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# Full-Length Telomerase Reverse Transcriptase Messenger RNA Is an Independent Prognostic Factor in Neuroblastoma

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**Telomerase activity (TA) is the most recently recognized prognostic factor in neuroblastoma, and its outstanding predictive power was documented by several studies. However, TA measurements require fresh tumor tissue that is not always available in daily clinical practice. We previously described a reverse transcriptase-polymerase chain reaction assay that we used to investigate the possible prognostic relevance of the telomerase catalytic subunit, hTERT, at the mRNA level. Because hTERT mRNA undergoes alternative splicing as a regulatory mechanism of TA, we discriminated between truncated and full-length hTERT transcripts. In a retrospective study on 124 neuroblastomas, 56 (45.2%) tumors showed spliced hTERT transcripts, whereas 30 (24.2%) contained full-length hTERT transcripts. The presence of both spliced and full-length hTERT transcripts was significantly associated with *MYCN* amplification. hTERT in general showed no correlation to other prognostic factors, ie, International Neuroblastoma Staging System stage, International Neuroblastoma Pathology classification grade, or age at diagnosis, whereas the presence of full-length transcripts was significantly associated with higher stages. The presence of any hTERT transcripts carried no significant prognostic information, yet full-length hTERT transcripts were highly predictive of poor outcome ($P < 0.0001$). In a multivariate analysis, full-length hTERT transcripts and International Neuroblastoma Pathology classification grade emerged as the sole independent predictors of event-free survival, with relative risks of 10.0 and 3.9, respectively. The strong statistical correlation of full-length hTERT transcripts with clinical outcome in neuroblastoma suggests that the reverse transcriptase-polymerase chain reaction analysis of hTERT transcripts may be equatable to TA measurements. Because this assay is well suited for archival material, it could become a useful adjunct in evaluating the prognosis of individual neuroblastoma cases.**
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Neuroblastoma, the most common extracranial solid tumor of infancy, is a highly heterogeneous disease. Histologically, some neuroblastomas present as totally undifferentiated tumors, but all degrees of differentiation up to benign mature ganglioneuroma may be seen. This morphological variability is mirrored by the biological behavior that covers a spectrum ranging from high aggressiveness with early metastatic spread to spontaneous regression. Because the clinical course of neuroblastoma is notoriously difficult to foresee, much work has been spent on the search for prognostic factors. Among these, the patient's age at diagnosis and clinical stage,[1] *MYCN* amplification,[2] deletion of the gene locus 1p32-36,[3] and gains of genetic material on chromosome 17q[4] have become recognized as reliable predictors of disease outcome.[5]

Recently, telomerase activity (TA) was shown to be a prognostic indicator of unusual predictive strength in neuroblastoma.[6,7] Although the relative level of TA carries prognostic information in a variety of tumors,[8,9] this correlation seems to be particularly accentuated in neuroblastoma. Earlier investigations had demonstrated that TA may discriminate between prognostically different subsets of neuroblastoma,[6,7,10] and in a most recent study of a large cohort, TA emerged as an independent predictor of clinical outcome with greater prognostic impact than the *MYCN* status and even clinical stage.[7]

Because shortening of telomeres, as it occurs in the course of aging, likely represents a control mechanism limiting the number of possible cell divisions, entry into senescence is normally triggered by intracellular signaling when a critical telomere length is reached. To overcome this hurdle and maintain a sufficient telomere length, rapidly dividing cells such as germ cells and tumor cells use telomerase, an enzyme capable of synthesizing telomeric DNA sequences and appending them to the chromosomal tips.[11,12] Accordingly, TA was detected in the majority of malignant tumors of both epithelial and mesenchymal origin.[13] In an important subset of

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neuroblastomas, however, down-regulation of TA might induce growth arrest and differentiation, which would explain the unusually close correlation between TA levels and clinical outcome. Neuroblastomas with sustained TA might therefore become a choice target for telomerase-directed therapy.[14,15]

