cells with telomerase activity. Recently, it was reported that telomere maintenance by the existence of telomerase activity is necessary for the proliferation of normal human cells (Masutomi *et al*, 2003). Thus, low levels of telomerase activity may reflect the proliferation of normal hepatocytes in children. To solve this false-positive problem, *in situ* evaluation is necessary to analyse the origin of telomerase expression in clinical samples using *hTERT* mRNA ISH (Chou *et al*, 2001; Kumaki *et al*, 2001; Kotoula *et al*, 2002) or hTERT immunohistochemistry (Yasui *et al*, 1999; Hiyama *et al*, 2001).

In summary, we show that an increased level of *hTERT* mRNA expression or telomerase activity is a prognostic indicator of poor outcome in patients with hepatoblastoma, independent of disease stage and histological classification. Although it would need large series to clarify the correlation between clinical variables and the levels of *hTERT* mRNA or telomerase activity, high telomerase activity may stratify patients that are likely to have cancer recurrence requiring postoperative aggressive chemoadjuvant therapy, or, in the future, telomerase-targeting therapy.

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REFERENCES

Albrecht S, von Schweinitz D, Waha A, Kraus JA, von Deimling A, Pietsch T (1994) Loss of maternal alleles on chromosome arm 11p in hepatoblastoma. *Cancer Res* **54**: 5041–5044

Blackburn EH (1991) Structure and function of telomeres. *Nature (London)* **350**: 569–573

Brown J, Perilongo G, Shafford E, Keeling J, Pritchard J, Brock P, Dicks-Mireaux C, Phillips A, Vos A, Plaschkes J (2000) Pretreatment prognostic factors for children with hepatoblastoma – results from the International Society of Paediatric Oncology (SIOP) study SIOPEL 1. *Eur J Cancer* **36**: 1418–1425

Chadeneau C, Hay K, Hirte HW, Gallinger S, Bacchetti S (1995) Telomerase activity associated with acquisition of malignancy in human colorectal cancer. *Cancer Res* **55**: 2533–2536

Chou SJ, Chen CM, Harn HJ, Chen CJ, Liu YC (2001) *In situ* detection of *hTERT* mRNA relates to Ki-67 labeling index in papillary thyroid carcinoma. *J Surg Res* **99**: 75–83

Chromzynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidium thiocyanate phenol-chloroform extraction. *Anal Biochem* **162**: 156–159

Dome JS, Chung S, Bergemann T, Umbricht CB, Saji M, Carey LA, Grundy PE, Perlman EJ, Breslow NE, Sukumar S (1999) High telomerase reverse transcriptase (*hTERT*) messenger RNA level correlates with tumor recurrence in patients with favorable histology Wilms' tumor. *Cancer Res* **59**: 4301–4307

Finegold MJ (2002) Chemotherapy for suspected hepatoblastoma without efforts at surgical resection is a bad practice. *Med Pediatr Oncol* **39**: 484–486

Fuchs J, Rydzynski J, Von Schweinitz D, Bode U, Hecker H, Weinel P, Burger D, Harms D, Erttmann R, Oldhafer K, Mildenberger H (2002) Pretreatment prognostic factors and treatment results in children with hepatoblastoma: a report from the German Cooperative Pediatric Liver Tumor Study HB 94. *Cancer* **95**: 172–182

Gupta J, Han LP, Wang P, Gallie BL, Bacchetti S (1996) Development of retinoblastoma in the absence of telomerase activity. *J Natl Cancer Inst* **88**: 1152–1157

Haas JE, Muczynski KA, Krailo M, Ablin A, Land V, Vietti TJ, Hammond GD (1989) Histopathology and prognosis in childhood hepatoblastoma and hepatocarcinoma. *Cancer* **64**: 1082–1095

Hata Y (1990) The clinical features and prognosis of hepatoblastoma: follow-up studies done on pediatric tumors enrolled in the Japanese pediatric tumor registry between 1971 and 1980. Part I. committee of malignant tumors, Japanese Society of Pediatric Surgeons. *Jpn J Surg* **20**: 498–502

Harley CB, Kim NW, Prowse KL, Weinrich SL, Hirsh KS, West MD, Bacchetti S, Hirre HW, Counter CM, Greider CW, Piatyszek MA, Wright WE, Shay JW (1994) Telomerase, cell immortality, and cancer. *Cold Spring Harbor Symp Quant Biol* **59**: 307–315

Hiyama E, Hiyama K (2002) Clinical utility of telomerase in cancer. *Oncogene* **21**: 643–649

Hiyama E, Hiyama K (2003) Telomerase as tumor marker. *Cancer Lett* **194**: 221–233

Hiyama E, Hiyama K, Ohtsu K, Yamaoka H, Ichikawa T, Shay JW, Yokoyama T (1997a) Telomerase activity in neuroblastoma: is it a prognostic indicator of clinical behavior? *Eur J Cancer* **33**: 1932–1936

Hiyama E, Hiyama K, Shay JW, Yokoyama T (2001) Immunohistochemical detection of telomerase (hTERT) protein in human cancer tissues and a subset of cells in normal tissues. *Neoplasia* **3**: 17–26

Hiyama E, Hiyama K, Yokoyama T, Matsuura Y, Piatyszek MA, Shay JW (1995a) Correlating telomerase activity levels with human neuroblastoma outcomes. *Nat Med* **1**: 249–255

Hiyama E, Kodama T, Sinbara K, Iwao T, Itoh M, Hiyama K, Shay JW, Matsuura Y, Yokoyama T (1997b) Telomerase activity is detected in pancreatic cancer but not in benign tumors. *Cancer Res* **57**: 326–331

Hiyama E, Yokoyama T, Tatsumoto N, Hiyama K, Imamura Y, Murakami Y, Kodama T, Piatyszek MA, Shay JW, Matsuura Y (1995b) Telomerase activity in gastric cancer. *Cancer Res* **55**: 3258–3262

Iwao T, Hiyama E, Yokoyama T, Tsuchida A, Hiyama K, Murakami Y, Shimamoto F, Shay JW, Kajiyama G (1997) Telomerase activity for the preoperative diagnosis of pancreatic cancer. *J Natl Cancer Inst* **89**: 1621–1623

Kim NW, Piatyszek MA, Prowse KR, Harley CB, West MD, Ho PLC, Coviello GM, Wright WE, Weinrich SL, Shay JW (1994) Specific association of human telomerase activity with immortal cells and cancer. *Science* **266**: 2011–2015

Koch A, Denkhaus D, Albrecht S, Leuschner I, von Schweinitz D, Pietsch T (1999) Childhood hepatoblastomas frequently carry a mutated degradation targeting box of the beta-catenin gene. *Cancer Res* **59**: 269–273

Kotoula V, Hytiroglou P, Pyrpasopoulou A, Saxena R, Thung SN, Papadimitriou CS (2002) Expression of human telomerase reverse transcriptase in regenerative and precancerous lesions of cirrhotic livers. *Liver* **22**: 57–69

Kumaki F, Kawai T, Hiroi S, Shinomiya N, Ozeki Y, Ferrans VJ, Torikata C (2001) Telomerase activity and expression of human telomerase RNA component and human telomerase reverse transcriptase in lung carcinomas. *Hum Pathol* **32**: 188–195

Marchetti A, Bertacca G, Buttitta F, Chella A, Quattrocolo G, Angeletti CA, Bevilacqua G (1999) Telomerase activity as a prognostic indicator in stage I non-small cell lung cancer. *Clin Cancer Res* **5**: 2077–2081

Masutomi K, Yu EY, Khurts S, Ben-Porath I, Currier JL, Metz GB, Brooks MW, Kaneko S, Murakami S, DeCaprio JA, Weinberg RA, Stewart SA, Hahn WC (2003) Telomerase maintains telomere structure in normal human cells. *Cell* **114**: 241–253

Naito Y, Takagi T, Handa O, Ishikawa T, Matsumoto N, Yoshida N, Kato H, Ando T, Takemura T, Itani K, Hisatomi H, Tsuchihashi Y, Yoshikawa T (2001) Telomerase activity and expression of telomerase RNA component and catalytic subunits in precancerous and cancerous colorectal lesions. *Tumour Biol* **22**: 374–382

Nakashio R, Kitamoto M, Tahara H, Nakanishi T, Ide T, Kajiyama G (1997) Significance of telomerase activity in the diagnosis of small differentiated hepatocellular carcinoma. *Int J Cancer* **74**: 141–147

Nishimura S, Sato T, Fujita N, Yamaoka H, Hiyama E, Yokoyama T, Ueda K (2002) High-dose chemotherapy in children with metastatic hepatoblastoma. *Pediatr Int* **44**: 300–305

Nouso K, Urabe Y, Higashi T, Nakatsukasa H, Hino N, Ashida K, Kinugasa N, Yoshida K, Uematsu S, Tsuji T (1996) Telomerase as a tool for the differential diagnosis of human hepatocellular carcinoma. *Cancer* **78**: 232–236

Piatyszek MA, Kim NW, Weinrich SL, Hiyama K, Hiyama E, Wright WE, Shay JW (1995) Detection of telomerase activity in human cells and tumors by a telomeric repeat amplification protocol (TRAP). *Methods Cell Sci* **17**: 1–15

Samuel DP, Tsokos M, DeBaun MR (1999) Hemihypertrophy and a poorly differentiated embryonal rhabdomyosarcoma of the pelvis. *Med Pediatr Oncol* **32**: 38–43

Saretzki G (2003) Telomerase inhibition as cancer therapy. *Cancer Lett* **194**: 209–219

Sasaki F, Matsunaga T, Iwafuchi M, Hayashi Y, Ohkawa H, Ohira M, Okamatsu T, Sugito T, Tsuchida Y, Toyosaka A, Nagahara N, Nishihira H, Hata Y, Uchino J, Misugi K, Ohnuma N (2002) Outcome of hepatoblastoma treated with the JPLT-1 (Japanese study group for pediatric liver tumor) protocol-1: a report from the Japanese study group for pediatric liver tumor. *J Pediatr Surg* **37**: 851–856

Shay JW (1995) Aging and cancer: are telomeres and telomerase the connection? *Molec Med Today* **1**: 378–384

Shay JW, Bacchetti S (1997) A survey of telomerase activity in human cancer. *Eur J Cancer* **33**: 787–791

Shay JW, Werbin H, Wright WE (1997) Telomerase assay in the diagnosis and prognosis of cancer. *Ciba Found Symp* **211**: 148–155

Takayasu H, Horie H, Hiyama E, Matsunaga T, Hayashi Y, Watanabe Y, Suita S, Kaneko M, Sasaki F, Hashizume K, Ozaki T, Furuuchi K, Tada M, Ohnuma N, Nakagawara A (2001) Frequent deletions and mutations of the beta-catenin gene are associated with overexpression of cyclin D1 and fibronectin and poorly differentiated histology in childhood hepatoblastoma. *Clin Cancer Res* **7**: 901–908

Tatsumoto N, Hiyama E, Murakami Y, Imamura Y, Shay JW, Matsuura Y, Yokoyama T (2000) High telomerase activity is an independent prognostic indicator of poor outcome in colorectal cancer. *Clin Cancer Res* **6**: 2696–2701

von Schweinitz D, Hecker H, Schmidt-von-Arndt G, Harms D (1997) Prognostic factors and staging systems in childhood hepatoblastoma. *Int J Cancer* **74**: 593–599

von Schweinitz D, Kraus JA, Albrecht S, Koch A, Fuchs J, Pietsch T (2002) Prognostic impact of molecular genetic alterations in hepatoblastoma. *Med Pediatr Oncol* **38**: 104–108

