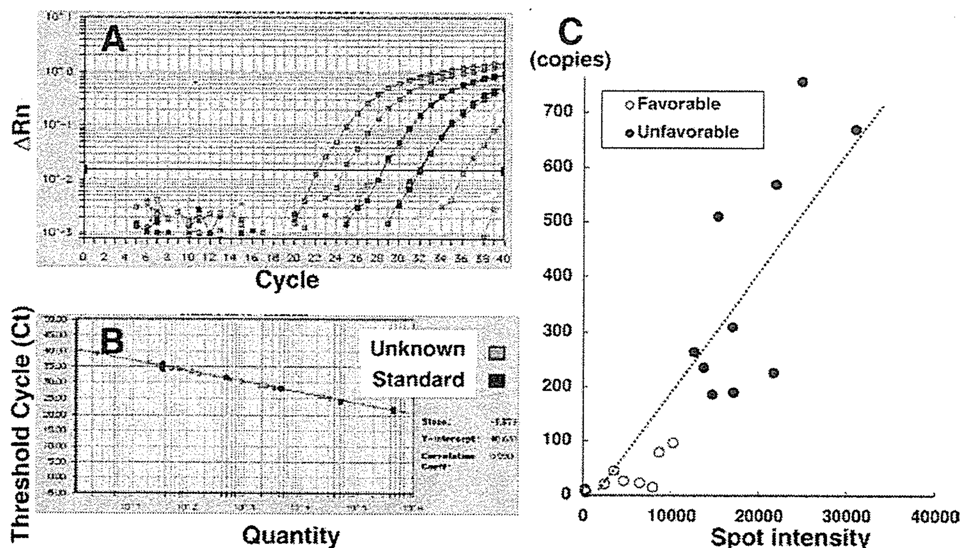

Fig. 2 Validation of *hTERT* mRNA expression levels in each tumor using real-time quantitative RT-PCR. **A** Representative *amplification plots* analyzed using the fluorescence of the PCR products. We prepared five serial diluted standard templates of *hTERT* (human telomerase reverse transcriptase) 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.

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Telomerase detection in the diagnosis and prognosis of cancer

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Abstract

Telomerase, a critical enzyme responsible 'for cellular immortality, is usually repressed in somatic cells except for lymphocytes and self-renewal cells, but is activated in approximately 85% of human cancer tissues. The human telomerase reverse transcriptase (hTERT) is the catalytic component of human telomerase. In cancers in which telomerase activation occurs at the early stages of the disease, telomerase activity and hTERT expression are useful markers for the detection of cancer cells. In other cancers in which telomerase becomes upregulated upon tumor progression, they are useful as prognostic indicators. However, careful attention should be paid to false–negative results caused by the instability of telomerase and of the *hTERT* mRNA and the presence of PCR inhibitors, as well as to false–positive results caused by the presence of alternatively spliced *hTERT* mRNA and normal cells with telomerase activity. Recently, methods for the *in situ* detection of the *hTERT* mRNA and protein have been developed. These methods should facilitate the unequivocal detection of cancer cells, even in tissues containing a background of normal telomerase-positive cells.

Abbreviations: BAL – bronchoalveolar lavage; FNA – fine needle aspiration; IHC – immunohistochemistry; ISH – in situ hybridization; hTERT – human telomerase reverse transcriptase; hTR – human telomerase RNA; TRAP – telomeric repeat amplification protocol.

Introduction

Mammalian telomeres are made of many hundreds to thousands units of the simple DNA repeat TTAGGG. Telomeres form a nucleoprotein that cap and protect the ends of linear chromosomes. Because the DNA replication machinery cannot fully replicate the ends of linear DNA molecules, telomeres progressively shorten with each cell division (Watson 1972). Eventually, when a critically short telomere length is reached, cells stop dividing and senesce (Greider 1990; Wright and Shay 1992). This phenomenon is thought to function as a 'mitotic clock' that limits the lifespan of individual cells. Telomerase is a specialized reverse transcriptase that synthesizes telomeric

repeats onto chromosomal ends and thus compensates for the progressive shortening of the telomeres caused by the end-replication problem (Lingner et al. 1995). This enzyme lacks from most somatic human cells and is typically restricted to certain specialized cell types, such as germ cells and stem/progenitor cells of self-renewal tissues, which must perform unusually large numbers of cell divisions (Hiyama et al. 1995c; Wright et al. 1996; Sakabe et al. 1998).

Cancer is a disease characterized by uncontrolled proliferation and invasion into surrounding tissues or distant organs. Most somatic human cells lack telomerase activity, have a limited life span, and require the activation of telomerase for unlimited proliferation. Although telomerase activation is not always concomitant with carcinogenesis, its presence in 85% of more than 3000 tumor samples tested makes telomerase activity the most universal maker of human cancers (Kim 1997; Shay and Gazdar 1997; Dhaene et al. 2000). Recent reports revealed that telomerase activity is upregulated during mouse tumorigenesis in spite of the fact that mice have very long telomeres (Blasco et al. 1996; Broccoli et al. 1996). This observation and others have suggested that telomerase may promote tumorigenesis independently of telomere length. By stabilizing telomeres and supporting the indefinite growth of most cancer cells, telomerase most certainly plays a crucial role in the progression and maintenance of tumors.

