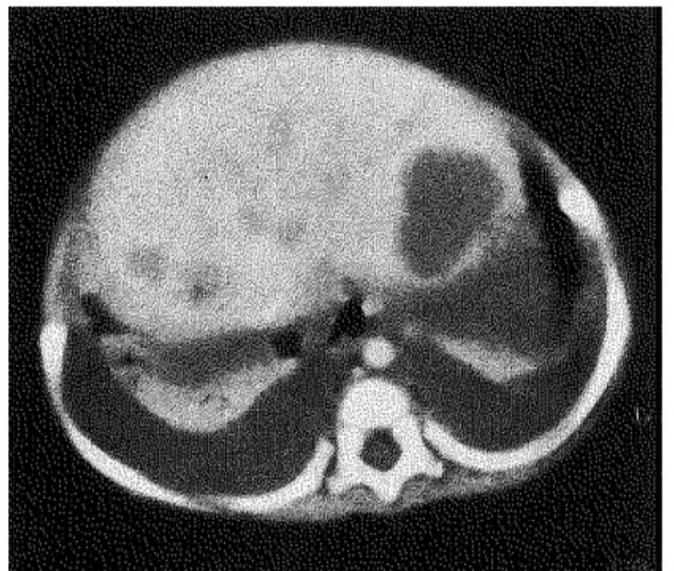


Figure. 11 Abdominal computed tomographic image showing massive pleural fluid and multiple hepatic tumor lesions in an established MP-MRT-AN cell line

## KP-MRT-YM cell line

The KP-MRT-YM cell line was established from a 5-month-old boy who had a thoracic mass without metastasis at the time of diagnosis (Figure 12). The tumor was completely resected, and histopathologic analysis demonstrated that the cells were round in shape with vesicular nucleoli and no typical eosinophilic cytoplasmic inclusions. The cells stained positive for vimentin and negative for desmin by immunohistochemistry. Expression of *MyoD*, *PAX3/FKHR*, *EWS/FLI1*, *EWS/ERG*, and *SSX/SYT* chimeric mRNA was absent in the tumor.

The patient received four cycles of doxorubicin, vincristine, and cyclophosphamide, alternating between ifosfamide and etoposide. However, after 18 months off-therapy, local recurrence was detected. He then underwent total resection, additional chemotherapy, and 30.6-Gy radiation therapy. No further recurrence has been observed. The patient is alive and well at 4 years post-onset [22, 23].

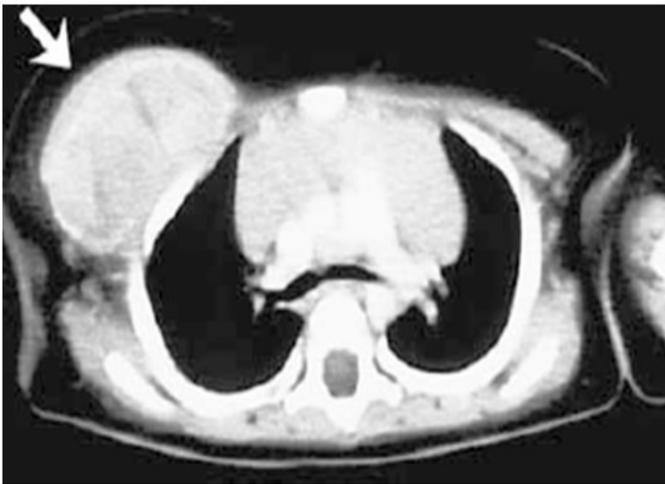


Figure 12 Chest computed tomographic image showing an enhanced thoracic mass on admission in an established KP-MRT-YM cell line

## Confirmation of the four cell lines as MRT cells

RT-PCR analysis of the four MRT cell lines did not detect *INI1CD1* in the KP-MRT-NS, KP-MRT-AN, or KP-MRT-YM cell lines. *INI1CD2* was also not detected in the KP-MRT-AN or KP-MRT-YM cell lines, and only a shortened form of this gene was detected in the KP-MRT-NS cell line (Figure 13A). These results were consistent with previous reports on MRT [19, 21–24]. However, both *INI1CD1* and *INI1CD2* were detected in our newly established KP-MRT-RY cell line, as well as in the HL60 cell line, which was used as a positive control. Therefore, we directly sequenced the amplicon corresponding to *INI1* exon 2. A mutation in exon 2 (C157T in codon 53) was observed in the

KP-MRT-RY cell line (Figure 13B). Furthermore, *INI1* protein expression was not detected in any of the four MRT cell lines by western blotting (Figure 13C), confirming that the four cell lines were MRT cells.

