

## Establishment and Characterization of a New Ewing's Sarcoma Cell Line From a Malignant Pleural Effusion

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**Abstract.** *Background:* Ewing's sarcoma cell lines may represent a good *in vitro* model for the understanding of tumor biology in this heterogeneous group of diseases. *In the present study, we report the establishment and characterization of a primary Ewing's sarcoma cell line (LDS-Falck 01). Materials and Methods:* LDS-Falck 01 was generated from a malignant pleural effusion of a patient with metastatic peripheral primitive neuroectodermal tumor arising from the chest wall. Extensive characterization of the cells was accomplished using immunocytochemical, RT-PCR and cytogenetic studies. *Results:* *In vitro* LDS-Falck 01 cells had both anchorage-dependent and -independent growth patterns. Immunocytochemical studies showed that cells were PAS-, vimentin-, CD99- and NSE-positive, EGFR- and CD117-negative. Cytogenetic analysis revealed a complex hyperdiploid karyotype with multiple chromosomal aberrations including an unbalanced translocation  $t(11;22)(q24;q12)$ . The EWS/FLI1 chimeric transcript type 1 was detected. *Conclusion:* This cell line may represent a valid tool for investigating the biomolecular characteristics of this group of neoplasms and their sensitivity to therapeutic agents.

The Ewing's sarcoma family of tumors (ESFT) include many related malignancies such as Ewing sarcoma (ES), Askin's tumor of the thoracic wall and peripheral primitive neuroectodermal tumor (pPNET) (1). They are the second most common type of sarcoma among children and young adults, arising more frequently in the bone and soft tissues

(2). Histologically the tumor cells are uniformly bland with a relatively low mitotic index (3) and a variable degree of neural differentiation, difficult to recognize through only a morphological approach (4).

The ESFT share the expression of the *MIC2* gene product, a glycoprotein also referred to as CD99, on their cell membrane (5), which is a diagnostic marker of the disease. In addition, almost 90% of cases show a typical balanced chromosomal translocation,  $t(11;22)(q24;q12)$  or  $t(21;22)$ , that combines the *N*-terminal region of the *EWS* gene (22 q12) with the *C*-terminal region of the *FLI1* (11q24) or *ERG* (21q22) genes, members of the ETS family of transcriptional factors (1, 6, 7). The translocation results in the production of a chimeric gene and the resulting fusion proteins are aberrant transcriptional activators involved in the pathogenesis of the tumor and may also correlate with the biological aggressiveness of the disease (8). The most common fusion protein is that of type 1 (joining EWS exon 7 in frame with FLI1 exon 6), which seems to be an independent positive prognostic factor (9), while non type 1 fusion proteins (present in 25% of cases) are thought to be more potent activators of the transcriptional process than type 1 protein (10). Some reports have described establishment of permanent cell lines from pPNET (11-15), useful for elucidating their histogenesis and studying sensitivity to anticancer drugs or radiation. Here, we describe a new cell line established from the pleural effusion of an adult patient with metastatic disease that shows most of the typical characteristics of the ESFT, but presents an alternative mechanism in the rearrangement of the *EWS-FLI1* gene other than the classic balanced translocation (16).

### Materials and Methods

**Case report.** A 56-year-old man presented with a fast-growing tumor mass of the right chest wall in March 2000. An incisional biopsy

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showed a monomorphic population of neoplastic cells with round nuclei and scant periodic acid-Schiff stain-positive cytoplasm; immunohistochemical staining was positive for CD99, vimentin and neuron-specific enolase (NSE); the histomorphological picture was diagnostic for pPNET. A bone scan showed multiple bone metastases, while the bone marrow trephine biopsy was negative. The patient was treated with combination chemotherapy including high-dose chemotherapy with hematopoietic stem cell transplantation, achieving a partial response. Soon after completion of the treatment, a malignant pleural effusion occurred. The tumor progressed rapidly despite systemic and local therapy and the patient eventually died one year from the initial diagnosis.

