

Short Communication

hTERT Gene Amplification and Increased mRNA Expression in Central Nervous System Embryonal Tumors

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High-level gains at 5p15, a chromosomal region including the human telomerase catalytic protein subunit (*bTERT*) gene, have been documented in several medulloblastomas. We therefore analyzed *bTERT* gene dosage in a group of medulloblastomas and other embryonal brain tumors using differential PCR. Amplification of the *bTERT* locus was detected in 15 of 36 (42%) tumors examined. To correlate gene amplification with message level, we used real-time quantitative PCR to measure *hTERT* mRNA in 50 embryonal brain tumors. *hTERT* mRNA was detected in all but one of these cases, and mRNA level correlated significantly with gene dosage ($r = 0.82$). Log-rank analysis of survival data revealed a trend toward poor clinical outcomes in patients with medulloblastomas containing high *hTERT* mRNA levels, but clinical follow-up was relatively short and the association was not statistically significant ($P = 0.078$). Comparative genomic hybridization was used to further analyze the tumor with the greatest *bTERT* gene dosage and mRNA level, a recurrent medulloepithelioma. *bTERT* was amplified in the recurrent tumor but not in the primary lesion, suggesting this locus can be involved in tumor progression. Our data indicate that *bTERT* gene amplification is relatively common in embryonal brain tumors, and that increased expression of *hTERT* mRNA may be associated with biologically aggressive tumor behavior. (*Am J Pathol* 2003, 162:1763–1769)

Brain tumors are the most common solid neoplasms that occur in childhood.¹ Among them, embryonal tumors are the most frequently encountered malignant lesions. In-

cluded in the current World Health Organization classification are medulloblastoma, supratentorial primitive neuroectodermal tumor (sPNET), atypical teratoid/rhabdoid tumor (AT/RT), and medulloepithelioma. The major molecular changes in central nervous system (CNS) embryonal tumors are only partially understood.² One gene commonly involved in carcinogenesis that has not yet been analyzed in a significant number of embryonal brain tumors is *hTERT*, which encodes the telomerase catalytic protein subunit.

Telomerase is an enzyme synthesizing the repetitive nucleotide sequence TTAGGG in telomeres. Human telomerase consists of an RNA subunit, (hTR), which is widely expressed, and a protein subunit, telomerase reverse transcriptase (hTERT), whose expression is more tightly regulated.^{3–5} The *hTERT* gene is located on chromosome 5 at 5p15.33; its expression is repressed in normal human somatic cells but is reactivated in most tumors (reviewed in⁶). In many neoplasms, increased telomerase activity is associated with poor clinical outcomes.^{6–11} While gene amplification has not generally been considered a common mechanism to increase telomerase activity in tumors, three recent reports have documented *hTERT* gene amplification in non-CNS primary tumors and tumor cell lines with concomitant increases in *hTERT* mRNA level.^{12–14}

Interestingly, high-level gains of chromosomal material in the 5p15 region have been detected in medulloblastomas, suggesting that the *hTERT* gene could be amplified in CNS embryonal tumors.^{15,16} Data on telomerase in these tumors is sparse. To our knowledge, *hTERT* gene dosage and mRNA levels have never been analyzed in

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Table 1. hTERT Molecular Analysis and Clinical Features in CNS Embryonal Tumors