The clinical application of TA measurements is nevertheless hampered by the fact that the telomeric repeat amplification protocol (TRAP) assay[16] requires well-preserved fresh or snap-frozen tumor tissue that is rarely available in a routine setting. It has therefore been proposed to assess hTERT expression as a substitute for TA measurements in archival material.[17–19] Indeed, telomerase is considered to constitute a ribonucleoprotein complex, of which three essential components have been identified: human telomerase reverse transcriptase (hTERT), an internal RNA strand (hTR), and an RNA-binding protein (hTEP1).[20–24] Expression of the catalytic subunit hTERT seems to closely correlate with TA in most tumors[25] whereas the other components seem to be expressed indifferently in tumors and normal tissue.[20,26]

We recently investigated the correlation between hTERT expression and TA in a small series of neuroblastomas from which fresh tissue was available.[27] Interestingly, the overall expression of hTERT showed only a limited correlation to TA, which was found to be attributable to alternate splicing of hTERT mRNA. Although we failed to detect TA in the absence of hTERT transcripts, expression of any hTERT transcripts was not sufficient to elicit TA. Indeed, a number of tumors with low or absent TA only contained truncated hTERT transcripts whereas tumors with high TA consistently exhibited the full-length mRNA. When analyzed in this way, specific hTERT splicing showed a correlation with disease outcome comparable to that obtained by TA measurements.

To verify these preliminary results on a larger scale, we now conducted a retrospective investigation on a cohort of 124 neuroblastoma cases. Formalin-fixed and paraffin-embedded specimens were assayed for the presence of different hTERT transcripts, and the results were correlated to clinical follow-up data. We will show that it is possible to detect the various hTERT transcripts by reverse transcriptase-polymerase chain reaction (RT-PCR) on archival material, and that the presence of full-length hTERT mRNA is a strong independent prognostic indicator for neuroblastoma when compared with established predictors of prognosis.

## Patients and Methods

### Patients

The initial cohort consisted of 137 patients enrolled in the German neuroblastoma trial and diagnosed between 1983 and 1999 who had not received chemotherapy before diagnostic biopsy. Of these 137 patients, 65 were male and 72 were female. Informed consent was obtained from their parents. The median age at diagnosis was 12 months (range, 0 to 191 months). The clinical stages, according to the International Neuroblastoma Staging System (INSS),[28] were as follows: 22 patients (16.1%) had stage 1 disease, 17 patients (12.4%) had stage 2, 32 patients (23.4%) had stage 3, 39 patients (28.5%) had stage 4, and 27 patients (19.7%) had stage 4S (special). Histological typing and grading were done according to the International Neuroblastoma Pathology classification (INPC).[29] Classified in this manner, the series comprised 105 neuroblastomas (32 undifferentiated, 34 poorly differentiated, and 39 differentiating) and 32 ganglioneuroblastomas (8 nodular and 24 intermixed), corresponding to 71 cases (52.5%) with favorable and 66 cases (47.5%) with unfavorable histology. The cohort was thus representative with respect to epidemiology and patient distribution, and comparable to other published series.

The patients were treated according to the guidelines set up by the German Neuroblastoma Trial (NB85, NB90, NB95-S, and NB97), with stratification criteria as described elsewhere.[30] According to the estimated risk, 50 patients received high-dose chemotherapy, 53 were treated with a standard regimen, and 34 were only observed (no therapy). The median follow-up period was 132.8 months (range, 6 to 251.1 months).

### RNA Isolation and RT-PCR

Paraffin-embedded tumor tissue was analyzed as follows: five 10-$\mu$m sections from representative areas of the tumor were cut from paraffin blocks and collected in a 1.5-ml sterile polypropylene tube. Paraffin was removed with two changes of xylene and two changes of absolute ethanol (both from Sigma, Deisenhofen, Germany). The remaining tissue pellet was air-dried and digested overnight at 42°C in 100 $\mu$l of 10 mmol/L Tris/HCl, pH 8.0, 0.1 mmol/L ethylenediaminetetraacetic acid, pH 8.0, 1% sodium dodecyl sulfate, and 500 $\mu$g proteinase K (Roche Molecular Biochemicals, Mannheim, Germany). After lysis the solution was mixed with 1 ml of Trizol (Life Technologies, Inc., Gaithersburg, MD) and RNA isolation was performed as indicated by the manufacturer. Finally, the RNA was digested with DNase I (Roche Molecular Biochemicals) for 15 minutes at 37°C.

