Ⅳ. 研究成果の刊行物・別刷

第Ⅳ章　同種細胞療法の合併症

3．移植治療における関連毒性の評価基準

はじめに

同種造血幹細胞移植に伴う毒性のうち，化学療法や放射線療法によるもの，感染症やGVHDに伴うものなどを含む総称を移植関連毒性（transplant-related toxicity：TRT）と呼び，そのうち移植前治療に直接伴うものを特に治療関連毒性（regimen-related toxicity：RRT）と呼ぶ。これらは時に重篤となるため，タイムリーかつ適切に評価して迅速に対応することが，移植の臨床成績を左右するといっても過言ではない。また，新しい移植療法の開発や複数の治療法の比較といった学術的な目的においても，客観的な毒性評価法を確立することが必要不可欠である。

1 Bearman基準

従来の同種造血幹細胞移植の前処置である大量化学療法および全身放射線療法においては，血液毒性はほとんどの場合にgrade 4となるため，重症度評価の対象とするのは非現実的である。さらに感染症やGVHDに伴う毒性は別個に評価し，特に非血液毒性だけを選択して評価することが多い。従来この目的のために最も広く用いられてきたBearman基準（表1）は，このような移植治療に伴う毒性の特殊性を鑑みて，独自の毒性評価システムとして開発されたものであり，day 28（例外として肺毒性についてはday 100）までの治療関連毒性を8つの臓器別に4段階に分けて評価している。Bearmanらは，195例の骨髄移植を受けた患者において，前処置関連であると思われる有害事象（RRT）について本評価法を用いて毒性評価を行った[1]。その結果を以下に要約する。

① 単変量解析を行うと，非寛解期移植，15.75 GyのTBI，同種骨髄，特にHLA不一致移植，シクロスポリン・メトトレキサート併用のGVHD予防をそれぞれ施された患者において，gradeⅢ～ⅣのRRTが有意に高頻度に認められた。

② ただし，上記の要素は互いに独立していないために多変量解析を行ったところ，同種移植患者においてはTBIの線量だけが有意な危険因子であった。

③ 一臓器における最大のRRT gradeを用いて層別化したday 100の生存率は，それぞれgradeⅠ：84.8%，gradeⅡ：74.2%，gradeⅢ：10.5%であり，特にgradeⅢ以上のRRTを示した患者において著明に低かった。

表1　Bearmanらによる RRT 評価システム

	grade Ⅰ	grade Ⅱ	grade Ⅲ
心毒性	治療を必要としない軽度心電図異常，もしくは無症状で見つかった胸部X線での心陰影拡大	治療を必要としかつそれに反応する中等度心電図異常，または治療は必要でないが持続監視を要する中等度心電図異常。またはジギタリス製剤，利尿薬に反応するうっ血性心不全	治療に全くもしくは少ししか反応しない重度の心電図異常。治療に全くもしくは少ししか反応しない心不全。もしくは50%を超える電位の低下
膀胱毒性	最後の化学療法から2日目以降に生じた肉眼的血尿で膀胱炎の自覚症状はなく，感染が原因でないもの	最後の化学療法から7日目以降に生じた肉眼的血尿。もしくは2日目以降に生じた自覚的な膀胱炎症状を有する肉眼的血尿で，感染が原因でないもの	明らかな血液を伴う出血性膀胱炎で，硬化性薬剤の注入や腎瘻などの外科的処置による局所治療が必要とされるもの
腎毒性	基準値（通常前処置の開始前の最後の検査値）から2倍までのクレアチニン上昇	基準値の2倍を超えるが透析を必要としないもの	透析が必要となるもの
肺毒性	胸部X線で異常がなく，感染や心不全を原因としない呼吸困難。もしくは胸部X線で孤立性浸潤影や中等度の間質性変化を示すが，無症状で感染や心不全を原因としないもの	呼吸困難を伴う広範な局所浸潤影や中等度の間質性変化で，感染や心不全を原因としないもの。人工呼吸もしくは酸素マスク（＞50%）を必要としないPaO_2(基準値より＞10%)の低下で，感染や心不全を原因としないもの	人工呼吸による補助もしくは酸素マスク(＞50%)を必要とする間質性変化で，感染や心不全を原因としないもの
肝毒性	ビリルビン値≧2.0 mg%から≦6.0 mg %の軽度肝障害。基準値から体重＞2.5%から＜5%の増加で心臓が原因でないもの。もしくは前処置開始前最低値から2倍を超え5倍未満のsGOT増加	ビリルビン値＞6 mg%から＜20 mg%の中等度肝障害。前処置開始前から5倍を超えるsGOT増加。臨床的な腹水か画像診断された100 mLを超える腹水。もしくは心臓を原因としない基準値から5%を超える体重増	ビリルビン値＞20 mg%を超える重度肝障害。肝性脳症。呼吸機能に障害を及ぼす腹水
中枢神経毒性	嗜眠傾向だが，簡単に覚醒し，覚醒後は見当識があるもの	嗜眠傾向で覚醒後も混乱を来しているもの。もしくは他の新たな客観的中枢神経症状が出現し，意識消失はなく，他の薬剤，出血，中枢神経感染では簡単に説明できないもの	痙攣発作または昏睡で，他の薬剤，出血，中枢神経感染では簡単に説明，証明できないもの
口腔粘膜毒性	麻薬性鎮痛剤の持続静注を必要としない疼痛，潰瘍	麻薬性鎮痛剤の持続静注（モルヒネ点滴）を必要とする疼痛，潰瘍	予防的挿管を必要とする重度の潰瘍または粘膜炎。もしくは挿管のあるなしにかかわらず明らかな誤嚥性肺炎に至るもの
胃腸毒性	連日，500 mLを超えるが2,000 mL未満の水様性下痢で，感染に関連しないもの	連日2,000 mLを超える水様性下痢で，感染に関連しないもの。心血管系に影響を与えない肉眼的血便で，感染が原因でないもの。サブイレウスで感染に関連しないもの	鼻胃管による吸引もしくは外科的治療を要するイレウスで，感染に関連しないもの。もしくは心血管系に影響を与え輸血を必要とする出血性腸管炎