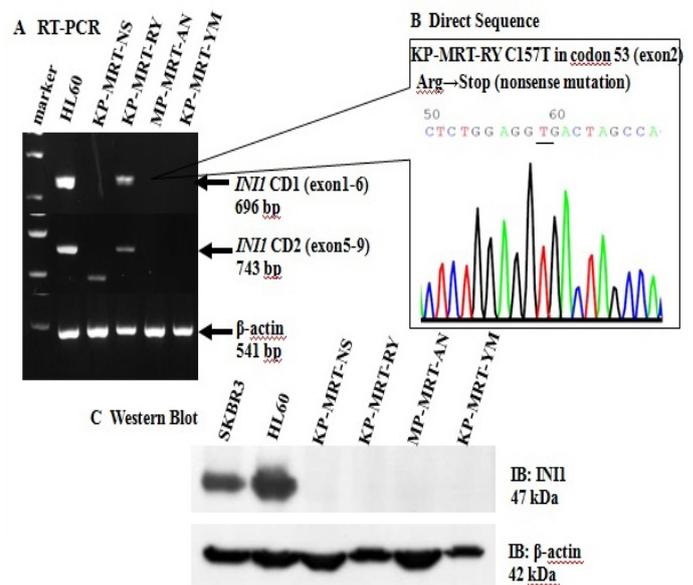


Figure 13 *INI1* gene analysis in four MRT cell lines

## Preclinical studies of molecular target therapy using gefitinide in MRT cells

The epidermal growth factor receptor (EGFR) was recently found to be expressed in MRT cell lines. Gefitinib (marketed as Iressa) is an oral and selective EGFR-tyrosine kinase inhibitor with demonstrated efficacy in inhibiting the proliferation of cancer cells in animal models *in vivo* as well as in clinical trials. These promising results encouraged us to examine the antitumor effects of gefitinib on our MRT cell lines *in vitro* and *in vivo*.

The expression of EGFR was confirmed in two MRT tumors and their respective established cell lines (MP-MRT-AN and KP-MRT-NS). Immunoblot analysis showed that gefitinib inhibited EGFR-phosphorylation [50% inhibitory concentration (IC<sub>50</sub>) < 0.1 μmol/L] (Figure 14). Moreover, gefitinib inhibited *in vitro* cell growth (IC<sub>50</sub> = 10–12 μmol/L), and a high concentration of gefitinib (20 μmol/L) induced apoptosis *in vitro* (42.9% MP-MRT-AN and 47.2% KP-MRT-NS) as determined by terminal deoxynucleotidyl transferase-mediated nick end labeling. Furthermore, gefitinib at 150 mg/kg had a cytostatic effect on established MRT xenografts in athymic mice (MP-MRT-AN, *P* = 0.039 and 0.0014; and KP-MRT-NS, *P* = 0.048 and 0.0086; Figure 15).