An important question, is when telomerase is activated during the multi-step process of carcinogenesis. In some instances, telomerase may already be ubiquitously expressed at the preneoplastic or *in situ* stage; while in other instances, the enzyme may be activated gradually with cancer progression (Shay and Bacchetti 1997). These differences are crucial in dictating whether telomerase might be clinically useful for either diagnostic or prognostic purposes. Although most somatic human cells lack telomerase activity, some tissues contain specialized cells, including germ cells, lymphocytes, stem cells, or certain epithelial cells, that display weak levels of telomerase activity, which can be upregulated concomitantly with growth signals. In such tissues, *in situ* immunohistochemical detection of telomerase may be necessary to determine whether telomerase expression is derived from normal telomerase-positive cells or from cancer cells. Taking these key points into account, telomerase is now being explored as a novel marker for early detection and/or the grading of malignant tumors. The present article reviews the use of human telomerase as a cancer diagnostic marker and as a prognostic tool for predicting the outcome of individual patients.

Detecting human telomerase in clinical materials

Telomerase can be measured by a PCR-based assay called telomeric repeat amplification protocol (TRAP) (Kim et al. 1994). The assay is quite sensitive and can detect as few as 10 telomerase positive cells (Wright et al. 1995). With this high sensitivity, telomerase activity can also be detected in certain normal somatic tissues, especially in proliferative and/or stem cells of self-renewing tissues (such as intestinal epithelium) and activated lymphocytes (Hiyama et al. 1995c, 1996b; Wright et al. 1996). Moreover, this activity is also detectable, albeit at low levels, in some benign tumors such as fibroadenomas of the breast (Hiyama et al. 1996a), hyperplastic nodule/adenomas of the thyroid (Matthews et al. 2001), and colon adenomas (Hiyama et al. 1996b). As a general rule, telomerase activity in normal somatic cells tends to be much lower in comparison to that detected in cancer cells. In clinical samples from tissues containing normal telomerase-positive cells, evidence of cancer cells requires levels of telomerase activity that are significantly higher than those of matched control tissues (Shay and Bacchetti 1997). As the TRAP assay is based on semi-quantitative PCR, a more precise method of quantification might be needed for such samples. To overcome this limitation, a real-time PCR assay (RTQ-TRAP) has been developed that allows quantitative measurements of telomerase activity in tissue samples (Hou et al. 2001). To avoid false–positive results due to contamination of cell samples with lymphocytes, we recommended using a thousand cell equivalents of cell lysate per assay, as proteins extracted from a thousand adult lymphocytes do not produce detectable telomerase activity (Hiyama et al. 1995c; Iwao et al. 1997). To avoid false–negative results, careful attention should be paid to the stability of telomerase and the presence of PCR inhibitors when examining clinical specimens.

Human telomerase activity is associated with the expression of two major components: human

telomerase RNA (*hTR*) (Feng et al. 1995) and human telomerase reverse transcriptase (hTERT) (Nakamura et al. 1997). Recent studies have targeted the expression of these two components as surrogates for telomerase activity and discussed their value as tumor markers. Since hTR is expressed at low levels in all cells, including cells that lack telomerase activity (Koyanagi et al. 2000), detection of the *hTERT* mRNA is believed to be a more reliable marker of the presence of cancer cells in clinical samples. However, the existence of splicing variants of the *hTERT* mRNA that fail to produce telomerase activity (Ulaner et al. 1998) can also be problematic for the use of the *hTERT* mRNA as surrogate for telomerase activity.

Telomerase as a diagnostic marker

Recently, there has been an increasing amount of experimental data on the detection of telomerase activity and/or hTERT expression in clinical materials as a diagnostic tool for various cancers (Table 1).

Head and neck tumors

Most cancer tissues in head and neck lesions show high levels of telomerase expression. Although the viability of cancer cells in these specimens is not particularly high, telomerase activity is often detected in oral washings of patients with oral malignancy (Califano et al. 1996; Sumida et al. 1998). In such specimens, it is difficult to avoid contamination by substances that interfere with PCR, such as necrotic tissue, leukocytes, erythrocytes, dental plaque, and bacteria. The presence of these substances in cancer samples can lead to false–negative results. Although currently limited in its sensitivity, the detection of telomerase activity or *hTERT* mRNA in oral washings is a novel marker indicating the presence of cancer cells shed from the upper aerodigestive tract.

In tumor biopsies, telomerase activity and the *hTERT* mRNA are almost always detected at high levels. Since low levels of telomerase activity are detected in the normal buccal epithelia and in approximately 20% of non-cancerous biopsy samples, a quantitative TRAP assay may be required for cancer diagnosis.

Thyroid and breast tumors

Because thyroid and breast lesions are easily palpable, fine needle aspiration (FNA) is widely used as a diagnostic tool for cancer detection in these lesions. For tumors of the thyroid gland, differential diagnosis between follicular adenoma and adenocarcinoma is difficult by FNA cytology alone. The detection of telomerase activity and/or *hTERT* mRNA has been found to be a useful tool for this differential diagnosis; as cancers gave positive signals while adenomas were negative for telomerase expression (Umbricht et al. 1997; Zeiger et al. 1999). However, thyroid tissues often contain lymphocytes, so that telomerase activity and *hTERT* mRNA derived from these inflammatory cells may also be detectable in certain benign diseases, such as Hashimoto thyroiditis (Haugen et al. 1997).