**Establishment of a cell line and determination of the cell population doubling time.** The LDS-Falck 01 cell line was derived from the malignant pleural effusion of the patient at the time of relapse. The cell population grew partly in suspension and partly attached to tissue culture flasks (Corning, Stone Staffordshire, UK) in Roswell Park Memorial Institute-1640 medium (RPMI-1640) containing 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 µg/ml streptomycin, and 100 µg/ml penicillin (Life Technologies Inc., Paisely, UK) at 37°C and 5% CO<sub>2</sub>. They were subcultured by trypsinization with 0.25% trypsin and 0.02% EDTA (Life Technologies Inc.) in a calcium/magnesium-free balanced solution. The culture medium was changed twice a week and cellular homogeneity was evaluated microscopically every 24-48 hours.

To determine the doubling time, 1x10<sup>5</sup> cells/ml were seeded in each well of 24-well dishes (Corning) with fresh medium and the number of viable cells were evaluated every 24 hours for 10 days. Experiments were performed in triplicates. The doubling time was determined from the slope of the best-fit line of a semi-log plot of cell counts *versus* days in culture.

The cell line has undergone more than 70 passages in culture, maintaining the same characteristics for morphology and antigen and protein expression as that of the initial cells.

**Clonogenic assay.** For this analysis, 3x10<sup>3</sup> cells were grown in a methylcellulose-based medium supplemented with fetal bovine serum (Methocult, StemCell Technologies, Vancouver, Canada) without cytokines. Colonies of more than 50 cells were counted after 10 days of culture. The percentage of clonogenic cells was calculated in four individual experiments as: (number of colonies/number of initial cells) x100.

**Immunohistochemistry.** Cultured cells were fixed in 10% neutral-buffered formalin, embedded in paraffin, sectioned and then stained with hematoxylin-eosin and with periodic acid-Schiff stain (PAS). Morphological examination of growing cultures was performed using a direct phase-contrast microscope. Immunocytochemical characteristics of LDS-Falck 01 were studied at the 10th passage, and every 20 passages thereafter, by indirect immunoenzymatic staining using monoclonal antibodies against CD99 (Dako, Glostrup, Denmark), vimentin/Clone V9 (Dako), NSE (Neomarkers, Fremont, CA, USA), EGFR (Neomarkers), HER2/neu (HerceptTest, Dako) and CD117 (Dako), according to the manufacturer's recommendations.

**Flow cytometry analysis.** Cell suspensions were immunostained with a panel of monoclonal antibodies FITC-conjugate (anti-CD57, -CD44, -CD56, -FAS, -CD71, -CD34 and -HLAABC), PE-

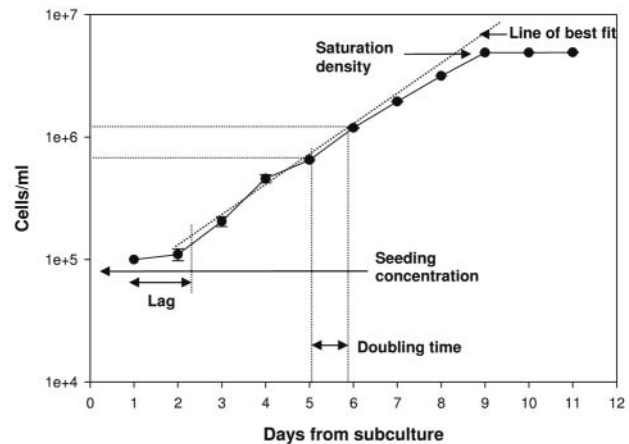


Figure 1. Growth curve of LDS-FALCK 01 cells. A semilog plot of the increase in cell density following subculture, from cultures sampled daily and counted. A straight line drawn through the part of the plot that represents the exponential phase derives the population-doubling time, which is less than 24 hours, from the middle region of this best-fit line. Representative data are shown from one of three independent experiments, all yielding similar results. Values represent the mean cell number  $\pm$  SEM.

conjugate (anti-CD 99, -CD117, -CD54, -CD133, -CD103, -CD49D, -CD29, -CD90, -CD138 and -CD86), PerCP-conjugate (anti CD45 and -HLADR) and APC-conjugate (anti-CD38) (BD Biosciences, San Jose, CA, USA). For each fluorochrome, a match isotype control antibody was used (BD Bioscience).