※ grade Ⅳ治療関連毒性は致死的なものと定義する。

④ 複数の臓器におけるそれぞれの最大 grade を総じた値（cumulative toxicities)を用いても，予後の層別化が可能であった。

⑤ 3臓器以上で grade Ⅱ以上の毒性を認めた患者は，そうでない患者に比して有意に day 100 の生存率が低かった。

表2　NCI-CTC (ver. 2.0) − Bearman 基準の項目に従い抜粋

有害事象	0	1	2	3	4
左室機能	正常	症状はなく，安静時駆出率が治療前値から≧10%，＜20%の低下；短縮率が≧24%，＜30%	症状はなく，安静時駆出率がLLN以下または安静時駆出率が治療前値より≧20%の低下；短縮率が＜24%	治療に反応するCHF	重症または難治性CHFまたは挿管の必要あり
排尿痛	なし	軽い症状で処置を要さない	症状があるが治療により軽快する	症状があり治療によっても軽快しない	−
血　尿	なし	顕微鏡的血尿のみ	時折の肉眼的出血：凝血塊なし	持続する肉眼的出血または凝血塊；カテーテルや器具の挿入また輸血を要する	緊急手術を要する大出血
クレアチニン	WNL	＞ULN～1.5×ULN	＞1.5～3.0×ULN	＞3.0～6.0×ULN	＞6.0×ULN
腎不全	なし	−	−	透析を要するが可逆性	透析を要し不可逆性
成人呼吸窮迫症候群(ARDS)	なし	−	−	−	あり
呼吸困難	正常	−	労作時呼吸困難	通常の活動レベルでの呼吸困難	安静時呼吸困難また人工呼吸器を要する
低酸素血症	正常	−	労作時の酸素飽和度の低下	安静時の酸素飽和度の低下，酸素吸入を要する	陽圧呼吸補助(CPAP)または補助換気を要する酸素飽和度の低下
肺臓炎・肺浸潤	なし	X線上の変化はあるが症状がないまたは症状はあるがステロイドを要さない	X線上の変化がありステロイドまたは利尿剤を要する	X線上の変化があり酸素吸入を要する	X線上の変化があり補助換気を要する
ALP	WNL	＞ULN～2.5×ULN	＞2.5～5.0×ULN	＞5.0～20.0×ULN	＞20.0×ULN
ビリルビン	WNL	＞ULN～1.5×ULN	＞1.5～3.0×ULN	＞3.0～10.0×ULN	＞10.0×ULN
GOT	WNL	＞ULN～2.6×ULN	＞2.5～5.0×ULN	＞5.0～20.0×ULN	＞20.0×ULN
GPT	WNL	＞ULN～2.5×ULN	＞2.5～5.0×ULN	＞5.0～20.0×ULN	＞20.0×ULN
体重増加	＜5.0%	5.0～9.9%	10.0～19.9%	≧20%	−
腹　水	なし	症状がない腹水	症状があり利尿剤を要する	症状があり治療的穿刺を要する	生命を脅かす病態
意識レベル低下	正常	傾眠または鎮静（意識清明でない）；機能障害なし	傾眠または鎮静（意識清明でない）；機能障害はあるが日常生活には支障なし	感覚鈍麻（刺激に対する反応低下）または昏迷；覚醒困難；日常生活に支障あり	昏睡
口内炎・咽頭炎	なし	疼痛がない潰瘍，紅斑または病変を特定できない軽度の疼痛	疼痛がある紅斑，浮腫，潰瘍，摂食・嚥下可能	疼痛がある紅斑，浮腫，潰瘍，静脈内輸液を要する	重症の潰瘍，経管栄養，経静脈または予防的挿管を要する
下　痢	なし	治療前に比し＜4回/日の排便回数増加	治療前に比し4～6回/日の排便回数増加または夜間排便	治療前に比し≧7回/日の排便回数増加または失禁または脱水に対する静脈内輸液を要する	集中治療を要する病態または循環動態の虚脱

CHF：慢性心不全

以上より，従来の骨髄破壊的な方法による同種造血細胞移植では，Bearman 分類は移植関連死亡の予測に有効であることが証明された。

2 NCI-CTC

一方近年，従来の移植に比べて毒性としての臓器障害が少ないといわれる RIST が確立され，高齢者や臓器合併症をもった患者に対する積極的な応用が期待されている。この RIST を受けた患者における毒性評価に，通常の骨髄破壊的移植を想定した尺度として開発された Bearman 基準を用いる妥当性は確立していない。そこで，Bearman 基準に比べて軽症な毒性を評価するのに優れていると考えられ，通常の化学療法領域で普及している National Cancer Institute の Commom Toxicity Criteria (NCI-CTC〔ver.2.0〕)(表 2)[3] を用いて，RIST の毒性評価を試みた結果を紹介する[3]。