Watson JD (1972) Origin of concatemeric T7 DNA. *Nat New Biol (London)* **239**: 197–201

Weinberg AG, Finegold MJ (1983) Primary hepatic tumors of childhood. *Hum Pathol* **14**: 512–537

Wick M, Zubov D, Hagen G (1999) Genomic organization and promoter characterization of the gene encoding the human telomerase reverse transcriptase (hTERT). *Gene* **232**: 97–106

Yasui W, Tahara E, Tahara H, Fujimoto J, Naka K, Nakayama J, Ishikawa F, Ide T (1999) Immunohistochemical detection of human telomerase reverse transcriptase in normal mucosa and precancerous lesions of the stomach. *Jpn J Cancer Res* **90**: 589–595

Differential gene expressions during immortalization of normal human fibroblasts and endothelial cells transfected with human telomerase reverse transcriptase gene

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Abstract. It is widely accepted that telomerase, which compensates for telomere shortening, is finally activated in almost all kinds of human malignant neoplasms, and ectopic expression of telomerase may endow some kinds of human somatic cells with indefinite proliferation capacity, i.e., immortality. To clarify the intrinsic responses required in acquiring immortality, we investigated the chronological changes in the expression levels of the cell cycle and apoptosis-related genes by real-time RT-PCR in human normal fibroblasts and endothelial cells after *hTERT* transfection. We found that fibroblast MJ90 required intrinsic responses including reversible upregulation of cell-cycle promoting genes and down-regulation of apoptosis-inducing genes in early phase after transfection, whereas the endothelial cell HUE142-2 did not. In addition, the microarray analysis of the fibroblast strains revealed that the dysregulated genes during cellular immortalization were different from those reported in fibroblasts probably having acquired telomere maintenance mechanism concomitant with *hTERT* induction. These findings indicate that cell-type specific differential gene expression after telomerase activation may be important to acquire telomere-maintenance capacity and immortality in some non-cancerous human cells. Investigation of these molecules may elucidate the differences in the capacity of acquiring immortality in cancer and normal somatic cells in future.

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Key words: cellular immortalization, telomerase, telomere, hTERT, fibroblast, endothelial cell, apoptosis

Introduction

Most cancer cells in advanced malignancies and established cancer cell lines have the capacity to undergo indefinite cell divisions. However, most normal somatic cells can divide no more than several dozens of times. Thus, cellular immortality is one of the hallmarks of cancer cells *in vivo* and *in vitro*.

Recent research has shown that telomerase, a highly conserved reverse transcriptase that adds G-rich nucleotide repeats onto the ends of chromosomal DNAs (i.e., telomeres), can endow some kinds of mortal cells with the immortal capacity (1,2). Telomerase expression has been observed in almost all immortal cell lines, proliferating germ-line cells, and about 85% of human tumors (3). Furthermore, normal somatic cells with self-renewal capacity over lifespan, such as lymphocytes and hematopoietic progenitor cells, can upregulate telomerase activity upon proliferation (4). Except for rare cases like alternative lengthening of telomere (ALT) cells (5), activation of telomerase is a prerequisite for human cells to acquire immortal capacity.

However, induction of telomerase activity by transfection of *hTERT* gene, which encodes telomerase protein component, does not always provide immortality (6). Although inactivation of the Rb/p16 pathway but not p53 was proposed to be necessary for human cultured cells to be immortalized (7), recent reports searching the genes responsible for the immortalization of *hTERT*-transfected somatic cells demonstrated different genes as candidates (8,9). As the dominancy of cellular senescence over the immortal phenotype was demonstrated (10), these findings indicate that induction of telomerase activity is not sufficient to acquire immortality but requires some intrinsic responses in each cell. Until now, upregulation of a subset of growth inhibiting genes or pro-apoptotic genes at the onset of the senescence and frequent

inactivation of cell cycle-related genes during immortalization have been proposed (11-13).

The above information led us to the hypothesis that differential expression of some apoptosis- or cell cycle-related genes in addition to the activation of telomerase are required for cellular immortalization. We therefore investigated the chronological changes in the expression levels of the genes involved in cellular proliferation and apoptosis, during cellular immortalization of human fibroblasts and endothelial cells.

Materials and methods

Cell culture. A normal neonatal human diploid fibroblast strain MJ90 was kindly provided by Dr J.R. Smith (University of Texas, Health Science Center, San Antonio, TX) and a normal human umbilical vein endothelial cell strain HUE142-2 was isolated by Dr Y. Mitsui. The fibroblasts were cultured in Dulbecco's modified Eagle's minimal essential medium (DMEM; Sigma-Aldrich Japan, Tokyo, Japan) supplemented with 2 mM L-glutamine (Life Technologies, Rockville, MD, USA) and 10% fetal calf serum (FCS; Life Technologies) as previously described (12). Endothelial cells were grown in MCDB151 medium (Sigma-Aldrich Japan) supplemented with 15% fetal bovine serum, 5 μg/ml of heparin (Sigma-Aldrich Japan), and 5 ng/ml of recombinant acidic fibroblast growth factor in a plastic flask (Corning, NY, USA) precoated with bovine fibronectin solution (1 μg/cm^2, Wako, Osaka, Japan) (14).

Transfection of hTERT. The human telomerase reverse transcriptase (*hTERT*) expression plasmids hTRT/FLAG and hTERTn2 were kindly provided by Dr F. Ishikawa (Kyoto University, Kyoto, Japan) (15). The 40 μg of plasmid hTRT/FLAG or hTERTn2 with single cut by a restriction enzyme *Bgl*II or *Nru*I, respectively, was transfected into 10^7 MJ90 cells at PDL (population doubling level) 41 and the HUE142-2 cells at PDL 35 by electroporation (Invitrogen, San Diego, CA, USA) at 330 V. Clones harboring the plasmids were obtained by 400 μg/ml of G418 selection (Wako) and named as TF1 from the MJ90 and nTE4-5 from the HUE142-2 cells. The TF1 cells at PDL 104, 148, and 216 and nTE4-5 cells at PDL 105, 145, and 216 were collected and stored at -80°C until used. Expression of *hTERT* and telomerase activity in TF1 and nTE4-5 cells and absence of them in MJ90 and HUE142-2 cells were confirmed by real-time RT-PCR and telomeric repeat amplification protocol (TRAP) assay, respectively.

Preparation of RNA. Total RNA was extracted from the cultured cells at each PDL using an RNAeasy™ Mini kit (Qiagen, Hilden, Germany) according to the protocol of the manufacturer. The extracted RNA was stored at -80°C until used.

TRAP assay (16). Using a TRAPeze™ telomerase detection kit (Serological Co., Gaithersburg, MD, USA), frozen cell pellet was lysed in ice-cold CHAPS lysis buffer™. After 30 min of incubation on ice, the lysate was centrifuged at 16,000 g for 20 min at 4°C, and the supernatant was stored at -80°C. An aliquot of the extract equivalent to ~2x10^4 cells was used for each TRAP assay using the TRAPeze kit: after 30 min of incubation at 30°C for telomerase mediated extension of the TS™ primer, the reaction mixture was subjected to PCR for 30 cycles of 94°C x 30 sec, 59°C x 30 sec, and 72°C x 30 sec. The PCR product was electrophoresed on a 12.5% acrylamide gel, and the telomerase signals were detected as 6-bp ladders after staining with SYBR™ Green (Molecular Probes, Eugene, OR, USA).

Southern blot analysis. Terminal restriction fragment (TRF) length was determined by Southern blot analysis. For MJ90 and TF1 strains, genomic DNA was extracted from the cell pellets using DNA Extractor WB Kit™ (Wako). DNA (5 μg) was digested to completion with *Hin*fI, subjected to electrophoresis on a 0.8% agarose gel, blotted onto a nitrocellulose filter, and then hybridized to a [γ-^{32}P]ATP labeled $(TTAGGG)_4$ probe at 50°C. The filter was washed in 4X SSC (1X = 0.15 M NaCl, 0.015 M sodium citrate) and 0.1% SDS at 55°C four times and exposed to X-ray film. For HUE142-2 and nTE4-5 strains, TRF lengths were measured as previously described (11). The mean length of TRFs was visually determined as the peak size of the smear signals and confirmed by BAS™ 2000 (Fuji, Tokyo, Japan).

Sequence analysis. For *TP53*, exons 1-11 were amplified from the cDNA of each strain using primers located in exons 1 and 11: 5'-TTCCACGACGGTGACACG-3' and 5'-GTGGG AGGCTGTCAGTGGGGAACAA-3'. After confirming the existence of 1.3-kb normal-size band, 1 μl aliquot of the first-step PCR product was subjected to the nested PCR for exons 5-8, the hot spots of mutations, to carry out sequence analysis using primers located in exons 4 and 9: 5'-TCTGTCCCTTC CCAGAAAACC-3' and 5'-AGAGGAGCTGGTGTTGTT GG-3'.

In addition, for MJ90 and TF1 cells, exons 3-4, 5-6, 7, 8-9 were amplified from the genomic DNA for confirmation using primers located in introns as follows: exons 3-4, 5'-GG ACTGACTTTCTGCTCTTG-3' and 5'-TGAAGTCTCATGG AAGCCAG-3'; exons 5-6, 5'-TGTTCACTTGTGCCCTGA CT-3' and 5'-GAGGTCAAATAAGCAGCAGG-3'; exon 7, 5'-CTTGCCACAGGTCTCCCCAA-3' and 5'-AGGGGTCAGC GGCAAGCAGA-3'; exons 8-9, 5'-TGGGACAGGTAGGAC CTGAT-3' and 5'-ACTTGATAAGAGGTCCCAAG-3'. The PCR products purified by QIAquick™ Gel Extraction Kit (Qiagen) were amplified using BigDye™ Terminator Cycle Sequencing Kit (Applied Biosystems, CA, USA), purified, and subjected to automated sequencer ABI PRISM™ 310 (Applied Biosystems), as recommended by the manufacturer.

For *RB1*, exons 10-27 were amplified from the cDNA of each cell strain using primers as follows: for exons 10-19, 5'-CTAATGGACTTCCAGAGGTT-3' and 5'-CGGAGATAGG CTAGCCGATA-3'; for exon 20-5'-site of exon 27, 5'-TACT GCAAATGCAGAGACAC-3' and 5'-GAAGAGGAAACAA TCTGCTA-3', to cover the tumor-suppressor pocket domain where most of the Rb-binding cellular and viral proteins make their primary contacts (17).