Briefly, cells were incubated with antibody at a concentration of 1 µg/10<sup>6</sup> cells for 20 minutes at 4°C and then washed with cold phosphate-buffered saline (PBS) with 0.1% sodium azide. Labelled cells were analysed using a FACSCalibur instrument (Becton Dickinson, San Jose, CA, USA). Cell data were collected using a four-decade log amplification scale on 20,000 to 75,000 viable gated cells, as determined by forward and side light scatter intensity and log fluorescence was displayed as single parameter histograms (17).

**Chromosome analysis.** Cytogenetic studies were performed from the cell line at the same time as the immunohistochemistry assay, according to standard techniques. Briefly, cells showing an exponential growth pattern were arrested in metaphase by treatment with 0.4 µg/ml colchicine for 1 h, followed by 0.075M KCl (0.075M) and subsequent fixation with a 3:1 mixture of methanol and glacial acetic acid. Quinacrine fluorescence (QFQ)-binding metaphases were examined to estimate the breakpoints, and C-banding induced by barium hydroxide and Giemsa (CBG) was used to evaluate the centromeric heterochromatin. Karyotype was determined according to the International System for Human Cytogenetic Nomenclature criteria (18).

**Detection of chimeric transcript EWS/FLI 1 by reverse transcriptase polymerase chain reaction (RT-PCR).** Total RNA was extracted from the cell line using Trizol reagent (GibcoBRL, Gaithersburg, MD, USA) according to the manufacturer's instructions. Reverse transcriptase-polymerase chain reaction was performed as described elsewhere(19). The integrity of the mRNA was assessed by an independent amplification using primers for the ubiquitously

expressed  $\beta$ -actin gene. The TC-71 cell line (ATCC, Rockville, MD, USA) was used as a positive control for one of the two most common types of t(11;22) fusion transcripts (20). One negative control, consisting of peripheral blood mononuclear cells (PBMC) from a healthy donor, was set at the time of each RNA extraction. All amplification products were fractionated through a 2% agarose gel together with a molecular weight marker (Marker VI, Boehringer Mannheim, Germany) and stained with ethidium bromide.

## Results

### *Establishment and propagation of the LDS-Falck 01 cell line.*

Tumor cells obtained from malignant pleural effusion were cultured in complete medium and reached subconfluence 2 weeks after initial cultivation in primary culture. Approximately 4 weeks later, at passage 6 to 7, the cells began to grow rapidly and thereafter could be serially subcultured at a dilution of 1:4 every 3 to 4 days. The mean doubling time was less than 24 hours (Figure 1). Optimal growth was obtained at  $1 \times 10^6$  cells/ml; after 4 days, the cell density ranged from 8 and  $10 \times 10^6$  cells/ml. For storage, cells were kept at a density of  $1 \times 10^7$  cells/ml in freezing medium which consisted of 90% FCS and 10% dimethyl sulphoxide (DMSO), and stored in liquid nitrogen, retaining their viability when cultures were re-established after thawing. Results of clonogenicity showed that  $95.5 \pm 1.01\%$  of cells were able to produce independent colonies in methylcellulose-supplemented medium. This new cell line was designated LDS-Falck 01 and has been maintained *in vitro* for more than 70 passages without any phenotypic differences between adherent and non-adherent cells.

*Morphological and phenotypic characterization.* Morphological examination show that the cells grew both adherent and in suspension, forming cell aggregates and multicellular tumor spheroids without any phenotypic differences between adherent and non-adherent cells. As assessed by light microscopy, the LDS-Falck 01 cells were uniform in appearance with a high nuclear:cytoplasmic ratio; the nuclei were round with finely dispersed chromatin. When cytoincluded and stained with hematoxylin-eosin, LDS-Falck 01 cells showed the characteristic cytological features of ESFT, with monomorphic, PAS-positive, small round cells with scant cytoplasm and hyperchromatic nuclei (Figure 2A). Like the primary tumor, this cell line was strongly positive for CD99/MIC2 (Figure 2B) and to a lesser extent for NSE and vimentin (data not shown). Immunocytochemical (ICC) staining for EGFR was negative while weakly positive for HER2/neu; CD117 (c-kit) showed focal positivity (data not shown).