国立がんセンター中央病院において 1999 年 9 月から 2002 年 4 月までに RIST を施行した 86 例において，前処置開始から移植後 28 日までを対象期間とし，Bearman 基準と NCI-CTC (ver. 2.0)による毒性評価を retrospective に行い，患者予後との相関を解析した。表 3 に患者背景を示す。Bearman 基準を用いた治療関連毒性では，grade Ⅲ以上の毒性は中枢神経 2 例，肺 4 例，膀胱 2 例と非常に少なく，grade Ⅱ以上の毒性は口内炎，肝毒性において多く認めた(図 1)。一方，NCI-CTC (ver. 2.0)では，grade 3 以上の毒性は腸管 (8 例)，肝 (27 例)，肺

表 3　患者背景

総患者数	86 人
年齢中央値 (範囲)	51 (4 ～ 67) 歳
男性 / 女性	57 / 29 人
conditioning regimen	
Flu-based / 2-CdA-based	64 / 22 人
ATG + / −	49 / 37 人
TBI + / −	3 / 83 人
GVHD 予防	
MTX あり / MTX なし	16 / 70 人
ドナーの背景	
related / unrelated	81 / 5 人
matched / mismatched	69 / 17 人
disease states	
low risk / high risk	53 / 33 人
診断名	
ALL	2 人
AML	26 人
CML	5 人
TLBL	2 人
ATL	3 人
MDS	11 人
NHL	16 人
その他	3 人
固形腫瘍	18 人

注：disease state は移植にあたっての risk と定義し，low risk は MDS の RA，ALL，AML の第 1，第 2 寛解期，CML の CP 1,2，リンパ腫の第 1，第 2 寛解期，部分寛解期，固形腫瘍とした。

Flu：フルダラビン，2-CdA：クラドリビン，ATG：抗胸腺細胞グロブリン，MTX：メトトレキサート，ALL：急性リンパ性白血病，AML：急性骨髄性白血病，ATL：成人 T 細胞白血病，CML：慢性骨髄性白血病，MDS：骨髄異形成症候群，NHL：非 Hodgkin リンパ腫，TLBL：T 細胞性リンパ芽球性リンパ腫

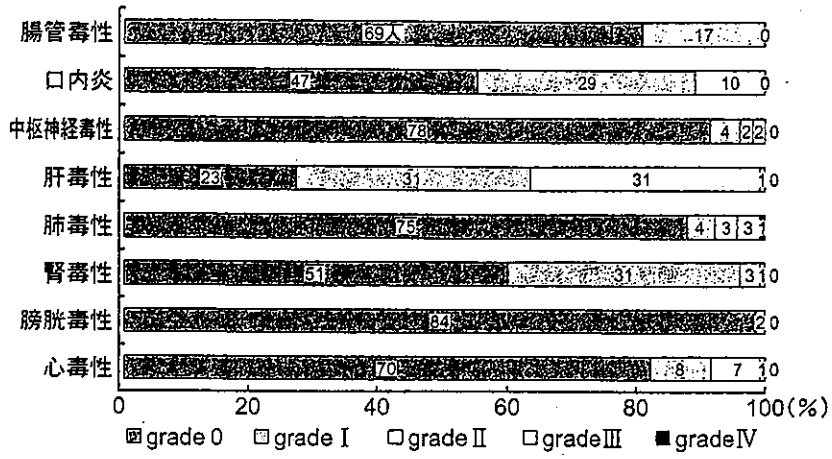


図1　Bearman 基準を用いた RRT の評価
grade Ⅲ以上の毒性は少なく，grade Ⅱ以上は口内炎，肝毒性において多く認めた。

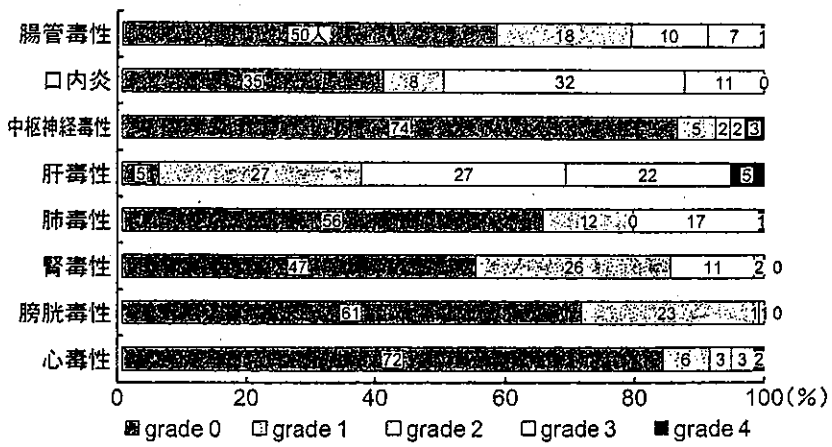


図2　NCI-CTC (ver. 2.0) を用いた RRT の評価
grade 3 以上の毒性は腸管毒性，肝毒性，肺毒性において多く認めた。

(18 例)において多く認められた(図 2)。grade 3 以上の毒性を生じた患者は，Bearman 基準では 5 例 (6 %)であるのに対して NCI-CTC (ver 2.0)では 43 例 (50%)であった。つまり，同一患者集団では Bearman 基準において毒性が低く評価される傾向が判明した。毒性別に生存率をみると，両基準とも移植関連毒性の重症度と予後との間に有意な相関を認めている（図 3，4)。

図 5 では，移植関連死亡を呈した 11 例についての検討結果を示した。Bearman 基準で grade Ⅲ以上を示した症例は 11 例中 3 例であったが，NCI-CTC(ver. 2.0)で grade 3 以上の毒性は 11 例中 10 例であり，NCI-CTC の方が移植関連死亡をより鋭敏に予測し得ることが示唆された。