In the breast, normal mammary tissue lacks detectable telomerase activity, while the activity is expressed in 80–90% of ductal carcinoma *in situ* (DCIS) lesions and 90% of invasive breast cancers (Hiyama et al. 1996a; Umbricht et al. 1999). *hTERT* mRNA is detected at high frequency in breast cancers, where its levels are relatively high (Bieche et al. 2000). One of the most common problems in using telomerase for breast cancer diagnosis is the presence of telomerase activity in benign fibroadenomas. Approximately 40% of fibroadenoma tissues display low-level telomerase activity (Hiyama et al. 1996a; Pearson et al. 1998). In combination with cytology and with careful attention to benign diseases, the screening of FNA samples for telomerase expression is likely to become a powerful tool for the detection of breast and thyroid cancers (Pearson et al. 1998; Mokbel et al. 1999; Poremba et al. 1999a; Hiyama et al. 2000).

Lung and mediastinum

Sputum, bronchoalveolar lavage (BAL), bronchial brushing, and bronchial washing samples have all been tested in the TRAP assay for the detection of lung cancer cells (Sen et al. 2001). Use of the TRAP assay on sputum samples might hold potential for the early and non-invasive diagnosis of lung cancer. However, since sputum contains an abundance of mucus, which interferes with PCR

Table 1. Telomerase/*hTERT* mRNA as a diagnostic marker.

Organs/samples	Telomerase activity		*hTERT* mRNA	
	Cancer positive (%)	Non-cancerous positive (%)	Cancer positive (%)	Non-cancerous positive (%)
Head & neck				
Oral/Washing	110/195 (56)	70/321 (22)	21/26 (81)	9/39 (23)
Oral/Biopsy	25/26 (96)	9/41 (22)	47/58 (81)	11/13 (85)
Thyroid & breast				
Thyroid/FNA	64/96 (67)	23/155 (15)	44/57 (77)	15/52 (29)
Breast/FNA	210/265 (79)	40/355 (11)		
Chest				
Lung/Sputum	15/42 (36)	0/10 (0)		
Lung/Brushing, BAL	123/188 (65)	16/211 (8)		
Lung/Biopsy	86/128 (67)	0/10 (0)		
Mediastinal LN/FNA			10/16 (63)	18/71 (25)
Pleural effusion	175/205 (85)	20/155 (13)	14/15 (93)	6/15 (40)
Digestive organs				
Esophagus/Biopsy	52/54 (96)	33/48 (69)		
Stomach/Biopsy	23/29 (79)	10/28 (36)	88/101 (87)	62/192 (32)
Colon/Washing	20/34 (59)	0/20 (0)		
Colon/Biopsy	110/126 (87)	57/148 (39)	32/32 (100)	17/49 (35)
Liver/Biopsy	53/86 (62)	17/58 (29)	21/23 (91)	17/63 (27)
Biliary duct/Bile	4/37 (11)	0/25 (0)	10/20 (50)	0/14 (0)
Biliary duct/Biopsy	20/26 (77)	0/10 (0)	6/10 (60)	0/6 (0)
Pancreas/Pancreatic juice	59/72 (82)	2/51 (4)	15/17 (88)	2/19 (11)
Pancreas/FNA	18/18 (100)			
Peritoneal Lavage	102/141 (72)	5/117 (4)		
Genitourinary organs				
Bladder/Voiding urine	374/637 (59)	44/488 (9)	159/179 (89)	6/169 (4)
Bladder/Washing urine	229/302 (76)	6/153 (4)	125/168 (74)	19/165 (12)
Bladder/Biopsy	46/54 (85)	30/56 (54)		
Prostate/Voiding urine[a]	21/33 (64)	1/21 (5)		
Prostate/Biopsy	130/166 (78)	19/136 (14)		
Uterus/Cervical scraping	105/273 (38)	37/233 (16)	14/17 (82)	11/44 (25)
Uterus/Biopsy	138/164 (84)	58/158 (37)	83/104 (80)	1/8 (13)
Others				
Skin/Biopsy	130/159 (82)	11/109 (10)		
Blood/Serum	59/95 (62)	0/80 (0)	4/16 (25)	0/23 (0)

These percentages were calculated from the review papers Dhaene et al. (2000), Hiyama and Hiyama (2002, 2003) and Orlando et al. (2001) and recent numerous reports in addition to our unpublished data.
Abbreviations: BAL, bronchoalveolar lavage; FNA, fine needle aspirates.
[a]Voiding urine after massage.

and other enzyme reactions, the sensitivity of the telomerase assay in sputum is unsatisfactory for the detection of cancer (Sen et al. 2001). In brushing or BAL samples, on the other hands, telomerase activity showed a relatively high sensitivity for the detection of lung cancer cells, but more so for squamous cell carcinoma than for adenocarcinoma. However, BAL samples can contain activated lymphocytes, which can give false–positive results in benign diseases. The clonal expansion of lymphocytes, in particular, can produce strong telomerase activity in BAL samples (Haruta et al. 1999), which may instead reflect the aggressiveness of autoimmunity in certain benign diseases (Hiyama et al. 1998).