Immunophenotyping of the cells was performed using FACS analysis. The surface antigens expressed were studied through the use of monoclonal antibodies with specificities against cells of lymphoid, myeloid and tumor lineage. Log

fluorescence was collected and displayed as single parameter histograms and fluorescence intensity was calculated as linear units (U) in logarithmic scale to take into account the weighting of the data distribution. Results of the cell surface marker analysis are shown in Table I. Cells were all CD99-positive and CD45-negative, in accordance with the diagnosis of ESFT. In addition, LDS-Falck 01 cells were highly positive for CD 90, positive for CD57, CD38 and CD49D, and weakly positive for CD117 and CD 56.

*Karyotype and molecular analyses.* Cytogenetic analysis of 15 metaphase cells revealed an abnormal karyotype in all cells examined at 450 band resolutions (Figure 3). The analysis at passage 10, showed a composite karyotype with 62 chromosomes: 62,XX,+1,+1,+7,+7,+8,+8,+18,+18,+20,+20,+21,+21,+der(22)t(11;22),(q24;q12)x2, +mar x2. The most consistent abnormalities included the translocation between chromosomes 11 and 22 with loss of chromosome 11, additional copies of chromosomes 1, 7, 8, 18, 20 and 21, and loss of chromosome Y. Some of the aberrations, such as gain of chromosomes 7, 20 and 8 (21) and, less frequent, loss of chromosome 11 (22), are known to be associated with Ewing's sarcoma. In order to determine if these cells maintained these chromosomal anomalies, cells from passage 30 were also analyzed (data not shown). These cells exhibited a karyotype that was essentially similar to the earlier passages.

RT-PCR results are shown in Figure 4. A 330 bp band, corresponding to the EWS/FLI-1 fusion transcript type 1 was detected both in LDS-Falck 01 and TC71 cell lines. A negative sample (healthy donor PBMC) yielded no EWS/FLI-1 amplification product but did generate a product for the  $\beta$ -actin mRNA, thereby indicating the presence of amplifiable RNA.

## Discussion

The difficulty of establishing long-term sarcoma cell lines represents a limitation for the understanding of tumor biology and the development of therapeutic models in this heterogeneous group of diseases. In this report, we described the establishment and characterization of a new ESFT cell line (LDS-Falck 01) whose morphological features and immunocytochemical staining are consistent with the original tumor. Despite the limited number of mitotic figures, LDS-Falck 01 cells have a short doubling time, grow well at low density, can form large colonies in a semisolid medium, and recover from cryopreservation quickly, making this cell line a favorable model for *in vitro* investigations.

Chromosome analyses indicate that this cell line was derived from highly malignant cells expressing cytogenetic abnormalities that are associated with Ewing's sarcoma. LDS-Falck 01 shows a very complex karyotype with a hyperdiploid chromosomal number and considerable

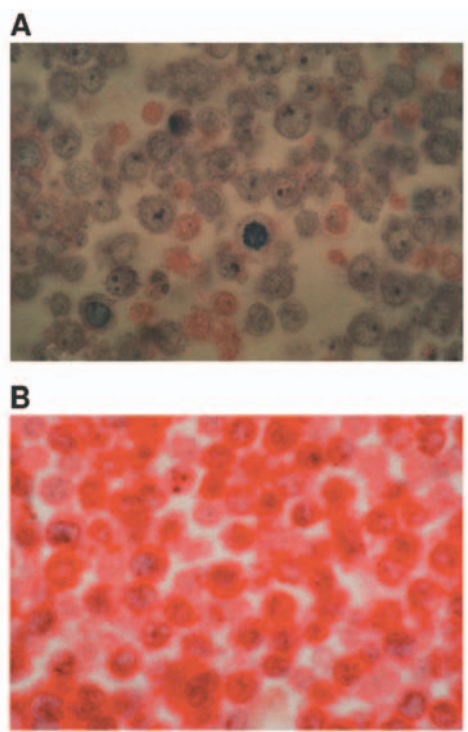


Figure 2. Morphological features of LDS-Falck 01 cells. Cytopins prepared from LDS-Falck 01 after 20 passages were stained using hematoxylin-eosin (A). Morphological analysis revealed the presence of a uniform population with a high nuclear:cytoplasmic ratio. The nuclei are round with finely dispersed chromatin and indistinct cytoplasm. (B) Staining for MIC2 antigen showed strong, diffuse staining in tumor cells (A, B: x1000 magnification, oil immersion).