Real-time RT-PCR. Total RNA (2 μg) extracted from each cell strain was reverse-transcribed using High-Capacity cDNA Archive™ Kit (Applied Biosystems). One hundred

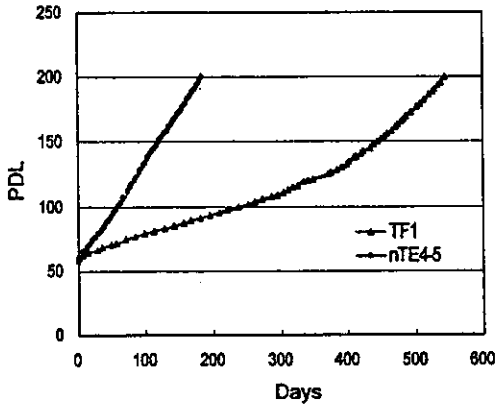

Figure 1. Growth curve of the TF1 and the nTE4-5 strains. The growth of the TF1 strain (closed triangles) was slow until ~120 PDL, +80 PDL after the *hTERT* transfection, and turned rapid thereafter. The growth of the nTE4-5 strain (closed circles) was comparably rapid at any PDL.

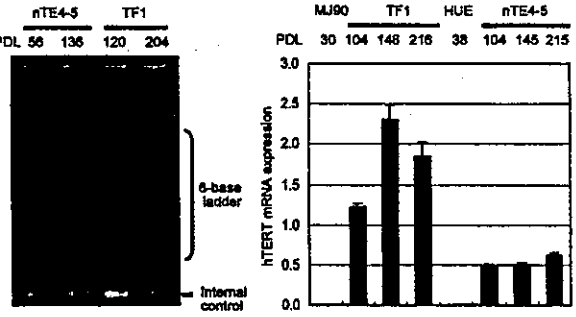

Figure 2. Telomerase activity and *hTERT* mRNA expression in *hTERT* transfected cells. The *hTERT* transfected endothelial cells (nTE4-5) and fibroblasts (TF1) showed 6-base ladder signals by TRAP assay (left) and *hTERT* mRNA expression by real-time RT-PCR using full-length mRNA specific primer set (right), while no *hTERT* mRNA was detected in either strain before transfection, MJ90 and HUE142-2.

aliquot of the cDNA (equivalent to 20 ng total RNA) was subjected to real-time RT-PCR using Assays-on-Demand™ Gene Expression products (Applied Biosystems) for *PCNA*, *RB1*, *TP53*, *APAF1*, *CDKN1A*, *CCND1*, and internal controls *ACTB*, *GAPD*, and *TBP*, Pre-Developed TaqMan™ Assay Reagents (Applied Biosystems) for *BAX*, *FAS*, *MYC*, *BCL2*, and *BCL2L1*, and originally designed TaqMan probe and primer set for *hTERT*. The feasibility of using *ACTB* as the internal control was confirmed by the comparison of the amount of total RNA and real-time RT-PCR results of *ACTB*, *GAPD* and *TBP* in each strain. mRNA expression level was calculated by the ratio in the value of target gene to *ACTB*, and relative quantity was expressed compared to that of control cDNA consisting of the same amount of cDNA of the 8 cell strains examined. Each reaction was carried out in duplicate using ABI PRISM 7900 or triplicate using ABI PRISM 7700 Sequence Detection System (Applied Biosystems).

Microarray analysis. Biotin-labeled cRNA was synthesized from 5 µg total RNA of each MJ90 or TF1 (PDL 216) strain using First and Second Strand cDNA Synthesis Kit™ (CodeLink), *In vitro* Transcription (IVT) Kit™ (CodeLink), and Biotin-11-UTP (Perkin-Elmer), according to the manufacturer's protocols. After purification, each cRNA sample was fragmented, and hybridized with a CodeLink UniSet Human 20K I Bioarray™ using Parallel Processing Kit™ (CodeLink) for 18 h at 37°C in an Innova™ 4080 incubator (New Brunswick Scientific) at 300 rpm. The hybridized arrays were rinsed with 0.75 x TNT buffer at 46°C for 1 h, labeled with Streptavidin-Cy5 at room temperature for 30 min, rinsed with TNT buffer and 0.05% Tween-20, dried by centrifugation, and then scanned using GenePix 4000B Array Scanner™ (Amersham Biosciences, Tokyo, Japan) and/or Agilent DNA Microarray Scanner™ (Agilent, Palo Alto, CA, USA). The experiment was carried out twice independently.

Statistical analysis. Statistical significance was analyzed using the software package Statview™ 5.0 (Abacus Concepts, Berkeley, CA, USA) for Student *T* analysis on the mRNA expression levels between the strains before and after the *hTERT* transfection.

Results

Cell growth. After the transfection of the *hTERT*-expression plasmids into the MJ90 cells at PDL 41 and the HUE142-2 cells at PDL 35, the isolated clones TF1 and nTE4-5 were serially passaged until 216 PDL. The growth curve of the TF1 strain was slow until ~PDL 120 and turned to rapid growth thereafter, while it was straight and rapid from just after the *hTERT* transfection in the nTE4-5 cells (Fig. 1).

Telomerase activity. By TRAP assay, the 6-bp ladders were observed in both TF1 and nTE4-5 strains (Fig. 2, left), indicating the existence of telomerase activity in *hTERT* transfected cells.

TRF length. The TRF length of each strain was measured by Southern blot analysis. The mean TRF lengths determined by the peak sizes of the TRF smear signals were 8.1, 6.1, 6.8, and 8.4 kb for MJ90, TF1 PDL 104, 148 and 216 cells, respectively (Fig. 3, left), while that in HUE142-2 was 8.0 kb and no significant change was observed after the *hTERT* transfection. The senescent HUE142-2 cells without *hTERT* transfection showed telomere shortening to 4.9 kb at 73 PDL (Fig. 3, right).

Differential gene expressions. In the experiment of real-time RT-PCR, *ACTB* was used as an internal control, after the confirmation of its feasibility comparing the amounts of *ACTB*, *GAPD*, and *TBP* mRNA estimated by the real-time RT-PCR with the amount of total RNA (data not shown). The *hTERT* mRNA expression was detected in TF1 PDL 104, 148 and 216 cells and nTE4-5 PDL 105, 145 and 216 cells but not in MJ90 and HUE142-2 cells, by real-time RT-PCR using originally designed TaqMan probe which is specific for the full length *hTERT* mRNA distinguishing the α- and ß-splice variants (18) (Fig. 2, right). While the *hTERT* expression levels in the nTE4-5 strains were comparable among the PDL 105, 145 and 216 cells, those of TF1 cells at PDL 148 and 216

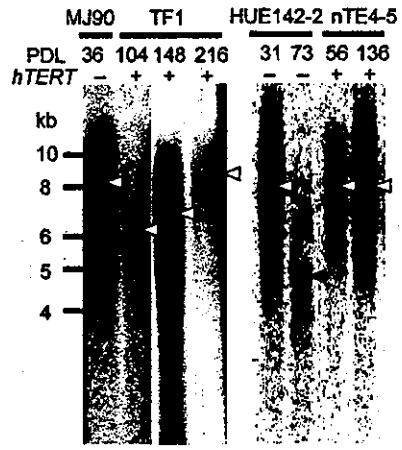

Figure 3. Sourthern blot analysis for TRF length. The peaks of the TRF smear signals are indicated by white arrowheads at 8.1, 6.1, 6.8 and 8.4 kb in MJ90, TF1 PDL 104, 148 and 216 cells, respectively (left), and 8.0 kb in HUE142-2 cells before and after *hTERT* transfection (right). That of the senescent HUE142-2 cells at 73 PDL is shown with black arrowhead at 4.9 kb.

were somewhat higher than that at PDL 104. This pattern was similar to the expression levels of PCNA (Fig. 4) and the growth curve (Fig. 1). Also, the expression level of TP53 was dramatically repressed at PDL 104 and gradually recovered thereafter in the TF1 strains, while such repression was not observed in the nTE4-5 strains (Fig. 4).

The expression levels of *RB1*, *BAX* and *MYC* were somewhat repressed in TF1 cells, while they were upregulated in general in nTE4-5 cells (Fig. 4). On the contrary, expression level of *BCL2* was apparently upregulated in the TF1 cells at PDL 104 and gradually decreased to the baseline, while no such transient upregulation was observed in nTE4-5 cells. Though, the upregulation of *BCL2L1* was only observed in nTE4-5 cells. The expression level of *CDKN2A*, encoding p16, was once upregulated at PDL 104 and dramatically down-regulated after then in TF1 strains. The expression levels of *FAS* and *APAF1* were comparable during cellular immortalization in both strains, fibroblasts and endothelial cells. Progressive down-regulation of *CDKN1A* was observed in TF1 cells, but not in nTE4-5 cells.

Sequence of RB1 and TP53. By the sequence analysis of the *RB1* cDNA from exon 10 to the 5'-site of exon 27 and *TP53* cDNA in exons 5-8 of both TF1 and nTE4-5 strains, no sequence abnormalities were observed at any PDL. The sequences of the *TP53* exons 4-9 and corresponding splice sites were also examined in the genomic DNA of the TF1 strains, which showed dramatic change in the *TP53* mRNA expression levels, and no abnormalities were detected.

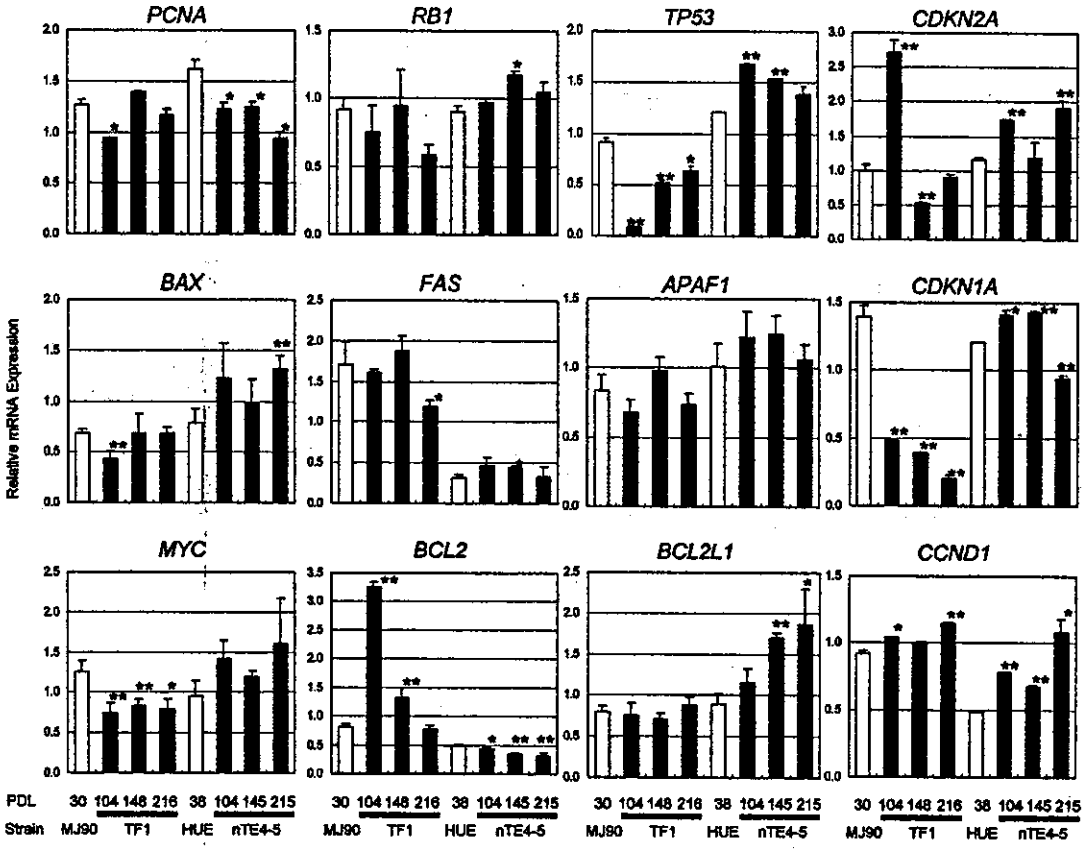

Figure 4. Relative levels of the mRNA expression in each strain. The expression level of each gene was evaluated by real-time RT-PCR using *ACTB* as an internal control, and expressed as mean + SD. The levels in a strain after *hTERT* transfection (closed bar) were statistically compared with those before transfection (open bar). $^{*}P<0.05$; $^{**}P<0.01$.