One-fourth of the RNA solution was reverse-transcribed using an RNA PCR kit (Perkin Elmer, Langen, Germany) and subjected to PCR as recommended by the manufacturer. As a polymerase we used 1.25 U/50 $\mu$l of Amplitaq Gold (Perkin Elmer), which must be activated by heating to 95°C for 10 minutes. This procedure avoids primer-dependent PCR artifacts. RNA integrity was ascertained by RT-PCR amplification of the ubiquitously expressed EWS mRNA with primers EWS 188 (5′ gatgtcagctatacccaggc 3′) and EWS 382 (5′ tgccatatgcagactgagct 3′) and an annealing temperature of 65°C. Two different regions of hTERT mRNA were amplified in parallel: primers hTERT 1784 (5′ cggaagagtgtctggagcaa 3′) and hTERT 1910 (5′ ggatgaagcggagtctgga 3′) amplify a 145-bp segment present in all transcripts (general hTERT transcripts), whereas the primers hTERT 2172 (5′ tgtacttgtcaaggtggatgtg 3′) and hTERT 2350 (5′ gtacggctggaggtctgtcaag 3′) were designed to amplify a 200-bp seg-

ment unique to the full-length hTERT transcripts. The annealing temperature was 60°C for hTERT 1784/1910 and 68°C for hTERT 2172/2350. Forty-three PCR cycles were run on a GeneAmp PCR System 2400 (Perkin Elmer). PCR products were separated on a 2% (w/v) agarose gel, stained with Sybr Green (Molecular Probes, Leiden, The Netherlands) and visualized by UV transillumination. To warrant detection of full-length hTERT transcripts in cases with low copy numbers, a seminested PCR with 1 $\mu$l of the first amplification product was run for another 40 cycles. The nested lower primer was hTERT 2280 (5′ ccttctggaccacggcatac 3′), yielding an amplicon of 128 bp. All PCR products were verified by automated sequencing on an AbiPrism 310 Genetic Analyzer (Perkin Elmer). Negative controls were obtained by omission of reverse transcriptase and by analysis of RNA from striated muscle.

Quantitative RT-PCR was done using the TeloTAGGG hTERT quantitation kit on a Light Cycler (both from Roche Molecular Biochemicals) with 200 ng of total RNA from a hyperplastic tonsil. Serial dilutions of tonsil RNA were then subjected to our RT-PCR assay and analyzed by agarose gel electrophoresis. Two hundred ng of genomic DNA instead of RNA served as negative control.

### *MYCN Amplification Analysis*

*MYCN* copy number was determined by Southern blotting of *Eco*RI-digested tumor DNA and subsequent hybridization with a probe complementary to the *MYCN* gene (AppligeneOncor, Illkirch, France) and a single copy gene (*β-globin*) as a control for potential hyperdiploidy.[31] In a portion of the cases, fluorescence *in situ* hybridization for *MYCN* was used in conjunction with a chromosome 2 centromeric probe.[27]

### *Statistics*

The SPSS 10.0 statistical software package run on a Pentium III PC was used for all calculations. Categories of variables were compared by means of chi-square statistics and Fisher's exact test. The Mann-Whitney *U*-test and the Kruskal-Wallis nonparametric analysis of variance were used to compare grouped continuous variables. Survival analysis was conducted with regard to event-free survival (EFS), any recurrence or progression in form of local tumor extension, distant metastasis, or tumor-related death being considered as an event. Patients who died from disease-unrelated causes before the occurrence of a specific event were considered as censored data. Cumulative probabilities of EFS were computed using the Kaplan-Meier product-limit method, and the significance level of the difference between risk groups was determined with the log-rank test. Statistical significance was assumed at $P < 0.05$. All factors achieving significance in the univariate survival analysis were further analyzed in a multivariate model using Cox's regression analysis for multiple proportional hazards with a forward conditional approach. In this analysis, the Wald test served to assess statistical significance.