さらに生存期間に影響を及ぼす因子を調べたところ，ドナー背景と移植関連毒性の重症度が

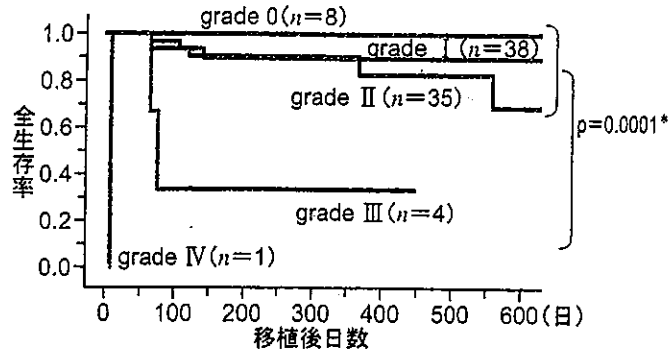


図3　Bearman 基準を用いた毒性別の生存率
毒性の grade と予後との間に有意な相関を認めた。

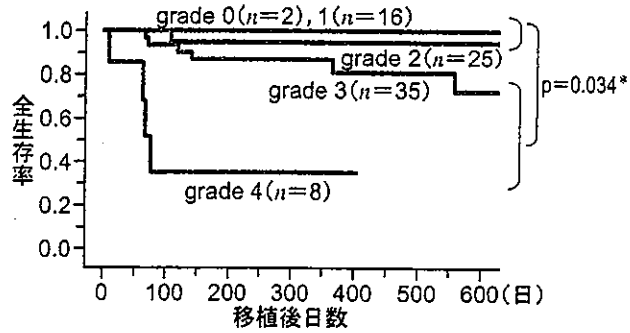


図4　NCI-CTC (ver. 2.0) を用いた毒性別の生存率
毒性の grade と予後との間に有意な相関を認めた。

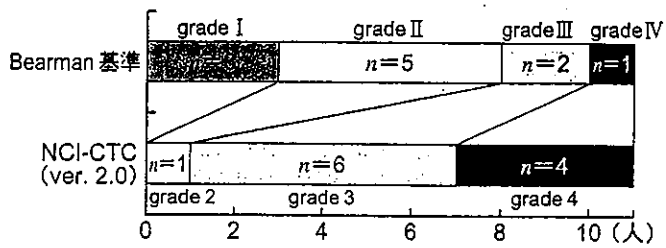


図5　移植関連死亡を呈した 11 例における毒性評価
移植関連死亡 11 例のうち，Bearman 基準と NCI-CTC (ver. 2.0) で grade 3 以上の毒性はそれぞれ 3 例と 10 例であった。

統計学的に有意に関連していた（表4）。

移植関連死亡の予測という点から毒性評価の閾値を検討すると，従来臨床的に問題とされている grade Ⅲ以上で，Bearman 基準は特異度が高くて感度は低かった。逆に NCI-CTC (ver. 2.0)の grade 3 以上では，感度は高いものの特異度は低かった（表5）。

おわりに

本研究における RIST の移植関連死亡は 12.8% (86 人中 11 人)であり，従来型の骨髄破壊的

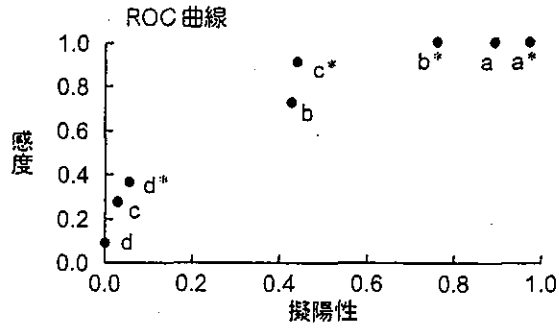
表4　生存期間に影響する因子（比例ハザードモデル，多変量解析）

	ハザード比	95%信頼区間	p 値
血縁 or 非血縁	7.502	1.715 ～ 32.814	0.0074 *
HLA 一致 or 不一致	3.838	1.143 ～ 12.885	0.0295 *
毒性 (NCI-CTC grade 3 ～ 4)	2.975	1.208 ～ 7.326	0.0177 *

ドナー背景と毒性の grade が有意に生存期間に影響していた。
*：統計学的有意

表5　移植関連死亡の予測における毒性評価基準の閾値の検討

		毒　性	感　度	特異度
Bearman 基準	a	grade Ⅰ以上	1.000	0.107
	b	grade Ⅱ以上	0.727	0.573
	c	grade Ⅲ以上	0.273	0.973
	d	grade Ⅳ	0.090	1.000
NCI-CTC (ver. 2.0)	a *	grade 1 以上	1.000	0.027
	b *	grade 2 以上	1.000	0.240
	c *	grade 3 以上	0.909	0.560
	d *	grade 4	0.364	0 947



移植と比べて低値であったものの，RIST のさらなる治療成績改善のためには，より優れた毒性評価法に基づいた治療法の改善が求められる。今後，RIST を含めて多様化する同種造血幹細胞移植の移植関連毒性の評価には，それぞれの移植手技に応じた，高感度かつ擬陽性が少ない新しい評価方法の開発が必須であると考えられる。

（崎山美知代，牧本　敦）

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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