Table I. Upregulated genes in TF1 at PDL 216 compared with MJ90 by microarray analysis.

Gene	UniGene ID	Cytogenetic
RAGE	Hs.104119	14q32
CLIC2	Hs.54570	Xq28
MCOLN3	Hs.49344	1p22.3
PODXL	Hs.16426	7q32-q33
SPP1	Hs.313	4q21-q25
CEB1	Hs.26663	4q22.1-q23
FOLR3	Hs.352	11q13
HLA-DRB1	Hs.308026	6p21.3
AK3	Hs.10862	9pter-p13
RAB33A	Hs.56294	Xq26.1
LGALS3BP	Hs.79339	17q25
LOC91752	Hs.159528	2q32.1
MX2	Hs.926	21q22.3

Microarray analysis. To investigate intrinsic responses required in the immortalization of fibroblast strain MJ90 other than the upregulation of cell-cycle promoting genes and down-regulation of apoptosis-inducing genes, microarray analysis using oligoarrays were carried out between the MJ90 cells, normal mortal cells, and the TF1 cells at PDL 216, definitely immortalized cells. After global normalization of the spot intensity, the TF1/MJ90 ratio was calculated for each spot. The genes whose TF1/MJ90 ratios of the normalized intensity were ≥4 or ≤0.25 are listed in Tables I (13 genes) and II (101 genes), respectively.

Discussion

By the transfection of *hTERT* gene into fibroblasts MJ90 and endothelial cells HUE142-2, immortal cell strains TF1 and nTE4-5 were obtained. During this immortalization course, no abnormalities were detected in the core regions of *RB1* or *TP53* genes, but the differential expression of the *TP53* was characteristic. It dramatically decreased at PDL 104 in the TF1 cells, but showed recovery after that, indicating that this down-regulation may not be due to a mutational event in the promoter region. The p53 is widely accepted to inhibit cell growth through activation of cell-cycle arrest and apoptosis, and its disruption with inactivation of Rb would provide normal human somatic cells to escape the Hayflick limit, the replicative senescence checkpoint, endowing the cells with elongated lifespan (2).

The TF1 at PDL 104 cells, where telomere maintenance mechanism seems insufficient with shortened telomeres, also showed transient down-regulation of *BAX* and upregulation of *BCL2*, which were not observed in nTE4-5 strains. *BAX* is a p53 primary-response gene (19) and was reported to be essential for death receptor-mediated apoptosis in cancer cells (20). *BCL2* was reported to control a cell-death pathway independently of Apaf-1, which was not differentially expressed either in the present study, in the upstream of initiator caspases in mice (21). Thus, the repression of *TP53* and *BAX* expression and transient upregulation of *BCL2* around PDL 104 might have promoted the resistance to apoptosis in the TF1 cells.

Down-regulation of *MYC* and *CDKN1A* was observed in all TF1 strains, which was absent in nTE4-5 strains. Whereas Myc is known to be a transcriptional activator of the *hTERT* promoter (22) and promotes cell growth and proliferation, it also induces apoptosis under certain conditions (23). More recently Myc was proved to switch the p53-dependent response of cancer cells to DNA damage from cytostatic to apoptotic, inhibiting p21(cip1/waf1) induction by p53 and other activators (24). The p21, encoded by *CDKN1A*, is the effector of p53 cell cycle control and both appeared to be essential for maintaining the G2 checkpoint (25).

The p16, encoded by *CDKN2A*, was once upregulated at PDL 104 and dramatically down-regulated thereafter in TF1 strains. The expression of p16 was previously found to be repressed during cellular immortalization in Epstein-Barr virus-transformed human B-lymphoblastoid cell lines (26). The dramatic down-regulation of the *CDKN2A* expression between PDL 104 and 148 in TF1 strains may indicate that cellular immortalization may have occurred between these PDLs, but the repression of this gene does not seem to be universally required in immortalization, e.g., in nTE4-5 strains.

Thus, repression of *MYC*, *CDKN1A*, *TP53* and/or *CDKN2A* in TF1 strains would have promoted their continuous proliferation and the resistance to apoptosis. Thus, the upregulation of anti-apoptotic genes and down-regulation of pro-apoptotic or apoptosis-related genes were observed in the TF1 cells in general, especially in early phase after *hTERT* transfection where telomere-maintenance mechanism has not been completed, while no such findings were observed in the nTE4-5 cells. Only the *CCND1*, which encodes cyclin D1 and is frequently overexpressed in a broad range of human tumor types as a positive regulator of progression through the G1 phase of the cell cycle interacting with cdk4, pRb, and some transcription factors (27), was overexpressed in both strains.

To investigate other intrinsic responses required in the immortalization of the fibroblast strain, microarray analysis was carried out between the MJ90 cells and the TF1 cells at PDL 216, and the 13 genes and 101 genes were found to be upregulated or down-regulated more than 4-fold (Tables I and II). The *CDKN1A*, whose expression was found to be dramatically repressed after immortalization in real-time RT-PCR analysis was also included as the down-regulated gene in microarray analysis (Table II), supporting the reliability of the analysis. The sequence of the *hTERT*-oligo spotted on the array located near the poly A site, which hybridizes both full length and splice variant *hTERT* mRNAs. So it is reasonable that the *hTERT* expression evaluated by oligoarray analysis was only slightly upregulated (data not shown), whereas it revealed to be clearly upregulated from none to positive after *hTERT* transfection by real-time RT-PCR analysis using full-length *hTERT*-specific primers.

Table II. Down-regulated genes in TF1 at PDL 216 compared with MJ90 by microarray analysis.

Gene	UniGene ID	Cytogenetic
NDN	Hs.50130	15q11.2-q12
PSMB3	Hs.82793	17q12
GDF15	Hs.296638	19p13.1-13.2
SEPP1	Hs.275775	5q31
CYBA	Hs.68877	16q24
KRT19	Hs.309517	17q21.2
PRG1	Hs.1908	10q22.1
PTPN12	Hs.62	7q11.23
FOXF1	Hs.155591	16q24
PPP1R14A	Hs.348037	19q13.1
LAMA4	Hs.437536	6q21
PDLIM4	Hs.424312	5q31.1
ALDH1A1	Hs.76392	9q21.13
TNA	Hs.65424	3p22-p21.3
COL3A1	Hs.443625	2q31
PTGIS	Hs.302085	20q13.11-q13.13
GABARAPL1	Hs.336429	12p13.31
IFITM1	Hs.458414	11p15.5
CCRL1	Hs.310512	3q22
AEBP1	Hs.439463	7p13
GALNT6	Hs.528445	12q13
F3	Hs.62192	1p22-p21
ACAA2	Hs.172506	18q21.1
BST1	Hs.169998	4p15
EFEMP1	Hs.76224	2p16
TAGLN	Hs.410977	11q23.2
CASC3	Hs.350229	17q11-q21.3
CDKN1A	Hs.370771	6p21.2
HAK	Hs.388674	18q21.31-q21.32
WNT2	Hs.89791	7q31
AQP10	Hs.259048	1q22
GPX7	Hs.43728	1p32
SERPINB2	Hs.75716	18q21.3
COX7A1	Hs.421621	19q13.1
BEX1	Hs.334370	Xq21-q23
TPK1	Hs.127548	7q34-q35
EPB41L3	Hs.103839	18p11.32
HAPLN1	Hs.2799	5q14.3
GAL	Hs.278959	11q13.1
T1A2	Hs.468675	1p36
GW112	Hs.273321	13q14.2
SERPING1	Hs.384598	11q12-q13.1
ROBO2	Hs.13305	3p13
POSTN	Hs.136348	13q13.3
SULF1	Hs.409602	8q13.2
SUCLA2	Hs.182217	13q12.2-q13.3
SLC12A7	Hs.172613	5p15
BGN	Hs.821	Xq28
DOK5	Hs.127751	20q13.2
KCNE4	Hs.348522	2q36.3

Table II. Continued.

Gene	UniGene ID	Cytogenetic
HOXD11	Hs.421136	2q31.1
NCAG1	Hs.124673	18q22.1
CDCP1	Hs.146170	3p21.32
RAC2	Hs.301175	22q13.1
PTGER1	Hs.159360	19p13.1
TRO	Hs.434971	Xp11.22-p11.21
CCDC8	Hs.97876	19q13.33
ARHGDIB	Hs.292738	12p12.3
MRVI1	Hs.251385	11p15
NET02	Hs.6823	16q11
LTC4S	Hs.456	5q35
QPCT	Hs.79033	2p22.3
MGC3036	Hs.284135	7q31-q35
POFUT1	Hs.178292	20q11
RAMP1	Hs.32989	2q36-q37.1
C20orf59	Hs.512686	20q13.33
GPR51	Hs.198612	9q22.1-q22.3
ISL1	Hs.505	5q11.2
DKFZP434B044	Hs.262958	16q24.1
FLJ11175	Hs.33368	15q26.1-q26.2
SH2D2A	Hs.103527	1q21
DF	Hs.155597	19p13.3
ZD52F10	Hs.417795	19q13.13
NLGN4	Hs.21107	Xp22.33
SLC38A5	Hs.195155	Xp11.23
FLJ10847	Hs.48403	17p11.2
MST4	Hs.23643	Xq26.2
C9orf67	Hs.134292	9q34.2-q34.3
NFATC1	Hs.96149	18q23
PTGDS	Hs.446429	9q34.2-q34.3
HSD17B2	Hs.155109	16q24.1-q24.2
STMN2	Hs.90005	8q21.11-q21.12
GAS1	Hs.65029	9q21.3-q22
GGTLA1	Hs.437156	22q11.23
STAP2	Hs.194385	19p13.3
IL17RB	Hs.5470	3p21.1
SUSD2	Hs.131819	22q11-q12
TSPAN-2	Hs.234863	1p13.1
ELN	Hs.252418	7q11.23
KISS1	Hs.95008	1q32
CXCL6	Hs.164021	4q21
LRRN1	Hs.126085	3p26.2
PTPRD	Hs.323079	9p23-p24.3
RGMA	Hs.271277	15q26.1
AGXT2L1	Hs.106576	4q25
C12orf14	Hs.356223	12p11
HOXD10	Hs.123070	2q31.1
EPSTI1	Hs.343800	13q13.3
ACVRL1	Hs.410104	12q11-q14
TU3A	Hs.8022	3p21.1
NTNG1	Hs.111224	1p13.3

Interestingly, among the 36 reportedly ≥4-fold dysregulated genes, including 11 upregulated and 25 down-regulated genes, during immortalization of normal human foreskin fibroblasts BJ cells (8), only *LAMA4*, *COL3A1* and *T1A2* were commonly down-regulated ≥4-fold and one third was oppositely dysregulated. Although both MJ90 and BJ cells are derived from the same organ, acquisition of telomere maintenance mechanism after *hTERT* transfection seems different between them, straightforward in the BJ cells acquiring telomere maintenance mechanism (1) and not straight in the MJ90 cells with telomere shortening (Fig. 3).