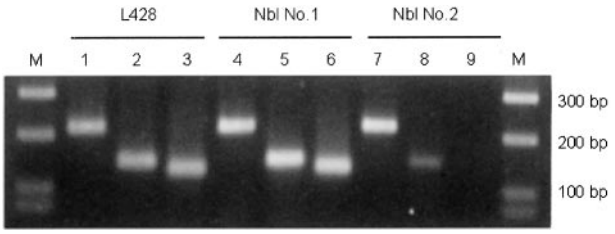


**Figure 1.** RT-PCR results of L428 cells and two neuroblastomas (Nbl). Positive controls (EWS transcripts in **lanes 1**, **4**, and **7**). **Lanes 2**, **5**, and **8** show general (145 bp) and **lanes 3**, **6**, and **9** show full-length (seminested PCR-products, 128 bp) hTERT transcripts. Note the presence of general hTERT transcripts and the lack of full-length hTERT transcripts in neuroblastoma no. 2. M, molecular weight marker.

To obtain a homogeneous cohort, the univariate analyses were conducted on the series of 124 cases for which information on hTERT was available. However, the *MYCN* status could be determined only in 116 of these 124 cases. Thus, when the cases with complete sets of data were included, 116 cases remained in the multivariate analysis. Given the small number of events in stage 1 tumors, the localized stages (1 and 2) were considered as one group in the survival analysis to enable an estimation of significance. Patient age was analyzed as a binary variable with a cutpoint at 12 months.

## *Results*

### *RNA Isolation and RT-PCR*

Our initial procedure for RNA isolation consisted of homogenization of deparaffinized tumor sections in the chaotropic reagent Trizol and further incubation overnight, as described previously.[27] This proved sufficient for most of the tumor samples, particularly for tissue blocks not older than 5 years. To increase the number of reactive tissue blocks, we performed an additional proteinase digestion step. With this approach, amplifiable RNA was obtained in 124 cases. RNA integrity was ascertained by amplification of the ubiquitously expressed EWS mRNA. We designed primers to amplify a PCR product of 212 bp to identify cases with advanced RNA degradation (shorter fragments) and to obtain comparable amplification conditions for both hTERT PCRs (Figure 1). Using this strategy, 56 of the 124 cases showed hTERT transcripts, and 68 cases were negative. Full-length hTERT transcripts were found in 30 cases, whereas the other 26 cases contained only truncated transcripts. Eighteen cases showed full-length hTERT transcripts after the first PCR run and 12 cases, most of which were older than 5 years, needed the seminested approach. The necessity for seminested PCR was independent of clinical, pathological, and molecular tumor characteristics, but is was significantly associated with the age of the paraffin blocks ($P = 0.036$). Because of the inclusion of a seminested PCR run for full-size hTERT transcripts with a relatively short amplification product of 128 bp, the percentage of hTERT-positive cases using older tissue blocks was identical to that obtained from more recent cases. As expected, all cases with full-size

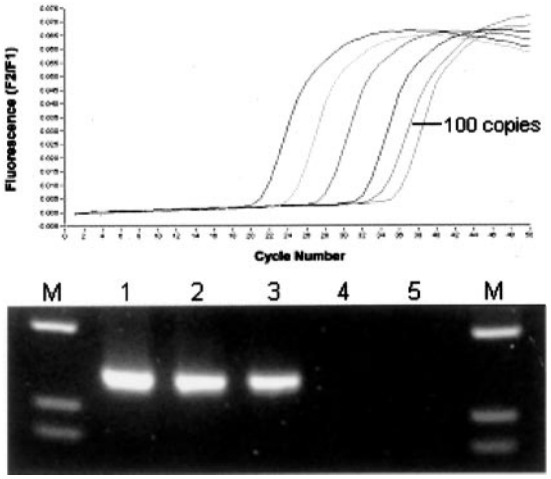


**Figure 2.** Quantitation of hTERT transcripts using Light Cycler chemistry (**top**) and detection threshold for the assay used in our study (**bottom**). Two hundred ng of tonsil RNA produced a signal corresponding to 100 copies of hTERT. The other curves show amplification of different copy numbers of hTERT-RNA for calibration of the assay (from **left** to **right**: $5 \times 10^5$, $4.5 \times 10^4$, $4 \times 10^3$, $4 \times 10^2$, $4 \times 10^1$ hTERT copies). Nested RT-PCR (**bottom**) detects 500 copies (**lane 1**), 50 copies (**lane 2**), and 5 copies (**lane 3**) of hTERT mRNA but yields no signal with one copy (**lane 4**) or 200 ng of genomic DNA (**lane 5**).