Samples	Sex	Age	Status	Follow-up (months)	Histology	hTERT RT-PCR	hTERT/5qSTS
1	M	9	P	A 18	Classic MB	0.0	2.0
2	M	21	P	A 1	Classic MB	0.5	1.3
3	M	2	P	D 10	PNET	0.7	0.8
4	F	13	P	A 58	Classic MB	1.1	2.6
5	F	9	P	A 4	Nodular MB	1.2	NA
6	F	9	P	A 20	Anaplastic MB-F	1.3	0.6
7	F	2	P	A 9	Nodular MB	1.3	NA
8	F	4	P	A 8	Classic MB	1.8	6.0
9	M	5	P	A 5M	PNET	2.2	2.2
10	M	3	P	A 15	Nodular MB	2.4	NA
11	F	16	P	A 24	Anaplastic MB-F	2.7	1.2
12	M	23	P	A 75	Classic MB	3.5	1.9
13	F	<1	P	D 0	PNET	4.9	0.4
14	F	18	P	A 28	Anaplastic MB	5.4	0.4
15	M	11	R	D 27	Anaplastic MB	5.5	1.0
16	F	4	P	D 14	PNET	7.0	0.8
17	M	1	P	A 14	Nodular MB	8.5	NA
18	M	4	P	A 12	Nodular MB	10.0	NA
19	F	43	P	A 23	Anaplastic MB	13.8	1.7
20	M	4	P	A 9	Nodular MB	14.1	3.4
21	M	6	P	A 49	Anaplastic MB-F	15.5	0.3
22	M	11	R	A 73	Anaplastic MB	22.1	0.8
23	M	5	R	A 21	Medulloepithelioma	22.6	2.3
24	F	7	P	A 19	Anaplastic MB-F	26.9	NA
25	F	5	P	A 3	Anaplastic MB-F	28.6	0.5
26	M	1	P	D 0	PNET	30.3	2.1
27	M	2	P	D 17	Classic MB	31.0	NA
28	M	5	R	D 7	Anaplastic MB	31.8	3.1
29	M	6	P	A 22	Classic MB	36.5	NA
30	M	12	P	A 14	Classic MB	37.4	0.1
31	F	3	P	D 2	PNET	41.0	3.6
32	F	2	P	A 32	Anaplastic MB-F	48.1	0.4
33	F	16	P	A 28	Nodular MB	50.7	NA
34	M	6	P	A 25	Nodular MB	52.0	NA
35	M	9	P	D 9	Anaplastic MB	52.4	3.2
36	F	3	R	A 122	PNET	52.5	5.1
37	F	10	P	A 82	Classic MB	59.7	3.7
38	F	3	P	A 5	Classic MB	64.5	0.4
39	M	9	P	A 21	Nodular MB	64.8	NA
40	F	12	P	A 21	Anaplastic MB-F	65.2	NA
41	F	1	P	A 14	Anaplastic MB-F	88.9	NA
42	M	12	P	A 32	Medullomyoblastoma	125.1	1.2
43	M	39	R	A 58	Classic MB	125.2	6.0
44	F	4	P	D 3	PNET	129.5	2.5
45	M	26	P	A 21	Nodular MB	132.0	1.0
46	M	5	P	D 11	Anaplastic MB	133.0	1.6
47	F	55	P	A 9	Classic MB	144.1	NA
48	F	22	P	A 70	Pineoblastoma	152.3	3.2
49	M	15	R	D 116	Classic MB	154.0	3.7
50	F	1	R	D 31	Medulloepithelioma	603.6	17.6

* A, alive; D, deceased; F, focal; MB, medulloblastoma; P, primary; PNET, supratentorial primitive neuroectodermal tumor; R, recurrent.

medulloblastoma or other CNS embryonal neoplasms. In a recent review of telomerase in brain tumors, Falchetti and colleagues¹⁷ identified fewer than 10 CNS embryonal tumors from three studies in which telomerase enzymatic activity had been analyzed. We therefore used differential PCR and real-time RT-PCR to determine the relationship between *hTERT* gene copy number, hTERT mRNA expression, and clinical outcome in CNS embryonal tumors. We show that the *hTERT* gene is amplified in a significant number of cases, and that medulloblastoma patients with increased hTERT expression in their tumors have a trend toward worse clinical outcomes.

Materials and Methods

Clinical Samples

Tissue from 50 embryonal tumors resected between 1992 and 2002 at either the Johns Hopkins Hospital, Emory University Hospital, or L'hôpital Ste-Justine were used in these studies (Table 1). The cases included 15 anaplastic medulloblastomas, 13 classic medulloblastomas, 10 nodular medulloblastomas, 8 supratentorial PNET, 2 medulloepitheliomas, 1 medullomyoblastoma, and 1 pineoblastoma. The median age of patients was 7 years

(range, 6 months to 55 years) and 82% of the cases occurred in patients 18 years of age or less. The median follow-up for all patients was 19 months; the median follow-up in the medulloblastoma patients used for survival analysis was 20 months.