Several attempts to detect telomerase activity or *hTERT* mRNA have been reported using pleural effusion and mediastinal lymph node aspiration samples (Yang et al. 1998; Braunschweig et al. 2001; Dejmek et al. 2001; Wallace et al. 2003). Because carcinomas from almost any tumor sites can metastasize to the pleura, pleural effusions

may contain cancer cells originating from other organs such as the breast, ovary, or gastrointestinal tract. In malignant pleural effusions diagnosed by either fluid cytology or pleural biopsy, Yang et al. (1998) detected telomerase activity in 91% of cases with a specificity of 94%, indicating that the measurement of telomerase activity is a useful adjunct to cytology for detecting cancer cells. In this study, the only false positives were three samples from patients with tuberculosis. Thus, in pleural effusions as well as BAL samples, the sensitivity of the telomerase assay for detecting cancer cells is relatively high, with most of the false–positive signals being caused by lymphocytes contamination of non-cancerous lesions.

Digestive organs and peritoneum

Cancers of the digestive system are frequently diagnosed by endoscopic examination. In biopsies of the esophagus, telomerase activity was present in almost all esophageal cancers but was also detected in more than half of non-cancerous tissues, where the activity can be found in the normal epithelial basal cells. Similar results were obtained for biopsies of the stomach and colon. The sensitivity of telomerase activity or *hTERT* mRNA expression for detecting cancerous lesions was high, but telomerase was also present, albeit at lower levels, in non-cancerous tissues, where it localizes to the basal cells of crypts (Hiyama et al. 1996b). Hence, a more precise measurement of the level of telomerase activity in biopsy samples of the gut may be necessary for the diagnosis of cancers. Cells derived from colon luminal washing can also be applied to the TRAP assay for cancer diagnosis. Because washing samples rarely contain basal crypt cells, the specificity of the TRAP assay for colon washing samples was remarkable but at the expense of sensitivity, which was found to be relatively low (Yoshida et al. 1997).

In liver biopsies, detection of telomerase activity or *hTERT* mRNA shows promises for cancer diagnosis, albeit low-level expression of these markers was also reported in non-cancerous tissues (Nagao et al. 1999). In pancreatico-biliary cancers, detection of telomerase activity or *hTERT* mRNA in biopsy samples displayed a high sensitivity for cancer diagnosis. With the exception of pancreatic juice, the assay had a sensitivity that was low for excretion and secretion samples, such as bile (Itoi et al. 1999, 2001). In patients with pancreatic ductal adenocarcinomas, the pancreatic juice contains freshly exfoliated ductal cells that carry very high levels of telomerase activity. Because of its high sensitivity and specificity, the detection of telomerase activity and/or *hTERT* mRNA in pancreatic juice has become a promising new application of cancer diagnosis (Hiyama et al. 1997b; Iwao et al. 1997; Suehara et al. 1997; Morales et al. 1998). Moreover, detection of telomerase activity in pancreatic juice was additionally useful for the differential diagnosis of benign and malignant intraductal papillary mucinous tumors (IPMT) of the pancreas, which can be difficult to distinguish preoperatively (Inoue et al. 2001; Uemura et al. 2003).

Because peritoneal dissemination usually occurs in advanced stages of digestive cancers, telomerase activity in peritoneal lavage samples also showed a high specificity for cancer cell detection. Tangkijvanich et al. (1999) measured telomerase activity in nonmalignant and malignancy-related ascites associated with hepatocellular carcinoma and peritoneal carcinomatosis. Both the sensitivity and specificity of the telomerase assay were higher than those of cytology for diagnosis of the malignancy. The incidence of false–positive for telomerase activity was only of 4%, and all of these false positives showed evidence of lymphocytic contamination. Duggan et al. (1998) also found telomerase activity to be more sensitive than cytology in ascitic samples obtained from patients with ovarian cancer.

Genitourinary organs

Among exfoliating materials, voiding urine is easiest to examine. For the detection of bladder cancer using voided urine samples obtained from bladder cancer patients and controls, the TRAP assay showed the highest sensitivity (67%) and specificity (99%) Ramakumar et al. (1999). Since the viability of cells in voided urine samples varies, the sensitivity of telomerase activity for cancer diagnosis was lower than specificity. As an alternative, high sensitivity could be obtained in urine samples by detection of the *hTERT* mRNA by RT-PCR (Ito et al. 1998; Fukui et al. 2001). While telomerase activity and *hTERT* mRNA might both be useful for the detection of cancer cells in

bladder washings, detection of the *hTERT* mRNA may be preferable for the screening of voided urine (Lee et al. 1998; Fukui et al. 2001).

In voided urine samples obtained after prostate massage, the sensitivity of telomerase activity was higher than that of cytologic examination for the detection of prostate cancer (Meid et al. 2001). As a surrogate for unstable telomerase, *hTERT* mRNA was an even more reliable marker. They reported that the addition of EDTA to a final concentration of 20 mmol/l stabilized the RNA for up to 2 h at 4 °C.

