研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Hiyama E, Hiyama K, Yamaoka H, Sueda T, Reynolds C. P, Yokoyama T.	Expression profiling of favorable and unfavorable neuroblastomas.	Pediatric Surgery International	20	33-38	2004
Hiyama E, Yamaoka H, Matsunaga T, Hayashi Y, Hayashi Y, Ando H, Suita S, Horie H, Kaneko M, Sasaki F, Hashizume K, Nakagawara A, Ohnuma N, Yokoyama T.	High expression of telomerase is an independent prognostic indicator of poor outcome in hepatoblastoma.	British Journal of Cancer	91	972-979	2004
Kumazaki T, Hiyama K, Takahashi T, Omatsu H, Tanimoto K, Noguchi T, Hiyama E, Mitsui Y, Nishiyama M.	Differential gene expressions during immortalization of normal human fibroblasts and endothelial cells transfected with human telomerase reverse transcriptase gene.	International Journal of Oncology	24	1435-1442	2004
Yamada S, Ohira M, Horie H, Ando K, Takayasu H, Suzuki Y, Sugano S, Hirata T, Goto T, Matsunaga T, Hiyama E, Hayashi Y, Ando H, Suita S, Kaneko M, Sasaki F, Hashizume K, Ohnuma N, Nakagawara A.	Expression profiling and differential screening between hepatoblastomas the corresponding normal livers: identification of high expression of the PLK1 oncogene as a poor-prognostic indicator of hepatoblastomas.	Oncogene	23	5901-5911	2004
Hiyama E, Hiyama K.	Telomerase detection in the diagnosis and prognosis of cancer.	Cytotechnology	45	61-74	2005
Morimoto K, Wakayama A, Yoshimine T, Nakayama M.	High-grade cord tumor with cerebellar and retroperitoneal extension	Pediatric Neurosurgery	40	149-50	2004
Kuwahara Y, Hosoi H, Osone S, Kita M, Iehara T, Kuroda H, Sugimoto T.	Antitumor activity of gefitinib in malignant rhabdoid tumor cells in vitro and in vivo	Clinical Cancer Research	10	5940-5948	2004
Osone S, Hosoi H, Kuwahara Y, Matsumoto Y, Iehara T, Sugimoto T.	Fenretinide induces sustained-activation of JNK/p38 MAPK and apoptosis in a reactive oxygen species-dependent manner in neuroblastoma cells	International Journal of Cancer	112	219-224	2004
Misawa A, Hosoi H, Imoto I, Iehara T, Sugimoto T, Inazawa J.	Translocation(1;22)(p36;q11.2) with concurrent del(22)(q11.2) resulted in homozygous deletion of SNF5/INI1 in a newly established cell line derived from extrarenal rhabdoid tumor	Journal of Human Genetics	in press		

Sato A, Imaizumi M, Chikaoka S, Niizuma H, Hoshi Y, Takeyama J, Fujii K, Nishio T, Watanabe M, Maesawa C, Hayashi Y, Iinuma K.	Acute renal failure due to leukemic cell infiltration followed by relapse at multiple extramedullary sites in a child with acute lymphoblastic leukemia	Leukemia and Lymphoma.	45	825-828	2004.
Sato A, Imaizumi M, Hoshi Y, Rikiishi T, Fujii K, Kizaki M, Kagechika H, Kakizuka A, Hayashi Y, Iinuma K.	Alteration in the cellular response to retinoic acid of a human acute promyelocytic leukemia cell line, UF-1, carrying a patient-derived mutant PML-RARalpha chimeric gene	Leukemia Research	28	959-967	2004
Lu L, Suzuki T, Yoshikawa Y, Murakami O, Miki Y, Moriya T, Bassett MH, Rainey WE, Hayashi Y, Sasano H.	Nur-related factor 1 and nerve growth factor-induced clone B in human adrenal cortex and its disorders	Journal of Clinical Endocrinology and Metabolism	89	4113-4118	2004
Oue T, Inoue M, Yoneda A, Kubota A, Okuyama H, Kawahara H, Nishikawa M, Nakayama M and Kawa K.	Profile of neuroblastoma detected by mass screening, resected after observation without treatment: Results of the Wait and See pilot study	Journal of Pediatric Surgery	40	359-363	2005
Kato C, Miyazaki K, Nakagawa A, Ohira M, Nakamura Y, Ozaki T, Imai T, Nakagawara A.	Low expression of human tubulin tyrosine ligase and suppressed tubulin tyrosination/detyrosination cycle are associated with impaired neuronal differentiation in neuroblastomas with poor prognosis	International Journal of Cancer	112	365-375	2004
Takahashi M, Ozaki T, Todo S, Nakagawara A.	Decreased expression of the candidate tumor suppressor gene ING1 is associated with poor prognosis in advanced neuroblastomas	Oncology Report	12	811-816	2004
Ando K, Ozaki T, Yamamoto H, Furuya K, Hosoda M, Hayashi S, Fukuzawa M, Nakagawara A.	Polo-like kinase 1 (Plk1) inhibits p53 function by physical interaction and phosphorylation	Journal of Biological Chemistry	279	25549-25561	2004
Wang YQ, Seimiya M, Kawamura K, Yu L, Ogi T, Takenaga K, Shishikura T, Nakagawara A, Sakiyama S, Tagawa M and O-Wang J.	Elevated expression of DNA polymerase K in human lung canceris associated with p53 inactivation: negative regulation of POLK promoter activity by p53	International Journal of Oncology	25	161-165	2004
Ohtori S, Isogai E, Hasue F, Ozaki T, Nakamura Y, Nakagawara A, Koseki H, Yuasa S, Hanaoka E, Shinbo J, Yamamoto T, Chiba H, Yamazaki M, Moriya H, Sakiyama S.	Reduced inflammatory pain in mice deficient in the differential screening-selected gene abrrative in neuroblastoma	Molecular and Cellular Neuroscience	25	504-514	2004
Hamano S, Ohira M, Isogai E, Nakada K, Nakagawara A.	Identification of novel human neuronal leucine-rich repeat (hNLRR) family genes and inverse association of expression of Nbla10449/hNLRR-1 and Nbla10677/hNLRR-3 with the prognosis of primary neuroblastomas	International Journal of Oncology	24	1457-1466	2004

Miyazaki K, Fujita T, Ozaki T, Kato C, Kurose Y, Sakamoto M, Kato S, Goto T, Itoyama Y, AokiM, Nakagawara A.	NEDL1, a novel ubiquitin-protein isopeptide ligase for Dishevelled-1, targets mutant superoxide dismutase-1	Journal of Biological Chemistry	279	11327-11335	2004
Nakagawara A, Ohira M.	Comprehensive genomics linking between neural development and cancer:Neuroblastoma as a model. In Special Issue: Neural development and cancer.	Cancer Letters	204	223-224	2004
Ohira M, Oba S, Nakamura Y, Isogai E, Kaneko S, Nakagawa A, Hirata T, Kubo H, Goto T,Yamada S, Yoshida Y, Fuchioka M, Ishii S, Nakagawara A.	Expression profiling using a tumor-specific cDNA microarray predicts the prognosis of intermediate-risk neuroblastomas	Cancer Cell	in press		
Ozaki T, Hosoda M, Miyazaki K, Hayashi S, Watanabe K, Nakagawa T, Nakagawara A.	Functionalimplication of p73 protein stability in neuronal cell survival and death	Cancer Letters	in press		
Lin L, Ozaki T, Takada Y, Kageyama H, Nakamura Y, Hata A, Zhang J-H, Simonds W, Nakagawara A, Koseki H.	Topors, a p53 and topoisomerase I-binding RING finger protein, is a co-activatorof p53 in growth suppression induced by DNA damage.	Oncogene	in press		
Ohira M, Oba S, Nakamura Y, Hirata T, Ishii S, Nakagawara A.	A review of DNA microarray analysis of human neuroblastomas	Cancer Letters	in press		
Abe M, Ohira M, Kaneda A, Yagi Y, Yamamoto S, Kitano Y, Takato T, Nakagawara A, Ushijima T.	CpG island methylator phenotype is a strong determinant of poor prognosis in neuroblastomas	Cancer Research	in press		
Kramer S, Ozaki T, Miyazaki K, Kato C, Hanamoto T, Nakagawara A.	Protein stability and function of p73 are modulated by a physical interaction with RanBPM in mammalian cultured cells	Oncogene	in press		
Yamakawa T, Toyabe S, Cao P, Akazawa K.	Web-based delivery of medical multimedia contents using an Motion Picture Experts Group (MPEG)-4 system	Computer methods and programs in biomedicine (Comput Methods Programs Biomed)	75	259-264	2004
Kaidu M, Toyabe S, Oda J, Okamoto K, Ozaki T, Shiina M, Sasai K, Akazawa K.	Development and evaluation of a teleradiology and videoconferencing system. J Telemed	Telecare	10	214-8	2004
Hasegawa T, Suzuki E, Koya T, Akazawa K, Sakagami T, Toyabe S, Koyanagi K, Kawano K, Haraguchi M, Toyama J, Arakawa M, Yoshizawa H, Satoh H, Gejyo F.	Analysis of Drug Compliance in Adult Patients with Bronchial Asthma	General Medicine	5	7-11	2004

