Establishment and Characterization of a Clonal Human Extraskeletal Ewing’s Sarcoma Cell Line, EES1

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Ewing’s sarcoma, a small round cell sarcoma arising in soft tissue as well as the bone, is one of the most malignant tumors in children and young adults. Few established cell lines of extraskeletal Ewing’s sarcoma (EES) have been reported, which made it difficult to examine the biological features of EES. Therefore, we have established a new clonal cell line of EES. We report its morphological characters, results of chromosomal and immunohistochemical analysis. A piece of tumor obtained from the 18-year-old female patient with EES was xenografted in a nude mouse. In vitro subcultured cells were then obtained from this xenograft. A clonal cell line was subsequently established by limiting dilution and designated EES1. EES1 cells had a doubling time of 24 hours. In the xenografted tumor, the cells expressed vimentin, CD99 (MIC2), neuron specific enolase (NSE) and cytokeratin. The original tumor cells also expressed vimentin, CD 99, and NSE, but was negative for cytokeratin. The morphological and immunohistochemical features of this cell line established, except for cytokeratin expression, were consistent with those of the primary tumor. Cytogenetic analysis of EES1 revealed chromosomal translocation of t(11; 12)(q24;ql2). The chimeric fusion of the Ewing’s sarcoma gene in band 22q12 with the Friend leukemia virus integration-1 gene in band 11q24 was also demonstrated. Fluorescence in situ hybridization further confirmed the presence of translocation involving the Ewing’s sarcoma gene in both the primary tumor and EES1 cells. In conclusion, we have established a human EES cell line EES1, which will provide a useful model for studying various aspects of human EES. ——— extraskeletal Ewing’s sarcoma; Ewing’s sarcoma; cell culture; immunohistochemistry; chromosomal translocation

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Ewing’s sarcoma, a highly malignant round cell tumor, was first reported by Sir James Ewing in 1921. This tumor is relatively uncommon accounting for 6-8% of all primary malignant bone tumors and is less common than other bone tumors such as multiple myeloma, osteosarcoma and chondrosarcoma (Ushigome et al. 2002). However, this tumor is one of the most common malignant tumors in children and young adults (Saitoh et al. 2000). The patients affected by this tumor are, on the average, younger than those with any other primary malignant tumors of bone. Ewing’s sarcoma is a rare malignancy that most often presents as an undifferentiated primary bone tumor; less commonly, it arises in various soft tissues (extraskeletal or extraosseous Ewing’s sarcoma [EES]) (Hatori et al. 2001; Isefuku et al. 2006). Both tumors constituted a spectrum of neoplastic diseases known as the Ewing’s sarcoma family of tumors (ESFT) (Horowit et al. 1993). Treatment of EES is similar to Ewing’s sarcoma of bone, with excision followed by radiation and chemotherapy. Metastases are common and predominantly to the lungs and bone. The histogenesis of EES has remained still controversial and few reports have been available on the establishment of EES cell lines in humans. Therefore, in this study, we have established a new clonal cell line of EES and reported its morphological characters and the results of cytogenetical and immunohistochemical analyses of the new cell line.

**Material and Methods**

**Patient**

An 18-year-old Japanese woman presented with a mass (Fig. 1) in her right posterior thigh. Skeletal survey by using computed tomography, magnetic resonance imaging and bone scan revealed no bone lesion or any other soft tissue lesions. The excised tumor was subsequently diagnosed as extraskeletal Ewing’s sarcoma. Soon after admission, metastasis was noted in the subcutaneous region on her right trunk. Despite chemotherapy and irradiation, the patient died of lung metastasis 11 months after admission.

