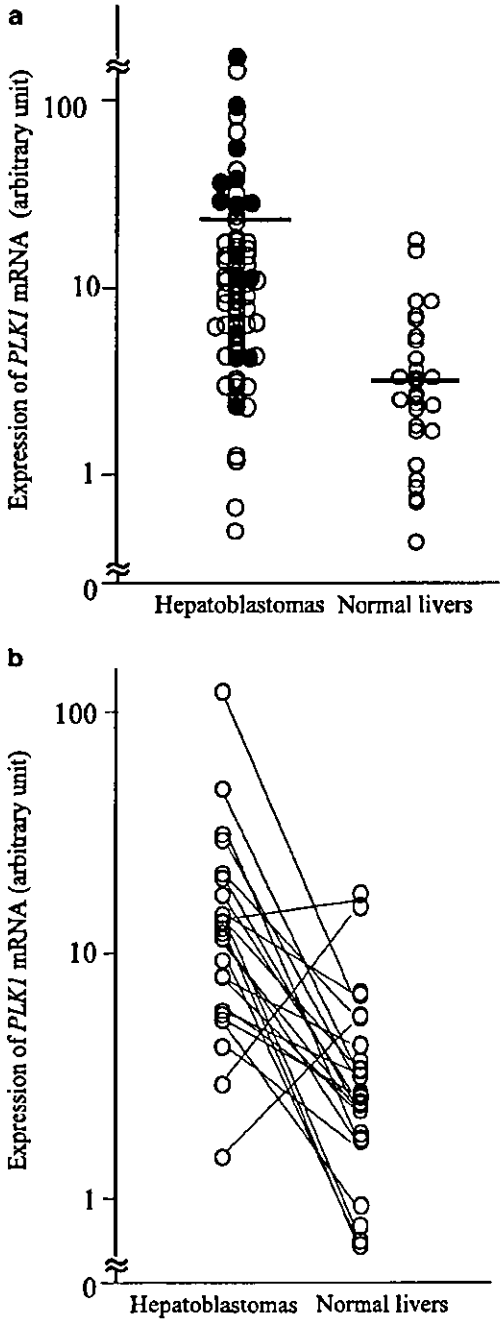


**Figure 3** mRNA expression of *PLK1* in HBLs and the corresponding normal livers measured by quantitative real-time RT-PCR. (**a**) The levels of *PLK1* mRNA expression in HBLs and normal livers. The expression levels of *PLK1* were determined by quantitative real-time RT-PCR analysis using 74 HBL tissues and 29 normal livers (see Materials and methods). The *PLK1* expression values were normalized by *GAPDH*. Open and closed circles represent alive and dead, respectively. Since the values of the *PLK1* expression were skewed, a log transformation was used for the expression values. The bars show mean values. (**b**) Correlation of *PLK1* expression between HBL and its corresponding normal liver in 24 paired samples

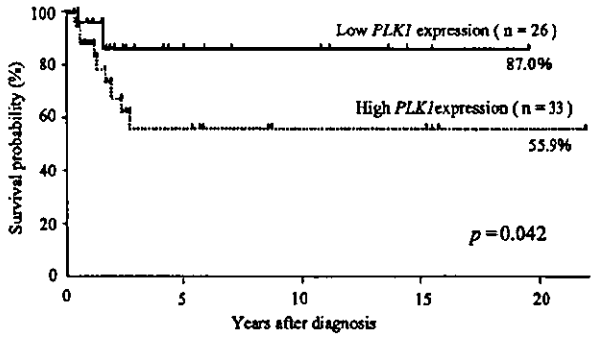


**Figure 4** Kaplan–Meier survival curves ($n = 59$) in relation to the expression levels of *PLK1* (median cutoff). The arbitrary median cutoff value was set as 13. The patients with high expression of *PLK1* represented significantly poor prognosis than those with its low expression

**Table 4** Univariate Cox regression analysis using *PLK1*(log) and dichotomous factors of *β-catenin* mutation, age, stage, and histology ($n = 59$)

| *Factor* | n | P-*value* | *HR (95% CI)* |
|---|---|---|---|
| *PLK1*(log) | 59 | 0.015 | 1.62 (1.10, 2.40) |
| *β-catenin* (mutant vs wild type) | 58 | 0.27 | 1.85 (0.62, .5.56) |
| Age (>1 vs ≤1 year) | 55 | 0.76 | 1.22 (0.33, 4.52) |
| Stage (3, 4 vs 1, 2) | 56 | 0.083 | 3.81 (0.84, 17.2) |
| Histology (poorly vs well) | 53 | 0.025 | 4.48 (1.21, 16.6) |

All variables with two categories, except *PLK1*(log); HR = hazard ratio shows the relative of death of first category relative to second; CI = confidence interval

or histology, but marginally related to survival after controlling both histology and stage (Table 5).

## Discussion

HBL is one of the embryonal tumors in close relation to the normal as well as abnormal tissue development. To understand the molecular basis of the genesis of HBL, here we randomly cloned a large number of genes expressed in HBLs with or without AFP production and in a non-tumorous infant's liver. Extensive screening for the differentially expressed genes between the tumors and their corresponding normal livers has successfully identified at least 86 genes including 40 with unknown function, which may potentially contribute to develop new therapeutic strategies against HBLs with poor prognosis.

### *HBL cDNA libraries*

We have identified the genes with unknown function in approximately 8% of the total 10431 clones obtained from our oligo-capping cDNA libraries. The comparison of the frequently appeared genes in each libraries shows that expression profile is relatively similar between AFP-positive HBL and the normal part of the infant's liver, whereas it is very different between AFP-positive and AFP-negative tumors, in which many genes

**Table 5** Multivariable Cox regression analysis using *PLK1*(log) and dichotomous factors of *β-catenin* mutation, age, stage, and histology ($n = 50$)

| *Variable* | P-*value* | *Variable* | P-*value* | *Variable* | P-*value* |
|---|---|---|---|---|---|
| *PLK1*(log) | 0.009 | *β-catenin* (mutant vs. wild type) | 0.51 | | |
| *PLK1*(log) | 0.005 | Age (>1 vs ≤1 year) | 0.92 | | |
| *PLK1*(log) | 0.019 | Stage (3, 4 vs 1, 2) | 0.46 | | |
| *PLK1*(log) | 0.027 | Histology (poorly vs well) | 0.12 | | |
| *PLK1*(log) | 0.052 | Histology (poorly vs well) | 0.12 | Stage (3, 4 vs 1, 2) | 0.47 |

All variables with two categories, except *PLK1*(log)

are downregulated (Table 2). In the library of the latter tumor, *vimentin*, *RNA-binding motif protein*, *Wnt inhibitory factor-1*, *dickkopf*, and *RAP1B* are frequently appeared, whereas they are hardly appeared in the other libraries. Wissmann *et al.* (2003) have recently reported that *WIF1* is downregulated in various cancers (prostate cancer, breast cancer, non-small-cell lung cancer, and bladder cancer), and suggested that loss of WIF1 expression may be an early event in tumorigenesis in those tissues. It is notable that, in contrast to AFP-positive HBLs, the patient's outcome of the tumor with negative AFP is very poor, though the incidence of the latter tumor is low (von Schweinitz *et al.*, 1995). This suggests that AFP-positive and AFP-negative HBLs have a different genetic as well as biological background. In addition, recent reports have demonstrated that frequent mutation of the *β-catenin* gene and nuclear accumulation of its protein product are one of the main causes of the tumorigenesis of HBL. The *APC* and *Axin* genes are also mutated in some HBLs (Oda *et al.*, 1996; Miao *et al.*, 2003; Thomas *et al.*, 2003), indicating that Wnt signaling pathway plays an important role in causing the tumors, most of which are AFP-positive. Therefore, the poor-prognostic HBL without producing AFP might be caused by the particular mechanism additional to or other than the abnormality of Wnt signaling pathway. Although the appearance frequency of the genes in each library does not always reflect the actual expression levels of each gene, it may at least in part show the differences among the tumor subsets with different genetic abnormalities. As our libraries contain many genes involved in liver development, normal liver functions, and carcinogenesis, they must be useful for making a liver-proper cDNA microarray to analyse expression profiles of HBL, viral infection-induced hepatitis, liver cirrhosis, and HCC.