Toyabe S, Cao P, Kurashima S, Nakayama Y, Ishii Y, Hosoyama N, Akazawa K.	Actual and estimated costs of disposable materials used during surgical procedures	Health Policy	in press		
Toyabe S, Nakamizo M, Uchiyama M, Akazawa K.	Circannual variation in the onset and relapse of steroid-sensitive nephrotic syndrome	Pediatric Nephrology	in press		
Arcana M, Ohtaki M.	Multi-target Models and their Application to Data Analysis	Hiroshima Journal of Medical Sciences	in press		
Hiyama K, Otani K, Ohtaki M, Satoh K, Kumazaki T, Takahashi T, Mitsui Y, Okazaki Y, Hayashizaki Y, Omatsu H, Noguchi T, Tanimoto K, Nishiyama M.	Differentially expressed genes throughout the cellular immortalization processes are quite different between normal fibroblasts and endothelial cells	International Journal of Oncology	in press		
檜山英三、金子道夫、家原知子.	抗がん剤使用ガイドライン：神経芽腫	がん治療学会誌	in press		
澤田淳、家原知子、松本良文、細井創、杉本徹.	神経芽腫マス・スクリーニング―過去と現在―日本のマススクリーニングが示したもの.	小児内科	36	1928-1932	2004
細井創、家原知子、松本良文、杉本徹、澤田淳.	神経芽細胞腫マス・スクリーニングの成果と問題点.	日本がん検診・診断学会誌	11	65-70	2004
井上雅美、大槻孝治、澤田明久、安井昌博、坂田尚己、窪田昭男、西川正則、森本静夫、桑江俊子、中山雅弘、河敬世.	難治性神経芽腫に対する造血幹細胞移植への工夫：移植1回法から2回法（自家－自家、自家－同種）へ.	日本小児がん学会雑誌	41	231-237	2004
田中丈夫、家原知子、細井 創、杉本 徹、水田祥代、澤田 淳.	生物学的指標によるマス・スクリーニング発見神経芽腫の腫瘍進展リスクの評価	小児がん	41巻1号	76-80	2004
杉本 徹、家原知子、細井創、澤田淳.	神経芽腫の早期発見・治療と子どものQOL：休止となった神経芽腫マス・スクリーニングの成果と問題点	京母衛誌	12巻	9-12	2004
福澤正洋.	小児悪性腫瘍の治療戦略	外科	66	428-435	2004
富所直子、高橋泰子、関口由紀子、瀬賀裕子、曹鳳宁、鳥谷部真一、赤澤宏平.	混合病棟と一般病棟の看護の質の差異を評価する.	看護管理	14(4)	303-307	2004
池田由紀子、上杉雅子、鳥谷部真一、吉村秀太郎、倉島幸子、赤澤宏平、瀬賀裕子.	心臓カテーテル検査における仙骨部圧迫予防効果の検討－透視台マットの改良－.	看護技術	51(2)	159-162	2005

厚生労働科学研究費補助金（子ども家庭総合研究事業）
神経芽細胞腫マススクリーニング研究

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書 籍 名	出版社名	出版地	出版年	ページ
Shay JW, Hiyama K, Hiyama E.	Telomerase and Lung Cancer	HI Pass, DP Carbone, DH Johnson, JD Minna, AT Turrisi III	Lung Cancer: Principles and Practice. 3rd ed. Vol.1	Lippincott Williams & Wilkins	Philadelphia, PA, USA	2005	160-171
Nakagawara A.	Neural crest development and neuroblastoma: the genetic and biological link.	Luigi Aloe and Laura Calza	NGF and Related Molecules in Health and Disease, Progress in Brain Research Vol. 146	Elsevier Science Publisher		2004	233-242
Nakagawara A.	Molecular and developmental biology of neuroblastoma.	S. Cohn & N-K. Cheung	Neuroblastoma	Springer-Verlag	Heidelberg	2005	in press
鳥谷部真一、赤澤宏平.	医療支援のためのデータ分析・評価	日本医療情報学会	医療情報（医療情報システム編）	篠原出版社	東京	2004	222-229
宮本正喜、近藤博史、河村徹郎、鳥谷部真一、赤澤宏平.	EBM（Evidence-based medicine）.	日本医療情報学会	医療情報（医療情報システム編）	篠原出版社	東京	2004	243-246
浜崎豊、秦順一、堀江弘、田中祐吉.	神経芽腫群腫瘍－国際分類INPCによる－	小児腫瘍組織分類委員会	小児腫瘍組織カラーアトラス	金原出版	東京	2004	1-83

厚生労働科学研究費補助金

（子ども家庭総合研究事業）

登録症例に基づく神経芽細胞腫マススクリーニングの効果判定と医療体制の確立

平成16年度研究報告書（2／2冊）

平成17年4月

主任研究者　檜山英三

厚生労働科学研究費補助金

(子ども家庭総合研究事業)

登録症例に基づく神経芽細胞腫マススクリーニングの効果判定と医療体制の確立

平成16年度研究報告書(2／2冊)

研究成果の刊行物・別刷

Pediatr Surg Int (2004) 20: 33–38
DOI 10.1007/s00383-003-1077-3

ORIGINAL ARTICLE

Eiso Hiyama · Keiko Hiyama · Hiroaki Yamaoka
Taijiro Sueda · C. Patrik Reynolds · Takashi Yokoyama

Expression profiling of favorable and unfavorable neuroblastomas

Published online: 23 December 2003

Abstract Neuroblastomas show remarkable biological heterogeneity, resulting in favorable or unfavorable outcomes. To survey the differences in gene expression profiles between favorable and unfavorable neuroblastomas, we analyzed ten favorable neuroblastoma samples from patients whose tumors consequently regressed or matured and ten unfavorable tumor samples from patients who consequently died of disease using the microarray technique. In each sample, total RNA was labeled with Cy3 or Cy5 in reverse-trancriptase reaction and hybridized with our original microarray prepared with a cDNA library of human fetal brain. Microarray analysis revealed that 43 genes, including *MYCN*, *hTERT*, *NME1* and cell cycle regulatory protein-coding genes, were highly expressed in unfavorable neuroblastomas, while another 80 genes were detected as highly expressed in favorable tumors, including neuronal differentiating genes and apoptotic inducing genes. Among favorable neuroblastoma samples, highly expressing genes in regressing tumors were different from those in maturing tumors. Expression profiling data revealed the existence of up-regulated and down-regulated gene clusters in favorable and unfavorable tumors. This cluster analysis is a powerful procedure to distinguish unfavorable tumors from favorable tumors as well as regressing tumors from maturing tumors among favorable tumors. The information obtained from expression profiling would clarify the key genes for cell growth, regression or maturation of neuroblastoma cells, and these genes will become diagnostic and therapeutic targets in human neuroblastoma in the future.

Keywords Neuroblastoma · Microarray · Outcome · Regression · Maturation

E. Hiyama (✉) · T. Yokoyama
Department of General Medicine,
Graduate School of Biomedical Sciences,
Hiroshima University, 1-2-3 Kasumi, Minami-ku,
734-8551 Hiroshima, Japan
E-mail: eiso@hiroshima-u.ac.jp
Tel.: +81-82-2575216
Fax: +81-82-2575219

K. Hiyama
Department of Translational Cancer Research,
Research Institute for Radiation Biology and Medicine,
Hiroshima University, Hiroshima, Japan

H. Yamaoka · T. Sueda
Department of Surgery,
Graduate School of Biomedical Sciences,
Hiroshima University, Hiroshima, Japan

C. P. Reynolds
Division of Hematology-Oncology,
Childrens' Hospital, Los Angeles, CA, USA

Introduction

Neuroblastomas show remarkable biological heterogeneity, resulting in favorable or unfavorable outcomes. Unfavorable tumors often have several genetic aberrations and grow aggressively, while favorable tumors regress or mature. To predict the malignant grade and the biological behavior of the individual tumor, several prognosis-predicting markers such as amplified *MYCN* gene, loss of heterozygosity (LOH) in the short arm of chromosome 1, expression levels of trk A and DNA ploidy have been proposed [1, 2]. However, each of these parameters appears to be insufficient to predict the outcome of each patient completely. Thus, massive efforts have been made to elucidate the genes affecting the mechanisms of tumor growth, regression or maturation. Recently, microarray techniques have been developed and extensively applied in the cancer field [3, 4, 5]. These technologies have emerged as indispensable research tools for gene expression profiling and detection of genetic aberrations. In the present study, to survey the differences in gene expression between unfavorable neuroblastomas and maturing/regressing neuroblastomas, we applied microarray techniques.

Table 1 Neuroblastoma cases. *m* month, *Ad* adrenal gland, *Ret* retroperitoneum, *UF* unfavorable type, *F* favorable type, *amp* amplification, *res* resection, *DOD* death of disease, *NED* no evidence of disease

Case	Age at diagnosis	Primary site	Stage[a] (INSS)	Histology[b]	*MYCN* amp (copy)	Surgical treatments before any other therapies	Outcome (period)
Unfavorable neuroblastoma							
N1	24 m	Ad	4	UF	40	Total res (primary)	DOD (13 m)
N3	72 m	Thorax	4	UF	1	Partial res (primary)	DOD (15 m)
N4	11 m	Ad	3	UF	80	Total res	DOD (9 m)
N7	72 m	Ad	4	UF	1	Total res (primary)	DOD (11 m)
N21	20 m	Ad	4	UF	25	Total res (primary)	DOD (14 m)
N29	9 m	Ad	4	UF	50	Partial res (primary)	DOD (11 m)
N51	31 m	Ad	4	UF	1	Biopsy	DOD (19 m)
N55	15 m	Ad	4	UF	200	Total res (primary)	DOD (2 m)
N66	15 m	Ad	4	UF	80	Biopsy	DOD (6 m)
N67	62 m	Ret	4	UF	1	Biopsy	DOD (27 m)
Favorable (maturing neuroblastoma)[c]							
N36	7 m	Ad	3	F	1	Partial res	NED (5 years)
N45	9 m	Thorax	3	F	1	Partial res	NED (12 years)
N57	10 m	Ret	3	F	1	Partial res	NED (11 years)
N141	13 m	Ret	2A	F	1	Partial res	NED (11 years)
N166	8 m	Thorax	2A	F	1	Partial res	NED (8 years)
N169	9 m	Thorax	3	F	1	Partial res	NED (10 years)
Favorable (regressing neuroblastoma)[d]							
N9	3 m	Ad	4S	F	1	Total res (primary)	NED (14 years)
N11	10 m	Ad	4S	F	1	Total res (primary)	NED (13 years)
N160	2 m	Ad	4S	F	1	Biopsy (liver meta)	NED (10 years)
N172	9 m	Ad	4S	F	1	Biopsy (liver meta)	NED (8 years)

[a]The disease staging was classified using INSS classification.
[b]Histology was classified according to Shimada's classification.
[c]In all six cases described as maturing neuroblastoma, maturation was confirmed by the pathological findings of biopsy samples, which were resected later.
[d]In all four cases described as regressing neuroblastoma, liver metastases spontaneously regressed until 5 years after diagnosis

Materials and methods

Tumor samples

During the past 2 decades, a total of 212 neuroblastoma cases whose tumors were obtained before any treatment were diagnosed at the Hiroshima University Medical Hospital or consulted for molecular analysis from other hospitals in Japan. Among these cases, ten were selected as unfavorable cases, with the patients dying of tumor progression (Table 1). In these tumors, six had *MYCN* gene amplification. In addition, we selected ten favorable tumors consisting of four regressing 4S tumor samples in which the remaining tumors consequently diminished and six maturing tumor samples obtained at biopsy or partial resection in which the remaining tumors consequently matured (Table 1). In the present study, we compared the data among these three groups: unfavorable tumors, regressing 4S tumors and maturing tumors.