hTERT transcripts also exhibited positivity with primers against general hTERT transcripts. To determine the sensitivity of our assay, serial dilutions of tonsil RNA with a known amount of full-length hTERT mRNA were tested. With the seminested PCR strategy, five copies of full-length hTERT-transcripts were detectable whereas our assay failed to produce a signal with one copy or with genomic DNA alone (Figure 2).

## *MYCN Amplification Analysis*

*MYCN* analysis was successful in 116 of the 124 cases with known hTERT status. Twenty-six cases (22.4%) with *MYCN* gene amplification were identified, the other 90 showing no change in copy number. *MYCN* amplification was slightly more frequent in undifferentiated and poorly differentiated tumors, but the association was not statistically significant ($P = 0.11$). In contrast, *MYCN* amplification correlated significantly with advanced tumor stages (stages 3 and 4, $P < 0.0001$) and with unfavorable INPC histology ($P = 0.016$).

## *Correlation of hTERT Transcripts with Clinical and Molecular Data*

The presence of any hTERT transcripts revealed no correlation to the patient's age at diagnosis, INSS stage, or INPC grade (all $P = 0.6$). This lack of correlation was partly verified for the full-length hTERT transcripts (Table 1). Although the median age was higher in children with full-length transcripts (32 months) than in those with splice variants (13 months), the difference of age did not attain statistical significance ($P = 0.09$, Mann-Whitney *U*-test). There also was a mere tendency toward an association with INPC unfavorable histology ($P = 0.12$). In contrast, full-length hTERT transcripts were more frequently observed in advanced stages whereas their prevalence was low in stage 4S ($P = 0.003$). Also, both types of hTERT transcripts were significantly more prevalent in the group of *MYCN*-amplified tumors ($P = 0.001$ and 0.002 for all transcripts and for the full-length transcripts, respectively, by Fisher's exact test).

**Table 1.** Distribution of hTERT in Subgroups of Neuroblastoma Defined by Patient Age, INSS Stage, *MYCN* Status, and INPC Grade

| | No full-length hTERT | Full-length hTERT | *P* value |
|---|---|---|---|
| Age | | | |
| ≤1 year | 46 | 10 | 0.14 |
| >1 year | 48 | 20 | |
| INSS stage | | | |
| 1 | 17 | 1 | 0.003 |
| 2 | 14 | 2 | |
| 3 | 23 | 7 | |
| 4 | 19 | 16 | |
| 4S | 21 | 4 | |
| *MYCN* | | | |
| Nonamplified | 75 | 13 | 0.002 |
| Amplified | 16 | 12 | |
| INPC | | | |
| Favorable histology | 53 | 12 | 0.12 |
| Unfavorable histology | 41 | 18 | |

## *Survival Analysis*

Tumor relapse or progression occurred in 53 (42.7%) of the 124 patients for whom complete follow-up data were available. The univariate survival analysis confirmed the relevance of established prognostic indicators to EFS. Thus, INSS stage, INPC grade, age at diagnosis, and the *MYCN* status were highly significant, whereas gender was not relevant ($P = 0.62$). Interestingly, the mere presence of hTERT transcripts did not have any apparent impact on prognosis ($P = 0.12$, Figure 3A), whereas if full-length transcripts were present, an adverse outcome could be predicted with high statistical significance ($P < 0.0001$, Figure 3B). Table 2 summarizes the clinical and pathological characteristics and the molecular genetic data in relation to EFS.