**Cell culture and histological studies**

A part of the excised tumor was cut into small pieces with a clean scissor. A 2-mm × 2-mm × 2-mm sized mass was xenografted into the subcutaneous tissue on the back of a six-week-old female BALB/c (nu/nu) nude mouse (CLEA, Tokyo). Two months after transplantation, a subcutaneous tumor grew to 30-mm × 30-mm sized mass. The tumor tissue obtained from the nude mouse at the first generation was cut into small pieces measuring about 0.5 × 0.5 × 0.5 mm with a scalpel, rinsed with phosphate-buffered saline (PBS) 3 times, and cultured in 24-well multiplates (Falcon, Los Angels, CA, USA) in modified Eagle medium (MEM: Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS) (Invitrogen), penicillin, streptomycin and fungizone (Bio Whittaker, Walkersville, MD, USA) in a humidified atmosphere with 5% CO₂ at 37°C. The medium was replaced every three days. On the 20th day of the primary culture, cells were detached by using 0.25% trypsin and were transferred into 25 cm² flasks (Falcon Plastics Corp., Oxnard, CA, USA) in modified Eagle medium (MEM: Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS) (Invitrogen), penicillin, streptomycin and fungizone (Bio Whittaker, Walkersville, MD, USA) in a humidified atmosphere with 5% CO₂ at 37°C. The medium was replaced every three days. On the 20th day of the primary culture, cells were detached by using 0.25% trypsin and were transferred into 25 cm² flasks (Falcon Plastics Corp., Oxnard, CA, USA). The cells were subcultured every 5-7 days at a 1:8 dilution until passage 10 (Doi et al. 2005). The subcultured cells were designated EES. Cloned cell lines were obtained from EES by limiting dilution in 96-well multiplates (Falcon) at Passage 10. One of cloned cell lines has been maintained continuous-ly in vitro for over 90 passages during more than 40 months and designated EES1. A suspension of 1 × 10⁶ EES1 cells in the 0.3 ml medium was injected subcutaneously into the back of a six-week-old female BALB/c (nu/nu) nude mouse (CLEA). The primary tumor and transplanted tumors were fixed in 10% buffered formalin, embedded in paraffin, sectioned and stained with hema-
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toxylin and eosin, and examined immunohistochemically by using avidin-biotin peroxidase complex (ABC) technique (Hsu et al. 1981). ABC Kit (Dako, Glostrup, Denmark) was used. The used primary antibodies were vimentin (1:100, DAKO), cytokeratin (1:50, IMMNOTECH), MIC 2 (1:500, DAKO), neuron specific enolase (NSE)(1:100, DAKO). Small pieces of the primary tumor fixed with 2.5% glutaraldehyde and 1% OsO4 for electron microscopic study. The cultured cells were morphologically examined under an inverted phase contrast microscope (Olympus IX 70, Olympus, Tokyo).

A sheet of tumor EES1 cells at confluence was gently detached from the plate and fixed with 2.5% glutaraldehyde and 1% OsO4 for electron microscopic study.

Cell growth analysis

In order to study cell growth, the cultured cells at Passage 50 were plated at a density of $2.0 \times 10^5$ cells/well in 24-well dishes with the medium. The cells harvested on days 1, 2, 3, 5 and 7 were counted on a hemocytometer (Burker-Turk deep, Elmo, Tokyo). Doubling time was calculated using a semilogarithmic graph.

Chromosome analysis

Chromosomal analysis of a total of 20 metaphase EES1 cells at passage 50 was made by a standard trypsin-Giemsa banding technique. The karyotype was analyzed according to the rules of the International System for Human Cytogenetic Nomenclature.

Reverse transcription and polymerase chain reaction analysis (RT-PCR)

Total RNA from EES1 cells at passage 80 were extracted using the method of guanidinium thiocynate followed by centrifugation in cesium chloride solutions (Sambrook et al. 1989).

Complementary DNA (cDNA) was generated from total RNA with a random primer using the first-strand cDNA synthesis kit (Pharmacia Biotech, Uppsala, Sweden). One to 5 μg of total RNA was transcribed to cDNA. PCR was carried out in a 100-μl reaction containing 2.5 μl cDNA template, 200 μM deoxynucleotide triphosphates, 0.5 μM of each oligonucleotide primer, and 2.5 unit of Taq polymerase in an 10 mM tris-HCl buffer, pH 8.8, containing 50 mM KCL, 1.5 mM MgCl2. Oligonucleotide primers used for PCR were EU-6 (Ewing’s sarcoma specific, tat gga cag cag agt agc tat ggt c) (Urano et al. 1996) and ESBP-2 (Friend leukemia virus integration-1 [FLI-1] specific, ceg ttg ctc tgt att ctt act ga) (May et al. 1993), for detecting EWS-FLI-1 fusion gene product; EU-6 and EU-15 (ERG specific, cat gta cgg gag gtc tga ggg gt) (Urano et al. 1996) for detecting EWS-ERG fusion gene product. Thirty five cycles of PCR were performed with the following parameters: denaturation step at 94°C for 1 min, annealing at 65°C for 1 min and elongation step at 72°C for 1 min. Amplified products were analyzed on 0.7% agarose gel. As a positive control for PCR, a part of EWS mRNA (from exon 1 to exon 5) was amplified as well.