Microarray analysis

We prepared target cDNA clones from a human fetal brain cDNA library. A set of 6,272 sequence-verified human fetal brain cDNA clones was PCR-amplified using M13 forward and reverse primers and spotted onto 24 poly L-lysine-coated glass slides using a custom robot arrayer (Genex 2000, Kaken Genetics, Chiba, Japan) according to the manufacturer's recommendations. Total cellular RNA was extracted from tumor samples by the acid-guanidium-phenol chloroform method. Ten micrograms of total RNA of each tumor sample was labeled with Cy3 or Cy5 and hybridized with the slide. Labeling was performed using random primer and oligo $d(T)_{18}$ primer by Cy 3- or Cy 5-labeled dUTP (Amersham Pharmacia Biotech Inc., Piscataway, N.J.). Hybridization was performed at 65°C for 10 h and then washed using Hybridization Station (Genomic Solutions, Ann Arbor, Mich.). After hybridization, slides were washed, dried and scanned using laser confocal scanner (Scan Array 5000, GSI Luminics, Ottawa, Ontario). This microarray analysis of the same sample was repeated using different fluorescence for validation of expression changes resulting from fluorescence and hybridization conditions. Hybridization intensity of each spot was normalized by the mean intensity of all non-control spots for each channel. In addition, the intensity of 16 ubiquitously expressed genes as control cDNA elements deposited at multiple locations on the microarray was examined. Quant array software was used for griding and calculation of signal intensities. The signals were analyzed from scatter plots and clustering using Software Quantarray (GSI Luminics) and GeneSpring (Silicon Genetics, Redwood City, Calif.).

TaqMan analysis

To confirm the difference of expression levels of the genes identified, we used fluorescent-based quantitative real-time RT-PCR using TaqMan probe. Each PT-PCR was performed in a 50-μl reaction mixture containing 25 μl of 2X TaqMan Universal RT-PCR master Mix (Applied Biosystems, Foster City, Calif.), 100–400 nM each primer and 200–400 nM TaqMan fluorogenic probe. Amplification reaction was carried out in a 96-well reaction plate (Applied Biosystems) in a spectrofluorimetric thermal cycler (ABI PRISM 7700 Sequence Detector, Applied Biosystems); after reverse transcription (30 min at 48°C) and denaturation (2 min at 95°C) amplification was performed with 40 cycles of 15 s at 95°C and 60 s at 60°C. Each sample was run in duplicate. A threshold cycle (Ct) for each sample was calculated by the point in which the fluorescence exceeded a threshold limit (tenfold the SD of the baseline) and the mean of the duplicate samples was used for calculation of the expression level. To normalize the samples for loading total RNA equivalent, the

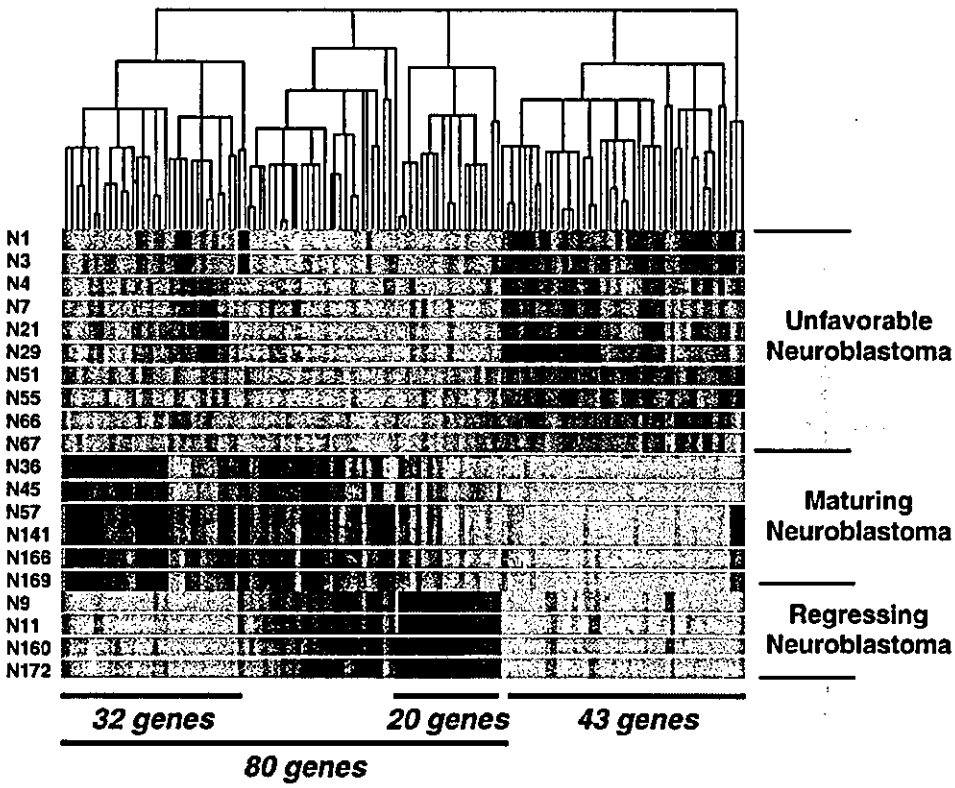

Fig. 1 A cluster analysis based on the expression of 123 genes that differ between favorable and unfavorable tumors. Unfavorable neuroblastomas showed overexpression of 43 genes and favorable tumors showed overexpression of another 80 genes. Among these 80 genes detected in favorable tumors, 32 and 20 genes were expressed in maturing tumors and regressing 4S tumors, respectively. The *dendrogram* indicates the degree of similarity of the expression profiles in these genes

second real-time PCR assay was performed targeting 18S ribosomal RNA gene. PCR conditions were the same as those mentioned above. A reference curve was made for each gene using a serial dilution of RNAs extracted from neuroblastoma cell line (SMS-SAN). Appropriate negative controls (distilled water and no probe/primers) were included in each set of experiments.

Statistical analysis

Comparison between the signal intensity of the microarray spot and the data of TaqMan analysis was calculated according to Spearman's rank correlation. A probability of less than 0.05 was accepted as a significant value.

Results

Array data

The total cellular RNA samples were labeled with Cy3 or Cy5, respectively, and two samples labeled by different fluorescence were hybridized onto the same slide. To make cut-offs in the analysis for the cDNA microarray, we calculated the mean intensity of each spot in the following two groups: unfavorable (UF, $n=10$) and favorable neuroblastoma (F, $n=10$). The following filter values were used: the value of the ratio (UF/F or F/UF) had to be greater than 2.0 and the signal intensity in at least one dye had to be greater than 10,000. In addition, we also calculated the mean intensity of each spot in regressing neuroblastoma (R, $n=4$) and maturing neuroblastoma (M, $n=6$) and compared with that in unfavorable tumors. If the value of the ratio (R/UF or M/UF) was greater than 2.0, this gene was analyzed further. Using these filters, cut-offs selected the number of clones from 6,727 to 517. The up-regulated clones in unfavorable or favorable tumors were 212 and 305, respectively. Among these 517 clones, we excluded unknown sequences and redundant sequences and obtained 123 previously known genes. Among these genes, 43 genes were overexpressed in the unfavorable tumors, and 80 genes were in the favorable tumors.

To analyze the correlation among the samples and genes, we applied a two-dimensional hierarchical clustering algorithm [6] using the data obtained from the 20 tumors (Fig. 1). Although no single gene could divide these tumors into three groups, we could divide these tumors into three groups using this clustering analysis. In unfavorable tumors, we identified 43 genes in this cluster including *CCND1*, *hTERT*, *NME1*, *MYCN* and other cell cycle regulatory genes (Table 2). In addition, we identified 20 genes including apoptosis-related genes such as *CASP 8* and *CASP 9* in regressing tumors and 32 genes including *MMP9* and neuronal growth-relating genes such as *NTRK1* and *BIRC1* in maturing tumors.