### *Differentially expressed genes between HBLs and the corresponding normal livers*

cDNA microarray, which is often applied to a comprehensive gene expression analysis, is able to detect many genes that are differentially expressed between tumors and normal tissues (Okabe *et al.*, 2001; Nagata *et al.*, 2003). However, it is expensive and needs further confirmation of the selected genes by a semi-quantitative RT–PCR or a real-time RT–PCR method. Therefore, using semi-quantitative RT–PCR and the specific primers of 1188 cDNAs, we have identified 86 genes differentially expressed between HBLs and their corresponding normal livers. Surprisingly, 75 out of 86 genes are preferentially expressed in the latter tissues, and only 11 including *RAN*, *PLK1*, *NPC1*, and *OLR1* known genes are expressed at high levels in HBLs. One of the reasons of this result may be that many gene products, which are necessary for full function in the matured liver metabolism, are dispensable for the malignant growth of the tumor except for the very limited genes. The results of some differentially expressed genes are consistent with those in the previous reports. von Horn *et al.* (2001) have shown that the mRNA levels of *insulin-like growth factor-binding proteins* including *IGFBP-3* are decreased in HBLs than in normal livers. Kinoshita and Miyata (2002) have also reported that *aldolase B* mRNA is downregulated in over 50% of 20 HCCs examined. They proposed that the measurement of aldolase activity in serum is useful to determine the number of collapsed hepatic cells in cirrhosis. Recently, evidences suggest that not only mutant β-catenin but also wild-type β-catenin localize in the cellular nuclei of HBL as well as some other cancers (Rimm *et al.*, 1999; Takayasu *et al.*, 2001). The increased expression of the *Ran* gene in HBLs might be correlated with the shuttling of β-catenin and/or other related proteins between cytoplasm and nucleus in the tumor cells.

Owing to constitutive activation of Wnt signaling in most of the HBLs, the 86 genes differentially expressed between the tumor and its corresponding normal liver were expected to contain downstream target genes of Wnt signaling pathway that might regulate early stage of the hepatic development. In this study, however, only the *lymphocyte alpha-kinase* (*LAK*) gene was found to be differentially expressed at high levels in HBLs with wild-type *β-catenin* and at low levels in those with *β-catenin* mutation. LAK is a new class of protein kinases with a novel catalytic domain, but its precise function is currently unknown (Ryazanov *et al.*, 1999). Thus, our result may suggest that the target genes of the Wnt signaling pathway are commonly affected in HBLs, regardless of the presence or absence of *β-catenin* mutation.

### *PLK1 as a prognostic indicator of HBL*

*PLK1* (*polo-like kinase 1*), the human counterpart of *polo* in *Drosophila melanogaster* and of *CDC5* in *Saccharomyces cerevisiae*, encodes a serine/threonine kinase with polo-box domains (Clay *et al.*, 1993). PLK1 is crucial for various events of mitotic progression including centrosome maturation (Lane and Nigg,

1996), spindle function (Glover *et al.*, 1996), activation of cyclin B/Cdc2 (Qian *et al.*, 1998; Toyoshima-Morimoto *et al.*, 2001), and regulation of anaphase-promoting complex (Kotani *et al.*, 1998; Nigg, 1998). Elevated expression of *PLK1* is also found in different types of adult cancers including non-small-cell lung cancer, head and neck tumors, esophageal carcinomas, melanomas, and colorectal cancers (Wolf *et al.*, 1997; Knecht *et al.*, 1999; Tokumitsu *et al.*, 1999; Dietzmann *et al.*, 2001; Takai *et al.*, 2001), implying its critical role in tumorigenesis. In the present study, we have found that *PLK1* is upregulated in primary HBLs, and that its mRNA expression levels are significantly correlated with poor outcome of the patients. Multivariate Cox regression analysis indicated that *PLK1* expression could be an independent prognostic factor from *β-catenin* mutation, age, stage, or histology. However, clinical stage did not show a significant correlation with *PLK1* expression, though it is one of the critical prognostic markers. One of the possible reasons may be that the 59 tumors we used for statistical analysis include two unusual patients, one had stage 4 tumor with good prognosis and another case had stage 1 tumor with poor prognosis. These might have reduced the significance of the tumor stage in patients' survival in our sample set.

It is notable that, among the 1188 genes we have screened for differential expression, *PLK1* is the only one known oncogene overexpressed in the HBL tissues. Smith *et al.* (1997) have reported that constitutive expression of *PLK1* in NIH3T3 cells causes oncogenic focus formation and forms tumors in nude mice. Furthermore, Liu and Erikson (2003) have recently shown that the application of small interfering RNA which specifically depletes *PLK1* expression in cancer cells inhibits cell proliferation, arrests cell cycle, and induces apoptosis. Thus, PLK1 may play a crucial role in causing HBL and other cancers. It may be interesting to examine whether *PLK1* is a target of β-catenin transported from the cytosol into the nucleus. The disruption of PLK1 function could be a future therapeutic tool for the aggressive type of hepatoblastomas.

In conclusion, our HBL cDNA project has provided a large number of genes related to liver development, metabolism, and carcinogenesis. We are currently applying these genes to the cDNA microarray system. Our cDNA resource should be an important tool to understand the molecular mechanism of the genesis of HBL as well as to develop new diagnostic and therapeutic strategies against the aggressive tumors in the future.

## Materials and methods

### *Clinical materials*

Tumor tissues and their corresponding normal liver tissues were frozen at the time of surgery and stored at −80°C until use. All specimens were provided from the Tissue Bank of the Japanese Study Group for Pediatric Liver Tumor (JPLT) (Uotani *et al.*, 1998). A total of 74 HBL samples (seven were classified as being stage 1, 17 as stage 2, 26 as stage 3, 15 as stage 4, and nine were unknown stages) were used in this study. The tumors were staged according to the Japanese histopathological classification of HBL (Hata, 1990). From 1991 to 1999, HBLs had been treated by combination chemotherapy using cisplatin and THP-adriamycin according to the JPLT-1 protocol (Sasaki *et al.*, 2002). After 2000, a more intensive chemotherapeutic regimen, ITEC (ifosfamide, THP-adriamycin, etoposide, and carboplatin), has been utilized for tumors that prove resistant to the combination chemotherapy in the JPLT-2 study. Among the 74 tumor samples we examined, 36 and 35 tumor tissues were obtained prior to and after chemotherapy, respectively, and the remaining three were unknown. In the same sample set, 59 tumors were accompanied by outcome information and used for making survival curves, among which 31 and 28 tissues were obtained prior to and after chemotherapy, respectively. Tumor histology was also classified according to Hata (1990). 'Poor histology' indicates 'poorly differentiated (embryonal type)', and 'well histology' indicates 'well-differentiated (fetal type)'. The informed consents were obtained in each institution or hospital. High molecular weight DNA and total RNA of these samples were prepared as described previously (Ichimiya *et al.*, 1999).

### *Construction of oligo-capping cDNA libraries*

Four oligo-capping cDNA libraries, two (HMFT, HYST) derived from HBLs with secretion of AFP, one (HKMT) from HBL without AFP secretion, and one (HMFN) from the corresponding normal liver, were constructed according to the method previously described (Suzuki *et al.*, 1997). These were approved by the institutional review board. The oligo-capping method enables full-length cDNA cloning with high efficiency. The 12000 cDNA clones in total were randomly picked up and single-run sequencing was performed. Nucleotide sequence of both ends for each cDNA clone was homology-searched against the public nucleotide database using the BLAST program at the National Center for Biotechnology Information (NCBI) (Genbank release 122, January 2001).

### *Differential screening of the genes by semi-quantitative RT–PCR*

The eight samples were selected as PCR templates to screen for the differentially expressed genes. Cases 58 and 81 were defined as stage 2 HBL, cases 10, 67, 78, and 85 were in stage 3, case 14 was in stage 4. Among those eight tumors, four (cases 14, 67, 78, and 81) had the mutant *β-catenin*, and the others (cases 10, 58, 77, and 85) not. Mutation analysis for *β-catenin* was performed as described previously (Takayasu *et al.*, 2001). The differential expression of the genes between the HBL and normal livers was confirmed at least twice using semi-quantitative RT–PCR. The individual gene-specific PCR primer sequences were determined by using Primer3 program (provided at Washington University). For cDNA templates, 5 μg of total RNA was converted to cDNA using random primers (Takara, Otsu, Japan) with SuperScript II RNaseH⁻ reverse transcriptase (Gibco BRL, Rockville, MD, USA). Those cDNAs were at first amplified with *GAPDH* primers for 27 cycles and the amounts of the PCR products were measured by ALF Express™ sequencer and normalized. The amplification was performed 35 or 40 cycles of 95°C for 30 s, 57 or 59 or 61°C for 15 s and 72°C for 60 s, and the final extension was at 72°C for 5 min, using a Perkin-Elmer Thermalcycler 9700 (Perkin-Elmer, Foster City, CA, USA). The PCR products

were run on 2% agarose gels and stained with ethidium bromide. We defined the gene as differentially expressed when it exhibits differential expression between the tumor and its corresponding normal liver in more than four out of the eight samples.