Molecular Analyses

DNA and total RNA were extracted from snap-frozen tumor tissues using TRIzol Reagent (Invitrogen, Carlsbad, CA) per the manufacturer's instructions. RNA was then treated with DNase and further purified using the RNeasy Protocol (Qiagen, Valencia, CA). Quantitative RT-PCR was performed using the ABI Prism 7700 Sequence Detector (Applied Biosystems, Weiterstadt, Germany) with TaqMan One-Step RT-PCR Master Mix reagents (Applied Biosystems) according to the manufacturer's instructions. PCR primers used for the analysis of *hTERT* expression were *hTERT*-1912F (forward primer: 5'-TACGTCGTGGGAGCCAGAAC-3') and *hTERT*-1978R (reverse primer: 5'-CCTTCACCCTCGAGGTGAGA-3'). The TaqMan probe *hTERT*-1933T (5'-TTC-CGCAGAGAAAAGAGGGCCGA-3') was labeled with 6-FAM and TAMRA. Amplicon length was 67 bp. Final concentration of primers was 0.5 $\mu\text{mol/L}$; final concentration of the TaqMan probe was 0.2 $\mu\text{mol/L}$. cDNA amount was standardized in each reaction using expression of β -actin according to the manufacturer's instructions (Applied Biosystems, Foster City, CA). All samples were analyzed in triplicate. Serial dilutions of total RNA from the D425 medulloblastoma cell line (ATCC) were used to generate standard curves and the expression of *hTERT* in all samples was calculated in relation to this. Gene copy number of *hTERT* was determined using differential PCR of sequences from *hTERT* on 5p and control sequences on 5q (5qSTS). The *hTERT* product was 260 bp long. The forward and reverse primers were 5'-GTGACCGTGGTTCTGTGTG-3' and 5'-GGGCCTCAGAGAGCTGAGTA-3' respectively. The 5qSTS fragment was 199 bp in length; forward and reverse primers were 5'-TTGGTCCCCAGTAACCTGATG-3' and 5'-TGTCCTAGGATCTTGCTCAG-3'. PCR conditions are available on request. Bands were visualized and quantitated using a Fluor-S Multiimager (Bio-Rad, Hercules, CA). CGH was performed as previously described.¹⁸ The SYSTAT 9 program was used for Kaplan-Meier survival curves and Breslow-Gehan log-rank analysis.

Results

The *hTERT* Gene is Amplified in 42% of CNS Embryonal Tumors

Amplification of the *hTERT* gene at 5p15.33 was detected using differential PCR following the methods of Waha and colleagues.¹⁹ The *hTERT*/5qSTS DNA ratios in the 36 tumors analyzed ranged from 0.1 to 17.6 (Table 1 and Figure 1). Experimental replicates were performed in selected cases and yielded similar results. Analysis of *hTERT*/5qSTS DNA ratios in 8 normal subjects produced

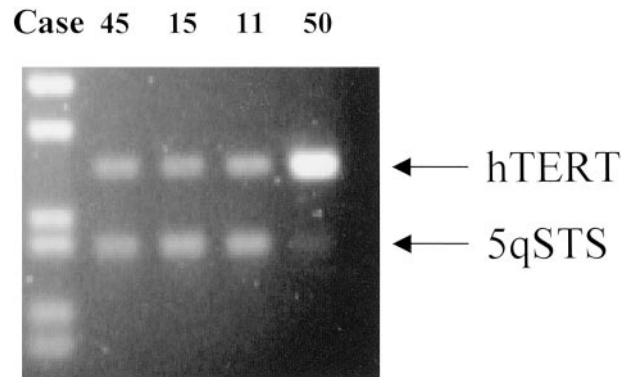


Figure 1. *hTERT* gene amplification detected by differential PCR. In cases 45, 15, and 11, *hTERT* and 5qSTS sequences are amplified in a roughly 1:1 ratio. In case 50, the *hTERT* product is 17-fold more abundant, consistent with gene amplification at the locus.