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, $P=0.0146$

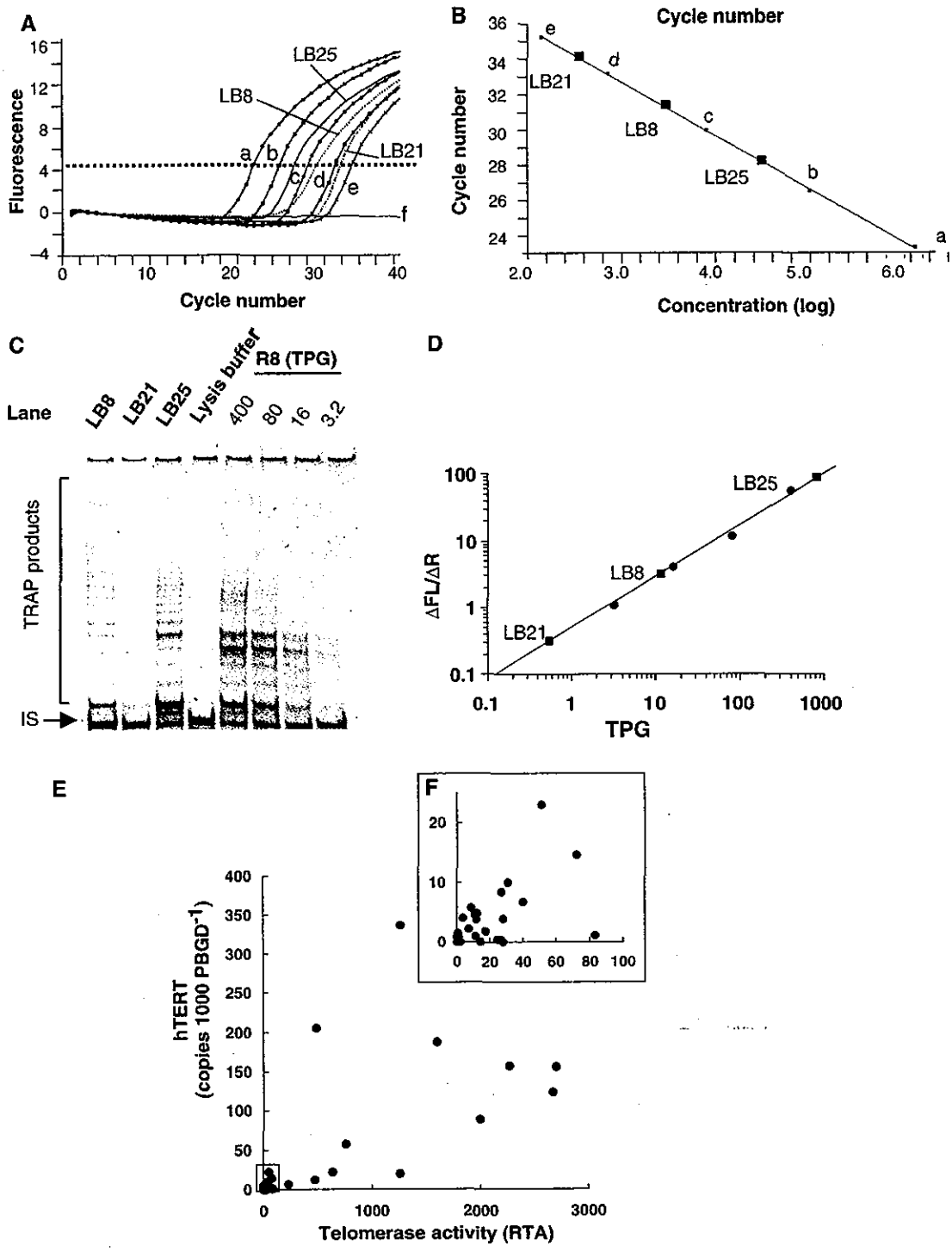

Figure 1 Detection of *hTERT* mRNA (**A**, **B**) and telomerase activity (**C**, **D**) and their relationship (**E**, **F**) in hepatoblastoma. (**A**) Amount of *hTERT* mRNA was measured by real-time RT–PCR analysis using LightCycler system in three representative hepatoblastoma samples (LB8, 21,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