*Northern blot analysis*

In all, 25 $\mu$g of total RNA from the primary HBLs, HCC, and their corresponding normal livers were subjected to Northern analysis. Total RNA was prepared according to the method of Chomczynski and Sacchi (1987). Total RNA was fractionated by electrophoresis on 1% agarose gel containing formaldehyde, transferred onto a nylon membrane filter, and immobilized by UV crosslinking. The hybridization cDNA probe was a 976-base pair human *PLK1* cDNA fragment and labeled with [$\alpha$-$^{32}$P]-dCTP using the BcaBEST random priming kit (Takara Biomedicals). The filter was hybridized at 65°C in a solution containing 1 M NaCl, 1% SDS, 7.5% dextran sulfate, 100 $\mu$g/ml of heat-denatured salmon sperm DNA, and radiolabeled probe DNA.

*Quantitative real-time RT–PCR of PLK1*

The primer set for amplification of the *PLK1* and probe sequence are as follows: forward primer, 5′-GCTGCACAAG AGGAGGAAA-3′; reverse primer, 5′-AGCTTGAGGTCTC-GATGAATAAC-3′; probe, 5′-CCTGACTGAGCCTGAGG CCCGATAC-TA-3′. Taqman *GAPDH* control reagents (Perkin-Elmer/Applied Biosystems) were used for the amplification of *GAPDH* as recommended by the manufacturer. PCR was performed using ABI Prism 7700 Sequence Detection System (Perkin-Elmer/Applied Biosystems). In all, 2 $\mu$l of cDNA was amplified in a final volume of 25 $\mu$l containing 1 × Taqman PCR reaction buffer, 200 $\mu$M each dNTP, 0.9 $\mu$M each primer, and 200 nM Taqman probe. The optional thermal cycling condition was as follows: 40 cycles of a two-step PCR (95°C for 15 s, 60°C for 60 s) after the initial denaturation (95°C for 10 min). Experiments were carried out in triplicate for each data point.

*Statistical analysis*

Statistical analyses were performed using Mann–Whitney's *U*-test and Cox regression. A *P*-value of less than 0.05 was considered significant.

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# Usefulness of Tyrosine Hydroxylase mRNA for Diagnosis and Detection of Minimal Residual Disease in Neuroblastoma

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**Neuroblastoma (NB) is the most common malignant solid tumor in childhood and, among all childhood malignancies, is second only to leukemia. NB originates before birth in the neural crest, which develops into the adrenal medullae and sympathetic ganglia. In the adrenal medulla, tyrosine hydroxylase (TH) is the first enzyme in the pathway of catecholamine synthesis. We used reverse transcription polymerase chain reaction (RT-PCR) to examine the expression of TH mRNA in NB and Ewing's sarcoma cell lines, small round cell tumors (SRCTs) containing NB, and other clinical tumor samples (osteosarcoma, osteochondroma, and Wilms' tumor). In total, we analyzed 33 clinical tumor samples. TH mRNA was expressed in all three NB cell lines examined, but not in two ES cell lines or in a breast cancer cell line. We detected TH mRNA in 23 of 25 NB tumor samples (92%), but in none of the SRCTs or other clinical tumor samples. This RT-PCR technique showed a sensitivity for TH mRNA of one NB cell per $10^5$ negative cells. Based on these results, the detection of TH mRNA is very useful both as a tumor marker for NB and for detecting minimal residual disease. Therefore, we can use this method to detect tumor cell contamination before hematopoietic stem cell transplantation.**

**Key words** neuroblastoma; tyrosine hydroxylase; hematopoietic stem cell transplantation; minimal residual disease; reverse transcription polymerase chain reaction

Neuroblastoma (NB) is a very common malignant solid tumor in childhood. NB belongs to the small round cell tumors (SRCTs), which include other solid tumors such as Ewing's sarcoma (ES), rhabdomyosarcoma, and malignant lymphoma.[1] SRCTs are histologically ambiguous, so it is necessary to analyze adequate tumor markers for an accurate diagnosis. Patients who are over the age of 1 year at diagnosis usually have poor prognoses.[2]

NB is also characterized by elevated levels of catecholamine production. Tyrosine hydroxylase (TH) is very important as the first and rate-limiting step in the synthesis of catecholamines.[3—6] Therefore, we used reverse transcription polymerase chain reaction (RT-PCR) to examine the expression of TH mRNA in some cell lines, SRCTs, and other clinical tumor samples, to assess whether we can use it as a tumor marker and detect cell contamination in hematopoietic stem cells. Specific TH mRNA could be detected in NB cell lines and clinical NB tumor samples, but not in other cell lines and tumor samples. Moreover, the technique had a high sensitivity of $1/10^5$.

We think that this method should be used for detecting minimal residual disease because the prognoses of patients in NBs depend on being positive or negative for TH mRNA in bone marrow (BM) samples within 4 months after chemotherapy.[7] Moreover, the risk of relapse after autologous peripheral blood stem cell (PBSC) or BM transplantation is high if there is NB cell contamination.[8,9]

Here, we describe a very useful method for detecting minimal residual disease. The method can also be used as a tumor marker.

## MATERIALS AND METHODS

**Cell Lines and Tumor Samples** Three NB cell lines (IMR-32,[10,12] SK-N-SH,[11,12] and NB-39,[12] two ES cell lines (NCR-EW2,[13] SCMC-ES1[14]), and one breast cancer cell line (MCF-7[15]) were examined. They were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 2 g/l sodium bicarbonate under 5% $CO_2$ at 37 °C. Thirty-three clinical tumor samples (25 NBs, 1 ES, 1 osteosarcoma, 1 osteochondroma, 1 Wilms' tumor, 1 malignant lymphoma, and 3 rhabdomyosarcoma) were examined. One of the three rhabdomyosarcoma samples was from a recurrent tumor. These tissues had been stored at −80 °C since collection. The clinical diagnoses for these patients were made by histopathology. Informed consent was obtained from all patients before they entered this study.

**RNA Extraction** Total RNA was extracted from the six cell lines using the acid-guanidium-phenol chloroform method after treatment with Catrimox-14™. Total RNA from the 33 clinical tumor samples was extracted by TRIZOL reagent (GIBCO BAL) based on the acid-guanidium-phenol chloroform extraction method.[16]

**Reverse Transcription Polymerase Chain Reaction** Total RNA (1 μg) was reverse-transcribed in a 10 μl reaction mixture with a first strand cDNA synthesis kit (Rever Tra-α-TM, TOYOBO). RT was performed with Oligo-dT. The mixture was annealing at 42 °C for 20 min, followed by incubated at 99 °C for 5 min, and then held at 4 °C. PCR amplification was carried out in 10× reaction mixture containing 1.2 pmol of the respective primers. We used a HOT START PCR kit from KOD-Plus- (TOYOBO).[17—19] The PCR conditions were one cycle of template denaturing at 94 °C for

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Table 1. PCR Primers

| Gene | Sequence | Location |
|---|---|---|
| TH sense | 5'-TGT CAG AGC TGG ACA AGT GT-3' | Exon 8 |
| TH anti-sense | 5'-GAT ATT GTC TTC CCG GTA GC-3' | Exon 9 |
| GAPDH sense | 5'-TCC TCT GAC TTC AAC AGC GAC ACC-3' | Exon 5 |
| GAPDH anti-sense | 5'-TCT CTC TTC CTC TTG TGC TCT TGG-3' | Exon 8 |

2 min, followed by 28 cycles of denaturing at 94 °C for 15 s, annealing at 60 °C for 90 s, extension at 68 °C for 20 s, and then holding at 4 °C. We used glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal marker and IMR-32, NB-39, and SK-N-SH neuroblastoma cell lines as positive controls. NCR-EW2, SCMC-ES1, and MCF-7 were examined as negative controls. The primer sequences are listed in Table 1.[20] The primers for TH are located in exons that are not affected by alternative splicing. To avoid contamination with genomic DNA, the reverse primers for both TH and GAPDH were located in successive exons.

**Analyses of PCR Products** The PCR products were electrophoresed through 2.0% agarose gel, stained with ethidium bromide (WAKO), and visualized under a UV lamp. We used a Bioanalyzer (Agilent Technologies) to accurately determine band sizes.

**Detection for Sensitivity** A NB cell line (IMR-32) was examined as a positive control and an ES cell line (NCR-EW2) as a negative control. We counted the number of each cell type and diluted them to make suspensions containing one NB cell per 10 ES cells, one per $10^2$, one per $10^3$, one per $10^4$, one per $10^5$, and one per $10^6$. We investigated the sensitivity of this RT-PCR technique for detecting TH mRNA.

## RESULTS AND DISCUSSION

We analyzed six cell lines for detecting a specific TH mRNA with the RT-PCR technique. The electrophoresis and Bioanalyzer results are presented in Figs. 1A and 1B. The band size for TH was 299 bp, and the internal marker GAPDH was 209 bp. TH mRNA was detected in three NB cell lines (IMR-32, NB-39, and SK-N-SH), but it was not detected in the ES cell lines (NCR-EW2, SCMC-ES1) or the breast cancer cell line (MCF-7).