To confirm the difference of expression levels of the genes identified by microarray we examined TaqMan quantitative real-time RT-PCR. The quantitative RT-PCR with TaqMan probe for *hTERT* (human telomerase reverse transcriptase) mRNA expression showed

Table 2 Genes overexpressed in each group of tumors

Unfavorable neuroblastoma	Favorable neuroblastoma
Bone morphogenetic protein 6	(Maturing neuroblstoma)
Bone morphogenetic protein 7	Aldolase A, fructose-bisphosphate
P-cadherin	Ankyrin 1 (*ANK1*)
Caveolin 2	BCL2-antagonist/killer 1
CD22 antigen	CD44 antigen
CD8 antigen polypeptide (p32)	CD27-binding (Siva) protein
Collagen, type IV	CD8 antigen, beta polypeptide 1 (p37)
Cyclin D1(*CCND1*)	Cell division cycle 25A
Cyclin E1 (*CCNE1*)	Chorionic gonadotropin, beta polypeptide
Cyclin-dependent kinase 4	Cytochrome P450, subfamily IIIA
Deoxythymidylate kinase	Early growth response 1
Developmentally regulated GTP-general transcription factor IIB	Fas-activated serine/threonine kinase
High-mobility group protein 1	FGF receptor 1
Apoptosis inhibitor 4 (*BIRC5*)	General transcription factor IIH
Human telomerase reverse transcriptase (hTERT)	Hexokinase 1
Integrin-β	IGF 2 (somatomedin A)
Interferon-γ	Insulin-like growth factor binding protein 2
Interleukin 1α	Interferon receptor 1
Interleukin 8	Interleukin 1 receptor antagonist
Keratin 18	Keratin 7
Matrix metalloproteinase 15	Matrix metalloproteinase 9 (*MMP9*)
Metastasis associated 1	Mitogen-activated protein kinase 7
Mitogen-activated protein kinase 10	NGF receptor (*NTRK1*)
NM23A (*NME1*)	Neural precursor cell expressed, developmentally down-regulated 4
NM23B (*NME2*)	NGFI-A binding protein 2
Neuronal apoptosis inhibitory protein (*BIRC1*)	p53-induced protein
Prefoldin 5	Profilin 1
Protease, inhibitor, serine, clade A	Protein tyrosine kinase 2 beta
Putative chemokine receptor GTP-binding protein	Rho GDP dissociation inhibitor (GDI) α
Ras homolog enriched in brain 2	Rho GTPase activating protein 1
Rho GTPase activating protein 4	Thrombospondin 4
Rho-GEF 1	TNF receptor-associated factor 5
Serine (or cysteine) proteinase inhibitor	Tubulin, alpha, brain-specific
Signal transducer and activator of transcription 1, 91 kD	Vascular endothelial growth factor
Small inducible cytokine B subfamily member 13	
Superoxide dismutase 1, soluble	
Transforming growth factor-α	(Regressing neruoblastoma)
TNF superfamily, member 2	Ancient ubiquitous protein 1
TNF receptor superfamily, member 6	BCL2-antagonist/killer 1
Tyrosine kinase with immunoglobulin	Bone morphogenetic protein receptor; type II (serine/threonine kinase)
Epidermal growth factor homology domains	Caspase 8 (*CASP8*)
Vascular endothelial growth factor C	Caspase 9 (*CASP9*)
N-myc (*MYCN*)	Collagen, type VII, alpha 1
Ras-related GTP binding protein	Contactin 2 (axonal)
	E74-like factor 4 (ets domain transcription factor)
	Galactosidase, beta 1
	Growth differentiation factor 10 (*GDF10*)
	High-mobility group (nonhistone chromosomal) protein 1
	Human DNA sequence from clone RP11–560A15 on chr 20
	Mitogen-activated protein kinase
	NGF-α (*NGFA*)
	Protease, serine, 22
	Rho GTPase activating protein 4
	TNF-related apoptosis inducing legand (*TNFSF10*)
	Transferrin receptor
	Transforming growth factor; beta 1
	Tubulin, alpha, brain-specific

that the levels of *hTERT* mRNA expression were correlated with the signal intensity obtained in the microarray (Fig. 2). We also used TaqMan analysis in 15 genes among the genes that showed different expression levels in the microarray analysis and obtained a good correlation of the expression levels between microarray analysis and TaqMan assay (data not shown).

Discussion

In the present study, we successfully identified 43 genes that were overexpressed in unfavorable tumors and 80 genes specifically overexpressed in favorable tumors (Fig. 1). The feasibility of this approach was shown in

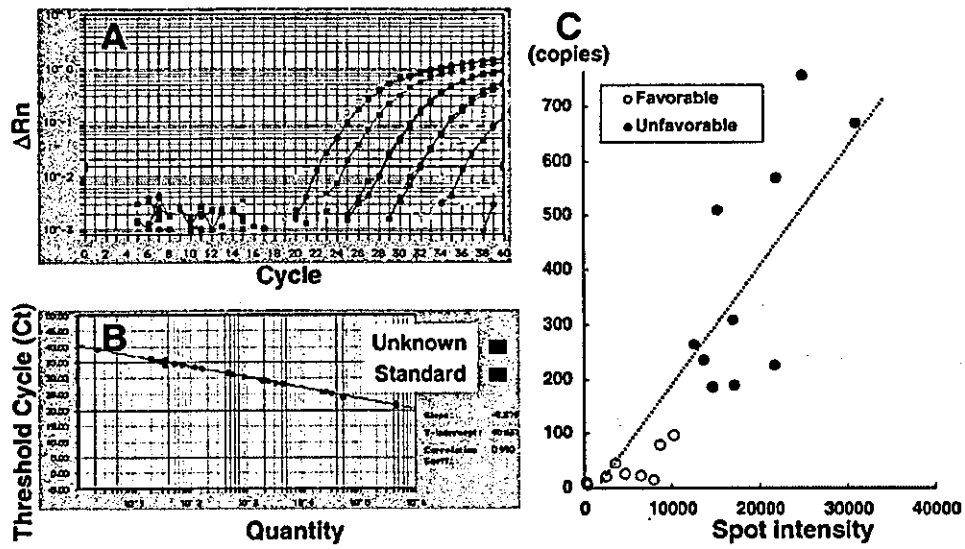

Fig. 2 Validation of *hTERT* mRNA expression levels in each tumor using real-time quantitative RT-PCR. A Representative *amplification plots* analyzed using the fluorescence of the PCR products. We prepared five serial diluted standard templates of *hTERT* (human telomerase reverse transcriptase) transcript. B *Linearity plots* of quantity and threshold cycle (*Ct*). This assay revealed a high correlation co-efficiency (0.990). Quantification of unknown samples was performed using the equation defined by this correlation. C Correlation between microarray spot signal and calculated copy number by TaqMan of *hTERT* mRNA in each sample. The levels of *hTERT* mRNA expression by these two different assays were correlated significantly ($\gamma^2 = 0.77$, $P < 0.0001$)

the finding that several genes such as *MYCN*, *BIRC5* and *hTERT*, which are well known to be highly expressed in aggressive neuroblastoma, were included in the 43 genes overexpressed in the unfavorable tumors. In addition, *NTRKI* was also included in the genes overexpressed in the favorable tumors. Real-time RT-PCR analysis revealed that our microarray analysis is available to estimate the expression levels of each gene.

In unfavorable tumors, the overexpressed genes involved cell-cycle regulation (*CCND1*, *CCNE1*, *E1*) apoptosis-escape (*BIRC5*, *BIRC1*), protein synthesis and transcription factors (Table 2). Moreover, overexpression of adhesion molecules such as P-cadherin, integrin-ß and matrix metalloproteinase might suggest the infiltration capacity of tumor cells [7]. Bone morphogenic proteins might correlate with a block of neuronal differentiation [8]. The expression of *NME1* genes, which locates at 17q, was up-regulated as the downstream signal of N-myc [9]. Overexpression of *hTERT* was considered to represent activation of telomerase in aggressive neuroblastoma [10, 11]. Approximately half of these selected genes have had their functions identified, but the remaining genes have not. Precise identification of the function of these genes will elucidate their novel functions and signal pathways in neuroblastoma. Identification of aberrant pathways and signal networks could lead to new therapeutic strategies in unfavorable neuroblastoma.

On the other hand, in favorable neuroblastoma, neuronal differentiation signals such as *CD44*, *IGF2*, *NTRK1* and *ANK1* were overexpressed in maturing tumors [12]. In regressing tumors, apoptosis-inducing signals (*CASP8*, *CASP 9*, *TNFSF10*) were overexpressed. The overexpression of these genes was compatible with the biological behaviors of these tumors. In the maturing tumors, apoptosis-inducing signals such as Fas-activated kinase and p53-induced protein were also expressed. In addition, the regressing tumors showed overexpression of differentiating factors (*NGFA* and *GDF10*). These results suggested that maturation or regression of favorable neuroblastoma depends on the balance of differentiating signals and apoptosis-inducing signals.

In the clustering analysis, three groups of tumors, unfavorable, regressing and maturing, were well classified. Since no single gene could completely classify these three groups, the clustering analysis using several number of key genes is necessary. What kinds of genes and how many key genes should be analyzed in future studies need to be clarified.

The detected genes in the present study, including cell-cycle regulatory, apoptosis-escape, protein synthesis and transcription factor genes as highly expressed in unfavorable tumors, would be candidates for new prognosis-predicting factors to decide therapeutic regimens and for new therapeutic targets in aggressive neuroblastoma. The detected genes including neuronal differentiation and apoptosis signals in favorable tumors would be the candidates for new regression/maturation-predicting factors and for new targets in differentiation-inducing therapy in neuroblastoma. Expression profiling is a powerful procedure to distinguish unfavorable tumors from favorable tumors in addition to regressing tumors from maturing tumors among favorable tumors. Genes whose expression correlated with outcome should be useful in risk assessment and as potential therapeutic targets in neuroblastoma. Prompt estimation of microarray data is required to

diagnose tumor biology of individual tumors and to enable appropriate therapy to be performed in each patient.

Acknowledgements This research was partially supported by a grant-in-aid for scientific research (A) (no. 13307050 and 15209058) from the Ministry of Education, Culture, Sports, Science and Technology and for Cancer Research (13-20) from the Ministry of Health, Labor and Welfare of the Government of Japan.