Fluorescence in situ hybridization (FISH)

FISH is a technique that utilizes fluorescently labeled DNA probes to detect the status of specific DNA sequences within the genome. Translocations of gene sequences are common abnormalities in Ewing’s sarcoma and can be detected by the FISH technique. FISH analysis was carried out by using formalin-fixed paraffin embedded tissues of the primary and xenografted EES1 tumors to confirm translocation. The FISH methodology for recognition of a EWSR1 gene translocation utilizes a dual-color, break-apart rearrangement probe. The LSI EWSR1 (22q12) dual-color, break-apart rearrangement probe consists of a mixture of two FISH DNA probes. The first probe, an approximately 500-kb probe labeled with SpectrumOrange, flanks the 5’ side of the EWSR1 gene and extends inward into intron 4. The second probe, an approximately 1,100-kb probe labeled with SpectrumGreen, flanks the 3’ side of the EWSR1 gene. There is a 7 kb gap between the 2 probes. This probe could identify a t(22q12) but not the specific translocation partner, such as 11q23, 21q22, or 7p22. All the probes were purchased from a commercial source (Vysis Inc., Downers Grove, IL, USA) and were used according to the manufacturer’s protocols (http://www.vysis.com). The test is conducted by noting the probe signal configuration within the cells and nuclei with rearrangements of 22q12. The abnormal cell hybridized with the LSI EWSR1 (22q12) Dual Color, Break Apart Rearrangement Probe shows the one fusion, one orange, and one green signal pattern indicative of a rearrangement of one copy of the EWSR1 region (Lee et al. 2005; Robert et al. 2006).

RESULTS

Histological findings

The primary tumor, microscopically, demonstrated a highly cellular tumor composed of small, round, uniform cells with round nuclei containing...
fine chromatin, scanty eosinophilic cytoplasm, and indistinct cytoplasmic membranes (Fig. 2), which is consistent with previously reported histological findings (Angervall and Enzinger 1975; Ahmad et al. 1999). Rosette-like structures were infrequently seen. Ultrastructurally the tumor was composed of round to oval tumor cells with large rounded or elongated nucleus and poorly developed cytoplasmic organelles. Phase contrast light microscopic observation demonstrated that the EES1 cells were small and polygonal in culture (Fig. 3A, B). Tumor cells contained periodic acid-Schiff staining (PAS) granules, which were digested with diastases were considered to contain glycogen. Ultrastructural observation revealed the EES1 cells piled up and multilayered at confluence. The closely packed small round cells with high N/C ratio had short processes. The nuclei were oval and showed invagination. They had very few cytoplasmic organelles and a poorly developed endoplasmic reticulum. No apparent neurotubules or neurosecretory granules were observed (Fig. 4). EES1 formed a tumor in the back of the nude mouse after injection of a suspension of $1 \times 10^6$ EES1 cells. There were no evidences of lung or liver metastasis in this model. Histological features of the heterotransplanted tumors in the nude mouse were similar to those of the primary tumor; the tumor cells formed solid sheets, separated by thin vascular stoma into irregular nests. The cells had small round to oval nuclei (Fig. 5). In the xenografted tumor, the cells expressed vimentin, CD99 (MIC2) (Fig. 6), NSE and cytokeratin. The primary tumor cells also expressed vimentin, CD99, and NSE, but not reactive for cytokeratin.

**Cell growth analysis**

EES1 cells had a doubling time of 24 hrs.