As a potential biomarker of cervical dysplasia, telomerase has also been the focus of intense investigations. In cervical cancers, whether telomerase is activated in pre-malignant lesions remains controversial. According to several studies published on cervical biopsies (Wisman et al. 1998; Zheng et al. 2000; Jarboe et al. 2002), telomerase activity is abnormally present in a remarkably high proportion of high-grade squamous intraepithelial lesions (HSILs), indicating that the activation of telomerase is an early event in the malignant progression of cervical lesions. Still, a more complex situation has been suggested by histochemical studies, which revealed that the hTERT protein was present in the lower suprabasal levels of the normal cervical mucosa (Frost et al. 2000). In cancers, the hTERT protein was relocalized to virtually all levels of the lesional epithelia, concomitantly with the aberrant reexpression of telomerase activity. Thus, for the diagnosis of cervical cancers, a more precise measurement of telomerase activity might be needed, which should be confirmed by the histochemical staining of mirror image specimens. Thus, the detection of *in situ* carcinomas and precancerous lesions in cervical biopsies would likely require the application of methodologies for the *in situ* detection of the *hTERT* mRNA or protein, as described later in this review.

Skin

Skin is a surface organ from which biopsy specimens are easily prepared. Investigations of telomerase activity as a marker of skin cancer showed that epidermal basal cells had low levels of telomerase activity; that telomerase was not activated in the vast majority of squamous cell carcinoma; but that most cutaneous malignant melanoma displayed high-levels of telomerase activity (Parris et al. 1999). To elucidate the correlation between carcinogenesis and telomerase activation in the skin, further studies on skin cancers and related lesions are necessary.

Circulating cancer cells

Irrespective of the tumor type, the blood of cancer patients is likely to contain circulating cancer cells that could potentially be detected using the telomerase assay (Gauthier et al. 2001). The detection of these rare cancer cells in whole blood samples would be predicted to be masked by the potential presence of activated lymphocytes expressing high levels of telomerase activity (Hiyama et al. 1995c; Haruta et al. 1999). To detect circulating carcinoma cells using the telomerase assay, immunomagnetic separation can first be used to isolate epithelial cells from peripheral blood mononuclear cells, after which point the harvested cells can be tested for telomerase activity. In one report, the harvested circulating epithelial cells showed telomerase activity in 70–80% of patients with advanced lung, colon, and breast cancers, suggesting that telomerase activity may become a useful clinical marker of circulating epithelial cancer cells (Gauthier et al. 2001).

One of the most routinely collected bodily fluids is blood plasma, which can easily be prepared by centrifugation of whole blood. If tumor cells undergo necrosis and release their contents, some tumor-specific molecules might be present in plasma that could be detected. Although several studies have addressed the detection of tumor-specific mRNA in plasma, this broader topic is beyond the scope of this review. While it is unlikely that intact telomerase might be detected in plasma, a recent report has detected the *hTERT* mRNA and hTR in plasma (Chen et al. 2000).

Hematopoietic malignancies

The presence of endogenous telomerase in normal hematopoietic stem cells and activated lymphocytes is an important confounding factor that can limit the value of the telomerase assay in the detection of hematopoietic malignancies. Blood samples from patients at the early stages of chronic

lymphoid leukemia (CLL) display low-level telomerase activity, which progressively increases over the course of the disease to reach levels that are much higher than those detected in normal blood samples. Moreover, this increase is accompanied by a net decrease in telomere length (Shay et al. 1996). A series of 58 patients with CLL showed that higher telomerase activity and shorter telomeres were associated with an adverse prognosis (Bechter et al. 1998). In chronic myeloid leukemia, telomerase activity is not increased over that of normal peripheral mononuclear cells, but decreases in telomere lengths are observed, which correlate with shorter intervals to the blast crisis phase (Iwama et al. 1997). In acute lymphoblastic leukemia, smaller studies have found telomerase activity to be variable (Shay et al. 1996). With the exceptions of Hodgkin's lymphoma and chronic myeloid leukemia, all of acute myelogenous leukemia, multiple myeloma, plasma cell leukemia and non-Hodgkin's lymphoma exhibited marked increases in telomerase activity that were well above that of normal peripheral mononuclear cells (Norrback et al. 1998; Xu et al. 2001).

In situ detection of the hTERT mRNA and protein

When using RT-PCR or the TRAP assay to detect the *hTERT* mRNA or telomerase activity, the presence of normal telomerase-positive cells, such as lymphocytes or basal epithelial cells, can cause false–positive results. Methodology for the *in situ* detection of telomerase in individual cells would be expected to solve this problem. An *in situ* TRAP assay had previously been developed to detect the telomerase activity, but this methodology could only be used on fresh viable cells (Ohyashiki et al. 1997b). The use of *in situ* hybridization (ISH) to detect components of the telomerase complex (hTR and *hTERT* mRNA), on the other hand, would be applicable to fixed tissues. However, hTR is also present at low levels in most cells lacking telomerase activity, and its level does not always correlate with telomerase activity. A better target for ISH detection would be the *hTERT* mRNA, whose levels appear to closely parallel those of telomerase activity.