We investigated 33 clinical tumor samples by the same method as that used for the cell lines. The electrophoresis and Bioanalyzer results are presented in Tables 2 and 3. In 23 of 25 NB tumor samples (92%), TH mRNA could be detected, but it was not detected in the T1 and T2 samples (Table 2). TH mRNA was not detected in the osteosarcoma (T26), osteochondroma (T27), Wilms' tumor (T28), and SRCT (T29—T33) samples (Table 3). The rhabdomyosarcoma from a recurrent tumor (T33) also did not express TH mRNA.

We examined the sensitivity for detecting minimal residual disease by this RT-PCR technique. We used a NB cell line (IMR-32) as a positive control and an ES cell line (NCR-EW2) as a negative control. The electrophoresis and Bioanalyzer results are presented in Figs. 2A and 2B. On the electrophoresis, a TH mRNA band could be seen in samples with concentrations down to one NB cell per $10^5$ ES cells, but not at a concentration of one NB cell per $10^6$ ES cells (Fig. 2A).

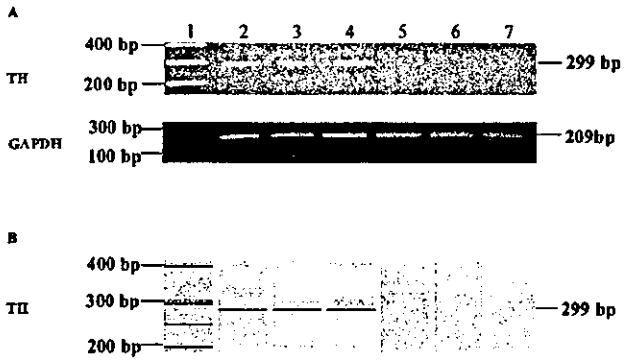


Fig. 1. RT-PCR Products Detected in Cell Lines

(A) Bands detected by electrophoresis. Lane 1, marker; lane 2, IMR-32; lane 3, NB-39; lane 4, SK-N-SH; lane 5, NCR-EW2; lane 6, SCMC-ES1; lane 7, MCF-7. (B) Bands detected by Bioanalyzer. Lanes are as for electrophoresis. A 299 bp RT-PCR product for TH was seen by both electrophoresis and Bioanalyzer, and a 209 bp product for GAPDH was also seen.

Table 2. NB Patients' Clinical Profile and TH mRNA Bands Detected by Electrophoresis and Bioanalyzer

| Tissue | Sex | Age | Diagnosis | TH mRNA |
|---|---|---|---|---|
| T1 | F | 1 y | NB | − |
| T2 | F | 10 m | NB | − |
| T3 | M | 10 m | NB | + |
| T4 | M | 10 m | NB | + |
| T5 | M | 9 m | NB | + |
| T6 | M | 3 y | NB | + |
| T7 | M | 7 m | NB | + |
| T8 | M | 4 m | NB | + |
| T9 | F | 10 m | NB | + |
| T10 | F | 1 y | NB | + |
| T11 | F | 6 y | NB | + |
| T12 | F | 1 y | NB | + |
| T13 | F | 8 m | NB | + |
| T14 | F | 6 m | NB | + |
| T15 | M | 4 y | NB | + |
| T16 | F | 3 y | NB | + |
| T17 | M | 4 y | NB | + |
| T18 | F | 10 y | NB | + |
| T19 | M | 9 y | NB | + |
| T20 | F | 7 m | NB | + |
| T21 | F | 9 m | NB | + |
| T22 | F | 10 m | NB | + |
| T23 | M | 3 y | NB | + |
| T24 | M | 5 y | NB | + |
| T25 | F | 5 y | NB | + |

NB: neuroblastoma.

Similarly, using the Bioanalyzer, TH mRNA could be detected at one NB cell per $10^5$ ES cells but not at one NB cell per $10^6$ ES cells (Fig. 2B).

Abnormal amplification and expression are well known for *MYCN*,[21—23] *trk-A*,[24—27] and protein gene product 9.5 (PGP9.5)[28] in NB. However, these markers are not very useful for diagnosis. Recently, by using monoclonal antibodies

Table 3. Other Tumor Patients' Clinical Profile and TH mRNA Bands Detected by Electrophoresis and Bioanalyzer

| Tissue | Sex | Age | Diagnosis | TH mRNA |
|---|---|---|---|---|
| T26 | M | 13 y | OS | - |
| T27 | F | 12 y | OC | - |
| T28 | M | 8 m | WT | - |
| T29 | F | 6 m | ML | - |
| T30 | M | 3 y | ES | - |
| T31 | M | 11 y | RMS | - |
| T32 | F | 3 y | RMS | - |
| T33 | M | 4 y | RMS | - |

OS: osteosarcoma, OC: osteochondroma, WT: Wilms' tumor, ML: malignant lymphoma, ES: Ewing's sarcoma, RMS: rhabdomyosarcoma.

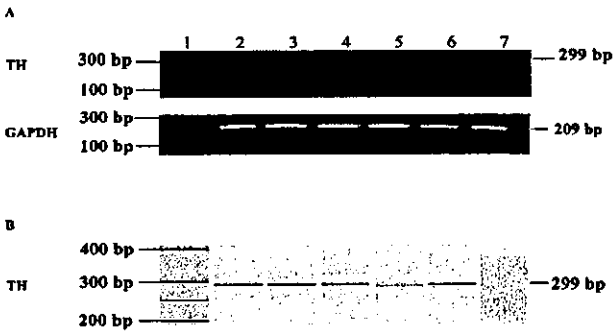


Fig. 2. Sensitivity of RT-PCR Technique for TH mRNA

Lane 1, marker; lane 2, one NB cell per 10 ES cells; lane 3, one NB cell per $10^2$ ES cells; lane 4, one NB cell per $10^3$ ES cells; lane 5, one NB cell per $10^4$ ES cells; lane 6, one NB cell per $10^5$ ES cells; lane 7, one NB cell per $10^6$ ES cells. (A) Bands detected by electrophoresis. (B) Bands detected by Bioanalyzer. The 299 bp RT-PCR product for TH was detected by both electrophoresis and Bioanalyzer, and a 209 bp product for GAPDH was also seen.

that react selectively to cells of neuroectodermal origin, the diagnostic usefulness has been improved. However, the monoclonal antibodies often produce false positive results,[29,30] and the clinical importance of detecting positive cells as evidence of infiltration is still debated.[31] As NB has a specific catecholamine metabolism, it may be more useful to detect TH, which is the first and rate-limiting enzyme of catecholamine synthesis. We found that TH mRNA was expressed in all NB cell lines (100%) and in 23 of 25 (92%) clinical NB tumor samples. On the other hand, it was not expressed in any of the other cell lines and clinical tumor samples.

Thus, we found that TH mRNA is expressed specifically in NB, and this specific expression can be use to distinguish NB from SRCTs. TH mRNA can also be used as a tumor marker for the accurate diagnosis of NB.

The expression of TH mRNA did not correlate with the patient's age or sex, and it might not be a prognostic factor for NB patients.

Another important problem is determining whether tumor cell contamination exists when a patient's PBSC or BM is used for autologous transplantation. Our method can detect contamination of one cell in $10^5$.

In future, our method may be very useful for diagnosing NB patients and detecting minimal residual disease in clinical samples.

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# Establishment and characterization of a biphasic synovial sarcoma cell line, SYO-1

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**Abstract**

We describe here the establishment of a new synovial sarcoma cell line, SYO-1, derived from a biphasic synovial sarcoma that developed in the groin of a 19-year-old female. The cell line was maintained for more than 70 passages (more than 24 months) in vitro. The SYO-1 cells in monolayer culture exhibited a spindle shape without conspicuous pleomorphism. Immunohistochemically, the cells were positive for vimentin, type IV collagen, S-100, mdm2, bcl-2, c-Met and c-Kit. Tumors developed by their implantation in nude mice histologically showed biphasic features that were composed of areas of fascicles of spindle cells and areas of compact proliferation of polygonal to ovoid cells, which occasionally formed epithelial plaque and expressed cytokeratin and EMA. SYO-1 cells harbored the characteristic t(X;18)(p11.2;q11.2) translocation by chromosome analysis and SYT-SSX2 chimeric transcript by RT-PCR. The SYO-1 cells, the first characterized cell line derived from biphasic synovial sarcoma retaining the characteristic genetic and phenotypic features of the tumor, will be useful for various investigations on synovial sarcoma, especially for its epithelial differentiation.


*Keywords:* Synovial sarcoma; Biphasic; Epithelial differentiation; Cell line; SYT-SSX

## 1. Introduction

Synovial sarcoma is characterized by its unique feature that displays, to varying degrees, an epithelial phenotype despite its connective tissue origin. Although the tumor is named 'synovial' sarcoma because of its histologic resemblance to normal synovial tissue, its exact histogenesis or line of differentiation remains unclear [1].