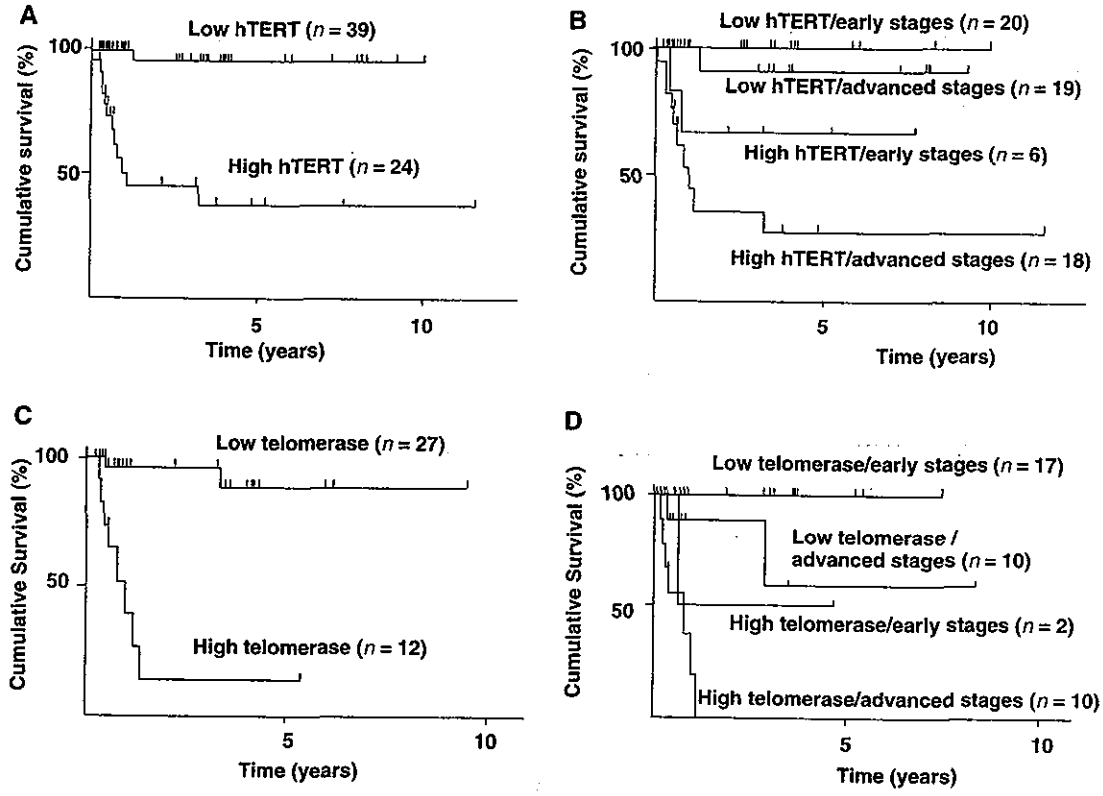

Figure 2 Kaplan–Meier cumulative survival spots for patients with hepatoblastoma. (**A**) Survival according to the levels of *hTERT* mRNA expression. High *hTERT*: *hTERT* mRNA $\geqslant$10 copies 1000 copies^{-1} of the *PBDG* genes, low *hTERT*: *hTERT* mRNA $<$10 copies 1000 copies^{-1} of the *PBDG* genes. The patients with tumours with high *hTERT* mRNA expression showed significantly worse survival ($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

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

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

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Expression of the Human Telomerase Reverse Transcriptase in Pheochromocytoma and Neuroblastoma Tissues

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Abstract. In an effort to clarify the role of telomerase in the pathogenesis of pheochromocytomas and neuroblastomas, and to test whether its component could serve as a marker of malignancy, we measured telomerase reverse transcriptase (TERT) mRNA in 31 human pheochromocytoma tissue samples (5 malignant, 23 benign and 3 suspected malignant) and 16 neuroblastoma tissues (9 unfavorable and 7 favorable). All cases were classified by both the clinical course and histopathological examination. Malignancy was defined as the presence of metastasis and/or extensive local invasion. TERT mRNA was determined by nested PCR and a real-time PCR system (LightCycler). By nested PCR methods, 5 of the 5 malignant pheochromocytoma samples were positive (sensitivity = 100%), and 21 of 23 benign pheochromocytoma samples were negative (specificity = 91%) in pheochromocytomas. Four out of five malignant tumors were positive for either hTERT expression or Ki-67/MIB-1 immunoreactivity. In the neuroblastoma tissues, 9 of the 9 unfavorable samples were positive (sensitivity = 100%), and only 2 of 7 favorable samples were negative (specificity = 29%). We also determined the expression of the hTERT mRNA by real-time PCR to quantitate the mRNA. The mean values of hTERT mRNA by real time PCR in benign and malignant pheochromocytomas were 2 and 26 arbitrary units (AU), respectively. The difference was not significant by the U-test. The mean values of hTERT mRNA in favorable and unfavorable neuroblastoma were 203 and 497 AU, respectively. This difference was also not significant (U-test). N-Myc mRNA expression correlated with the expression of hTERT mRNA in the neuroblastoma samples ($r = 0.534$, $p = 0.0317$). Thus, hTERT mRNA might be a potential marker for estimating the malignancy of pheochromocytomas and neuroblastomas.

Key words: hTERT, mRNA, Malignancy, Pheochromocytoma, Neuroblastoma

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TELOMERASE is a ribonucleoprotein complex that extends and maintains the telomeres by the addition of telomeric repeats to the ends of linear chromosomes. The human telomerase complex includes telomerase RNA (hTR), human telomerase reverse transcriptase (hTERT) and a number of associated proteins. hTR acts as a template for the telomeric repeat synthesis, and hTERT has reverse transcriptase activity [1, 2]. It has been suggested that telomerase activity may be a useful marker in cancer diagnosis because it can be detected in approximately 85% of the most common cancers [3, 4]. Moreover, telomerase activity is a useful indicator for determining the degree of malignancy in several tumors. For example, in meningiomas and similar tumors, it is difficult to distinguish benign tumors from malignant ones by histopathological examinations [5, 6]. In these tumors, the levels of telomerase activity correlate with the prognosis of

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the patients.

hTR is sometimes expressed in cells without telomerase activity; in contrast, the degree of telomerase activity correlates with the expression of hTERT. Thus, most somatic human cells lack telomerase activity because they do not express the hTERT gene, but most cancer cells express hTERT [7]. Therefore, detection of hTERT mRNA expression by RT-PCR analysis is also useful for cancer diagnosis.

Pheochromocytomas are tumors arising most often from the adrenal medulla. There is no tumor excretory profile that is predictive of malignancy. Reliable prediction of malignant behavior on the basis of histopathology is also notoriously difficult. DNA ploidy can be a helpful indicator, although aneuploidy *per se* is not considered to be a specific marker of malignancy. Mutations in the p53 gene have been reported to occur frequently in cases of benign pheochromocytoma [8]. Kinoshita *et al.* and Kubota *et al.* [9, 10] reported that analysis of telomerase activity is a powerful tool for the diagnosis of malignant pheochromocytoma. On the other hand, Bamberger *et al.* [11] reported that telomerase activity may not be a suitable marker for malignancy in the adrenal gland.