References

1. Westermann F, Schwab M (2002) Genetic parameters of neuroblastomas. Cancer Lett 184:127–147
2. Brodeur GM (2003) Neuroblastoma: biological insights into a clinical enigma. Nat Rev Cancer 3:203–216
3. Schena M, Shalon D, Davis RW, et al (1995) Quantitative monitoring of gene expression patterns with a complementary DNA microarray. Science 270:467–470
4. Osin P, Shipley J, Lu YJ, et al (1998) Experimental pathology and breast cancer genetics: new technologies. Recent Results Cancer Res 152:35–48
5. Hiyama E, Hiyama K (2002) Clinical application of microarrays as a tool of understanding cancer biology. In: Pandalai SG (ed) Recent research developments in cancer. Transworld Research Network, Kerala, India 4, pp 675–692
6. Zhang K, Zhao H (2000) Assessing reliability of gene clusters from gene expression data. Funct Integr Genomics 1:156–173
7. Gluer S, Zense M, von Schweinitz D (1998) Cell adhesion molecules and intermediate filaments on embryonal childhood tumors. Pathol Res Pract 194:773–780
8. Gomez-Santos C, Ambrosio S, Ventura F, et al (2002) TGF-beta1 increases tyrosine hydroxylase expression by a mechanism blocked by BMP-2 in human neuroblastoma SH-SY5Y cells. Brain Res 958:152–160
9. Godfried MB, Veenstra M, Sluis P, et al (2002) The N-myc and c-myc downstream pathways include the chromosome 17q genes nm23-H1 and nm23-H2. Oncogene 21:2097–2101
10. Hiyama E, Hiyama K, Yokoyama T, et al (1995) Correlating telomerase activity levels with human neuroblastoma outcomes. Nature Med 1:249–255
11. Hiyama E, Hiyama K, Ohtsu K, et al (1997) Telomerase activity in neuroblastoma: is it a prognostic indicator of clinical behavior? Eur J Cancer 33:1932–1936
12. Hoehner JC, Hedborg F, Eriksson L, et al (1998) Developmental gene expression of sympathetic nervous system tumors reflects their histogenesis. Lab Invest 78:29–45
13. Kushner BH, Cheung NK, LaQuaglia MP, et al (1996) International neuroblastoma staging system stage 1 neuroblastoma: a prospective study and literature review. J Clin Oncol 14:2174–2180
14. Shimada H, Chatten J, Newton WJ, et al (1984) Histopathologic prognostic factors in neuroblastic tumors: definition of subtypes of ganglioneuroblastoma and an age-linked classification of neuroblastomas. J Natl Cancer Inst 73:405–416

British Journal of Cancer (2004) 91, 972–979

www.bjcancer.com

High expression of telomerase is an independent prognostic indicator of poor outcome in hepatoblastoma

E Hiyama[*,1,2,3], H Yamaoka[2], T Matsunaga[3], Y Hayashi[3], H Ando[3], S Suita[3], H Horie[3], M Kaneko[3], F Sasaki[3], K Hashizume[3], A Nakagawara[3], N Ohnuma[3] and T Yokoyama[2]

[1]Natural Science Center for Basic Research and Development, Hiroshima University, Hiroshima, Japan; [2]Department of Surgery, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan; [3]Japanese Study Group for Pediatric Liver Tumor, Japan

Telomerase, an enzyme related with cellular immortality, has been extensively studied in many kinds of malignant tumours for clinical diagnostic or prognostic utilities. Telomerase activity is mainly regulated by the expression of hTERT (human telomerase reverse transcriptase), which is a catalytic component of human telomerase. To evaluate whether the levels of *hTERT* mRNA provides a molecular marker of hepatoblastoma malignancy, we examined *hTERT* mRNA expression levels in the primary hepatoblastoma tissues by fluorescent RT–PCR using LightCycler technology and followed up the clinical outcomes in 63 patients listed in the Japanese Study Group of Pediatric Liver Tumor between 1991 and 2002. The *hTERT* mRNA expression was detected in 61 (96.8%) specimens and their expression levels ranged between 0.1/1000 and 745.1/1000 copies of PBGD gene that was used as an internal control. Among these cases, frozen 39 tumour samples and 14 adjacent noncancerous liver tissues were analysed for semiquantitative telomerase assay. In the 39 tumour samples, the levels of telomerase activity ranged between 0.11 and 2709 TPG and 12 (30.7%) had high telomerase activity (>100 TPG), whereas only nine of 14 noncancerous liver tissue samples showed telomerase activity which was less than 1.0 TPG. The levels of telomerase activity were significantly correlated with the levels of *hTERT* mRNA expression ($P<0.001$). The frequency of high *hTERT* mRNA expression and/or high telomerase activity did not significantly associate with the clinicopathological factors except for stage of disease. The prognosis of the patients with high *hTERT* mRNA expression was significantly worse than that of others ($P<0.01$), as was the patients with high telomerase activity ($P<0.01$). Multivariate analysis indicated that high levels of *hTERT* mRNA expression as well as telomerase activity are independent prognosis-predicting factors in patients with hepatoblastoma.

British Journal of Cancer (2004) **91**, 972–979. doi:10.1038/sj.bjc.6602054 www.bjcancer.com
Published online 27 July 2004

Keywords: human telomerase reverse transcriptase; telomerase; hepatoblastoma; prognosis; indicator; LightCycler

Hepatoblastoma is one of the common paediatric tumours and more than 70% of the tumours are diagnosed in children less than 2 years old (Weinberg and Finegold, 1983). This tumour, which is derived from hepatic precursor cells, is morphologically similar to immature hepatocytes and the prognosis of the patients is various. In the previous reports, tumour distribution, stage of tumour, and complete tumour resection were proposed to be the prognostic indicators in hepatoblastoma (Brown *et al*, 2000; Fuchs *et al*, 2002). The prognosis of children with hepatoblastoma has been improved significantly during the past two decades. Several multicentric trials such as the International Society of Pediatric Oncology (SIOP), United States-Intergroup, and our JPLT (the Japanese Study Group for Pediatric Liver Tumors) group studies, revealed that the successful reduction of large hepatoblastoma tumours by preoperative chemotherapy and complete resection are possible in many patients. In other instances, some tumours grow aggressively regardless of the use of preoperative chemotherapy. The latter tumours are considered to have high-grade malignancy. In advanced tumours with a low malignant grade, standard chemotherapeutic regimens are effective to reduce the primary tumour and to diminish metastatic tumours, resulting in patients' long survival, while new aggressive chemotherapy such as high-dose chemotherapy with stem cell transplantation is needed to cure the tumours with a high-grade malignancy (Nishimura *et al*, 2002). Thus, evaluation of the malignant grade of hepatoblastoma is necessary to improve the outcome of patients with advanced hepatoblastoma. Several molecular markers have been analysed to identify hepatoblastomas with high malignant potential: loss of heterozygosity (LOH) of chromosome 11p15.5, which is often affected in nephroblastoma and rhabdomyosarcoma in children, may contain a putative tumour suppressor gene for hepatoblastoma (Albrecht *et al*, 1994), but is unlikely to be a prognostic marker (Samuel *et al*, 1999; von Schweinitz *et al*, 2002). The mutation or deletion of the β-catenin gene exon 3 is frequently detected in hepatoblastoma, suggesting overactivation of the wingless/WNT signal pathway (Koch *et al*, 1999). This plays an important role in the pathogenesis of hepatoblastoma, but is not

*Correspondence: E Hiyama, Natural Science Center for Basic Research and Development, Hiroshima University, Hiroshima 1-2-3, Kasumi, Minami-ku, Hiroshima 734-8551, Japan; E-mail: eiso@hiroshima-u.ac.jp
Received 26 January 2004; revised 25 May 2004; accepted 7 June 2004; published online 27 July 2004

considered to be a good molecular marker to distinguish high-risk tumours from others (Takayasu *et al*, 2001; von Schweinitz *et al*, 2002).

In Japan, JPLT was opened to enrollment in 1991 and more than 150 patients were treated by JPLT protocols (Sasaki *et al*, 2002). The event-free survival (EFS) rate of patients with advanced stages was under 50%. Except for stage of disease, there are few markers to predict the prognosis of patients or to evaluate the malignant grade of hepatoblastoma. Elucidation of the useful prognosis-predicting factors is necessary to improve the prognosis of patients with hepatoblastoma.

Telomeres, which are specialised structures containing unique guanine-rich hexameric repeat sequences at the ends of human chromosomes (Blackburn, 1991), cannot be completely synthesised (referred to as the end-replication problem) with each cell division (Watson, 1972) and it is proposed that the loss of telomere eventually induces antiproliferative signals that result in cellular senescence (Shay, 1995). Telomerase is activated to maintain telomere length to compensate for the end-replication problem in germlines and immortal cells, but repressed in almost all human somatic cells. The activation of telomerase and the stabilisation of telomeres appear to be concomitant with the attainment of immortality in cancer cells (Harley *et al*, 1994; Kim *et al*, 1994; Shay, 1995). Telomerase activity has been found in approximately 85% of the cancer tissues examined, covering a large variety of cancer types including neuroblastoma, Wilms' tumour, and retinoblastoma (Hiyama *et al*, 1995a; Gupta *et al*, 1996; Dome *et al*, 1999; Hiyama and Hiyama, 2002). In some kinds of tumours, in which telomerase activity increases according to tumour progression, such as in neuroblastoma, non-small lung carcinoma, and colorectal cancer, the level of telomerase activity is a useful prognostic marker of the patients (Tatsumoto *et al*, 2000; Hiyama and Hiyama, 2002, 2003). Major components of telomerase are the RNA template (human telomerase RNA component: hTR) and the catalytic subunit (human telomerase reverse transcriptase: hTERT). hTR is expressed in the tissues with or without telomerase activity and is not correlated with the detection of telomerase activity, while hTERT expression is correlated with the detection of telomerase activity (Naito *et al*, 2001; Hiyama and Hiyama, 2002). Although *hTERT* transcripts show several splicing variants which have no telomerase activity (Wick *et al*, 1999), a system to detect full-length-*hTERT* mRNA alone has been developed.

To evaluate whether the levels of *hTERT* mRNA provides a molecular marker of hepatoblastoma malignancy, in the present study, we examined *hTERT* mRNA expression with this system and telomerase activity in hepatoblastoma specimens and compared the levels of their expression and the clinicopathological features and outcomes of the patients.

MATERIALS AND METHODS

Tissue samples

Hepatoblastoma tissue samples were obtained at surgery, immediately frozen, and stored at −80°C in the Tissue Bank of the JPLT or in the Hiroshima University Medical Hospital. In all, 63 tumours having total RNA samples available were enrolled in this study. The patients with these tumours were treated in the various hospitals or institutes under the framework of the JPLT between 1991 and 2001. Most patients were treated in the JPLT-1 study (Sasaki *et al*, 2002), which consisted of two different protocols: protocol 91A for patients with stage I or II hepatoblastoma and protocol 91B for patients with stage III or IV tumours. In these cases, 39 tumour samples with 14 corresponding normal liver tissues were stored at −80°C as frozen tissues and the remaining 24 samples were stored as total RNA samples.