values ranging from 0.99 to 1.12, with a mean of 1.02. The *hTERT*/5qSTS DNA ratio threshold for amplification was therefore set at 2.17, which represents twice the mean ratio of the normal subjects plus three standard deviations, a standard established in earlier reports on this method.^{19,20} Using this cut-off, *hTERT* gene amplification was detected in 15 of 36 (42%) CNS embryonal tumors (Table 1). The *hTERT*/5qSTS ratios in amplified cases ranged from 2.2 to 17.6.

hTERT mRNA Expression Level and Clinical Factors

We used real-time RT-PCR to measure the level of *hTERT* in mRNA extracted from snap-frozen tumor tissue. *hTERT* mRNA was detected in 49 of 50 CNS embryonal tumors examined using this method. We divided these tumors into those with above-median *hTERT* expression, high *hTERT*, and below-median *hTERT* level, low *hTERT* (Table 1). *hTERT* mRNA expression levels did not distinguish between different types of embryonal tumors, eg, PNET, medulloepithelioma, medulloblastoma, etc. The nodular, classic, and anaplastic medulloblastoma subtypes were also distributed roughly evenly with respect to *hTERT* level. However, as has been observed in other tumor types, high levels of *hTERT* were more common in patients who died from their disease. In the combined group of embryonal tumor patients, 9 of 25 (36%) with above-median *hTERT* died, compared to 4 of 25 (17%) with below-median *hTERT*. The survival difference was more pronounced within the diagnostically uniform group of medulloblastoma patients. Only one medulloblastoma patient with low *hTERT* died from his disease, compared to five deaths in the high *hTERT* medulloblastoma group. However, perhaps because of the relatively short follow-up time, log-rank analysis of Kaplan-Meier survival curves did not confirm significantly worse clinical outcomes in the high *hTERT* group of medulloblastoma patients ($P = 0.078$; Figure 2).

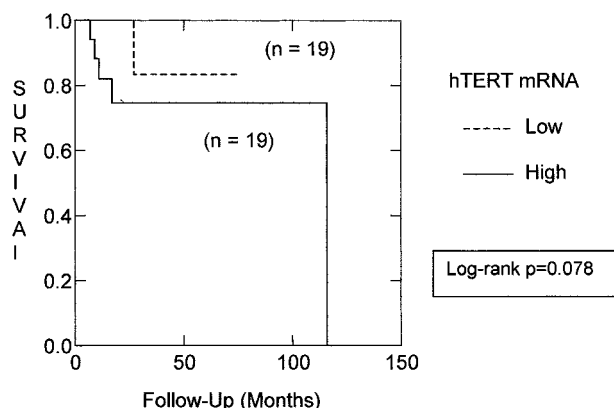


Figure 2. High *hTERT* expression is associated with poor clinical outcome. Log-rank analysis of Kaplan-Meier survival curves for patients with above or below median *hTERT* mRNA level reveals a trend toward better clinical outcomes for patients in the low *hTERT* group ($P = 0.078$).

Gene Amplification is Associated with Increased *hTERT* mRNA Expression

Gene amplification often results in increased expression from the amplified locus. We therefore correlated *hTERT* copy number with *hTERT* mRNA expression. Linear regression analysis showed a strong association between increasing *hTERT* DNA copy number and increasing *hTERT* mRNA level. When comparing *hTERT*/5qSTS ratio to *hTERT* mRNA, the correlation coefficient was 0.82, a statistically significant value for this number of samples ($P < 0.01$). However, the copy number of *hTERT* at the DNA level did not appear to correlate with survival, and log-rank analysis of survival in patients with tumors amplified or nonamplified at the *hTERT* locus revealed no trend toward worse outcomes in cases with *hTERT* amplification.

CGH Analysis of a Medulloepithelioma with *hTERT* Gene Amplification

We chose to further investigate the molecular alterations in case 50, which represented the most extreme example of *hTERT* gene amplification and had a very high *hTERT* message level as well. The *hTERT*/5qSTS DNA ratio and *hTERT* mRNA level were several-fold higher in this recurrent medulloepithelioma than in any other CNS embryonal tumor. The patient was a one-year-old white female diagnosed with a medulloepithelioma located in the left cerebellar hemisphere when she was 16 months old. Initial therapy consisted of cisplatin, oral VP-16, and craniospinal radiation followed by peripheral blood autologous stem cell transplant. Seventeen months after the first operation, the tumor recurred in the posterior fossa and was again resected. Despite additional therapy, the tumor continued to progress locally and the patient died three months later.