Table 1. Antigenic expression on the established LDS-Falck 01 cell line. Fluorescence intensity was evaluated as linear units (U) in logarithmic scale using the geometric mean (geo mean), a statistical parameter used when events are not symmetrically distributed. Mean cell fluorescence was evaluated by comparing the geometric mean fluorescence of the control antibody-labelled sample with geometric mean fluorescence of the specific antibody-labelled sample. Fluorescence intensity: (-) no expression; (+/-) low expression; (+) moderate expression; (++) high expression; (+++) very high expression.

Antigenic markers	Geo mean (U)	Fluorescence intensity
CD99	930.81	++
CD45	41.70	-
CD44	15.73	-
CD117	144.53	+/-
HLADR	21.88	-
CD56	182.61	+/-
CD54	65.57	+/-
CD133	97.78	+/-
FAS	60.41	+/-
CD71	103.51	+/-
CD103	7.81	-
CD34	18.83	-
CD49D	99.82	+
HLAABC	41.06	-
CD29	34.98	+/-
CD90	5212.20	+++
CD138	71.07	+/-
CD86	46.49	+/-
CD38	24.13	+
CD57	296.59	+

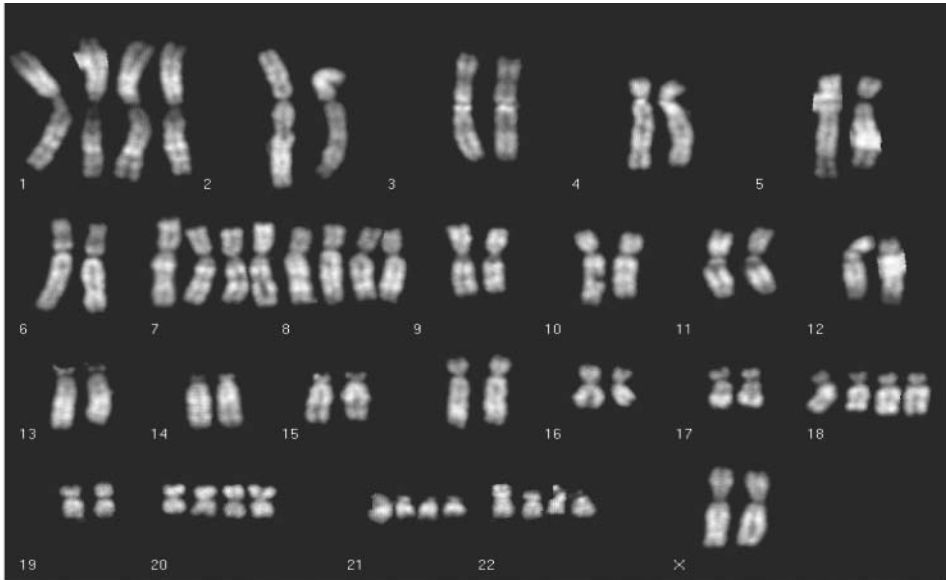


Figure 3. G-banded karyotype analysis of LDS-Falck 01 cells performed at passage 20. 62,XX,+1,+1,+7,+7,+8,+8,+18,+18,+20,+20,+21,+21,+der(22)t(11;22),(q24;q12)x2,+mar x2.



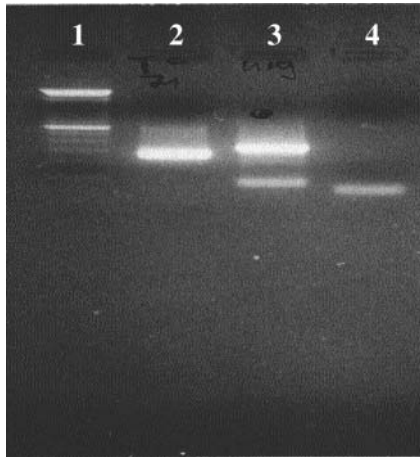


Figure 4. Identification of amplified transcript EWS-FLI1. Reverse transcriptase polymerase chain reaction (RT-PCR) detection of one chimeric transcript (fusion protein type 1, 330 bp). 1) Marker ladder 100 bp, 2) positive control (TC-71 cell line), 3) LDS-Falck 01 cell line, 4) negative control (healthy donor peripheral blood mononuclear cells).

heterogeneity as determined by the extent of the abnormalities and the number of each of the abnormal chromosomes. The most consistent abnormality is the unbalanced translocation between chromosome 11 and 22, usually balanced in ES (7), with the loss of chromosome 11. While the t(11;22) is usually balanced in ESFT, the presence of fusion on derivative 22 may be sufficient to explain the presence of fusion transcript product. There are also additional copies of various chromosomes, including chromosome 8, which are considered to be associated with tumor progression and poor clinical outcome (21, 22).