The inconsistency of the dysregulated genes during *hTERT*-induced immortalization between the cell strains derived from the same origin with differences in the acquisition of telomere maintenance mechanism may also support the idea that some specific intrinsic responses are necessary for *hTERT*-induced immortalization in some kinds of non-cancerous cells. Since no report has found that once telomerase-activated cancer cells lose their proliferation capacity without artificial manipulation, the intrinsic responses required in the immortalization of *hTERT*-induced non-cancerous cells may be the specific characteristics that distinguish cancerous and non-cancerous cells.

It was reported that the telomerase catalytic subunit *hTERT* was expressed at low level in cycling human fibroblasts, which were previously believed to lack *hTERT* expression and telomerase activity (28), indicating that expression of *hTERT* is not necessarily the rate-limiting step for cellular immortalization. Moreover, it was reported that there are at least three tumor suppressor pathways regulating *hTERT* expression (29), suggesting that it is not straightforward but there are different settings for human somatic cells to acquire immortality through telomerase activation.

In conclusion, we propose that immortalization of normal human somatic cells by *hTERT* expression is not straightforward but requires some specific intrinsic responses including upregulation of cell-cycle promoting genes and down-regulation of apoptosis-inducing genes, not in a mutational event manner, in early phase after transfection in some non-cancerous cells. These requirements seem to be decreased after continuous culture concomitant with acquisition of telomere-elongation capacity, and may be the specific characteristics that distinguish non-cancerous cells from cancer cells.

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References

1. Bodnar AG, Ouellette M, Frolkis M, Holt SE, Chiu CP, Morin GB, Harley CB, Shay JW, Lichtsteiner S and Wright WE: Extension of life-span by introduction of telomerase into normal human cells. Science 279: 349-352, 1998.
2. Harley CB: Telomerase is not an oncogene. Oncogene 21: 494-502, 2002.
3. Hiyama E and Hiyama K: Telomerase as tumor marker. Cancer Lett 194: 221-233, 2003.
4. Hiyama K, Hirai Y, Kyoizumi S, Akiyama M, Hiyama E, Piatyszek MA, Shay JW, Ishioka S and Yamakido M: Activation of telomerase in human lymphocytes and hematopoietic progenitor cells. J Immunol 155: 3711-3715, 1995.
5. Bryan TM, Englezou A, Dalla PL, Dunham MA and Reddel RR: Evidence for an alternative mechanism for maintaining telomere length in human tumors and tumor-derived cell lines. Nat Med 3: 1271-1274, 1997.
6. Lundberg AS, Randell SH, Stewart SA, Elenbaas B, Hartwell KA, Brooks MW, Fleming MD, Olsen JC, Miller SW, Weinberg RA and Hahn WC: Immortalization and transformation of primary human airway epithelial cells by gene transfer. Oncogene 21: 4577-4586, 2002.
7. Kiyono T, Foster SA, Koop JI, McDougall JK, Galloway DA and Klingelhutz AJ: Both Rb/p16INK4a inactivation and telomerase activity are required to immortalize human epithelial cells. Nature 396: 84-88, 1998.
8. Lindvall C, Hou M, Komurasaki T, Zheng C, Henriksson M, Sedivy JM, Bjorkholm M, Teh BT, Nordenskjold M and Xu D: Molecular characterization of human telomerase reverse transcriptase-immortalized human fibroblasts by gene expression profiling: activation of the epiregulin gene. Cancer Res 63: 1743-1747, 2003.
9. Smith LL, Coller HA and Roberts JM: Telomerase modulates expression of growth-controlling genes and enhances cell proliferation. Nat Cell Biol 5: 474-479, 2003.
10. Tominaga K, Olgun A, Smith JR and Pereira-Smith OM: Genetics of cellular senescence. Mech Ageing Dev 123: 927-936, 2002.
11. Kumazaki T, Sasaki M, Nishiyama M, Teranishi Y, Sumida H and Mitsui Y: Effect of BCL-2 down-regulation on cellular life span. Biogerontology 3: 291-300, 2002.
12. Kumazaki T, Sasaki M, Nishiyama M, Teranishi Y, Sumida H, Eboshida A and Mitsui Y: Life span shortening of normal fibroblasts by overexpression of BCL-2: a result of potent increase in cell death. Exp Cell Res 285: 299-308, 2003.
13. Schwarze SR, Shi Y, Fu VX, Watson PA and Jarrard DF: Role of cyclin-dependent kinase inhibitors in the growth arrest at senescence in human prostate epithelial and uroepithelial cells. Oncogene 20: 8184-8192, 2001.
14. Kumazaki T, Kobayashi M and Mitsui Y: Enhanced expression of fibronectin during *in vivo* cellular aging of human vascular endothelial cells and skin fibroblasts. Exp Cell Res 205: 396-402, 1993.
15. Nakayama J, Tahara H, Tahara E, Saito M, Ito K, Nakamura H, Nakanishi T, Tahara E, Ide T and Ishikawa F: Telomerase activation by hTRT in human normal fibroblasts and hepatocellular carcinomas. Nat Genet 18: 65-68, 1998.
16. Kim NW, Piatyszek MA, Prowse KR, Harley CB, West MD, Ho PLC, Coviello GM, Wright WE, Weinrich SL and Shay JW: Specific association of human telomerase activity with immortal cells and cancer. Science 266: 2011-2015, 1994.
17. Lee JO, Russo AA and Pavletich NP: Structure of the retinoblastoma tumour-suppressor pocket domain bound to a peptide from HPV E7. Nature 391: 859-865, 1998.
18. Ulaner GA, Hu JF, Vu TH, Giudice LC and Hoffman AR: Telomerase activity in human development is regulated by human telomerase reverse transcriptase (hTERT) transcription and by alternate splicing of hTERT transcripts. Cancer Res 58: 4168-4172, 1998.
19. Miyashita T and Reed JC: Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. Cell 80: 293-299, 1995.
20. LeBlanc H, Lawrence D, Varfolomeev E, Totpal K, Morlan J, Schow P, Fong S, Schwall R, Sinicropi D and Ashkenazi A: Tumor-cell resistance to death receptor-induced apoptosis through mutational inactivation of the proapoptotic Bcl-2 homolog Bax. Nat Med 8: 274-281, 2002.
21. Marsden VS, O'Connor L, O'Reilly LA, Silke J, Metcalf D, Ekert PG, Huang DC, Cecconi F, Kuida K, Tomaselli KJ, Roy S, Nicholson DW, Vaux DL, Bouillet P, Adams JM and Strasser A: Apoptosis initiated by Bcl-2-regulated caspase activation independently of the cytochrome c/Apaf-1/caspase-9 apoptosome. Nature 419: 634-637, 2002.
22. Takakura M, Kyo S, Kanaya T, Tanaka M and Inoue M: Expression of human telomerase subunits and correlation with telomerase activity in cervical cancer. Cancer Res 58: 1558-1561, 1998.

23. Grandori C, Cowley SM, James LP and Eisenman RN: The Myc/Max/Mad network and the transcriptional control of cell behavior. Annu Rev Cell Dev Biol 16: 653-699, 2000.
24. Seoane J, Le HV and Massague J: Myc suppression of the p21(Cip1) Cdk inhibitor influences the outcome of the p53 response to DNA damage. Nature 419: 729-734, 2002.
25. Bunz F, Dutriaux A, Lengauer C, Waldman T, Zhou S, Brown JP, Sedivy JM, Kinzler KW and Vogelstein B: Requirement for p53 and p21 to sustain G2 arrest after DNA damage. Science 282: 1497-1501, 1998.
26. Takahashi T, Kawabe T, Okazaki Y, Itoh C, Noda K, Tajima M, Satoh M, Goto M, Mitsui Y, Tahara H, Ide T, Furuichi Y and Sugimoto M: *In vitro* establishment of tumorigenic human B-lymphoblastoid cell lines transformed by Epstein-Barr virus. DNA Cell Biol 22: 727-735, 2003.
27. Lamb J, Ramaswamy S, Ford HL, Contreras B, Martinez RV, Kittrell FS, Zahnow CA, Patterson N, Golub TR and Ewen ME: A mechanism of cyclin D1 action encoded in the patterns of gene expression in human cancer. Cell 114: 323-334, 2003.
28. Masutomi K, Yu EY, Khurts S, Ben-Porath I, Currier JL, Metz GB, Brooks MW, Kaneko S, Murakami S, De Caprio JA, Weinberg RA, Stewart SA and Hahn WC: Telomerase maintains telomere structure in normal human cells. Cell 114: 241-253, 2003.
29. Lin SY and Elledge SJ: Multiple tumor suppressor pathways negatively regulate telomerase. Cell 113: 881-889, 2003.

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Expression profiling and differential screening between hepatoblastomas and the corresponding normal livers: identification of high expression of the *PLK1* oncogene as a poor-prognostic indicator of hepatoblastomas

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Hepatoblastoma is one of the most common malignant liver tumors in young children. Recent evidences have suggested that the abnormalities in Wnt signaling pathway, as seen in frequent mutation of the *β-catenin* gene, may play a role in the genesis of hepatoblastoma. However, the precise mechanism to cause the tumor has been elusive. To identify novel hepatoblastoma-related genes for unveiling the molecular mechanism of the tumorigenesis, a large-scale cloning of cDNAs and differential screening of their expression between hepatoblastomas and the corresponding normal livers were performed. We constructed four full-length-enriched cDNA libraries using an oligo-capping method from the primary tissues which included two hepatoblastomas with high levels of alpha-fetoprotein (AFP), a hepatoblastoma without production of AFP, and a normal liver tissue corresponded to the tumor. Among the 10431 cDNAs randomly picked up and successfully sequenced, 847 (8.1%) were the genes with unknown function. Of interest, the expression profile among the two subsets of hepatoblastoma and a normal liver was extremely different. A semiquantitative RT–PCR analysis showed that 86 out of 1188 genes tested were differentially expressed between hepatoblastomas and the corresponding normal livers, but that only 11 of those were expressed at high levels in the tumors. Notably, *PLK1* oncogene was expressed at very high levels in hepatoblastomas as compared to the normal infant's livers. Quantitative real-time RT–PCR analysis for the *PLK1* mRNA levels in 74 primary hepatoblastomas and 29 corresponding nontumorous livers indicated that the patients with hepatoblastoma with high expression of *PLK1* represented significantly poorer outcome than those with its low expression (5-year survival rate: 55.9 vs 87.0%, respectively, $p=0.042$), suggesting that the level of *PLK1* expression is a novel marker to predict the prognosis of hepatoblastoma. Thus, the differentially expressed genes we have identified may become a useful tool to develop new diagnostic as well as therapeutic strategies of hepatoblastoma.
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Introduction

Hepatoblastoma (HBL) is the most common hepatic cancer in children (Exelby *et al.*, 1975; Weinberg and Finegold, 1983). However, the etiology of HBL has been unclear in contrast to the adult hepatocellular carcinoma (HCC), in which preceding infection of hepatitis virus is often found (Buendia, 1992; Idilman *et al.*, 1998). Although most HBLs are sporadic, it is sometimes associated with certain hereditary diseases such as Beckwith–Wiedemann syndrome (Albrecht *et al.*, 1994) and familial adenomatous polyposis (Li *et al.*, 1987; Giardiello *et al.*, 1996; Kinzler and Vogelstein, 1996). In the former, loss of heterozygosity of chromosome 11p15.5 is frequently observed, and the abnormal regulation of the *insulin-like growth factor 2* (*IGF2*) and the *H19* genes at this locus may contribute to the disease (Albrecht *et al.*, 1994; Montagna *et al.*, 1994; Li *et al.*, 1995; Rainier *et al.*, 1995; Yun *et al.*, 1998; Fukuzawa *et al.*, 1999). In the latter, the *APC* gene, which is one of the key molecules in Wnt signaling, was found to be constitutively mutated (Kinzler and Vogelstein, 1996).