**Chromosome analysis**

Chromosome analyses of all the 20 Giemsa-banded metaphase cells revealed chromosome number of 55. Karyotype analysis indicated 55, XX, +i(1)(q10), +7, +8, +8, +9, t(11;22)(q24;q12),...
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+12, +16, +18, +20 (Fig. 7).

Reverse transcription and Polymerase chain reaction analysis

EES1 expressed specific chimeric gene of EWS-FLI-1 but not EWS-v-ets erythroblastosis virus E26 oncogene like (avian) (ERG) (Fig. 8).

Fluorescence in situ hybridization

One fused signal and one split signal of orange and green, indicating the rearrangement of the EWS gene, were detected in the primary tumor (Fig. 9A) and EES1 cells (Fig. 9B).

DISCUSSION

EES is a rare tumor and only few data have been available in its etiology or pathogenesis (Ahmad et al. 1999). Angervall and Enzinger (1975) first reported the pathological features and the behavior of EES, which are round, or oval cell sarcomas occurring in the soft tissues, and they also reported that this tumor was histologically indistinguishable from Ewing’s sarcoma of bone. The tumors affect mostly young adults (median age 20 years) and most commonly involve the soft tissues of the lower extremity and the para-vertebral region. Primitive neuroectodermal
tumor (PNET) is one of the Ewing’s sarcoma family of tumors (ESFT). Ewing’s sarcoma and PNET are defined as round cell sarcomas that show varying degrees of neuroectodermal differentiation. The term Ewing’s sarcoma has been used for those tumors that lack evidence of neuroectodermal differentiations by light microscopy and immunohistochemistry, and electron microscopy, whereas the term PNET has been employed for tumors which demonstrate neuroectodermal features as evaluated by these modalities (Ushigome et al. 2002). However, there is considerable clinical and histological overlap between PNET and EES. It is true that many pathologists no longer make a distinction between EES and PNET (Llombart-Bosch et al. 1990;
These tumors have similar patterns of biochemical and oncogene expression and are considered to be all derived from the same primordial stem cell (Denny 1996). The primary tumor of the present case was diagnosed as EES because of the absence of an involvement of bones and of the fact that the histological features lack apparent neuroectodermal differentiation.

There have been few reports regarding establishment and characterization of EES or PNET cell lines. The extensive literature survey reveals that only ten cell lines of EES or PNET have been reported so far (Whang-Peng et al. 1986; Fujii et al. 1989; Homma et al. 1989; Llombart-Bosch et al. 1990; Hara et al. 1991). Whang-Peng et al. (1986) evaluated long cultured EES and the other small, round, blue-cell tumors including rhabdomyosarcoma, Ewing’s sarcoma of bone, and mesenchymal chondrosarcoma. Fujii et al. (1989) described a cell line of EES obtained from the chest wall lesion in a 14-year-old girl. Homma et al. (1989) reported a cell line designated SCCH-196 established from an extraskeletal small round cell sarcoma developed in a 16-year-old Japanese girl. The cells grew as a monolayer, and have been continuously propagated by serial subcultures during the past 26 months. Llombart-Bosch et al. (1990) established cultured cell lines and nude mice xenografts from original neoplasms of EES or from their metastases. Hara et al. (1991) investigated the capability of neural differentiation of ES by using the established EES cell lines. However, there have been no published reports on a clonal EES cell line.

In this report, we have established a clonal cell line retaining significant features of EES.
The xenografted tumors in the nude mice, microscopically, are composed of solidly packed small, round, or ovoid cells of great uniformity, arranged in lobules separated by strands of fibrous connective tissue. It is well known that the cytoplasm of EES frequently contains PAS-positive glycogen (Ushigome et al. 2002). The established EES1 cells also contain PAS-positive glycogen. Ultrastructural observation is useful in making a diagnosis of EES because the ultrastructural features are distinctive to allow separation from other small cell malignant neoplasms in the majority of cases (Mahoney et al. 1978; Gillespie et al. 1979). EES has varying amounts of glycogen are aggregated in the scanty ill-defined cytoplasm, which usually has a paucity of organelles (Friedman and Gold 1968). These ultrastructural features were also detected in the cell line, EES1. In the previously established cell lines, several ultrastructural features of neural type were detected; the same were also seen on culture cell lines (Llombart-Bosch et al. 1990). In EES1, cellular processes were observed but no apparent neurosecretory granules were detected.