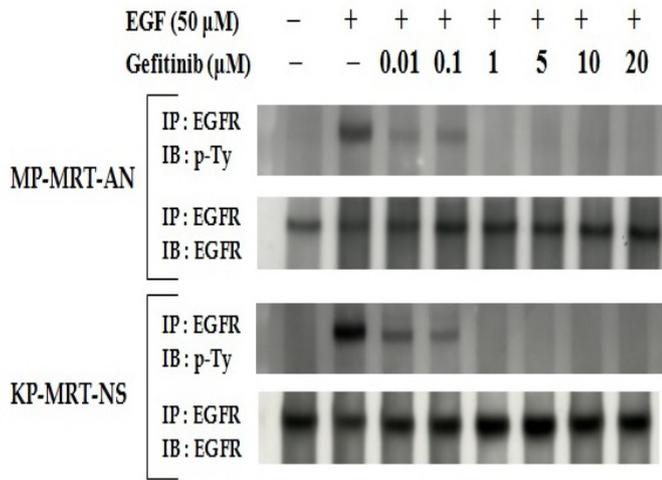


Figure. 14 Effect of gefitinib on two MRT cell lines

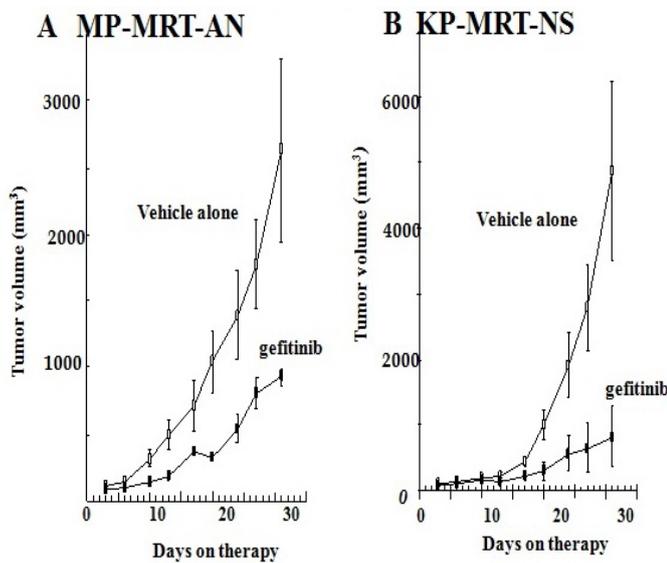


Figure. 15 Inhibition of tumor growth by gefitinib in MRT cell lines

Overall, these results demonstrated that gefitinib has antitumor effects in MRT cells *in vitro* and *in vivo*, and thus has promise as a novel and therapeutic strategy for MRT [24].

### Preclinical studies on the activation of antibody-dependent cellular cytotoxicity by trastuzumab against MRT cells

Trastuzumab, a humanized monoclonal antibody against human epidermal growth factor receptor-2 (HER-2), has been shown to be effective against breast cancer and other cancers. Therefore, we also examined the expression of HER-2 in our four MRT cell lines by indirect immunofluorescence, flow cytometry, and immunohistochemistry [20].

All four MRT cell lines (KP-MRT-NS, KP-MRT-RY, MP-MRT-AN, KP-MRT-YM) expressed the HER-2 protein. Treatment of trastuzumab alone did not reduce the viability

of the MRT cell lines, whereas the cytotoxicity of trastuzumab against each of the MRT cell lines was significantly increased by the presence of allogeneic and autologous peripheral blood mononuclear cells ( $P < 0.01$ ). There was a strong correlation coefficient ( $r = 0.825$ ) between HER-2 expression and the cytotoxicity enhanced by trastuzumab (Figure 16). Moreover, trastuzumab in combination with peripheral blood mononuclear cells augmented by interleukin (IL)-2 was significantly more cytotoxic than trastuzumab alone or IL-2 alone ( $P < 0.01$ ) (Figure17) [20].

These results indicated that (1) trastuzumab can exert antitumor effects on MRT cells using the antibody-dependent cellular cytotoxicity of effector cells and (2) IL-2 can enhance the cytotoxicity of trastuzumab against MRT cells [20].

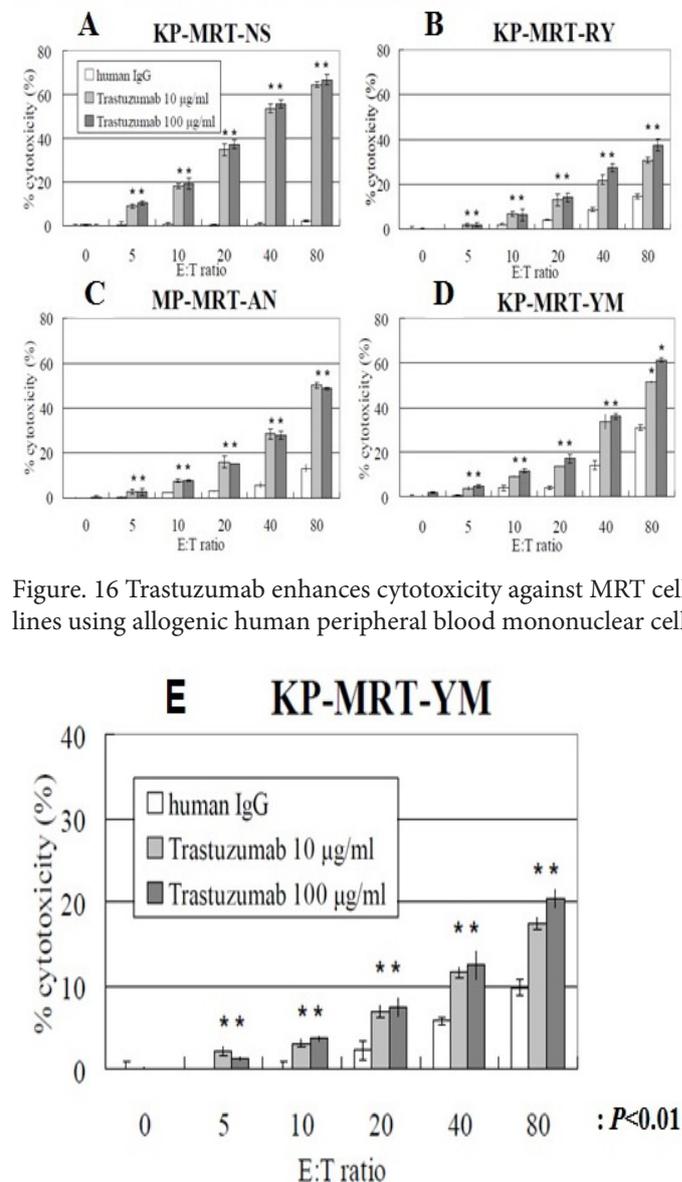


Figure. 16 Trastuzumab enhances cytotoxicity against MRT cell lines using allogeneic human peripheral blood mononuclear cells