For evaluating hTERT expression, several studies have reported the successful use of *hTERT* mRNA ISH (Chou et al. 2001; Kumaki et al. 2001). While it is necessary to target sequences specific to the full-length mRNA to avoid false positives resulting from splice variants of *hTERT* mRNA, the ISH detection of the *hTERT* mRNA is predicted to become a powerful tool of cancer detection. For detecting hTERT expression, immunohistochemistry (IHC) can also be used to reveal the presence of the hTERT protein in a wide variety of clinical samples, including archival paraffin-embedded specimens (Hiyama et al. 2001; Kumaki et al. 2001, 2002). In spite of its very low abundance, the hTERT protein can now be detected in paraffin-embedded samples and core biopsies with the use of polyclonal or monoclonal antibodies in conjunction with appropriate antigen retrieval (Figure 1) and/or the highly sensitive tyramide-based method of signal amplification (Frost et al. 2000). Since IHC does not require specialized equipment for detection, hTERT IHC is predicted to become a powerful new technology for cancer detection. In most cancer tissues, the hTERT protein is heterogeneously distributed and, in some cases, can display regional variability. In most cancer specimens, the signal intensity of individual hTERT-positive cells did not differ substantially between tumors with high and low telomerase activity, and the level of telomerase activity was mainly dependent on the percentage of cells displaying hTERT expression (Hiyama et al. 2001). This heterogeneity in telomerase expression appears to be an important factor dictating the overall levels of telomerase activity in tumors. With the availability of hTERT IHC, telomerase-positive cancer cells can now be detected in tissues containing a background of normal telomerase-positive cells. Likewise, Frost et al. (2000) have observed changes in the tissue distribution of the hTERT protein in cervical cancers: while the hTERT protein was limited to the lower suprabasal cells of the normal cervix, it was present at all levels of the lesional epithelium in moderate to severe dysplasia.

Telomerase in cancer prognosis and the grading of malignant tumors

In certain types of cancers, telomerase activity is upregulated during tumor progression, so that the level of telomerase activity can be used to evaluate the malignant grade of tumors and predict patient

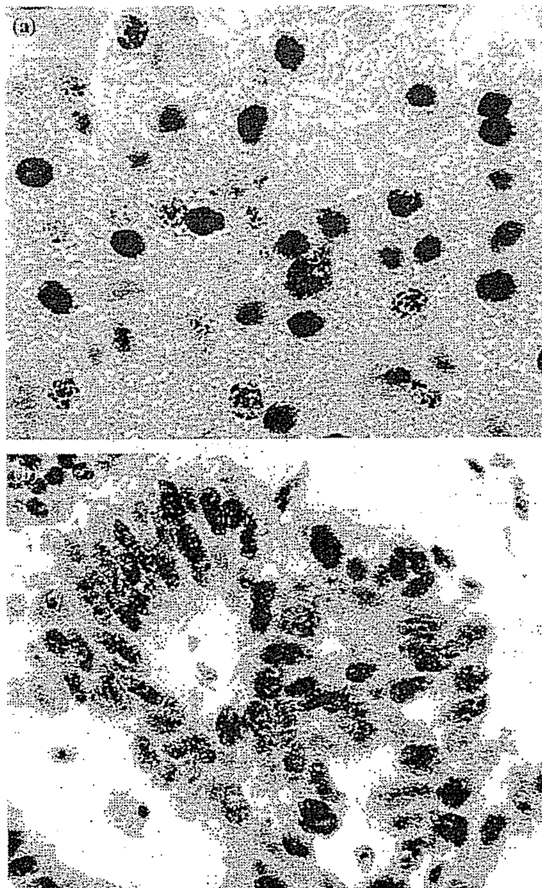

Figure 1. Immunohistochemical detection of hTERT in cancer samples. An anti-hTERT sera (EST21-A™, Alpha Diagnostic Int. Co., San Antonio, TX) was employed to reveal the presence of the hTERT protein in a FNA sample of a duct cell adenocarcinoma of the breast (a) and in a biopsy derived from an adenocarcinoma of the sigmoid colon (b). Tumor cells are revealed by the presence of brown pigments in the nucleus of hTERT-positive cells. Staining with 3,3′-diaminobenzidine (DAB) was preformed as described previously (Hiyama et al. 2001). The cells obtained from FNA sample were formalin-fixed and paraffin-embedded. For both samples, heat-based antigen retrieval was performed using a citrate buffer.

prognosis (Table 2). In certain cancers of the adults, the activation of telomerase correlates with advanced disease and poor prognosis, as in the cases of non-small cell lung cancer, gastric cancer, colorectal cancer, soft tissue tumors, and myelodysplastic disease (Chadeneau et al. 1995; Hiyama et al. 1995b; Marchetti et al. 1999; Tahara et al. 1999; Tatsumoto et al. 2000; Tomoda et al. 2002; Kido et al. 2003). In patients with colorectal cancer (Tatsumoto et al. 2000), including those undergoing curative resection of liver metastases (Smith et al. 2004), both telomerase activity and *hTERT* mRNA expression could be used as independent prognostic factors. In a retrospective study of a large number of breast cancer patients, telomerase activity correlated with a more aggressive tumor phenotype and its level was highly predictive of clinical outcomes (Clark et al. 1997). These findings suggest that telomerase activity is an useful indicator for identifying patients that would benefit from postoperative adjuvant chemotherapy.