To confirm *hTERT* gene amplification, and to identify chromosomal changes occurring in medulloepithelioma, we analyzed DNA from both the original and the recurrent tumor. The chromosomal regions with DNA copy number

alterations in the primary and recurrent medulloepithelioma are illustrated in Figure 3A. There were 5 chromosomal alterations in the primary tumor and 17 in the recurrent lesion. The gains in the primary medulloepithelioma were localized to 3p13-22, 6p21.2-21.3, 14q24-qter, 15q15-25, and 20q; gains in the recurrent medulloepithelioma were at 1q32-qter, 3p14-22, 3q13-25, 5p14-pter, 7p15-q11, 9q34-qter, 10p, 12p, 14q, 15q24-qter, 17q21-qter, and 20q (Figure 3). There were two high-level gains in the recurrent tumor on chromosome 5p14-pter and 20q. Chromosome 5 profiles from the primary and recurrent tumor are shown in Figure 3B, where each line to the right or left of the central, dark band represents an incremental difference ratio of 0.25. Thus the difference ratio at the *hTERT* locus in the amplified recurrent tumor is over 2.0, a change equal to or higher than what we have previously observed in medulloblastomas highly amplified at the *myc* loci.¹⁵ There was no loss found in the primary medulloepithelioma. The losses in the recurrent medulloepithelioma involved 4p14-q28, 4q34-qter, 5q, 13q, and 18q12-qter.

Discussion

The *hTERT* gene is located on chromosome 5 at 5p15.33, a region that is sometimes gained in CNS embryonal tumors such as medulloblastoma.^{15,16} Recently, two groups demonstrated that the *hTERT* gene is amplified in several types of malignancy^{13,14}; however, to date, no information on *hTERT* amplification in solid tumors of the CNS has been published. We therefore analyzed *hTERT* gene dosage in a series of CNS embryonal tumors using comparative PCR, and identified *hTERT* gene amplification in 42% of the 36 samples examined. To our knowledge, this is the first analysis of *hTERT* gene copy number in CNS embryonal tumors, and one of only a few reports on *hTERT* gene amplification in any tumor type.

We identify a somewhat higher percentage of *hTERT* amplification in CNS embryonal tumors than previously reported for other tumor types. Using FISH, Zhang and colleagues¹³ found amplification of the *hTERT* gene in 31% of 26 tumor cell lines and 29% of 58 primary tumors examined, including lung tumors, cervical tumors, breast carcinoma, and neuroblastomas. The same group also described *hTERT* gene amplification in 24% of 88 cervical carcinomas, and an association between elevated *hTERT* protein expression and gene amplification.¹² A second group recently analyzed *hTERT* in lung cancer cell lines using several methods, documenting increased *hTERT* gene dosage in 6 of 20 samples. They also found a correlation between increased *hTERT* gene dosage, *hTERT* mRNA expression and telomerase activity.¹⁴

High *hTERT* expression or increased telomerase activity has correlated with clinical factors in a number of tumors, including Wilms' tumor, lymphoma, breast cancer, non-small-cell lung cancer, and urothelial carcinoma.¹¹⁻¹⁴ We therefore evaluated *hTERT* message levels in CNS embryonal tumors using quantitative RT-PCR, detecting mRNA in 49 of 50 tumors. In both the full group of CNS embryonal tumors and the clinically more homogeneous subset of individuals with medulloblastomas,