Increasing evidence suggests that Wnt signaling pathway also plays an important role in the genesis of sporadic hepatoblastomas. A high frequency (more than 60% in some reports) of somatic mutations in the *β-catenin* gene has recently been reported in sporadic tumors (Koch *et al.*, 1999; Wei *et al.*, 2000; Takayasu

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et al., 2001; Buendia, 2002). Mutant β-catenin proteins accumulate in the nucleus, resulting in stimulating transcription of the target genes such as *c-myc* and *cyclin D1* (Morin *et al.*, 1997; Polakis, 1999). Mutation in the *Axin* gene, whose product is an antagonist of nuclear accumulation of β-catenin, has also been found in HBL and may contribute to the pathogenesis of the tumors without *β-catenin* mutation (Taniguchi *et al.*, 2002; Miao *et al.*, 2003). However, the molecular mechanism underlying the pathogenesis of HBL is still largely unknown.

Recent progress in therapeutic strategies including intensive chemotherapy and liver transplantation improved the outcome of the patients with HBL. However, the prognosis of a significant fraction of the tumors still remains poor. The clinical markers currently used for HBL include staging, which is a major instrument for assessing prognosis (Hata, 1990), serum alpha-fetoprotein (AFP) (Mann *et al.*, 1978), mitotic activity (Haas *et al.*, 1989), DNA ploidy (Hata *et al.*, 1991), nuclear localization of β-catenin (Park *et al.*, 2001), p53 mutation (Oda *et al.*, 1995), and chromosomal alteration (Weber *et al.*, 2000). Serum AFP level is used as a diagnostic marker to monitor the tumor progression, responsiveness to the therapy, and recurrence after the treatment. Extremely high levels of serum AFP are reported to be associated with aggressiveness of the tumors with unfavorable outcome (van Tornout *et al.*, 1997), except some reports showing that there is no significant relationship between initial serum AFP levels and prognosis of the patients with HBL (Ortega *et al.*, 1991; von Schweinitz *et al.*, 1994). Moreover, the tumor with low levels of serum AFP often grows rapidly and is often reluctant to chemotherapy (von Schweinitz *et al.*, 1995). The other genetic markers including DNA ploidy, chromosomal aberration, and *p53* mutation are not so powerful clinical indicators. Even the nuclear localization of β-catenin and/or mutation of the *β-catenin* gene appear to lose their impact as a prognostic factor when combined with the grade of histological differentiation because of its close correlation with the latter (Takayasu *et al.*, 2001). Therefore, we may need to find novel markers to predict the patient's outcome in a comprehensive way.

To understand the molecular mechanism of the genesis and progression of HBL, as well as to develop a novel diagnostic and therapeutic system for the tumor, we have randomly cloned 10431 cDNAs expressed in primary HBL tissues and a normal infant's liver by using full-length-enriched oligo-capping cDNA libraries. In the present study, we have identified 86 genes differentially expressed between HBLs and their corresponding normal livers. One of such genes, *PLK1*, showed a significantly high expression in the formers as compared with the latters, and its high expression was significantly associated with poor prognosis of HBLs.

Results

Expression profiles of primary HBLs and a normal liver

To obtain the genes expressed in primary HBLs and normal infant's liver, we constructed oligo-capping cDNA libraries from two primary HBLs with increased AFP secretion (HMFT, HYST), a primary HBL without AFP secretion (HKMT), and a corresponding normal liver (HMFN). After cloning 3000 cDNAs from each of the four cDNA libraries, 2289, 2837, 2537, and 2768 clones from the libraries of HMFT, HYST, HKMT, and HMFN, respectively, were successfully end-sequenced. Homology search against the public databases of those 10431 clones by BLAST program revealed that 847 clones (8.1%) in total contained novel sequences which had not been annotated (Table 1).

To elucidate the gene expression pattern in each cDNA library, we compared expression profile of the known genes that appeared in three different kinds of libraries, a HBL with positive AFP (HMFT), a HBL with negative AFP (HKMT), and an infant's liver (HMFN) (Table 2). BodyMap (Okubo *et al.*, 1992) and a serial analysis of gene expression (SAGE) (Velculescu *et al.*, 2000) are very good methods to quickly provide quantification of the levels of all mRNAs in certain tissues and cell types by high throughput end-sequencing of cDNA clones. In this study, we applied the former method by counting cDNA clones to show each expression profile of HBL tumors or a non-tumorous tissue. Although each library consists of 3000 clones, which may be a rather small number, the frequency of each cDNA appearance provides a hint to understand each tissue's genetic background.

Overall, the most frequently appeared gene was *albumin* as expected, which was extremely low in the tumor with negative AFP. Genes involved in cellular structure and/or maintenance, glucose and lipid metabolisms, and a part of protein synthesis and its transport were frequently found in the normal liver library. On the

Table 1 Summary of the number of genes cloned from the cDNA libraries of hepatoblastomas and a normal infant liver of hepatoblastomas

Oligo-capping cDNA library	*No. of the clones*	*No. of the genes successfully end-sequenced*	*No. of the genes with unknown function*
Hepatoblastomas with positive AFP	6000	5126	323 (6.3%)
Hepatoblastoma with negative AFP	3000	2537	262 (10.3%)
Infant's liver	3000	2768	262 (9.5%)
Total	12000	10431	847 (8.1%)

Table 2 Comparison of the known genes frequently appeared in hepatoblastomas with or without secretion of AFP and a non-tumorous infant's liver

Gene symbol	*Acc. no.*	*Gene name*	*No. of appearance of the genes*		
			HBL with positive AFP	*Normal infant's liver*	*HBL with negative AFP*
Total number of genes			2289	2768	2537
Protein synthesis, metabolism, transport					
ALB	NM_000477	Albumin	558	482	8
AFP	NM_001134	Alpha-feto protein	67	0	0
AGT	NM_000029	Angiotensinogen	43	16	0
EEF1A1	X03558	Eukaryotic translation elongation factor 1 alpha 1	35	20	87
RPL27A	NM_000990	60S ribosomal protein L27a	31	4	52
FTL	M11147	Ferritin	24	11	3
FGA	NM_021871	Fibrinogen, A alpha polypeptide	20	38	2
HP	K01763	Haptoglobin	19	6	1
ORM1	X02544	Orosomucoid-1	12	8	0
RPS27	NM_001030	Ribosomal protein S27	11	4	31
F2	J00307	Coagulation factor 2	11	26	0
TF	NM_001063	Transferrin	8	6	0
PAH	U49897	Phenylalanine hydroxylase	6	6	0
PLG	NM_000301	Plasminogen	5	8	0
SERPINA1	X01683	Serine proteinase inhibitor, clade A, member 1	5	6	0
GC	NM_000583	Group-specific component	4	21	1
RPS29	NM_001032	Ribosomal protein S29	3	1	0
CTSB	NM_147783	Cathepsin B	2	5	3
SERPING1	BC011171	Serine proteinase inhibitor, clade G, menber 1	2	33	0
CRP	X56692	C-reactive protein	1	8	0
ITIH2	NM_002216	Inter-alpha (globulin) inhibitor, H2 polypeptide	0	25	0
Growth factor					
MST1	M74178	Macrophage stimulating 1	8	16	0
Cell signaling					
WIF1	NM_007191	Wnt inhibitory factor 1	0	0	11
DKK1	NM_012242	Dickkopf	0	0	7
Cell structure, adhesion					
VTN	NM_000638	Vitronectin	7	30	0
ACTB	BC013380	Actin	6	17	6
LRG	AF403428	Leucine-rich alpha-2-glycoprotein	6	11	0
VIM	NM_003380	Vimentin	0	3	38
Cell cycle					
RBM4	NM_002896	RNA binding motif protein	2	0	21
RAP1B	NM_015646	RAP1B	0	0	11
Organism defense					
BF	L15702	B-factor, properdin	5	13	0
GPX1	NM_000581	Glutathione peroxidase	4	0	0
C1R	NM_001733	Complement component 1	1	21	1
Glycometabolism					
LDHA	NM_005566	Lactate dehydrogenase	19	28	7
ADH1B	AF153821	Alcohol dehydrogenase	15	29	1
CES1	L07764	Carboxylesterase	9	22	2
ALDH1A1	NM_000689	Aldehyde dehydrogenase	2	13	2
Lipid metabolism					
EPHX1	NM_000120	Epoxide hydrolase 1	7	12	0
APOA2	NM_001643	Apolipoprotein A-II	6	2	0
ADFP	BC005127	Adipose differentiation-related protein	5	14	1
Heat shock protein, metabolic enzyme					
UGT2B4	Y00317	UDP-glucuronosyltransferase	11	32	2
HSPA8	NM_006597	Heat shock 70 kDa protein	1	6	1
Unknown, others					
ATP5A1	NM_004046	ATP synthase	18	11	23
SEPP1	NM_005410	Selenoprotein P	7	10	2

Table 2 (*continued*)

Gene symbol	*Acc. no.*	*Gene name*	*No. of appearance of the genes*		
			HBL with positive AFP	*Normal infant's liver*	*HBL with negative AFP*
CYP3A4	M18907	P450	6	81	3
AHSG	M16961	Alpha-2-HS-glycoprotein	6	5	2
TPT1	X16064	Translationally controlled tumor protein	6	0	3
CYP2C9	M61855	P4502C9	1	10	1

other hand, genes involved in protein synthesis such as elongation factors and ribosomal proteins were observed more frequently in HBLs than in normal liver. The expression profile in the library of the tumor without AFP secretion was very different from that with positive AFP (HMFT *vs* HKMT). As expected, *AFP* gene did not appear in the HKMT library. Intriguingly, *Wnt Inhibitory factor-1* and *dickkopf*, both of which are inhibitors of Wnt signaling (Hsieh *et al*., 1999; Wang *et al*., 2000), frequently appeared in the HKMT library. In addition, *vimentin*, *RNA-binding motif protein*, and *RAP1B* also frequently appeared in the HKMT library, but hardly in the HMFT library with AFP secretion. Thus, HBL with positive AFP and that with negative AFP seem to have a distinct gene expression profile, resulting in different biological characteristics.