Clinical course and disease status

The clinicopathological parameters and outcomes for these 63 patients were analysed. The clinical stages of disease were determined at the time of initial biopsy or resection according to the classification of the Japanese Society of Pediatric Surgeons, which was based on the number of liver segments involved, the extent of local invasion, the extent of regional lymph node involvement, and the presence of distant metastases (Hata, 1990). The PRETEXT system (intrahepatic tumour extension) is based on hepatic surgical anatomy which is divided into four sectors, namely anterior and posterior sectors on the right and medial and lateral sectors on the left (Brown *et al*, 2000). Histological subtypes were diagnosed according to the classification of Haas *et al* and the Japanese Society of Pathology (Haas *et al*, 1989; Hata, 1990). Their criteria classified the tumours into four subtypes: a well-differentiated (fetal), a poorly differentiated (embryonal), immature (anaplastic) and other (including macrotrabecular pattern) types.

Quantification of telomerase activity

Extraction of telomerase protein and evaluation of its activity were done by the TRAP (telomeric repeat amplification protocol) assay as described earlier (Kim *et al*, 1994; Piatyszek *et al*, 1995). Briefly, 50–100 mg of tumour or noncancerous liver tissues were homogenised in approximately 50–100 μl of CHAPS lysis buffer. After 25 min of incubation on ice, the lysates were centrifuged at 16 000 g for 20 min at 4°C and the supernatant was rapidly frozen in liquid nitrogen and stored at −80°C. An aliquot of extract containing 0.5 μg of protein was used for each assay. The levels of telomerase activity was measured using a commercial kit, the TRAPeze XL kit (Serological Co., Gaithersburg, MD, USA), which is a quantitative fluorescent-labelled PCR system for the estimation of relative telomerase activity with the use of a PCR internal control. The PCR product was measured in the fluorescent plate reader (Wallac, Perkin-Elmer, Wellesley, MA, USA) to detect the levels of fluorescein and sulphorhodamine by using the appropriate excitation and emission filters. The levels of telomerase activity were quantified by the ratio of the fluorescein intensity of the entire TRAP ladder to the sulphorhodamine intensity of the internal control after the correction of each fluorescent intensity for the negative control and the background, respectively, and were expressed as Total Product Generated (TPG) units.

Quantification of *hTERT* mRNA expression

Using the acid-guanidium-phenol-chloroform method (Chromzynski and Sacchi, 1987), total cellular RNA was extracted. Quantitative detection of *hTERT* mRNA was performed with the LightCycler TeloTAGGG hTERT Quantification Kit (Roche Diagnostics, Mannheim, Germany) using the LightCycler instrument (Roche Molecular Systems, Alameda, CA, USA). For each sample, 100 ng of total RNA was prepared in a 20 μl mixture containing 2 μl of reaction mix, 0.1 μl of reverse-transcriptase, and 2 μl of hTERT or porphobilinogen deaminase (PBGD) detection mix. RT-PCR for the mRNA encoding the housekeeping gene *PBGD* was equally processed in a separate tube as a reference for relative quantification of *hTERT* mRNA expression. The mixture without template was examined as the negative control. These mixtures were reverse-transcribed for 10 min at 60°C, followed by denaturation (30 s at 95°C) and amplification of the 198-bp fragment of the *hTERT* mRNA sequence in 40 PCR cycles (0.5 s at 95°C, 10 s at 60°C, and 10 s at 72°C) using specific primers in a one-step RT-PCR. To establish a standard curve, five standards with *in vitro*-transcribed *hTERT* mRNA containing five different copy numbers were included in each experiment. The copy number of *hTERT* mRNA in each sample was normalised on the basis of its PBGD

mRNA content according to the formula: *hTERT* mRNA expression level = *hTERT* mRNA copies/1000 PBGD mRNA copies.

Statistical analysis

Correlations between the *hTERT* mRNA expression and telomerase activity levels or each of the other factors were analysed using Wilcoxon's *t*-test, χ^2-test, or Fisher's exact test where appropriate. The overall survival curves for each group of patients were estimated by the Kaplan–Meier method and the resulting curves were compared using the Cox–Mantel test. Multivariate survival analysis using the Cox proportional hazard regression model was carried out to assess the independent contribution of each variable to disease-free survival. Differences were considered significant at $P<0.05$. A Computer program package (StatView 5.0; Abacus Concepts, Berkeley, CA, USA) was used for all of the statistical testing.

RESULTS

Clinicopathological findings (Table 1)

Among the 63 patients studied, the ages at diagnosis ranged between 0 and 13 years (mean 3 years and 6 months). They included nine stage I cases, 17 stage II, 13 stage IIIA, 10 stage IIIB, and 14 stage IV cases. Overall, 39 (61.9%) cases underwent curative surgery. Surgical resection was considered curative when no distant metastasis was evident and the clearance of cancer was complete as determined by standard histological analysis. The remaining 24 cases underwent noncurative surgery due to distant metastasis or extensive occupation of primary tumour. Totally, 34 cases underwent preoperative chemotherapy and all cases underwent postoperative chemotherapy.

In histological classification according to the pathological criteria of the Japanese Society of Pathology, 33 were classified as the well-differentiated type, 27 as the poorly differentiated type, two as immature and the remaining one case as other types. Serum levels of alpha-fetoprotein (AFP) ranged between 5 and 3 657 247 ng ml^{-1} and 56 cases showed more than 1000 ng ml^{-1} of AFP.

Among these patients, 11 died of disease, two showed recurrence of tumour and 50 are alive disease free. The survival periods ranged from 0 to 288 months (mean 74 months).

Out of 39 cases whose frozen tumour samples were available included six stage I cases, 13 stage II, five stage IIIA, eight stage IIIB, and seven stage IV cases. Among them 30 (75.9%) cases underwent curative surgery. Clinicopathological findings in these 39 cases were not significantly different from those in the whole cases (Table 1).

Levels of *hTERT* mRNA expression and telomerase activity in hepatoblastoma specimens

Among the 63 primary hepatoblastoma specimens obtained, 58 (92%) specimens displayed apparent *hTERT* mRNA expression using the quantitative *hTERT* mRNA expression assay (Figure 1A–C). The levels of *hTERT* mRNA expression ranged from 0.008 to 745.1 (mean 49.5) copies 1000 copies^{-1} of the *PBDG* mRNA. In these 58 cases, 24 (38.1%) showed high levels of *hTERT* mRNA expression (more than 10 *hTERT* mRNA copies 1000 copies^{-1} of the *PBDG* mRNA). In the 14 noncancerous liver specimens examined, only two samples derived from two patients under 1-year old showed *hTERT* mRNA expression, but their levels were low (0.42 and 0.78). Among these cases, telomerase activity was examined in 39 cases. Using quantitative TRAP assay (Figure 1D), telomerase activity ranged between 0.11 and 2669 TPG (mean 432.7 TPG). As previously described (Tatsumoto *et al*, 2000), more than 100 TPG was defined as high telomerase activity. Overall, 12 cases (30.8%) showed high telomerase activity. Figure 1E shows the correlation between *hTERT* mRNA expression levels and telomerase activity levels. There was a significant correlation between these two expression levels ($\gamma = 0.87$, $P<0.01$).

Table 1 Patients and tumour characteristics

	(Cases)	*hTERT* mRNA (copies)	(Cases)	Telomerase activity (TPG)
Sex				
Male	44	61.74±130.94	27	445.8±792.5
Female	19	37.48±90.80	12	403.4±809.6
Age				
0–11 months	12	27.28±62.94	7	458.1±990.0
12–23 months	18	57.85±97.72	12	722.3±1214.3
2–3 years	20	21.14±48.34	10	122.8±269.1
4–14 years	13	130.32±210.93	10	687.5±689.8
PRETEXT				
I	6	2.56±4.88	5	101.5±207.3
II	22	56.02±160.07	15	184.4±514.1
III	20	62.84±90.01	8	624.4±998.1
IV	11	65.55±106.82	9	579.7±1045.6
Unknown	4	25.76±50.09	2	630.1±890.6
Stage				
I	9	1.83±4.03	6	86.4±189.0
II	17	17.49±46.34	13	174.1±545.8
IIIA	13	39.63±61.47	5	333.1±706.7
IIIB	10	105.50±134.93	8	915.0±1072.0
IV	14	104.41±197.70	7	729.9±969.6
Histology				
Well	33	58.63±138.63	20	574.5±878.2
Poorly	27	51.21±96.00	17	312.8±704.3
Others	3	3.57±3.37	2	34.1±8.37
Preoperative chemotherapy				
Yes	34	63.61±149.14	19	486.6±802.7
No	29	40.18±65.60	20	381.6±789.7
Curative surgery				
Yes	39	56.91±130.29	30	397.4±758.8
No	24	46.19±96.98	9	550.3±914.6
Prognosis				
Survived with evidence-free	50	33.25±72.11	30	252.7±604.2
Recurrence/died of disease	13	128.11±207.25	9	1032.8±1046.0

Levels of *hTERT* mRNA expression or telomerase activity and the clinicopathological features of the patients

Table 1 shows the correlation between *hTERT* mRNA expression or telomerase activity levels and the clinicopathological features of the patients. Regarding age at diagnosis, the levels of *hTERT* mRNA expression and of telomerase activity were high in the elder patients, but not significantly. In histological classification, there was no significant difference of the levels of *hTERT* mRNA expression or telomerase activity between well- and poorly differentiated types. In PRETEXT classification, the levels of *hTERT* mRNA expression increased in PRETEXT 2, 3, and 4 tumours but not significantly ($P=0.116$). The levels of telomerase activity in the PRETEXT 2, 3, and 4 tumours were significantly higher than in the PRETEXT 1 tumours ($P=0.025$). The levels of *hTERT* mRNA expression and telomerase activity significantly increased in advanced stages (stages IIIA, IIIB, and IV, $P=0.0146$