Neuroblastomas are pediatric tumors that display a well-documented relationship between tumor biology and patient outcome. In these tumors, poor prognosis is associated with high levels of telomerase activity and full-length *hTERT* mRNA expression (Hiyama et al. 1995a; Hiyama et al. 1997a; Poremba et al. 1999b; Streutker et al. 2001; Krams et al. 2003). Interestingly, in stage 4S neuroblastoma, which represents a unique entity characterized by a high frequency of spontaneous regression, telomeres were shortened and telomerase activity was undetectable (Hiyama et al. 1995a, 1997a). Telomerase activity can also predict the outcomes of patients with gliomas, brain tumors that are consistently difficult to assign as either benign or malignant. Studies have shown that telomerase activity is present in most cases of malignant gliomas but is undetectable in grade I gliomas, making it a useful indicator of the malignant grade of gliomas (Nakatani et al. 1997; Huang et al. 1999). In tumors of the thyroid gland, telomerase activity may be useful to distinguish benign from malignant tumors and might provide a useful indicator of prognosis (Haugen et al. 1997; Saji et al. 1997). In pituitary adenoma, detection of telomerase expression may also correlate with biological aggressiveness and potential for regrowth (Yoshino et al. 2003). Telomerase activity in bone marrow has recently been reported to be a highly significant prognostic factor in pediatric patients with acute myeloid leukemia (Verstovsek et al. 2003). Collectively, these findings demonstrate that telomerase activity and hTERT expression are markers that can be used successfully to predict the outcome of cancer patients and take decisions on the appropriate treatments.

Table 2. Telomerase activity/*hTERT* mRNA as a prognostic marker.

Site	Tumor type	Correlated with prognosis	Correlated with other markers
Brain	Central nervous system malignant lymphoma; Pituitary tumor	Harada et al. (1999)[a,b] and Yoshino et al. (2003)[a]	
Head & Neck	Head & neck cancer; Oral cavity and oropharynx postchemotherapeutic tumors	Patel et al. (2002) and Ogawa et al. (1998)[a]	
Lung	Non-small cell lung cancer	Gonzalez-Quevedo et al. (2002), Hirashima et al. (2000), Marchetti et al. (1999), Taga et al. (1999)[a] and Hara et al. (2001)[b]	Dysplasia in smokers Soria et al. (2001)[b]
Breast	Invasive duct cell carcinoma		Proliferative index Carey et al. (1999)[a]; Relapse-free period Bieche et al. (2000)[a]
	Node-positive breast cancer	Clark et al. (1997)[a,c]	
Thyroid	Papillary carcinoma		Extrathyroidal extension Okayasu et al. (1997)[a]
Stomach	Adenocarcinoma	Hiyama et al. (1995b), Kakeji et al. (2001) and Usselmann et al. (2001)[a]	
Colon	Adenocarcinoma	Tatsumoto et al. (2000)[a]	Advanced stages Okayasu et al. (1998), Yoshida et al. (1999)[a], Boldrini et al. (2002), Naito et al. (2001) and Niiyama et al. (2001)[b]; Risk for metastasis Shoji et al. (2000)[a]
	Hepatic metastasis of colorectal cancer	Smith et al. (2004)	
Liver	Hepatocellular carcinoma	Kishimoto et al. (1998), Hisatomi et al. (1999) and Shimada et al. (2000)[a]	Recurrence risk (Suda et al. 1998)[a]
Pancreas	Endocrine tumors	Pearson et al. (2000)[a]	
Urogenital	Renal cell carcinoma		Tumor grade Hara et al. (2001)[a]; Advanced stage Paradis et al. (2001)[a]
	Transitional cell carcinoma	De Kok et al. (2000) and Nakanishi et al. (1999)[b]	Tumor relapse Lancelin et al. (2000)[a]
Prostate	Prostate cancer		Advanced stage Engelhardt et al. (1997)[a, b]
Uterus	Endometrial carcinoma		Recurrence risk Bonatz et al. (2001)[a]
Soft tissue	Osteosarcoma	Sangiorgi et al. (2001)[a]	Response to chemotherapy Kido et al. (2003)[a]
	Soft tissue sarcoma	Schneider-Stock et al. (2000) and Wurl et al. (2002)[a]	Recurrence and metastasis Tomoda et al. (2002)[a, b]
	Liposarcomas	Schneider-Stock et al. (2000)[b]	
Blood	Acute leukemia, B-cell Lymphocytic leukemia, Adult T-cell leukemia, Acute myelogenous leukaemia (AML)	Shay et al. (1996), Ohyashiki et al. (1997a), Uchida et al. (1999), Verstovsek et al. (2003)[a] and Xu et al. (1998)[a,b]	Recurrence in MDS Ohshima et al. (2003)[a]
	Pediatric AML	Verstovsek et al. (2003)	
	Multiple myeloma	Wu et al. (2003)	
Childhood tumor	Neuroblastoma	Hiyama et al. (1997a), Hiyama et al. (1995a), Poremba et al. (1999b)[a] and Krams et al. (2003)[b]	Cytogenetic abnormalities Brinkschmidt et al. (1998), Hiyama et al. (1997a), Hiyama et al. (1995a) and Wu et al. (2003)[a]
	Wilms tumor		Recurrent risk Dome et al. (1999)[b]
	Hepatoblastoma	Hiyama et al. (2004)[a,b]	

[a]Telomerase activity.
[b]*hTERT* mRNA.
[c]Conflicting findings have been reported.