In a breakdown by grade and stage, the presence of full-size hTERT transcripts remained significant in tumors with both favorable and unfavorable INPC histology ($P = 0.031$ and $P < 0.0001$, respectively) and in clinical stages 1 and 2 (grouped), 3 and 4 (all $P < 0.002$). In 4S tumors the presence of full-length hTERT transcripts did not show any association with EFS, probably because of the small number of events in this stage. In addition, full-length hTERT transcripts were a highly significant prognosticator in both age groups (both $P < 0.001$). Most remarkably, however, the presence of full-length hTERT transcripts contributed to a refined prognosis in tumors with and without *MYCN* amplification (both $P < 0.005$).

The *MYCN* status, on the other hand, failed to provide prognostic information beyond that supplied by full-length hTERT transcripts. Not only was there generally a lesser divergence between the survival curves defined

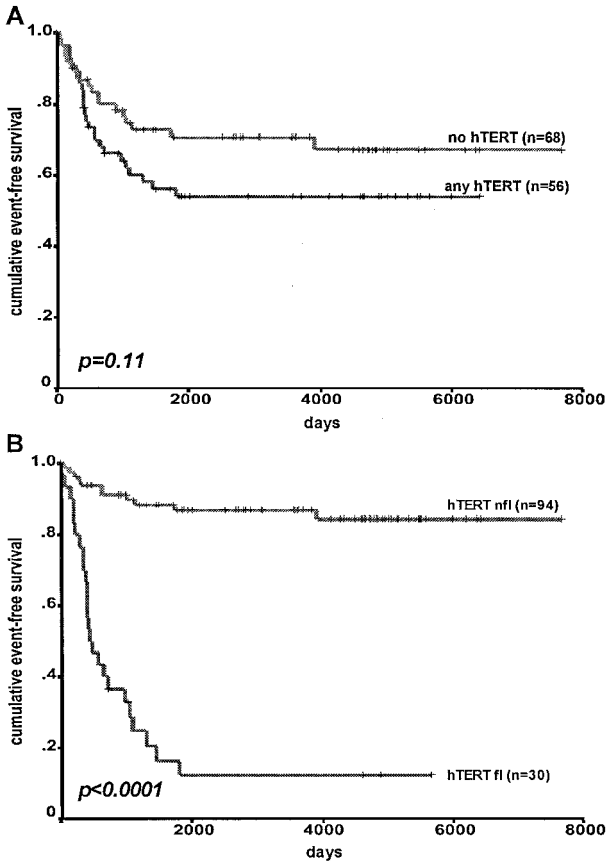


**Figure 3.** Kaplan-Meier analysis of EFS in 124 neuroblastoma patients with respect to general hTERT transcripts (**top**) and the presence of full-length hTERT (hTERT fl) transcripts (**bottom**).