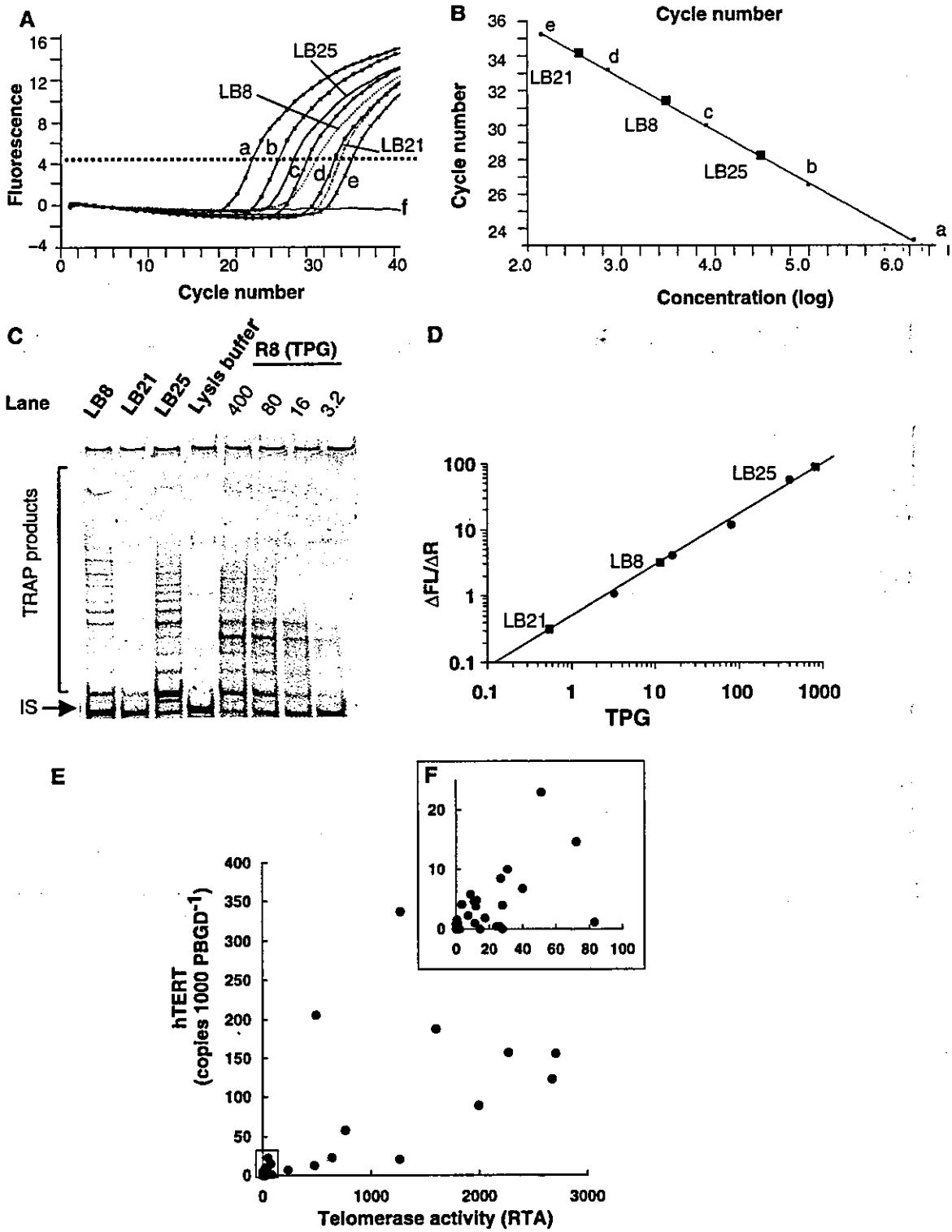

Figure 1 Detection of *hTERT* mRNA (**A**, **B**) and telomerase activity (**C**, **D**) and their relationship (**E**, **F**) in hepatoblastoma. (**A**) Amount of *hTERT* mRNA was measured by real-time RT–PCR analysis using LightCycler system in three representative hepatoblastoma samples (LB8, 21, and 25) with five external *hTERT* mRNA standards (a–e) and a negative control (f). (**B**) *hTERT* mRNA levels of three representative samples were calculated by the standard curve of the external *hTERT* RNA standards (a–e). (**C**) Detection of telomerase activity was done using the TRAPeze XL kit (Serological Co., Gaithersburg, MD, USA), which is a quantitative fluorescence-labelled PCR system for the estimation of relative telomerase activity with the use of a PCR internal control (IS). Positive controls included serial diluted control template (R8), oligonucleotides with eight telomeric repeats $AG(GGTTAG)_7$, to produce a standard curve. (**D**) The levels of telomerase activity were quantified by the ratio of the fluorescein intensity (ΔFL) of the entire TRAP ladder to the sulphorhodamine intensity (ΔR) of the internal control, and were expressed as Total Product Generated units (TPG). Levels of telomerase activity in the three representative samples (LB8, 21, and 25) were calculated by the standard curve using ΔFL/ΔR of the external standard R8. The levels of telomerase activity in LB8, LB21, and LB25 were calculated as 31.1, 0.37, and 761.7 TPG, respectively. (**E**, **F**) The correlation between the levels of *hTERT* mRNA expression normalised to the internal control PBDG and those of telomerase activity in overall 39 hepatoblastoma samples (**E**) and those with low telomerase activity (**F**). There is a significant correlation between these two parameters ($P<0.0001$).

and 0.0234, respectively) and in tumours with distant metastasis (stage IV *vs* others), but not significantly. The levels of *hTERT* mRNA expression (mean 49.9, $n=22$) and telomerase activity (mean 369.1 TPG, $n=14$) in tumours obtained after preoperative chemotherapy did not significantly differ from those in tumours obtained without any therapies (mean 54.4, $n=42$ and mean 435.4, TPG, $n=25$, respectively). There was no significant correlation between the levels of serum AFP and *hTERT* mRNA expression or telomerase activity.

Correlation between *hTERT* mRNA expression and prognosis of the patients

The median follow-up in the series of patients examined was 74 months (range, 1–288 months). Kaplan–Meier event-free survival (EFS) curves of all patients (Figure 2A) show that the 10-year EFS rate in the patients with high *hTERT* mRNA expression ($\geqslant 10$ copies 1000 copies^{-1} of the *PBDG* gene) was 38%, while that in the remaining patients was approximately 90%. The prognosis of the patients with high expression of *hTERT* was significantly worse than that of other patients ($\chi^2=23.40$, $P<0.0001$). Since the levels of *hTERT* mRNA were significantly correlated with advanced stages of tumour, the correlation between *hTERT* mRNA expression and prognosis was examined in tumours in early stages (stage I or II), and those in advanced stages (stage III or IV), separately (Figure 2B). The prognosis of the patients with high levels of *hTERT* mRNA expression was significantly poor in advanced tumours ($\chi^2=26.03$, $P<0.0001$). In 26 patients with early tumours, all 20 patients with low levels of *hTERT* mRNA expression are alive disease free and two out of six patients with high levels of *hTERT* mRNA expression showed poor prognosis ($\chi^2=3.291$, $P=0.046$).

Correlation between the levels of telomerase activity and prognosis of the patients

This study attempted to determine the effect of telomerase activity and *hTERT* mRNA expression on the prognosis of patients with hepatoblastoma. Telomerase activity was investigated in only 39 cases because frozen tumour tissue was unavailable in the remaining 24 cases. Kaplan–Meier EFS curves of these 39 patients (Figure 2C) show that the 10-year EFS rate in the patients with high telomerase activity (TPG $\geqslant 100$) was approximately 40%, while that in the remaining patients was approximately 90%. The prognosis of the patients with high telomerase activity (TPG $\geqslant 100$) was significantly worse than that of other patients ($P=0.0003$). Since the levels of telomerase activity were significantly correlated with advanced stages of tumour, the correlation between telomerase activity and prognosis was examined in the tumours in early stages (stage I or II) and those in advanced stages (stage III or IV), separately (Figure 2D). The prognosis of the patients with high telomerase activity was significantly poor in advanced tumours ($\chi^2=27.12$, $P<0.0001$). In early tumours, one of two patients with high telomerase activity showed poor prognosis, while all patients with low telomerase activity are alive disease free.