Figure. 17 Trastuzumab enhances cytotoxicity against KP-MRT-YM cells

**CCS cell lines**

**Case patient SY in CCS**

CCS was first described by Enzinger in 1965 [25] as a rare melanin-producing soft tissue sarcoma. Although CCS is also referred to as malignant melanoma of soft parts, it is clinically, genetically, and biologically distinct from cutaneous melanoma, despite certain histological similarities. CCS of soft parts mainly arises from the tendon or aponeurosis in adults 20–40 years of age. Local recurrence is occasionally distinct from cutaneous melanoma, despite certain histological characteristics observed, and this tumor is insensitive to chemotherapy with a poor prognosis.

A 13-year-old SY girl was referred to us in December 1994 with a left-Achilles tendon tumor that had been growing for the past eight months (Figure 18). The patient received four courses of chemotherapy, and wide resection of the primary tumor was performed. Furthermore, mega therapy with autologous peripheral blood stem cell transplantation was performed in November 1995. Despite intensive therapy, the disease gradually progressed, and the tumor metastasized to the left popliteal lymph nodes, right lung, and left the femoral bone. Although a transient minor response was observed, the patient passed away due to progressive disease in December 1999.

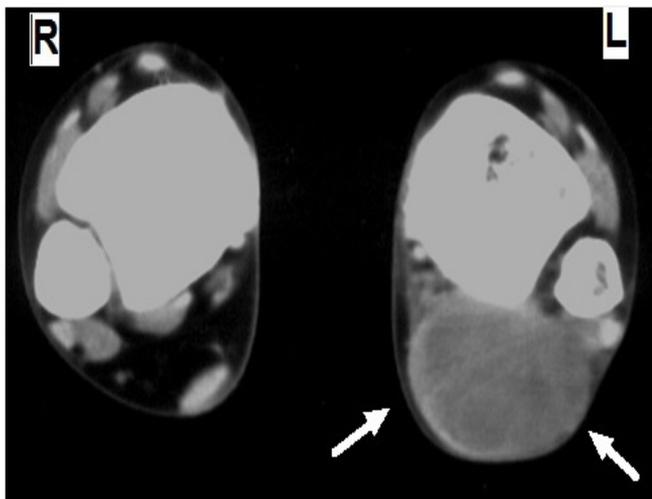
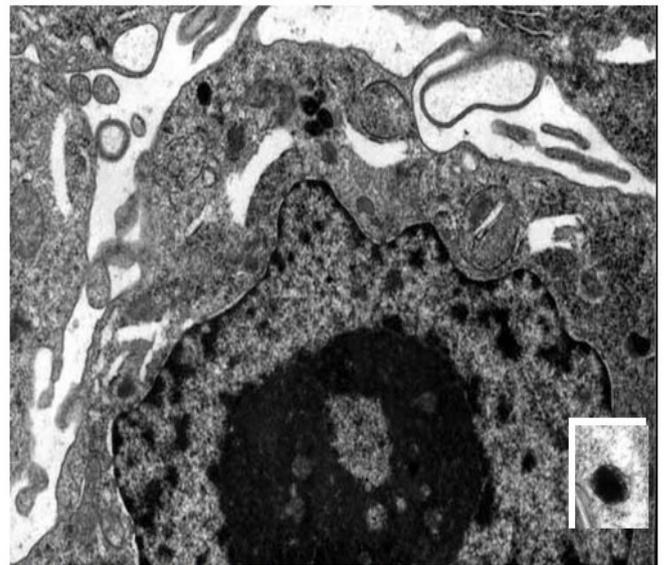


Figure. 18 Enhanced computed tomography scan of left and right Achilles tendons of a patient with clear cell sarcoma of soft parts

**Establishment of the MP-CCS-SY cell line**

To date, there are only 10 established cell lines of CCS [26, 27]. We established the MP-CCS-SY cell line from metastasis of the left femoral bone tumor in a 17-year-old SY girl (Figure 18).