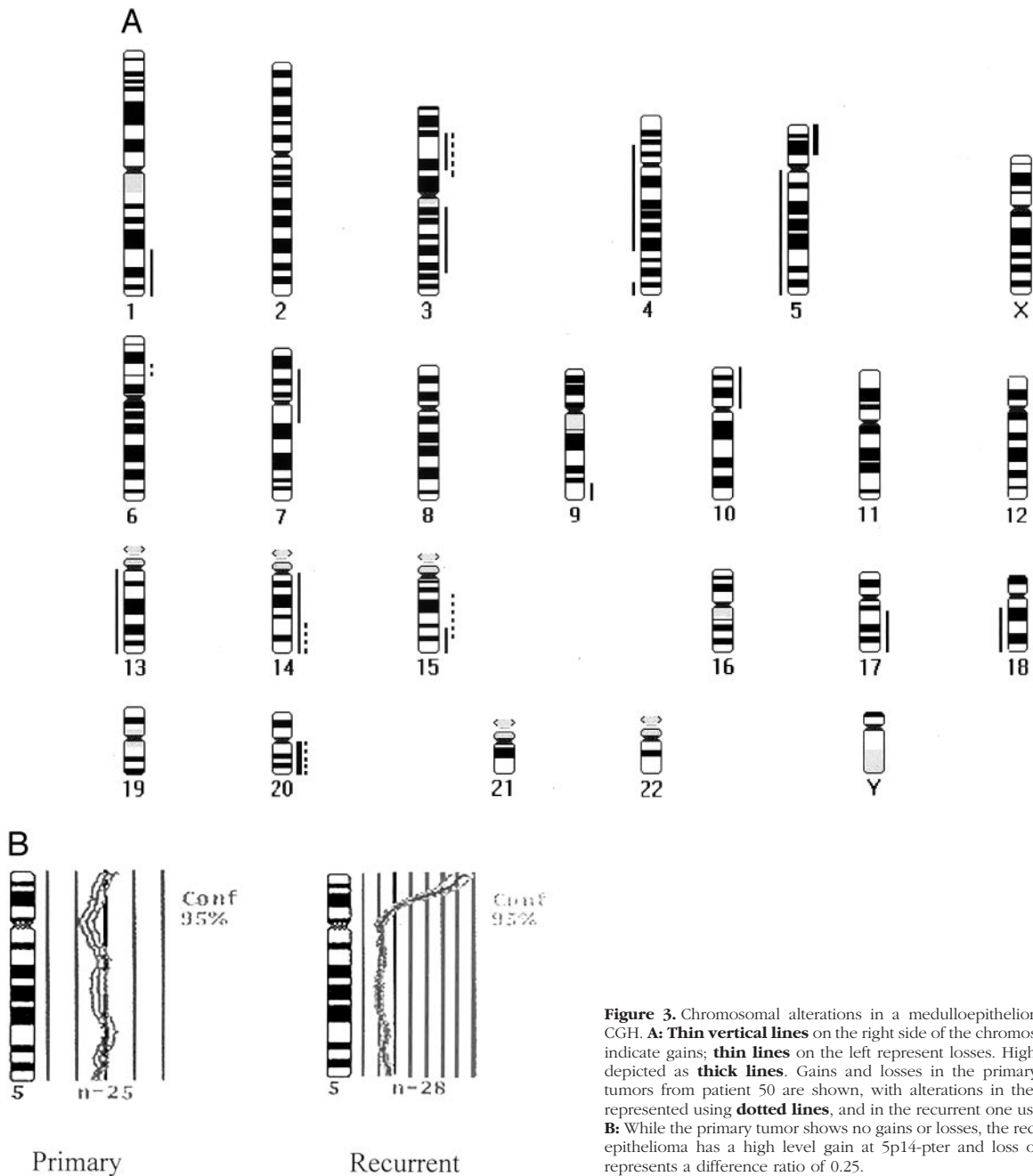


Figure 3. Chromosomal alterations in a medulloepithelioma detected by CGH. **A:** **Thin vertical lines** on the right side of the chromosome ideograms indicate gains; **thin lines** on the left represent losses. High-level gains are depicted as **thick lines**. Gains and losses in the primary and recurrent tumors from patient 50 are shown, with alterations in the primary lesion represented using **dotted lines**, and in the recurrent one using **solid lines**. **B:** While the primary tumor shows no gains or losses, the recurrent medulloepithelioma has a high level gain at 5p14-pter and loss of 5q. Each line represents a difference ratio of 0.25.

more than twice as many deaths were seen when tumors contained high levels of *hTERT* mRNA. However, log-rank analysis of survival curves did not confirm the significance of this difference ($P = 0.078$). Most of the patients involved in our study had their tumors removed within the last few years, and the follow-up available is therefore relatively short (median 20 months). This short follow-up period may have contributed to the lack of significance in our study, and additional investigations will be needed to confirm the prognostic power of *hTERT* expression in these CNS tumors.