Cytogenetic and molecular studies have revealed that synovial sarcoma harbors a characteristic chromosomal translocation t(X;18)(p11.2;q11.2), which results in fusion of the SYT gene at 18q11 and either of the SSX1 or the SSX2 gene (or rarely, SSX4) at Xp11 [2–4]. Because of its presence in a high

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proportion (greater than 90%) of synovial sarcomas, the generation of this translocation was suggested to be a primary causal event in tumor development. The SYT-SSX fusion product is thought to regulate the expression of a particular set of genes that may eventually develop the tumor. However, these putative SYT-SSX target genes and the mechanisms underlying oncogenesis are currently unknown.

Histologically, synovial sarcoma is classified into two major types, according to the presence or absence of gland-like structures: the biphasic type, which shows epithelial elements arranged in gland-like structures scattered against a uniform spindle-cell background, and the monophasic fibrous type, which is entirely composed of spindle tumor cells [1]. The type of the SSX genes involved in the SYT-SSX fusion transcript is associated with histopathological and clinical features of synovial sarcoma [5–8]. The majority of biphasic synovial sarcomas contain SYT-SSX1 fusion transcript, and patients with tumors having the SYT-SSX1 fusion transcript show a significantly reduced metastasis-free survival. The hepatocyte growth factor (HGF)/c-Met and stem cell factor (SCF)/c-Kit tyrosine kinase signaling systems also have been implicated in the histopathological features of synovial sarcoma, particularly in relation to its epithelial differentiation [9–12]. However, the mechanisms by which these genetic alterations produce phenotypically distinct types of tumors also remain unknown.

A cell line retaining the unique features of synovial sarcoma is valuable for studying these questions about the tumor. However, only a small number of established cell lines have been reported [13–19], and none of them are derived from biphasic synovial sarcoma, that is particularly useful for studying the epithelial differentiation of the tumor. We describe here the establishment of a new synovial sarcoma cell line, SYO-1, derived from a biphasic synovial sarcoma that exhibits the distinct features of the tumor.

## 2. Materials and methods

### 2.1. Establishment of cell line

A histologically confirmed biphasic synovial sarcoma which showed both features of proliferation of spindle tumor cells and epithelial cells arranged in glandular structures that developed in the groin of a 19-year-old female was the original tumor (Fig. 1). At the time of definitive wide excision (no preoperative chemotherapy), a part of the surgically excised tumor was minced with scissors and seeded in 25-$cm^2$ tissue culture flasks (Becton Dickinson Lab., Bedford, MA) in a humidified atmosphere containing 5% $CO_2$ at 37 °C. The culture medium was Dulbecco's modified minimal essential medium supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin G and 100 μg/ml streptomycin (Invitrogen Co., Carlsbad, CA). When the cultured cells became confluent, the cells were dispersed with 0.25% trypsin solution and seeded in new flasks for passage. These procedures were serially performed until establishment of a cell line.

To determine the doubling time, $1.0 \times 10^4$ cells were seeded in each well of 24-well dishes (Corning Inc., Corning, NY) with fresh medium containing 10% FCS and the number of viable cells was counted every 48 h for 12 days.

The tumorigenicity was examined after subcutaneous injection of $5.0 \times 10^7$ cells into the back of BALB/c (nu/nu) nude mice (Charles River Japan, Inc., Yokohama, Japan).

### 2.2. Pathologic studies

Cells growing on chamber slides (Becton Dickinson Lab.) in addition to in vivo tumor tissues were examined. The cultured cells were fixed with 95% ethanol and the tumor tissues were fixed with 10% formalin. They were stained with hematoxylin and eosin and immunostained with the avidin–biotin–peroxidase complex (ABC) method (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA). The HistoMouse™-Plus kit (Zymed Laboratories, San Francisco, CA) was used for detection of mouse primary antibodies on the tumors transplanted in nude mice.

The primary antibodies used were mouse monoclonal antibodies against vimentin (V9; Dako, Glostrup, Denmark), cytokeratin (AE1/AE3; Dako), epithelial membrane antigen (EMA; E29; Dako), type IV collagen ( × 100, Chemicon Int., Temecula, CA), α-smooth muscle actin (1A4; Dako), muscle specific actin (HHF35; Dako), p53 (DO-7, × 50; Dako), mdm2 (SMP14, × 50; Dako), bcl-2 (124, × 50,


Fig. 1. Light microscopic findings of the original tumor ( × 200). The tumor is composed of fascicles of spindle tumor cells (A) and areas of glandular differentiation (B). The epithelial cells arranged in glandular structures exhibit strong immunoreactivity for cytokeratin (C).

Dako), bcl-x (44, × 50; Transduction Lab., Lexington, KY), rabbit polyclonal antibodies against S-100 (Dako), bax (Ab-1, × 50, Oncogene Research Products, San Diego, CA), c-Met ( × 50, IBL Lab., Gumma, Japan), c-Kit (C-19, × 400; Santa Cruz Biotechnology, Santa Cruz, CA), as well as goat polyclonal antibodies against HGF ( × 100; R&D Systems, Minneapolis, MN) and SCF (N-19, × 200, Santa Cruz Biotechnology). The antibodies of vimentin (V9), cytokeratin (AE1/AE3), EMA, α-smooth muscle actin (1A4), muscle specific actin (HHF35), and S-100 were optimally diluted by the manufacture.

### 2.3. Cytogenetic analysis

Cultured cells at passage 27 were used for cytogenetic analysis. Chromosomal preparations were prepared by the air-drying method and karyotype analyses were performed by the conventional trypsin-Giemsa banding methods.

### 2.4. Reverse transcriptase polymerase chain reaction (RT-PCR)

The expressions of SYT-SSX fusion transcript and mRNAs of HGF, c-Met, SCF and c-Kit, were examined by RT-PCR. One microgram of total RNA from each material was reverse transcribed using the first strand cDNA synthesis kit (ReverTra Ace-α-™; TOYOBO, Osaka, Japan). One microliter of the cDNA samples was applied to PCR in 20 μl of reaction solution containing 0.4 μM of specific primers, 10 mM Tris–HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTP and 0.5 units of Taq polymerase (Takara, Ohtsu, Japan). The PCR profile comprised 35 cycles of denaturation at 94 °C for 1 min, annealing at 64 °C for 45 s, and extension at 72 °C for 1 min, with a final extension at 72 °C for 10 min.

To detect the SYT-SSX fusion transcripts, the following primers were used: forward primer (SYT): 5′-CAACAGCAAGATGCATACCA-3′, reverse primers (SSX): 5′-CACTTGCTATGCACCTGATG-3′, (SSX1): 5′-GGTGCAGTTGTTTCCCATCG-3′, and (SSX2): 5′-GGCACAGCTCTTTCCCATCA-3′. Using the consensus SYT-SSX primer pair, the PCR product had a 585-bp fragment, and using

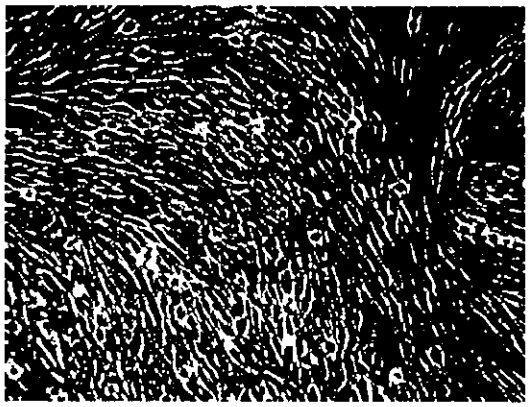

Fig. 2. Light microscopic finding of SYO-1 cells in vitro (phase contrast microscope, × 100). SYO-1 cells are spindle-shaped and possess oval nuclei with small distinct nucleoli.

a SYT-SSX1 or SYT-SSX2 specific primer pair, it yielded a 331-bp fragment. To detect the HGF transcripts, the forward primer: 5′-GGGAAATGAGAAATGCAGCCAG-3′, and the reverse primer: 5′-AGTTGTATTGGTGGGTGCTTC-3′, were used to yield a 316-bp product. To detect the c-Met transcripts, the forward primer: 5′-CATGCCGACAAGTGCAGTA-3′, and the reverse primer: 5′-TCTTGCCATCATTGTCCAA-3′, were used to yield a 252-bp PCR product. Primers used for SCF were 5′-ATTCAAGAGCCCAGAACCCA-3′ (forward) and 5′-CTGTTACCAGCCAATGTACG-3′ (reverse), to yield 409- and 494-bp products. Primers used for c-Kit were 5′-GAGTTGGCCCTAGACTTAGA-3′ (forward) and 5′-CCTGGAGTTGGATGCAAGTT-3′ (reverse), to yield a 749-bp PCR product.