The antigenic pattern of the LDS-Falck 01 cell line is consistent with Ewing's sarcoma; in particular they are positive for CD99/MIC2, NSE and vimentin. As for the tyrosine kinase pathway, this cell line was weakly positive for HER2/neu, while negative for EGFR and CD117 (c-kit). These results, in keeping with previous reports (23, 24), do not exclude the possibility that different mechanisms of aberrant signalling in the tyrosine kinase or other signalling pathways may play a role in the pathogenesis and progression of ESFT (25).

In conclusion, we have established and characterized a new human cell line derived from the malignant pleural effusion of a patient with primitive neuroectodermal tumor. This cell line may represent a valid tool for investigating both the biological and molecular characteristics of this group of neoplasms and their sensitivity to therapeutic agents.

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## References

- 1 de Alava E and Gerald WL: Molecular biology of the Ewing's sarcoma/primitive neuroectodermal tumor family. *J Clin Oncol* 18: 204-213, 2000.
- 2 Bernstein M, Kovar H, Paulussen M, Randall RL, Schuck A, Teot LA and Juergens H: Ewing's sarcoma family of tumors: current management. *Oncologist* 11: 503-519, 2006.
- 3 Cohen N, Betts DR, Tavori U, Toren A, Ram T, Costantini S, Grotzer MA, Amariglio N, Rechavi G and Trakhtenbrot L: Karyotypic evolution pathways in medulloblastoma/primitive neuroectodermal tumor determined with a combination of spectral karyotyping, G-banding, and fluorescence *in situ* hybridization. *Cancer Genet Cytogenet* 149: 44-52, 2004.
- 4 Parham DM, Hijazi Y, Steinberg SM, Meyer WH, Horowitz M, Tzen CY, Wexler LH and Tsokos M: Neuroectodermal differentiation in Ewing's sarcoma family of tumors does not predict tumor behaviour. *Hum Pathol* 30: 911-918, 1999.
- 5 Halliday BE, Slagel DD, Elsheikh TE and Silverman JF: Diagnostic utility of MIC-2 immunocytochemical staining in the differential diagnosis of small blue cell tumors. *Diagn Cytopathol* 19: 410-416, 1998.
- 6 Kawauchi S, Fukuda T, Miyamoto S, Yoshioka J, Shirahama S, Saito T and Tsukamoto N: Peripheral primitive neuroectodermal tumor of the ovary confirmed by CD99 immunostaining, karyotypic analysis, and RT-PCR for EWS/FLI-1 chimeric mRNA. *Am J Surg Pathol* 22: 1417-1422, 1998.
- 7 Peter M, Couturier J, Pacquement H, Michon J, Thomas G, Magdelenat H and Delattre O: A new member of the ETS family fused to EWS in Ewing tumors. *Oncogene* 14: 1159-1164, 1997.
- 8 Kovar H: Progress in the molecular biology of Ewing tumors. *Sarcoma* 2: 3-17, 1998.
- 9 de Alava E, Kawai A, Healey JH, Fligman I, Meyers PA, Huvos AG, Gerald WL, Jhanwar SC, Argani P, Antonescu CR, Pardo-Mindan FJ, Ginsberg J, Womer R, Lawlor ER, Wunder J, Andrulis I, Sorensen PH, Barr FG and Ladanyi M: EWS-FLI1 fusion transcript structure is an independent determinant of prognosis in Ewing's sarcoma. *J Clin Oncol* 16: 1248-1255, 1998.
- 10 Aryee DN, Sommergruber W, Muehlbacher K, Dockhorn-Dworniczak B, Zoubek A and Kovar H: Variability in gene expression pattern of Ewing tumor cell lines differing in EWS-FLI1 fusion type. *Lab Invest* 60: 1833-1844, 2000.
- 11 Moritake H, Sugimoto T, Kuroda H, Hidaka F, Takahashi Y, Tsuneyoshi M, Yoshida MA, Cui Q, Akiyoshi K, Izumi T and Nunoi H: Newly established Askin tumor cell line and overexpression of focal adhesion kinase in Ewing sarcoma family of tumors cell lines. *Cancer Genet Cytogenet* 146: 102-109, 2003.
- 12 Kodama K, Doi O, Higashiyama M, Mori Y, Horai T, Tateishi R, Aoki Y and Misawa S: Establishment and characterization of a new Ewing's sarcoma cell line. *Cancer Genet Cytogenet* 57: 19-30, 1991.
- 13 Nishio J, Iwasaki H, Hshiguro M, Ohjimi Y, Fujita C, Yanai F, Nibu K, Mitsudome A, Kaneko Y and Kikuchi M: Establishment and characterization of a novel human desmoplastic small round cell tumor cell line, JN-DSRCT-1. *Lab Invest* 82: 1175-1182, 2002.
- 14 Metwally H, Cheng J, Maruyama S, Ohshiro K, Suzuki I, Hoshina Y and Saku T: Establishment and characterization of new cell lines derived from melanotic neuroectodermal tumor of infancy arising in the mandible. *Pathol Int* 55: 331-342, 2005.