For many years, final diagnosis of Ewing’s sarcoma/PNET was essentially based on an immunohistochemical diagnosis of exclusion. More recently, however, numerous findings have been published regarding the product of the MIC2 gene (CD99) in this group of tumors (Weiss and Goldblum 2001). Both Ewing’s sarcoma/PNET cells express the MIC2 gene in high amounts, which represents a highly selective and almost unique feature of these cells, making an assignment of these tumors in one entity even more likely (Ambros et al. 1991). Khoury (2005) stated that MIC2 expression is identified in nearly all ESFT and constitutes a useful positive marker when used as part of a panel of immunostains that can help rule out other differential diagnostic considerations. In the present tumor, both of the primary and the nude mouse xenografted tumor tissues expressed vimentin and NSE. Of interest, Ewing’s sarcoma/PNET has been demonstrated to be positive for cytokeratin, a manifestation of epithelial differentiation, in some cases (Ushigome et al. 2002). Gu et al. (2000) reported that cytokeratin immunoreactivity was detected in 10 out of 50 cases (20%), in five diffusely and five focally. There were no significant associations between cytokeratin expression and each of the following parameters: patient age, sex, skeletal and extraskeletal primary site. Fujii et al. (1989) and Llombart-Bosch et al. (1990) reported positivity of their established EES cells for cytokeratin. In the present case, the primary tumor did not express keratin but the xenograft was reactive with keratin. This result supports that Ewing’s sarcoma may represent the stage of either very early pluripotential cells or primitive neuroectodermal cells that can differentiate along a neuronal, glial, Schwannian, melanocytic, neuroendocrine, or even ectomesenchymal pathway (Sohn et al. 1998).

The ESFT comprises morphologically heterogeneous tumors that are characterized by nonrandom chromosomal translocations involving the EWS gene and one of several members of the ESFT of transcription factors (Khoury 2005). The expression of EWS/FLI-1 or EWS/ERG chimeric genes was found to be generated through a t(11;22)(q24;q12) or a t(21;22)(q22;q12) translocation (Urano et al. 1996). Classical Ewing’s sarcoma and PNET are now known to be the same tumor with variable differentiation, defined by a translocation between the EWS gene on chromosome 22q12 with one of three ETS-like genes, especially the FLI-1 gene on chromosome 11 or ERG on chromosome 21q22. Molecular techniques used to identify this translocation along with the knowledge that the protein product of the MIC2 gene is highly expressed on the cell surface have greatly improved diagnostic abilities in the ESFT (Grier 1997). The translocation t(11;22)(q24;q12) is the most common feature (about 90%) and results in the formation of the EWS-FLI-1 fusion protein (Khoury 2005). About 10% of cases with ESFT express EWS-ERG (Kaneko et al. 1997). The translocation
t(11;22)(q24;q12) was also detected in the reported EES cell lines (Whang-Peng et al. 1986; Fujii et al. 1989; Homma et al. 1989). Karyotype analysis of our established EES1 revealed chromosomes with translocation of t(11;22)(q24;q12). RT-PCR assay confirmed EES1 expressed specific chimeric fusion gene of EWS-FLI-1 but not EWS-ERG. The reason of different amplicon sizes in lanes 2 and 3 in Fig. 9 is that there are several kinds of EWS-FLI-1 fusion gene products and the length of each fusion gene is different. The fusion gene products in lanes 2 and 3 are thought to be different types of EWS-FLI-1. Although RT-PCR provides additional information regarding fusion transcript subtype, FISH is also very sensitive and reliable ancillary technique for the diagnosis of Ewing’s sarcoma/PNET in formalin-fixed paraffin-embedded tissue (Bridge et al. 2006). This analysis confirmed the presence of translocation involving the EWS gene on both the primary tumor and EES1. The unique translocation seen in virtually all of ESFT is a potential target for a “magic bullet” therapy, because the protein product of this translocation is present only in the malignant cells (Grier 1997).

In conclusion, EES1 established in this study demonstrates unique cellular properties, which makes it a useful model for studying various aspects of the biology of human EES.

References