As an internal control for PCR and for quality assessment of the RNAs, a 450-bp portion of the ubiquitously expressed glyceraldehyde 3-phosphate dehydrogenase (G3PDH) transcript was amplified with primers 5′-ACCACAGTCCATGCCATCAC-3′ (forward) and 5′-TCCACCACCCTGTTGCTGTA-3′ (reverse). In each PCR procedure, negative controls lacking RNA and lacking reverse transcriptase were included.

The PCR products were fractionated on 2.0% agarose gels and visualized with ethidium bromide.

## 3. Results

### 3.1. Establishment of cell line

Three weeks after initial cultivation, small foci consisting of polygonal or spindle-shaped tumor cells appeared among the loosely proliferating fibroblastic cells and gradually increased in size. These foci were selectively digested with 0.25% trypsin solution for 5 min at 37 °C, and collected cells were plated in 25-cm$^2$ plastic flasks. Thereafter, they were serially subcultured at a dilution of 1:3 when they became confluent. After five to six passages, these cells began to grow stably, and thereafter could be subcultured every week. The cell line, designated as SYO-1 (Fig. 2), has been stably maintained for more than 70 passages (more than 24 months) in vitro. The doubling time of the cells in logarithmic growth phase was 28 h.

Immunohistochemically, the cultured SYO-1 cells were positive for vimentin, type IV collagen, S-100, mdm2, bcl-2, c-Met and c-Kit (Table 1). Cytokeratin and EMA were negative, while they gave positive results in the original tumor.

### 3.2. Tumorigenicity

SYO-1 cells inoculated into the back of nude mice developed tumors within 2 months (5/5 mice).

Table 1
Immunohistochemical staining of SYO-1 cells *in vitro* and *in vivo*

| Antigen | SYO-1 cells | | Original tumor |
|---|---|---|---|
| | *In vitro* | *In vivo* | |
| Vimentin | + | + | + |
| Cytokeratin | − | + | + |
| EMA | − | + | + |
| Type IV collagen | + | + | + |
| α-smooth muscle actin | − | − | − |
| Muscle specific actin | − | − | − |
| S-100 | + | + | + |
| p53 | − | − | − |
| mdm2 | + | + | − |
| Bcl-2 | + | + | + |
| Bcl-x | − | − | − |
| Bax | − | + | + |
| c-Met | + | + | + |
| c-Kit | + | + | + |
| HGF | − | − | − |
| SCF | − | − | − |

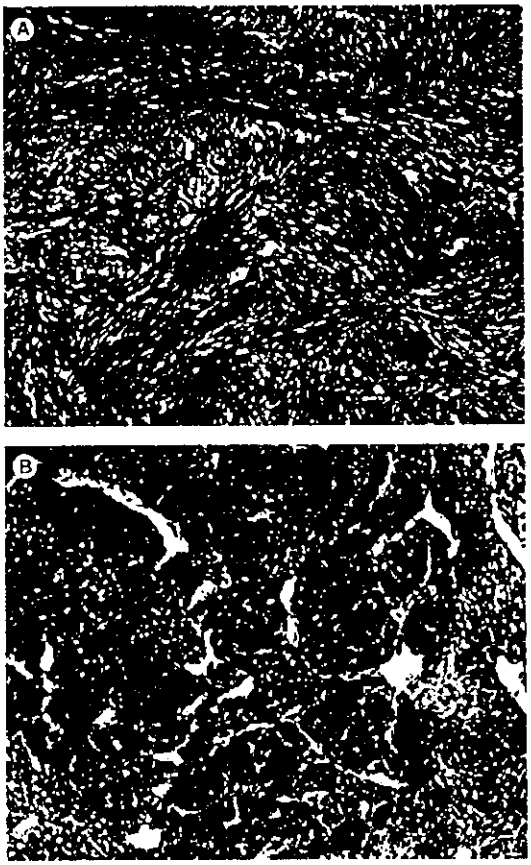


Fig. 3. Light microscopic findings of the tumor produced by SYO-1 cells in vivo ( × 200). The tumor shows biphasic features consisting of proliferation of spindle cells (A) and areas of polygonal epithelial cells (B).

The histologic features of these tumors resembled those of the original one. In some areas, the tumor consisted of proliferation of spindle cells having darkly stained nuclei and indistinct cytoplasm (Fig. 3A). Areas consisting of polygonal epithelial cells arranging around branching vessels and clefts were also observed (Fig. 3B).

Immunohistochemically, the tumor cells exhibited positive staining for vimentin, cytokeratin, EMA, type IV collagen, S-100, mdm2, bcl-2, bax, c-Met, and c-Kit (Table 1). The polygonal cells forming epithelial plaque were strongly positive for cytokeratin and EMA.

### 3.3. Cytogenetic findings

Chromosome analysis showed that consistent chromosomal abnormalities were present. The modal chromosome number was 47 (50 cells counted). G-banding analysis showed a characteristic reciprocal translocation involving chromosomes X and 18, t(X;18)(p11.2;q11.2), in every metaphase cell examined (Fig. 4).

### 3.4. RT-PCR

On RT-PCR, the SYO-1 cells showed a 585-bp product using the consensus SYT-SSX primer pair and a 331-bp product using the SYT-SSX2 specific primer pair, confirming that this cell line had the SYT-SSX2 fusion transcript (Fig. 5). The SYO-1 cells also expressed c-Met (Fig. 6), SCF and c-Kit mRNA (Fig. 7).

## 4. Discussion

Histologically, synovial sarcoma is classified into two major types according to the presence or absence of epithelial elements arranged in gland-like structures: the biphasic type and the monophasic fibrous type. Immunohistochemically, both the epithelial markers cytokeratin and EMA and the mesenchymal marker vimentin are positive in the majority of synovial sarcomas, irrespective of the histologic subtypes [1]. Soft tissue sarcomas other than synovial sarcoma and epithelioid sarcoma ordinarily do not react with these epithelial markers. Based on these findings, some studies have considered synovial sarcoma as a carcinosarcoma-like tumor of soft tissue [20,21].

A recent progress in the understanding of the molecular mechanisms of tumorigenesis of synovial sarcoma is the discovery of the SYT-SSX fusion gene resulting from the chromosomal translocation t(X;18)(p11.2;q11.2) [2,3]. The aberrant SYT-SSX fusion protein was suggested to lead to neoplasia perhaps as a result of transactivation of other target genes not normally transcribed. Current evidence suggests that the SYT-SSX protein acts as a transcriptional co-factor, through a repression domain, SSX-RD, on the C-terminus of SSX and an activation domain, the QPGY domain, on the SYT

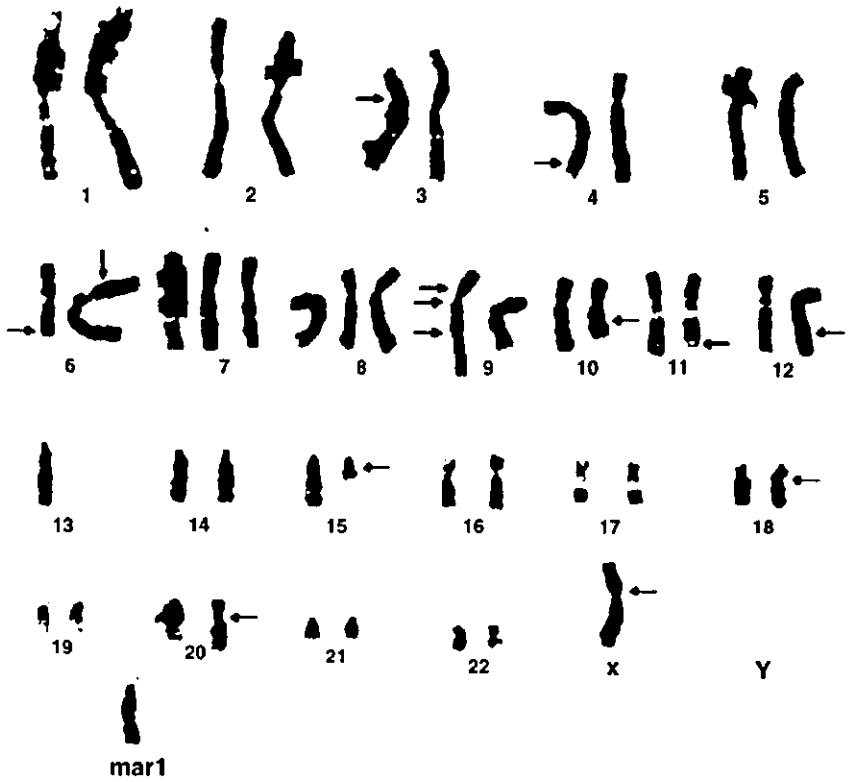


Fig. 4. A representative G-banding karyotype of SYO-1 cells, revealing 47, t(X;18) (p11.2;q11.2), −X or −Y, del (3) (p13q21), t(4;12)(q?31.3;q?24.1), t(6;6) (p23;q21), +7, +8, der(9) inv(9) (p12q13) t(9;15) (q32;q?13), t(10;20) (q22;q11.2), del(11) (q23), −13, der(15)t(9;15) (q32;q?13), +mar1. Arrows indicate abnormal chromosomes.

portion of the fusion protein [22,23]. However, the SYT-SSX fusion protein lacks a DNA-binding domain and the downstream transcriptional targets are presently unknown. Clinically, as this abnormality is specific for synovial sarcoma and has been observed in essentially all cases of the tumor, detection of this fusion either by chromosome analysis or by RT-PCR is useful for diagnosis of synovial sarcoma, especially for diagnostically difficult cases and tumors in unusual locations [24,25].