It will also be important to correlate *hTERT* mRNA expression with telomerase enzymatic activity in CNS embryonal tumors, as the link between these remains uncertain. While

all of the telomerase positive brain tumors studied by Cabuy and de Ridder²¹ contained *hTERT* message, they failed to detect telomerase activity in some *hTERT*-expressing tumors. It has been suggested that the expression of non-functional *hTERT* splice variants can result in *hTERT* positive tumors that lack telomerase enzymatic activity.²² However, many authors report good correlations between *hTERT* message level and telomerase enzymatic activity.²³⁻²⁵

Intratumoral heterogeneity of *hTERT* expression and telomerase activity could have contributed to some of the differences reported in these earlier studies. Regional variations in *hTERT* expression and telomerase activity have been detected in some CNS gliomas.^{21,26} Similar heterogeneity at the level of *hTERT* DNA amplification or

hTERT expression could be present in the embryonal tumors we analyzed. We attempted to extend our studies of hTERT expression to the protein level using immunohistochemistry, which would also enable localization of expression to specific tumor regions. However, while the highly amplified medulloepithelioma was diffusely immunopositive for hTERT, in the other 13 cases we found no correlation between DNA, RNA and protein levels (data not shown).

Gene amplification in tumors often results in increased mRNA expression from the amplified locus. In CNS embryonal tumors, we found a significant positive correlation between increasing gene dosage and hTERT message level. In some individual cases gene amplification was not associated with elevated message level, a finding similar to that observed for other oncogenes. For example, Grotzer and colleagues²⁷ recently found that *c-myc* gene amplification was only associated with high mRNA levels in a subset of medulloblastomas. Similarly, we have identified medulloblastomas amplified at the *N-myc* locus, but with low levels of *N-myc* mRNA (unpublished results). In 20 CNS embryonal tumors we detected hTERT mRNA, but no amplification was found at the locus by differential PCR. Presumably, other transcriptional regulators direct hTERT expression in these tumors. *c-Myc*,^{28–30} Sp1,³¹ and estrogen³² have all been implicated as hTERT transcriptional regulators (reviewed in³³), and could play a role in the diverse hTERT message levels we detected. The large number of embryonal tumors we found with unamplified *hTERT* and low-level hTERT mRNA expression also suggests that only modest telomerase expression levels may be required for formation of these neoplasms. Indeed, it is possible that telomerase-independent mechanisms of transformation are operative in PNET, as has been suggested for CNS gliomas.³⁴

We examined chromosomal alterations in a recurrent medulloepithelioma with very high hTERT levels using CGH. High-level DNA gains were detected at 5p15, confirming the *hTERT* amplification identified using differential PCR. Interestingly, CGH analysis of the primary lesion from this patient detected no gains of 5p, indicating that *hTERT* amplification can first occur during tumor progression. However, most of the embryonal brain tumors with *hTERT* amplification in our study were not recurrent lesions, suggesting that as is the case in gliomas these alterations can occur early in tumorigenesis as well.³⁴ The overall number of chromosomal abnormalities was much higher in the recurrent medulloepithelioma than in the primary one, suggesting molecular progression can occur in these rare embryonal neoplasms. A similar accumulation of chromosomal changes has been observed in recurrence or metastasis of medulloblastomas.^{15,35} Thus our CGH analysis of this medulloepithelioma adds to the growing body of evidence suggesting CNS embryonal tumors can progress both microscopically and molecularly.

In conclusion, we have shown that the *hTERT* oncogene is amplified in a significant proportion of medulloblastomas and other CNS embryonal neoplasms. This gene amplification correlates with increased expression of hTERT mRNA, and implicates, for the first time, changes at the *hTERT* locus

in the evolution of primitive neuroepithelial tumors of the CNS. Finally, our correlation of hTERT expression with survival suggests hTERT message level could be a useful molecular prognostic marker.

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