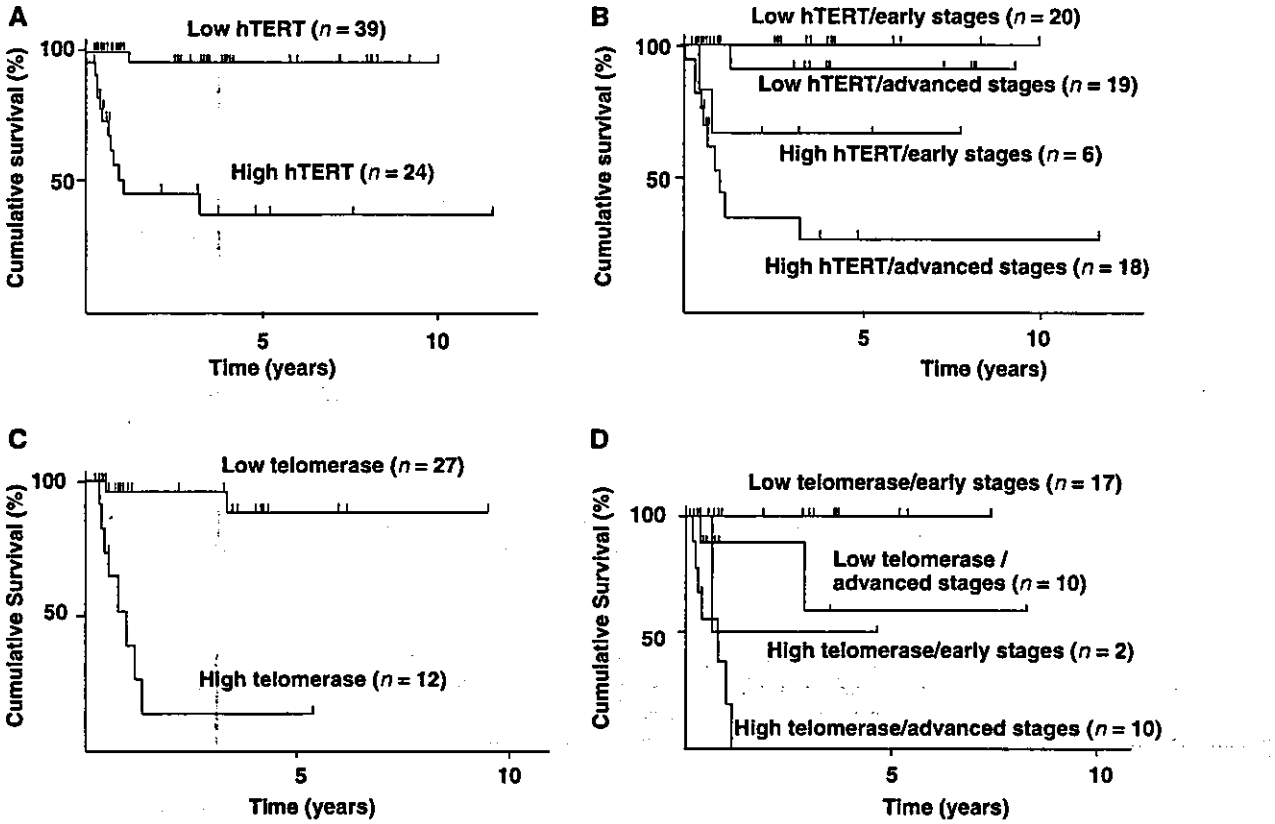

Figure 2 Kaplan–Meier cumulative survival spots for patients with hepatoblastoma. (**A**) Survival according to the levels of *hTERT* mRNA expression. High *hTERT*: *hTERT* mRNA $\geqslant$ 10 copies 1000 copies^{-1} of the *PBDG* genes, low *hTERT*: *hTERT* mRNA $<$ 10 copies 1000 copies^{-1} of the *PBDG* genes. The patients with tumours with high *hTERT* mRNA expression showed significantly worse survival ($P<0.0001$). (**B**) Survival according to the levels of *hTERT* mRNA expression and stages. Early stages: I and II, advanced stages: III and IV in the stage classification of the Japanese Society of Pediatric Surgeons. Patients having advanced tumours with high *hTERT* mRNA expression showed significantly worse survival ($P<0.0001$). (**C**) Survival according to the levels of telomerase activity. High telomerase: TPG value $\geqslant$100, low telomerase: TPG value $<$100. The patients having tumours with high telomerase activity showed significantly worse survival ($P<0.0001$). (**D**) Survival according to the levels of telomerase activity and stages. The patients having advanced tumours with high telomerase activity showed significantly worse survival ($P<0.0001$).

Prognostic factors

By univariate analysis, we analysed clinical parameters such as PRETEXT classification, distant metastasis, stage classification, serum levels of AFP, histological classification, preoperative chemotherapy, and total surgical resection, for the correlation with prognosis of the patients. PRETEXT 2, 3, and 4 tumours and the tumours with distant metastasis showed poorer prognosis, but not significantly. On the other hand, advanced stages (stages 3 and 4) were significantly correlated with poor prognosis of the patients ($P=0.022$). Thus, both telomerase activation and advanced stages were correlated with poor prognosis of patients.

To identify which independent factors had a significant influence on survival, multivariate survival analysis using the Cox proportional hazard regression model was performed. In this multivariate analysis, we assessed the prognostic value for event-free survival of the parameters that were significant in univariate analysis: stage and *hTERT* mRNA expression. For this multivariate analysis, variables with *P*-value lower than 0.30 in the univariate analysis were also selected: gender, age at diagnosis, curative surgery, stage/PRETEXT classification, histology, and hTERT/telomerase activity. As stage classification was significantly associated with PRETEXT and distant metastasis, we used stage classification in this multivariate analysis. As the levels of *hTERT* mRNA expression were significantly correlated with the levels of telomerase activity, and telomerase activity could not be analysed in 24 cases, we analysed these two factors separately in different multivariate analysis sheets. In the multivariate analysis including *hTERT* mRNA expression and the other five factors for 63 cases, *hTERT* mRNA and poorly differentiated histology were independent predictors of EFS. The hazard ratios were 50.0 (95% confidence interval of 5.07–492.9, $P=0.0008$) and 5.11 (95% confidence interval of 1.16–22.5, $P=0.031$). In the multivariate analysis including telomerase activity and the other five factors for 39 cases, the level of telomerase activity was also an independent predictor of EFS. The hazard ratio of telomerase activity levels was 17.7 (95% confidence interval of 2.16–120.1, $P=0.0032$). In advanced stages, the hazard ratios for *hTERT* mRNA and telomerase activity levels were 9.221 ($P=0.043$) and 5.248 ($P=0.188$), respectively.

DISCUSSION

Clinical investigation revealed that the prognosis of children with hepatoblastoma correlates with multifocal growth in the liver, invasion of blood vessels, distant metastasis, and either very low or high levels of serum AFP (von Schweinitz *et al*, 1997; Brown *et al*, 2000). Survival rates of children with more than two of these factors were less than 10%. Thus, these findings discriminate a subgroup of hepatoblastoma with more aggressive biological properties, which correlate with a poor prognosis. However, these factors are not sufficient to predict the prognosis of children with hepatoblastoma. Recently, high-dose chemotherapy with stem cell transplantation has become effective in some patients with aggressive hepatoblastoma with metastasis (Nishimura *et al*, 2002). Thus, to identify such high-risk patients with hepatoblastoma, we need additional useful prognostic markers for evaluation of aggressive biological properties.

Telomerase activity has been reported in many kinds of malignant tumours, including gastric cancer (Hiyama *et al*, 1995b), hepatocellular carcinoma (Nouso *et al*, 1996; Nakashio *et al*, 1997), pancreatic cancer (Hiyama *et al*, 1997b; Iwao *et al*, 1997), and colorectal cancer (Chadeneau *et al*, 1995; Naito *et al*, 2001). Approximately 80–90% of these malignant tumours showed telomerase activity (Shay and Bacchetti, 1997). In some kinds of tumours, high telomerase activity has been reported as a marker of tumour aggressiveness and poor prognosis (Hiyama *et al*, 1995a, b; Shay *et al*, 1997; Marchetti *et al*, 1999). In childhood tumours, telomerase activity and *hTERT* mRNA expression were also detected in a majority of cases of neuroblastoma, retinoblastoma, and nephroblastoma. In neuroblastoma, we have already reported a significant correlation between high telomerase activity and poor outcomes of patients (Hiyama *et al*, 1995a, 1997a). In retinoblastoma, telomerase activity was detected in about 50% of the tumours and such tumours showed a high recurrence rate (Gupta *et al*, 1996). In the present study, *hTERT* mRNA expression and telomerase activity were correlated with poor prognosis of patients, indicating these factors are useful prognosis-predicting factors in hepatoblastoma. Thus, activation of telomerase may correlate with malignant potential in most childhood malignant tumours including hepatoblastoma, neuroblastoma, and retinoblastoma.

This is the first report to show an association between the levels of *hTERT* mRNA expression or telomerase activity and patient prognosis in hepatoblastoma. In the multivariate analysis, activation of telomerase, stage of disease, and histological type were significantly correlated with the outcome of patients. In these three independent parameters, the risk of *hTERT* mRNA or telomerase activation was highest, indicating that telomerase reactivation is the most useful prognosis-associating factor in hepatoblastoma. In the present study, four (15.4%) of the 26 cases with early stage hepatoblastoma showed recurrence of tumours, and all four cases showed high telomerase activity (TPG $\geqslant$100 or *hTERT* mRNA $\geqslant$100 copies). In contrast, three (16.7%) of the 18 advanced cases with high telomerase activity remain disease-free. Since all stage 4 cases underwent different chemotherapeutic regimen in the JPLT study (Sasaki *et al*, 2002), one explanation for this result is that the high-dose chemotherapy might have been effective in preventing recurrence in these four early cases. Thus, in early stage tumours, selection of patients for high-dose chemotherapy based on high telomerase activity (TPG $\geqslant$100) might be an effective method to improve the prognosis of this category of patient. Moreover, the exclusion of low-risk patients from postoperative chemotherapy could spare some of its serious side effects. In advanced hepatoblastoma with low malignancy, complete resection and chemotherapy should be performed, but in such tumours with high malignancy, complete resection and chemotherapy might be insufficient and new aggressive strategies should be implemented. The observations in our study suggest that telomerase inhibition is an effective strategy for the reversal of tumour growth. Since most somatic cells do not have detectable telomerase activity and telomerase shows a tumour-specific expression in general, telomerase is an important target for new anticancer therapy. A number of different approaches have been developed for telomerase inhibition in human cancer. Different components and type of inhibitors targeting various regulatory levels have been regarded as useful telomerase inhibitors and seem to be most efficient when combined with conventional chemotherapy (Saretzki, 2003). Telomerase inhibition, which may be involved in triggering apoptosis, may be a new strategy for curing hepatoblastoma in the future.

In the present study, we analysed the clinical variables of hepatoblastoma cases, but did not find significant correlation between the levels of *hTERT* mRNA or telomerase activity and these variables except for PRETEXT system and disease-stage. It is well-known that prognosis of the cases with pure-foetal histology is good (Finegold, 2002). In the present study, we had only two pure-foetal subtypes in 33 well-differentiated tumours. Although the levels of *hTERT* mRNA or telomerase activity in them were relatively low, further study with large number of this subtype is necessary to analyse statistically.

Some noncancerous childhood liver tissues showed low levels of telomerase activity and *hTERT* mRNA expression. In childhood liver tissue infiltrating lymphocytes, multipotential stem cells, and their daughter cells might have telomerase activity, resulting in positive results by the contamination of lymphocytes and stem