The pathognomonic SYT-SSX fusion appears to be related not only to oncogenesis, but also to the epithelial differentiation of synovial sarcoma [5,6]. The majority of biphasic synovial sarcomas contain the SYT-SSX1 fusion transcript, whereas the monophasic tumors have both fusion transcripts. This suggests a model in which SYT-SSX1 may be more

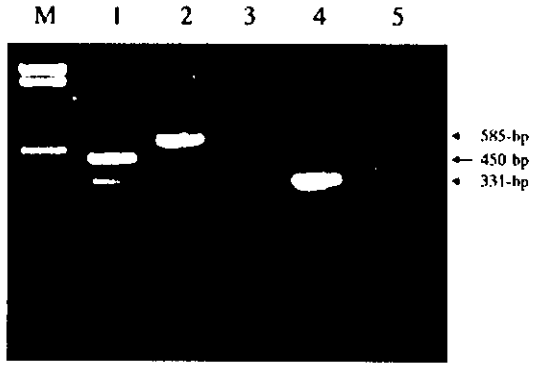


Fig. 5. Detection of SYT-SSX fusion transcripts by RT-PCR. M: 100-bp DNA ladder, 1: G3PDH, 2: SYT-SSX, 3: SYT-SSX1, 4: SYT-SSX2, 5: negative control. The SYO-1 cells express a 585-bp product with the SYT-SSX primer pair and a 331-bp product with the SYT-SSX2 specific primer pair, confirming the presence of the SYT-SSX2 fusion transcript.

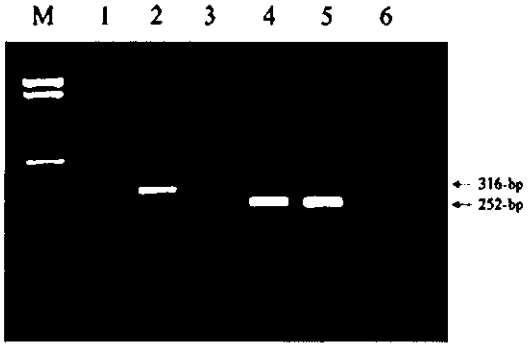


Fig. 6. Detection of HGF and c-Met mRNA by RT-PCR. M: 100-bp DNA ladder, 1, 4: SYO-1 cells, 2, 5: KAO cells (clear cell sarcoma, HGF and c-Met positive control), 3, 6: negative control. SYO-1 cells generated a 252-bp c-Met product, but not a HGF product.

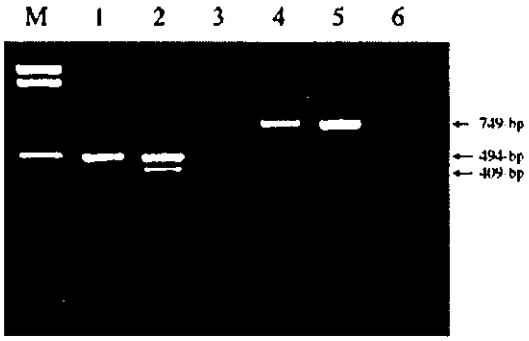


Fig. 7. Detection of SCF and c-Kit mRNA by RT-PCR. M: 100-bp DNA ladder, 1, 4: SYO-1 cells, 2, 5: SaOS2 cells (osteosarcoma, SCF and c-Kit positive control), 3, 6: negative control. SYO-1 cells generated both SCF products (409, 494 bp) and a 749-bp c-Kit product.

permissive for glandular morphogenesis or SYT-SSX2 may be a repressor of this differentiation pathway. However, the relation is not absolute, because rare SYT-SSX2-bearing biphasic tumors with clear glandular formation have been reported, as one case found in our previous study and the case of SYO-1 cells [24].

HGF/c-Met and SCF/c-Kit signaling systems were also reported to be involved in the epithelial differentiation of synovial sarcoma [9,10,12]. In line with the epithelial differentiating role of c-Met in the HGF/c-Met system, prominent expression of c-Met in the epithelial component of synovial sarcomas was reported [9,10]. On the other hand, Oda et al. [11] reported that the co-expression of HGF and c-Met was correlated rather with progression of the tumors. A strong expression of other types of tyrosine kinase receptor c-Kit mainly in the epithelial component of synovial sarcoma was recently reported [12]. In SYO-1 cells, the expressions of c-Met and c-Kit were diffusely positive in the in vitro cultured conditions and were observed both in the epithelial and spindle-cell components in the in vivo tumors. The expression of SCF/c-Kit in synovial sarcoma is potentially interesting because the incorporation of this signaling system has also been observed in neuroectodermal tumors [26], which may share some phenotypic and genotypic characteristics with synovial sarcomas [27,28].

Further studies to clarify the link between the SYT-SSX fusion, HGF/c-Met and SCF/c-Kit signaling systems and the nature of synovial sarcoma will provide an important insight into the biology of the tumor. A cell line that retains the characteristic feature of synovial sarcoma will be valuable for these studies. Although a few cell lines have been established from synovial sarcoma [13–19], they have not always been fully characterized, and none of them are derived from biphasic synovial sarcoma (Table 2). A2243 was established in 1982 by Aaronson, but detailed characteristics of the cell line and the histological findings of original tumor have not been published. SYO-1 is the first well-characterized cell line derived from biphasic synovial sarcoma, which might be particularly useful for studying the epithelial differentiation of the tumor.

Four of nine reported synovial sarcoma cell lines showed SYT-SSX1 fusion transcript (containing HS-SY-3 that showed the truncated form of SYT-SSX1), and five cell lines expressed SYT-SSX2 fusion transcript (Table 2). Of the limited number of established cell lines, there was no clear relation between the types of fusion transcript and the histologic types of the tumor. In addition, the impact of SYT-SSX fusion types on the in vitro growth characters or in vivo tumorigenicity of the cells could not be answered with the available data about the established cell lines.

From the observation of the SN-SY-1 cells, Noguchi et al. [16] proposed that the in vitro growth pattern of synovial sarcoma had an epithelial morphology even though the original tumors were

Table 2
Documented synovial sarcoma cell lines

| Cell line | Age | Sex | Site | Histology | SYT-SSX | D.T. (hours) | Tumorigenicity |
|---|---|---|---|---|---|---|---|
| A2243 (Aaronson, 1982)[a] | – | – | – | – | SYT-SSX2 | – | – |
| SS255 (Reeves et al., 1989) | 24 | F | Thigh | Monophasic fibrous | SYT-SSX2 | – | – |
| HS-SY-II (Sonobe et al., 1992) | 43 | M | Forearm | Monophasic fibrous | SYT-SSX1 | 60 | Yes |
| CME-1 (Renwick et al., 1995) | 18 | F | Thigh | Monophasic fibrous | SYT-SSX2 | – | – |
| SN-SY-1 (Noguchi et al., 1997) | 21 | M | Abdominal wall | Monophasic fibrous | SYT-SSX1 | 167 | Yes |
| HS-SY-3 (Sonobe et al., 1999) | 86 | F | Popliteal fossa | Monophasic fibrous | SYT-SSX1, truncated | 120 | No |
| KU-SS-1 (Yakushiji et al., 2000) | 27 | F | Popliteal fossa | Monophasic fibrous | SYT-SSX2 | 93.8 | Yes |
| FU-SY-1 (Nishio et al., 2002) | 31 | F | Supinator | Monophasic fibrous | SYT-SSX1 | 96 | No |
| SYO-1 (Present study) | 19 | F | Groin | Biphasic | SYT-SSX2 | 28 | Yes |

[a] Data not published.

classified as monophasic fibrous type. Under the in vitro monolayer culture condition, the SYO-1 cells showed relatively uniform short spindle shapes. On the other hand, the in vivo tumors developed by their implantation showed biphasic features composed of areas of fascicles of spindle tumor cells and areas of a compact proliferation of polygonal to ovoid tumor cells, which occasionally formed epithelial plaque. Immunohistochemically, despite the lack of positivity for cytokeratin and EMA in the in vitro cultured SYO-1 cells, these antigens were positive in the original tumor and in vivo SYO-1 transplanted tumors. Although it is unclear why there were such discrepancies, alteration of the differentiation phenotypes depending on the surrounding conditions has frequently been observed in cultured human cells [14,16,18]. In KU-SS-1 cells, positive immunostaining for cytokeratin and EMA were observed only in the late passages in vitro [18].