Neuroblastoma is an embryonic tumor of neuroectodermal cells derived from the neural crest and destined for the adrenal medulla and sympathetic nervous system. Neuroblastoma is the most common extracranial solid tumor in children. Its biological behavior is notoriously unpredictable and comprises a spectrum ranging from spontaneous regression to rapid metastatic spread. Hiyama *et al.* [12] reported the clinical utility of telomerase as a prognostic marker in neuroblastoma.

In this report we present our study of the expression of the hTERT mRNAs in pheochromocytoma and neuroblastoma tissues.

Materials and Methods

Tissue samples

Freshly excised samples of pheochromocytoma tissues and neuroblastic tumors were snap-frozen in liquid nitrogen and stored at –80°C.

A total of 31 pheochromocytoma tissues (5 malignant, 23 benign, and 3 suspected malignant) and 16 neuroblastoma tissues (9 unfavorable and 7 favorable) were obtained. All cases were classified by the clinical course and the histopathological examination as reported by Linnolia *et al.* [13]. Malignancy was defined as the presence of metastasis and/or extensive local invasion. A signed informed consent was obtained from patients wanting to participate in the study. This study was approved by the ethics committee of the Medical Faculty of Tsukuba University, Tsukuba, Japan.

The no. 5 sample of pheochromocytomas was diagnosed as malignant by the clinical course after the detection of hTERT mRNA. Cases 6, 7 and 8 are suspected malignant based on pathological criteria, but they did not have metastases during the study period.

Two regions of the samples were assayed by nested PCR.

RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated from frozen tumor samples by the guanidine thiocyanate-phenol-chloroform method, using isogen reagent (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Total RNA (1 μg)was used for the synthesis of single-strand complementary DNA (cDNA) with random primer and reverse transcriptase (Takara, Tokyo, Japan) in a total volume of 20 μl, and then 1 μl was used as a template for PCR. The primers used in this study for hTERT mRNA are shown in Table 1. As positive controls for PCR, the primers for GAPDH were used. For hTERT, nested PCR methods were performed with the inner primers. The designed primers are as reported by Nakamura *et al.* [2]. The thermal cycler profile consisted of an initial denaturation at 94°C for 3 min, followed by 35 cycles with a 30 s denaturation at 94°C, a 30 s annealing of primers at 55°C, and 60 s extension at 72°C. The second steps

Table 1. Primers and probes used in this study

Name	Sequence (5'-3')
hTERT-of	AGC CAG TCT CAC CTT CAA CCG C
hTERT-or	GGA GTA GCA GAG GGA GGC CG
hTERT-if	GCT GGG AGG AAC ATG CGT CG
hTERT-ir	GGG AGG CCG TGT CAG AGA TG
N-Myc-891F	CGA CCA CAA GGC CCT CAG TA
N-Myc-1031R	ACA GCC TTG GTG TTG GAG GA
N-Myc-probe	5'-FAM-TCA TCT GAA TCG CTC AGG GTG TCC T3'-TMR

of nested PCR for hTERT were performed using 1 μl of products from the first PCR according to the same program. After PCR, 5 μl aliquots of the products were subjected to 2% agarose gel electrophoresis and stained with ethidium bromide.

Quantitation of hTERT mRNA

The numbers of hTERT (+α+β) mRNA molecules were determined using 0.05–0.1 μg of total RNA from each sample by real-time RT-PCR using the Light-Cycler and LightCycler TeloTAGGG hTERT Kit from Roche Molecular Biochemicals according to the manufacturer's instructions. The primers and fluorescent probes in this assay are designed so that only the potentially functional +α+β form of hTERT is measured. The assay allows determination of the relative telomerase expression levels by comparing them to the expression level of the housekeeping gene porphobilinogen deaminase (PBGD). The unit shows hTERT mRNA expression divided by PBGD mRNA expression.

Quantitation of N-Myc mRNA

The PCR reaction mixture was prepared using a Taqman PCR master reagent kit (PE Applied Biosystems, CA) according to the manufacturer's instructions. The thermal cycling protocol was 2 min at 50°C and 10 min at 95°C, which was followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Thermal cycling, fluorescence detection, and data analysis were performed on the ABI PRISM 7700 Sequence Detector using the software provided with the instrument. The sequences of synthetic oligonucleotide primers and probe are shown in Table 1. The expression of N-Myc mRNA was normalized by the expression of the housekeeping gene GAPDH. For GAPDH detection, we used Taqman GAPDH Detection Reagents (PE Applied Biosystems, CA).

Immunohistochemistry

Immunohistochemical analyses of the proportion of Ki-67/MIB-1 immunoreactive cells were performed using a Simple-stein method (Nichirei, Tokyo, Japan) according to the manufacturer's instructions. Paraffin sections 5 um thick were incubated overnight at +8°C with monoclonal antibodies against Ki-67 (clone MIB-1, diluted 1:200; Immunotech, Marseille, France). To intensify the immunoreaction, the sections were autoclaved at 121°C for ten minutes. Known positive samples of breast cancer tissue were included as positive controls.

The proliferative activity, given as the percentage of Ki-67/MIB-1 immunoreactive cells, was calculated with the aid of an 10 × 10-square ocular grid. To estimate the cell density of each tumor section, the total number of cells (positive and negative) was counted in one row of the grid (10 squares) and multiplied by 10. Then the total number of immunoreactive cell nuclei was recounted at 10 different locations in the areas with the highest proliferative activity.

Statistical analysis

Statistical significance was determined by the Mann-Whitney U-test and Pearson's correlation test. The significance level was set at $p<0.05$.