Identification of the differentially expressed genes between HBLs and normal livers

To identify differentially expressed genes between HBLs and their corresponding normal livers, 1188 independent genes which included all of the 847 genes with unknown function and 341 known genes that were related to cellular functions including cell growth and differentiation among the 10431 cDNAs were selected and subjected to semiquantitative RT–PCR analysis (Figure 1a). The complementary DNAs reverse-transcribed from total RNA obtained from eight tumors and their corresponding normal livers were used as PCR templates after normalization with *GAPDH* expression. As a result, we found that 75 genes were expressed at higher levels in normal livers than in HBLs, whereas only 11 genes were expressed at higher levels in the tumors than in normal livers. Figure 1a shows the representatives of the results of differential screening using semi-quantitative RT–PCR and Table 3 lists 46 differentially expressed genes with known functions. We classified those differentially expressed genes into 12 categories according to their known functions. The genes preferentially expressed in normal liver showed the profiles which reflected normal liver function. Consistent with the previous reports about HBL and hepatocellular carcinoma (von Horn *et al*., 2001; Xu *et al*., 2001; Kinoshita and Miyata, 2002), *Insulin-like growth factor binding protein-3* (*IGFBP-3*), *aldolase B*, *ceruloplasmin*, and *c-reactive protein* were downregulated in HBLs as compared with the normal livers. The expression of *IGF2*, whose product has mitogenic

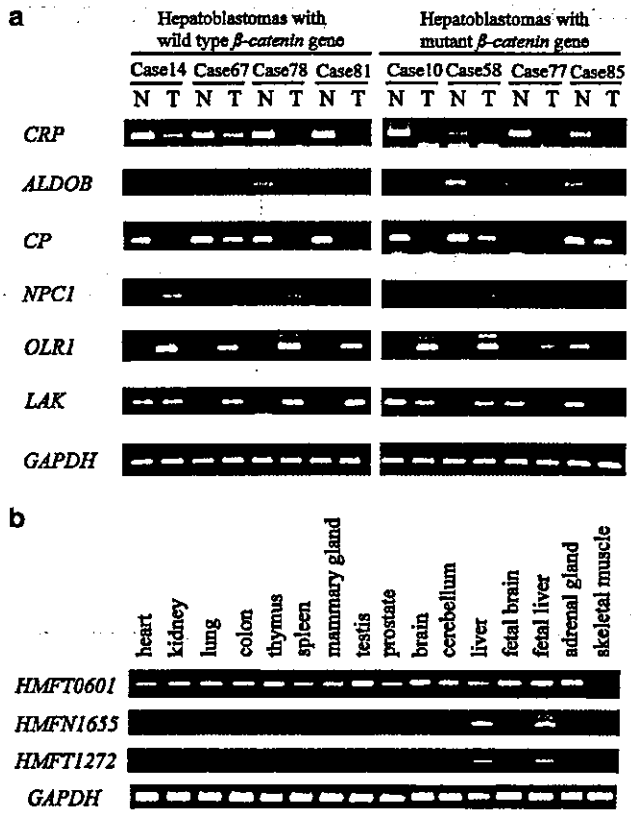

Figure 1 Expression of the representative genes by semi-quantitative RT–PCR. (**a**) Differentially expressed genes between HBLs with or without *β-catenin* mutation and the corresponding normal livers. cDNA was synthesized from RNAs prepared from eight pairs of tumors and their corresponding normal livers, and was used as a PCR template. Amount of cDNAs was normalized to that of *GAPDH*. Four tumors (cases 14, 67, 78, and 81) were with wild-type *β-catenin* gene, while the other four tumors (cases 10, 58, 77, and 85) were with mutant *β-catenin* gene. Gene symbols were shown on the left; *CRP*: C-reactive protein, *ALDOB*: aldolase, *CP*: ceruloplasmin, *NPC1*: Niemann–Pick disease, type C1, *OLR1*: oxidized low-density lipoprotein receptor 1, *LAK*: lymphocyte alpha-kinase. N: normal, T: tumor. (**b**) Semiquantitative RT–PCR of multiple human tissues. *HMFT0601* exhibited ubiquitous expression in all tissues examined, whereas *HMFN1655* and *HMFT1272* showed specific expression in liver and fetal liver

activity, is upregulated in HBLs, suggesting that the IGF axis may be involved in development of the tumor (Gray *et al*., 2000).

Four known genes which were expressed at high levels in HBLs (tumor>normal liver) include GTP-binding

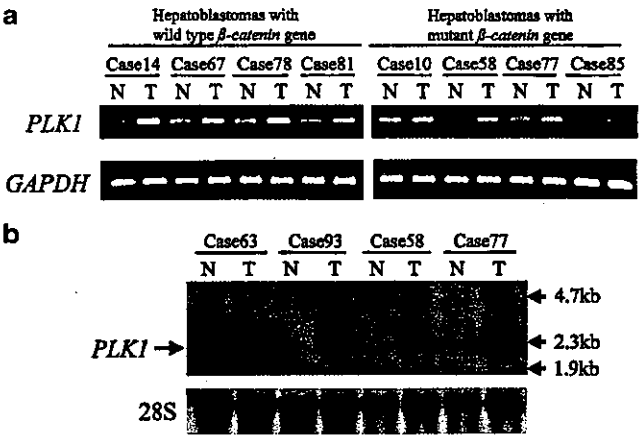

Figure 2 Increased expression of *PLK1* in HBLs. (**a**) Semiquantitative RT–PCR of *PLK1* gene in eight HBL cases. Preferential expression of the *PLK1* was seen in all sample pairs with and without *β-catenin* mutation. (**b**) Northern blot analysis of *PLK1* in primary HBLs, The 28S ribosomal band is shown as a control of each RNA amount

nuclear protein gene *RAN*, *PLK1* onocgene, and two cholesterol metabolism-associated protein genes, *low-density lipoprotein (LDL) receptor 1* and *Niemann-Pick disease type C1 (NPC1)*. The RAN protein is involved in the control of nucleo-cytoplasmic traffic of many nuclear proteins through formation of the transport nuclear pore complex (Ribbeck *et al.*, 1998). Nagata *et al.* (2003) also reported that *RAN* is upregulated in HBLs by oligonucleotide DNA array experiment. The LDL receptor 1 binds LDL, a major plasma cholesterol-carrying lipoprotein, and plays an important role in cholesterol homeostasis (Sudhof *et al.*, 1987; Goldstein and Brown, 1990; Hamanaka *et al.*, 1992). *NPC1* is a causal gene of Niemann–Pick type C disease which is an autosomal recessive lipid storage disorder that affects the viscera and central nervous system (Brady *et al.*, 1989). It encodes a protein with sequence similarity to the morphogen receptor 'patched', and to the cholesterol-sensing regions of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (Loftus *et al.*, 1997) and is involved in the intracellular trafficking of cholesterol. Concerning the differentially expressed genes which contained unknown sequences, those cDNA sequences have been submitted to the public database (Genbank/DDBJ Accession numbers: AB07 3346-AB073347, AB073382-AB073387, AB073599-AB0 73614, and AB075869-AB075881). Interestingly, only one known gene, *lymphocyte alpha-kinase (LAK)*, showed distinct expression pattern between HBLs with mutant *β-catenin* and those with wild type *β-catenin* (Figure 1a).

We next examined expression pattern of the novel genes in human multiple tissues by semi-quantitative RT–PCR and found that at least five genes were specifically expressed in the liver (a part of the data is shown in Figure 1b). Since the oncogene *PLK1* (*polo-like kinase-1*) was expressed in HBLs at significantly high levels as compared with the corresponding normal livers, we further examined the role of its expression in HBL.

PLK1 oncogene is overexpressed in HBLs

Recent studies have demonstrated that the preferential expression of *PLK1* mRNA is associated with some cancers including non-small-cell lung cancer (Wolf *et al.*, 1997), squamous cell carcinoma of the head and neck (Knecht *et al.*, 1999), and esophageal carcinoma (Tokumitsu *et al.*, 1999). However, the role of *PLK1* in HBL has never been reported. As indicated by semi-quantitative RT–PCR described above, we found that *PLK1* mRNA expression in HBLs is higher than in normal livers (Figure 2a). Northern blot analysis also confirmed its higher expression in HBLs (Figure 2b). We also performed Southern blot analysis by using the genomic DNAs obtained from primary HBLs and human placenta as a control, and probed with the *PLK1*-specific DNA fragment. However, we failed to find any clue of rearrangements or amplification of the *PLK1* gene locus (data not shown).

To examine the clinical significance of the expression level of *PLK1*, we performed quantitative real-time RT–PCR analysis using 74 primary hepatoblastomas and 29 corresponding normal liver samples (Figure 3a). The average arbitrary values of *PLK1* expression in HBLs and normal livers were 28.9 ± 6.7 and 4.1 ± 0.76, respectively (mean $\pm$ s.e.m., $P < 0.01$). The average values in alive and dead cases were 21.7 ± 5.2 ($n = 61$) and 62.4 ± 28.2 ($n = 13$), respectively ($p = 0.021$). When we compared the expression levels of *PLK1* between 24-paired HBLs and their corresponding normal livers, the former in HBL samples was significantly higher in comparison with the latter ($P < 0.01$) (Figure 3b). We also examined the relationship between the expression levels of *PLK1* and clinicopathological data of HBLs. Statistically significant correlation was observed only between histology and *PLK1* expression ($p = 0.041$). The expression level of *PLK1* in the tumors with poorly differentiated histology was higher than those with the well-differentiated one. The other clinicopathological factors such as age, clinical stage, and *β-catenin* mutation did not show a statistical significance with *PLK1* expression.

To further examine whether the *PLK1* expression was associated with the outcome of the patients with HBL, we performed a Kaplan–Meier analysis (Figure 4). The distinction between high and low levels of *PLK1* expression was based on the median value (low, *PLK1* < 13 d.u.; high, *PLK1* $\geq$ 13 d.u.). Since the overall survivals of 15 out of 74 cases were unknown, 59 cases were applied to the analysis. The 5-year survival rates of the groups with high and low *PLK1* expression were 55.9 and 87.0%, respectively ($P = 0.042$). The univariate analysis showed that both *PLK1* expression ($P = 0.015$) and histology ($P = 0.025$) have a significant prognostic importance (Table 4). The multivariate analysis demonstrated that *PLK1* expression was significantly related to survival, after controlling *β-catenin* mutation, age, stage,

Table 3 The known genes differentially expressed between hepatoblastomas and normal livers