Using microdissection techniques, Kasai et al. [29] and Nishio et al. [30] found that the SYT-SSX fusion transcripts were present in both epithelial and spindle cell areas of biphasic synovial sarcoma and suggested the monoclonarity of the tumor. The biphasic feature of the tumors developed by SYO-1 cells may support the theory that the tumor cells of biphasic synovial sarcoma have a dual differentiation capacity.

In conclusion, the current study established a cell line, SYO-1, derived from a biphasic synovial sarcoma. The SYO-1 cells, the first characterized cell line derived from biphasic synovial sarcoma retaining the characteristic genetic and phenotypic features of the tumor, will be an important tool for various investigations of synovial sarcoma, especially for its epithelial differentiation.

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# LETTER TO THE EDITOR
# Extraovarian Primary Peritoneal Carcinoma in a Child†

**Key words:** extraovarian primary peritoneal carcinoma; ovarian cancer; CA125; childhood cancer

Extraovarian primary peritoneal carcinoma (EOPPC) was first described in 1959 by Swerdlow [1]. It is an adenocarcinoma that develops from the peritoneum lining the pelvis and abdomen and is characterized by abdominal carcinomatosis, uninvolved or minimally involved ovaries, and no identifiable primary tumor. It accounts for ~10% of cases with a presumed diagnosis of ovarian cancer [2]. It is similar to ovarian serous carcinoma with respect to clinical presentation, histologic appearance, and response to chemotherapy [3]. The median age of recorded cases is 57–66 years [3] and there has been no prior report in a child. Our experience in such a case is of interest.

Our patient was 13-year-old girl, who was admitted to St. Luke's International Hospital, Tokyo, Japan, because of a huge abdominal mass. Her chief complaint was abdominal pain and constipation. A hard mass, about 5 cm × 6 cm in size, was palpable in the lower abdomen upon physical examination. Thyroid gland was not enlarged. Laboratory data were within normal limits except for CA125, which was 453.8 U/ml. Abdominal CT and MRI revealed a large, solid mass that occupied the pelvic cavity (Fig. 1), and no other tumor lesions were detected. The diagnosis before operation was a primary ovarian tumor. An open biopsy was performed and the histologic diagnosis was serous adenocarcinoma (Fig. 2). The size of the tumor did not decrease at all after three course of chemotherapy consisting of vindesine, doxorubicin, and fluorouracil (5-FU). Surgery performed 3 months after the biopsy removed the tumor together with the uterus, adnexa, and a part of the rectum and the sigmoid colon because the tumor directly invaded the uterus and large bowel. An endometrial cyst, 5 cm in diameter and attached to the neoplasm was also resected. The excised mass measured 9.0 cm × 6.0 cm × 4.5 cm (Fig. 3, arrowheads). The left ovary was 4.0 cm × 1.5 cm × 1.0 cm (Fig. 3, arrow) and no tumor cells were seen in the ovarian tissue. The size of the right ovary was 4.5 cm × 4.0 cm × 2.5 cm. There were adenocarcinoma components within the ovarian stroma. Since the ovary was not in continuity with the tumor, it was considered to be a metastatic lesion. Both fallopian tubes were normal. From the operative findings and histologic examination, it appeared that tumor originated from extraovarian tissue. It was, therefore, diagnosed as an EOPPC. Seventeen months after the operation, a vaginal recurrence was noted. Colpectomy was carried out and radiation therapy (total 50 Gy) was added. Six months after radiation, the tumor recurred in the pelvic cavity and abdominal wall. Management then became palliative to control pain by morphine sulfate, instead of aggressive chemotherapy and repeat surgery. She died 50 months after the diagnosis.

Most malignant ovarian tumors in adults are epithelial in type, but in children and adolescents, 60% are of germ cell origin [4]. Ovarian carcinoma is rare, and extraovarian carcinoma is extremely rare in children. EOPPC should nonetheless be added to the differential diagnosis when a tumor occupying the abdominal cavity and considered of ovarian origin is encountered in a child.

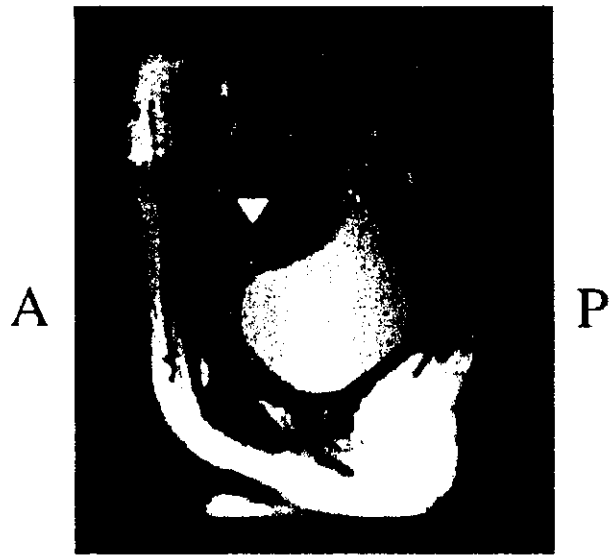


Fig. 1. Sagittal $T_2$-weighted abdominal MRI. A large, solid tumor, 10 cm × 10 cm × 8 cm in size, occupied the pelvic cavity. A small, cystic mass lesion lay above the solid tumor (arrowhead). This mass turned out to be an endometrial cyst at surgery. A; anterior, P; posterior.

The differential diagnosis should also include desmoplastic small round cell tumor in which elevated serum CA125 has been reported [5,6]. However, the pathologic findings of our case were completely different. No psammoma bodies or papillary and glandular structures are to be found in desmoplastic small round cell tumors.

It has been reported that levels of the tumor marker CA125 were elevated (>35 U/ml) in most of the EOPPC

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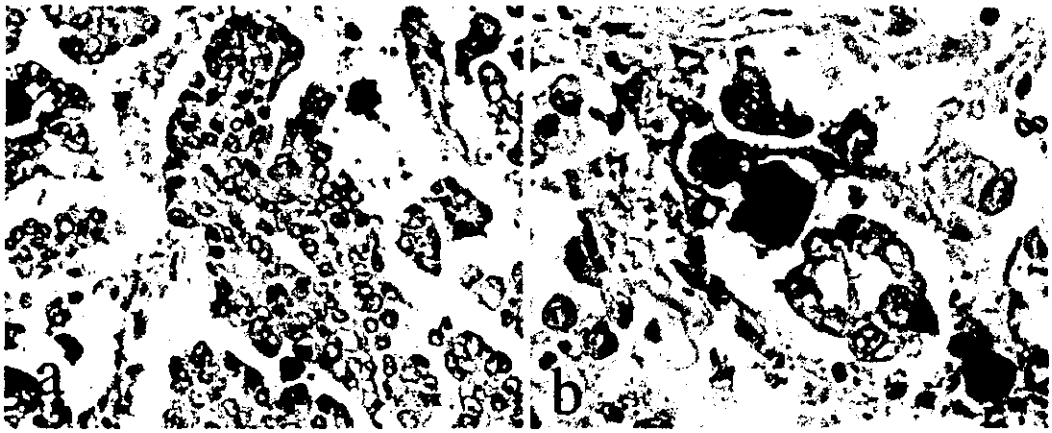


Fig. 2. Hematoxylin and eosin stain. a: The tumor cells with nuclear atypia showed papillary growth (original magnification × 200). b: Psammoma bodies were seen in the tumor tissue (original magnification × 400).

patients [7,8]. In our patient, the CA125 levels correlated with the clinical status of the disease and response to therapy. This finding has also been reported in reference [7]. It is important to measure CA125 not only during chemotherapy but also thereafter.

The response to chemotherapy and the prognosis of EOPPC is said to be similar to serous ovarian carcinoma [3]. However, there have been some reports that the prognosis for EOPPC is worse than for ovarian serous cancers with comparable peritoneal spread [2,8]. Reported median survival durations vary between 7.0 and 27.8 months and 5-year survival rates range from 0 to 26.5% among EOPPC patients [3]. Our patient suffered repeated relapses in spite of tumor excision, chemotherapy, and radiation therapy. We chose "quality of life" management after chemotherapy and surgery failed to cure the tumor, so that the patient could go back to high school and travel abroad with her family.

In summary, we here report the first case of EOPPC in a child. Aggressive combined modality therapy failed to cure the girl.

Fig. 3. Gross appearance of the resected tumor and ovaries. The tumor (arrowheads) was solid and its color was yellow and white with degeneration and necrosis. It measured 9.0 cm × 6.0 cm × 4.5 cm. The left ovary (arrow) was 4.0 cm × 1.5 cm × 1.0 cm in size. No tumor cells were seen in the ovarian tissue.

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