Results

hTERT mRNA expression in pheochromocytomas

The tumor weights or sizes, malignancy, the expressions of hTERT mRNA and Ki-67 and follow up periods in 31 pheochromocytomas are listed in Table 2.

By the nested PCR methods, 5 of the 5 malignant samples were positive (sensitivity = 100%), 21 of 23 benign samples were negative (specificity = 91%) for hTERT mRNA detection.

We also determined the expression of the hTERT mRNA by real-time PCR. The mean values of hTERT mRNA by real time PCR in benign and malignant pheochromocytomas were 2 and 26 AU, respectively. The difference was not significant by the U-test.

MIB-1/Ki-67 Immunoreactivity in pheochromocytomas

All of the benign and suspected malignant tumors showed <1% proliferative activity, as measured by Ki-67/MIB-1 staining (Table 2). Four of 4 tumors classified as malignant showed ≥1% proliferative activity. However, strongly increased proliferative activity was observed in only one sample.

Table 2. Expression of hTERT mRNAs in the 31 pheochromocytomas

No.	Age/Sex	Diagnosis	weight (g) or size (cm)	pathology	hTERT mRNA	Ki-67	Follow-up (mo)	Relapse location
1	15/M	pheo	39 g, 5 × 4 × 3.5 cm	M	+	1%	death	local recurrence
2	27/M	pheo	193 g	M	+	5%	death	liver, bone
3	51/M	para	ϕ2 cm	M	+	22%	death	liver
4	76/M	pheo	ϕ4 cm	M	+	ND	death	local recurrence
5	61/F	pheo	700 g, 12.5 × 10 × 11 cm	?	+	7%	73	liver
6	48/M	para, vRH	5.5 × 5.0 × 3.0 cm	?	–	<1%	83	
7	49/M	pheo	643 g, 11.0 × 7.5 × 3.5 cm	?	+	<1%	35	
8	33/F	pheo	16 g, 3.6 × 4.0 × 2.5 cm	?	–	<1%	27	
9	72/M	pheo	53 g, ϕ3.5 cm		–	<1%	56	
10	62/F	pheo	5 × 5.5 × 6 cm		+	<1%	116	
11	55/M	pheo	18.5 g, 4.0 × 2.2 cm		–	<1%	61	
12	37/F	MEN IIA	7 × 4 × 3 cm		–	<1%	76	
13	70/M	pheo, diabetes	5 × 4 × 3 cm		–	<1%	37	
14	49/F	pheo, hypertension	5.5 × 5.0 × 4.5 cm		–	<1%	45	
15	33/F	para	ϕ2 cm		–	<1%	30	
16	24/M	para	58 g, 7.5 × 6.5 × 2.5 cm		–	<1%	128	
17	47/M	para, diabetes	5 × 6 cm		–	<1%	49	
18	50/F	pheo	120 g, 7.5 × 5.0 × 4.5 cm		–	<1%	86	
19	61/F	pheo	30 g, 5.0 × 3.5 × 4.0 cm		–	<1%	78	
20	23/F	pheo	17 g, 2.8 × 3.0 × 2.5 cm		–	<1%	70	
21	58/M	pheo	57 g, 2.6 × 4.3 × 5.8 cm		+	<1%	70	
22	61/M	pheo	4.5 × 2.5 × 3.5 cm		–	<1%	68	
23	36/F	pheo	3 × 3 × 2 cm		–	<1%	50	
24	30/F	pheo	80 g, 4 × 5 × 6 cm		–	<1%	50	
25	28/M	pheo	50 g, 3.5 × 5.3 × 4.0 cm		–	<1%	41	
26	49/M	para	49 g, 3.0 × 3.6 × 4.0 cm		–	<1%	41	
27	51/M	pheo	5 × 5 × 6 cm		–	<1%	39	
28	67/M	pheo	18g, 3.3 × 2.5 × 2.8 cm		–	<1%	29	
29	42/F	pheo			–	<1%	24	
30	30/M	pheo, diabetes	7.5 × 6.0 × 3.8 cm		+	<1%	24	
31	41/M	pheo, hyperlipidemia	18 g, 4.0 × 3.0 × 2.5 cm		–	<1%	23	

pheo = pheochromocytoma; para = paraganglioma; vRH = von Recklinghausen disease; M = malignant; ND = not done

hTERT mRNA expression in neuroblastomas

Age at diagnosis, status, Shimada classification, the expressions of hTERT mRNA and its isoforms, and N-myc mRNA in 16 neuroblastomas are listed in Table 3. Eight of 9 unfavorable neuroblastomas were diagnosed in patients older than one year.

By the RT-PCR methods, 9 of the 9 malignant samples were positive (sensitivity = 100%), and only 2 of 7 benign samples were negative (specificity = 29%) in the neuroblastoma tissues we examined.

We also determined the expression of the hTERT mRNA by real-time PCR in the samples. The mean values of hTERT mRNA by real time PCR in benign and progressive neuroblastoma were 203 and 497 AU, respectively. This difference was not significant (U-test).

N-Myc gene amplification is a prognostic marker [14], so we examined the correlation between the expressions of N-Myc mRNA and hTERT mRNA. The N-Myc mRNA expression correlated with hTERT mRNA expression($r = 0.534$, $p = 0.0317$).

Discussion

The hTERT mRNA detection by the nested PCR was satisfactory for discriminating malignant tumors in the pheochromocytomas we examined, but the full-length type of hTERT mRNA by real-time PCR was less informative because of lower sensitivity.

The no. 5 case of pheochromocytoma was diagnosed as malignant due to development of metastasis after the examination of hTERT mRNA expression.