Conclusion

In conclusion, measurement of telomerase activity and/or hTERT expression has several clinical utilities: for the early detection of cancer cells (in tumors that acquire telomerase activity at the early stages); as a prognostic indicator (in tumors that acquire telomerase activity upon progression); a marker that can distinguish malignancies from benign tumors; and a marker for detecting circulating cancer cells in the blood. *In situ* hybridization and the immunohistochemical detection of hTERT can now be used to identify telomerase-positive cancer cells in a background of non-cancerous cells. In the near future, methods for the *in situ* detection of hTERT are likely to become of common use in the clinics for both the diagnosis of cancers and the grading of malignancies.

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

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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.

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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

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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

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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, $P=0.0146$

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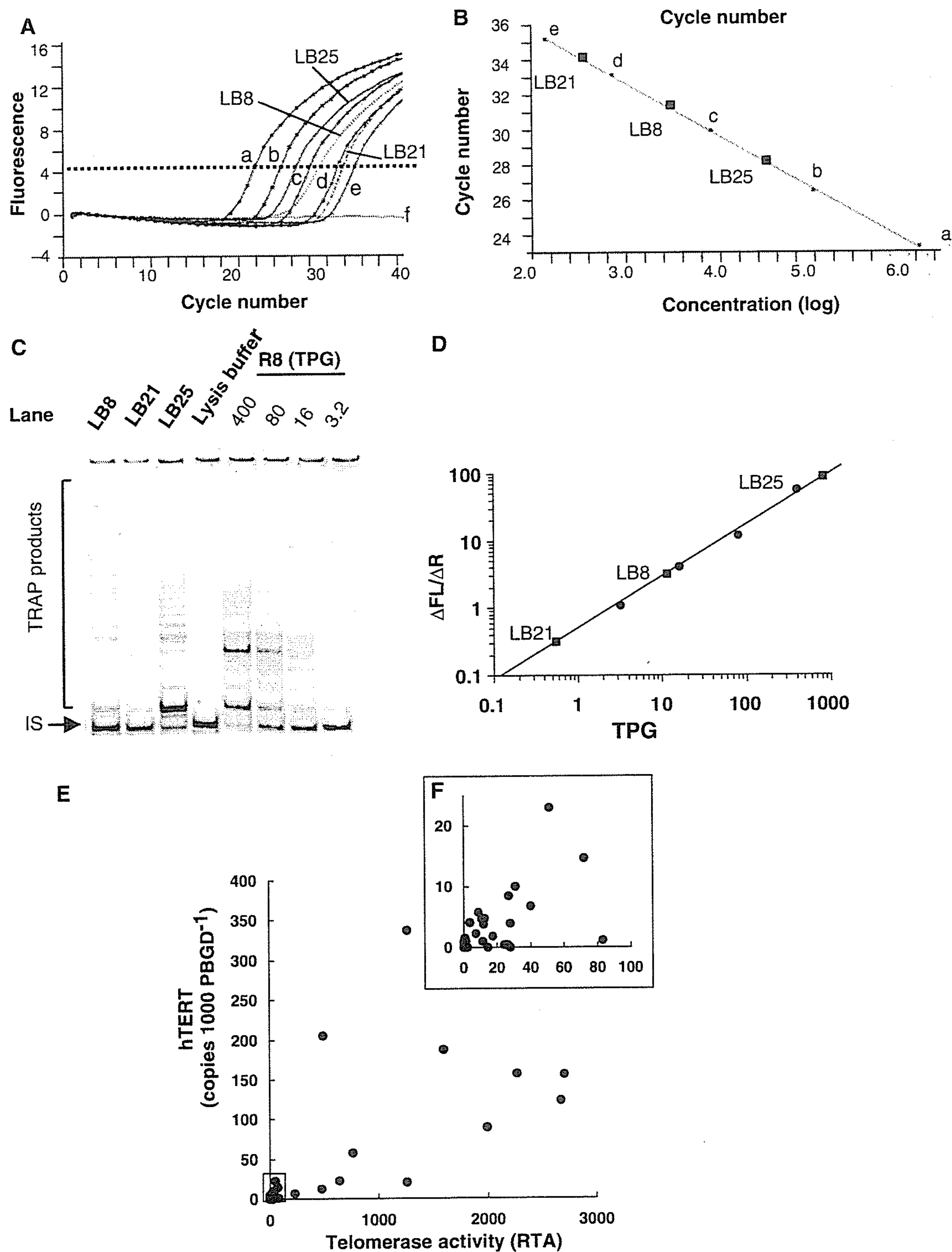

Figure 1 Detection of *hTERT* mRNA (**A**, **B**) and telomerase activity (**C**, **D**) and their relationship (**E**, **F**) in hepatoblastoma. (**A**) Amount of *hTERT* mRNA was measured by real-time RT–PCR analysis using LightCycler system in three representative hepatoblastoma samples (LB8, 21, 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 ($P<0.0001$).

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