The tumor cells grew as an adherent monolayer. A small number of melanosomes were detected in the cytoplasm by electron microscopy (Figure 19), which immunoreacted with two melanoma-associated antibodies, HMB45, and Melan-A (Figure 20A, B). Western blot analysis further demonstrated the existence of a Melan-A antigen in this cell line (Figure 20C) [26].



Mature melanosomes were observed (x25,000).

Figure. 19 Electron microphotograph of MP-CCS-SY cell line

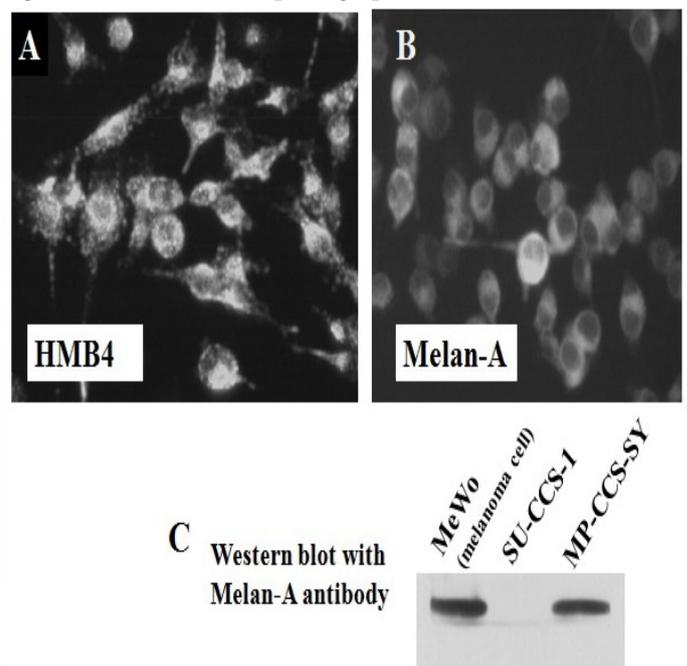


Figure. 20 Immunofluorescence and western blot with HMB45 and Melan-A antibodies in MP-CCS-SY cell line

The chimeric *EWS/ATF1* transcript was detected in both the SY tumor and MP-CCS-SY cell line (Figure 21A) [26], as well as in another CCS cell line, SU-CSS-1 (Figure 21A) [27] by RT-PCR. Overexpression of *c-Myc* mRNA was detected in both the SY tumor and MP-CCS-SY cell line by northern blot analysis (Figure 21B) indicating a potential role in the malignant progression of CCS [26]. The availability of this MP-CCS-SY cell line will help to improve understanding of the molecular biology of this malignancy and should serve as a useful tool for developing boron neutron capture therapy [28].

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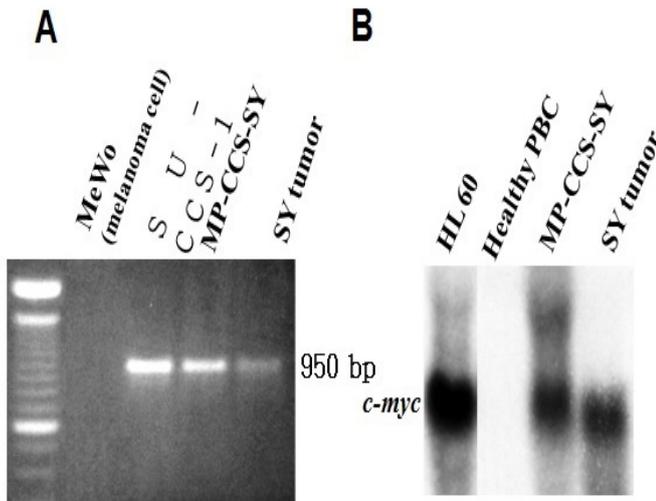


Figure. 21 *EWS/ATF1* chimeric gene (a) and *c-myc* mRNA in MP-CCS-SY cell line and SY tumor cells

## Conclusion

Since August 1984, our frozen vials have been routinely thawed and cultured for basic and clinical *in vitro* and *in vivo* studies in oncology. In the previous review, we discussed the establishment of 16 cell lines from 12 neuroblastoma patients and their characteristics [29]. In the present review, we provided an overview of the establishment of 13 soft-tissue sarcoma cell lines, including two alveolar RMS cell lines and one *MYCN*-amplified and overexpressed RMS cell line. In addition, we highlight the establishment of one Askin sarcoma cell line and four rare MRT cell lines. Two molecular target therapies involving gefitinide and trastuzumab have been attempted for the refractory MRT cell lines. The rare CCS cell line of soft parts offers a possible tool for developing a boron neutron capture therapy. Overall, these established childhood soft-tissue sarcoma cell lines are expected to provide novel insights into tumor biology.

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