	Gene symbol	*Acc. no*	*Gene name*
Protein synthesis, metabolism, transport			
T>N	RAN	NM_006325	GTP-binding nuclear protein RAN
N>T	LBP	AF105067	Lipopolysaccharide-binding protein
N>T	TDO2	BC005355	Tryptophan 2,3-dioxygenase
N>T	CRP	X56692	C-reactive protein
N>T	GC	NM_000583	Group-specific component
N>T	HP	K01763	Haptoglobin
N>T	HPX	NM_000613	Hemopexin
N>T	SQSTM1	NM_003900	Sequestosome 1
N>T	PHDGH	AF171237	A2-53-73 3-phosphoglycerate dehydrogenase
N>T	PPP1R3C	XM_005398	Protein phosphatase 1, regulatory (inhibitor) subunit 3C
N>T	ITIH4	D38595	Inter-alpha-trypsin inhibitor family heavy chain-related protein
N>T	G1P2	M13755	Interferon-induced 17-kDa/15-kDa protein
Cytokine, growth factor, hormones			
N>T	HABP2	D49742	Hyaluronan binding protein 2
N>T	IGFBP3	NM_000598	Insulin-like growth factor binding protein 3
N>T	GOT1	AF052153	Glutamic-oxaloacetic transaminase 1
Cell signaling			
N>T	CSNK2B	M30448	Casein kinase II, beta polypeptide
N>T	TPD52	NM_005079	Tumor protein D52
cell cycle			
T>N	PLK1	X73458	PLK1
Cell structure, adhesion			
N>T	LRG	AF403428	Leucine-rich alpha-2-glycoprotein
N>T	PGRP-L	AF384856	Peptidoglycan recognition protein L precursor
N>T	CLDN4	NM_001305	Claudin4
N>T	VTN	NM_000638	Vitronection
Organism defense			
N>T	RODH-4	NM_003708	Retinol dehydrogenase 4
N>T	MASP1	AF284421	Mannan-binding lectin serine protease 1
N>T	C4BPA	M31452	Complement component 4 binding protein, alpha
Glycometabolism			
N>T	ADH1B	AF153821	Alcohol dehydrogenase 1B, beta polypeptide
N>T	ALDOB	M15657	Aldolase B
Lipid metabolism			
T>N	NPC1	NM_000271	Niemann–Pick disease, type C1
T>N	OLR1	NM_002543	Oxidized low density lipoprotein (lectin-like) receptor 1
N>T	DGAT2	AF384161	Diacylglycerol acyltransferase
N>T	SCP2	NM_002979	Sterol carrier protein 2
N>T	APOA5	AF202890	Apolipoprotein A-V
N>T	AADAC	L32179	Arylacetamide deacetylase
N>T	SAA4	M81349	Amyloid A protein
Transcription			
N>T	BZW1	NM_014670	Basic leucine zipper and W2 domains 1
N>T	CREB-H	NM_032607	CREB/ATF family transcription factor
RNA biogenesis, metabolism			
N>T	HNRPDL	AB017018	Heterogeneous nuclear ribonucleoprotein D-like
Homeostasis, heat shock protein, metabolic enzymes			
N>T	UGT1A	AF297093	UGT1 gene locus
N>T	ALPL	X14174	Liver-type alkaline phosphatase
N>T	SLC10A1	L21893	Solute carrier family 10
N>T	CES1	AF177775	Carboxylestelase
N>T	AKR1D1	Z28339	Aldo-keto reductase family 1, member D1
N>T	AKR1C2	U05598	Aldo-keto reductase family 1, member C2
N>T	CP	D45045	Ceruloplasmin
Others			
N>T	DGCR6L	NM_033257	DiGeorge syndrome critical region gene 6 like
N>T	A1BG	AF414429	Alpha-1-B glycoprotein

T>N: highly expressed in the tumors as compared to normal livers. N>T: highly expressed in normal livers as compared to the tumors

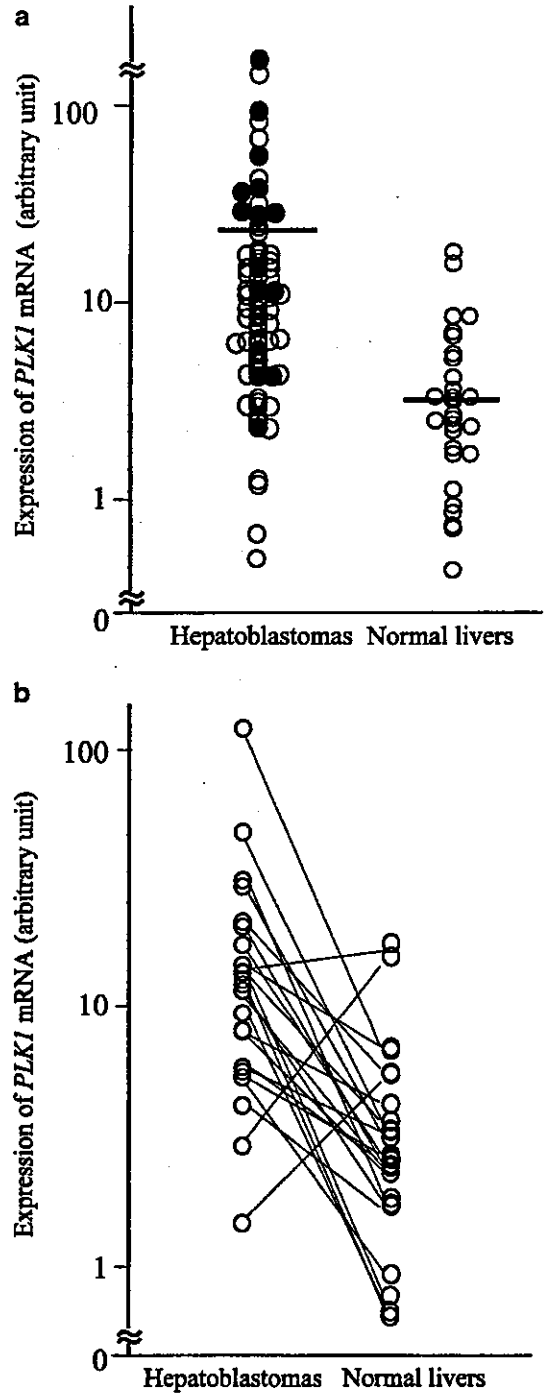

Figure 3 mRNA expression of *PLK1* in HBLs and the corresponding normal livers measured by quantitative real-time RT–PCR. (**a**) The levels of *PLK1* mRNA expression in HBLs and normal livers. The expression levels of *PLK1* were determined by quantitative real-time RT–PCR analysis using 74 HBL tissues 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

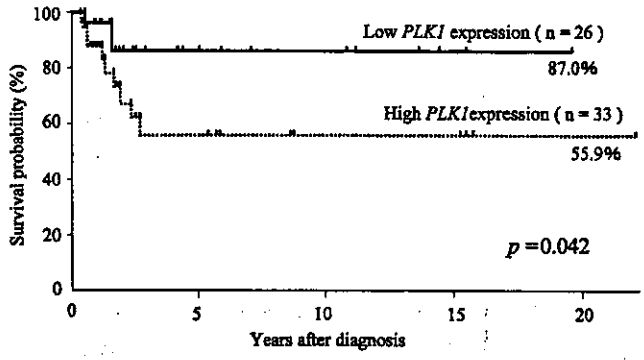

Figure 4 Kaplan–Meier survival curves ($n = 59$) in relation to the expression levels of *PLK1* (median cutoff). The arbitrary median cutoff value was set as 13. The patients with high expression of *PLK1* represented significantly poor prognosis than those with its low expression

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 12 000 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 μg 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 [α-^{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 μg/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 μl of cDNA was amplified in a final volume of 25 μl containing 1 × Taqman PCR reaction buffer, 200 μM each dNTP, 0.9 μM 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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References

Albrecht S, Von Schweinitz D, Waha A, Kraus JA, Von Deimling A and Pietsch T. (1994). *Cancer Res.*, **54**, 5041–5044.
Brady RO, Filling-Katz MR, Barton NW and Pentchev PG. (1989). *Neurol. Clin.*, **7**, 75–88.
Buendia MA. (1992). *Adv. Cancer Res.*, **59**, 167–226.
Buendia MA. (2002). *Med. Pediatr. Oncol.*, **39**, 530–535.
Chomczynski P and Sacchi N. (1987). *Anal. Biochem.*, **162**, 156–159.
Clay FJ, McEwen SJ, Bertoncello I, Wilks AF and Dunn AR. (1993). *Proc. Natl. Acad. Sci. USA*, **90**, 4882–4886.
Dietzmann K, Kirches E, Von Bossanyi, Jachau K and Mawrin C. (2001). *J. Neurooncol.*, **53**, 1–11.
Exelby PR, Filler RM and Grosfeld JL. (1975). *J. Pediatr. Surg.*, **10**, 329–337.
Fukuzawa R, Umezawa A, Ochi K, Urano F, Ikeda H and Hata J. (1999). *Int. J. Cancer*, **82**, 490–497.
Giardiello FM, Petersen GM, Brensinger JD, Luce MC, Cayouette MC, Bacon J, Booker SV and Hamilton SR. (1996). *Gut*, **39**, 867–869.
Glover DM, Ohkura H and Tavares A. (1996). *J. Cell Biol.*, **135**, 1681–1684.
Goldstein JL and Brown MS. (1990). *Nature*, **343**, 425–430.
Gray SG, Eriksson T, Ekstrom C, Holm S, Von Schweinitz D, Kogner P, Sandstedt B, Pietsch T and Ekstrom TJ. (2000). *Br. J. Cancer*, **82**, 1561–1567.
Haas JE, Muczynski KA, Krailo M, Ablin A, Land V, Vietti TJ and Hammond GD. (1989). *Cancer*, **64**, 1082–1095.
Hamanaka R, Kohno K, Seguchi T, Okamura K, Morimoto A, Ono M, Ogata J and Kuwano M. (1992). *J. Biol. Chem.*, **267**, 13160–13165.
Hata Y. (1990). *Jpn. J. Surg.*, **20**, 498–502.
Hata Y, Ishizu H, Ohmori K, Hamada H, Sasaki F, Uchino J, Inoue K, Naitoh H, Fujita M, Kobayashi T and Yokoyama S. (1991). *Cancer*, **68**, 2566–2570.
Hsieh JC, Kodjabachian L, Rebbert ML, Rattner A, Smallwood PM, Samos CH, Nusse R, Dawid IB and Nathans J. (1999). *Nature*, **398**, 431–436.
Ichimiya S, Nimura Y, Kageyama H, Takada N, Sunahara M, Shishikura T, Nakamura Y, Sakiyama S, Seki N, Ohira M, Kaneko Y, McKeon F, Caput D and Nakagawara A. (1999). *Oncogene*, **18**, 1061–1066.
Idilman R, De Maria N, Colantoni A and Van Thiel DH. (1998). *J. Viral. Hepat.*, **5**, 285–299.
Kinoshita M and Miyata M. (2002). *Hepatology*, **36**, 433–438.
Kinzler KW and Vogelstein B. (1996). *Cell*, **87**, 159–170.
Knecht R, Elez R, Oechler M, Solbach C, Von Ilberg C and Strebhardt K. (1999). *Cancer Res.*, **59**, 2794–2797.
Koch A, Denkhaus D, Albrecht S, Leuschner I, Von Schweinitz D and Pietsch T. (1999). *Cancer Res.*, **59**, 269–273.
Kotani S, Tugendreich S, Fujii M, Jorgensen PM, Watanabe N, Hoog C, Hieter P and Todokoro K. (1998). *Mol. Cell*, **1**, 371–380.
Lane HA and Nigg EA. (1996). *J. Cell Biol.*, **135**, 1701–1713.
Li FP, Wendy AT, Seddon J and Holmes GE. (1987). *J. Am. Med. Assoc.*, **257**, 2475–2477.
Li X, Adam G, Cui H, Sandstedt B, Ohlsson R and Ekstrom TJ. (1995). *Oncogene*, **11**, 221–229.
Liu X and Erikson RL. (2003). *Proc. Natl. Acad. Sci. USA*, **100**, 5789–5794.