- 15 O'Regan S, Diebler MF, Meunier FM and Vyas S: A Ewing's sarcoma cell line showing some, but not all, of the traits of a cholinergic neuron. *J Neurochem* 64: 69-76, 1995.
- 16 Batanian JR, Bridge JA, Wickert R, Vogler C, Gadre B and Huang Y: EWS/FLI-1 fusion signal inserted into chromosome 11 in one patient with morphologic features of Ewing sarcoma, but lacking t(11;22). *Cancer Genet Cytogenet* 133: 72-75, 2002.
- 17 Brando B, Barnett D, Janossy G, Mandy F, Autran B, Rothe G, Scarpati B, D'Avanzo G, D'Hautcourt JL, Lenkei R, Schimtz G, Kunkl A, Chianese R, Papa S and Gratama JW: Cytofluorimetric methods for assessing absolute numbers of cell subsets in blood. *Cytometry (Comm Clin Cytom)* 42: 327-346, 2000.
- 18 Shaffer LG and Tommerup N (eds.). *ISCN 2005: An International System for Human Cytogenetic Nomenclature*. Basel, Switzerland: S. Karger. 2005
- 19 West DC, Grier HE, Swallow MM, Demetri GD, Granowetter L and Sklar J: Detection of circulating tumor cells in patients with Ewing's sarcoma and peripheral primitive neuroectodermal tumor. *J Clin Oncol* 15: 583-588, 1997.
- 20 Bandres E, Malumbres R, Escalada A, Cubedo E, Gonzales I, Honorato B, Zarate R, Garcia-Foncillas J and de Alava E: Gene expression profile of ewing sarcoma cell lines differing in their EWS-FLI1 fusion type. *J Pediatr Hematol Oncol* 27: 537-542, 2005.
- 21 Hattinger CM, Rupler S, Strehl S, Ambros IM, Zoubek A, Potschger U, Gadner H and Ambros PF: Prognostic impact of deletion at 1p36 and numerical aberration in Ewing tumors. *Genes Chrom Cancer* 24: 243-254, 1999.
- 22 Udayakumar AM and Sundareshan TS: Cytogenetic characterization of Ewing tumors: further update on 20 cases. *Cancer Genet and Cytogenet* 133: 102-103, 2002.
- 23 Zhou Z, Jia SF, Hung MC and Kleinerman ES: E1A sensitizes HER2/neu overexpressing Ewing's sarcoma cells to topoisomerase II-targeting anticancer drugs. *Cancer Res* 61: 3394-3398, 2001.
- 24 Thomas DG, Giordano TJ, Sanders D, Biermann JS and Baker L: Absence of HER2/neu gene expression in osteosarcoma and Ewing's sarcoma. *Clin Cancer Res* 8: 788-793, 2002.
- 25 Kraus DS and Van Etten RA: Tyrosine kinases as targets for cancer therapy. *New Engl J Med* 353: 172-187, 2005.

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