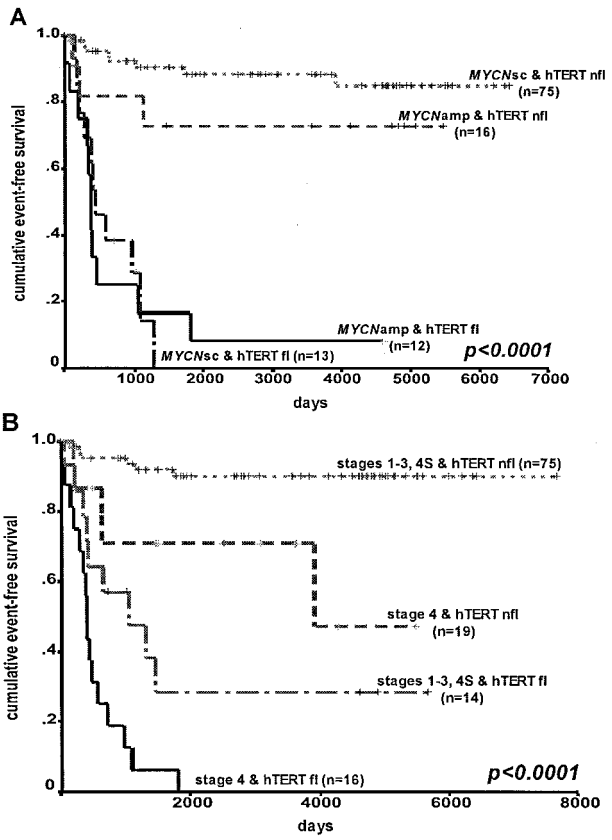


**Figure 4.** Kaplan-Meier analysis of the impact of hTERT transcripts on EFS in subgroups of neuroblastoma patients defined by the *MYCN* status (**A**) and INSS stage (**B**). To obtain sufficient sample sizes, stages 1, 2, 3, and 4S were grouped and compared to stage 4. sc, single copy; amp, amplified; fl, full-length transcripts; nfl, no full-length transcripts. For **A** and **B**, *P* is <0.0001.

**Table 2.** Clinical, Pathological, and Molecular Characteristics of the Tumors in This Study, and Corresponding Rates of Event-Free Survival (EFS) with Significance Levels

| | *n* | EFS ± SE | *P* value |
|---|---|---|---|
| Age | | | |
| ≤1 year | 63 | 0.69 ± 0.05 | 0.019 |
| >1 year | 61 | 0.45 ± 0.06 | |
| Gender | | | |
| Male | 56 | 0.56 ± 0.06 | 0.62 |
| Female | 68 | 0.58 ± 0.06 | |
| INSS stage | | | |
| 1 | 18 | 0.93 ± 0.07 | <0.0001 |
| 2 | 16 | 0.79 ± 0.09 | |
| 3 | 30 | 0.73 ± 0.07 | |
| 4 | 35 | 0.15 ± 0.06 | |
| 4S | 25 | 0.72 ± 0.08 | |
| INPC | | | |
| Favorable histology | 63 | 0.79 ± 0.06 | 0.0001 |
| Unfavorable histology | 61 | 0.42 ± 0.07 | |
| *MYCN* | | | |
| Nonamplified | 90 | 0.66 ± 0.05 | <0.0001 |
| Amplified | 26 | 0.29 ± 0.08 | |
| hTERT | | | |
| No transcripts | 68 | 0.67 ± 0.07 | 0.11 |
| Any transcripts | 56 | 0.54 ± 0.07 | |
| hTERT | | | |
| No full length | 94 | 0.84 ± 0.05 | <0.0001 |
| Full length | 30 | 0.12 ± 0.07 | |

by the *MYCN* status, but *MYCN* also lacked significance in low tumor stages (1 to 3, data not shown). It should be noted that in stages 1 and 2, none of the tumors with normal *MYCN* copy numbers progressed, yet this result did not approach statistical significance ($P = 0.38$).

To further corroborate the prognostic value of hTERT transcripts, we investigated their influence in subgroups defined by patient age at diagnosis, INSS stage, INPC grade, and *MYCN* amplification. In children aged 1 year or less, the probability for EFS was 92% for tumors without, and 45% in tumors with full-length hTERT. Similarly, 77% of the children older than 1 year survived event-free when full-length transcripts were absent, in contrast to 0% of the children with full-length hTERT ($P < 0.0001$). INPC-favorable tumors without full-length hTERT had an EFS probability of 91% in contrast to 51% when full-length transcripts were present. An INPC unfavorable histology in the absence of full-length hTERT was associated with a 76% probability for EFS, whereas all patients with an INPC unfavorable grade and full-length transcripts relapsed ($P < 0.0001$).

The corresponding data for the *MYCN* status and INSS stage are illustrated in Figure 4. In tumors without full-length hTERT, the EFS probability was not markedly reduced when *MYCN* was amplified, whereas the presence of full-length transcripts predicted an equally poor out-

**Table 3.** Independent Prognostic Factors for the Event-Free Survival Selected in a Multivariate Analysis Including the Patient's Age at Diagnosis, INSS Stage, INPC Grade, the *MYCN* Status, and Differential Splicing of hTERT mRNA

| | Relative risk (95% confidence interval) | *P* value |
|---|---|---|
| hTERT full length | 10.0 (4.16–24.0) | <0.0001 |
| INPC unfavorable histology | 3.9 (1.3–11.7) | 0.015 |

come in tumors with and without *MYCN* amplification ($P < 0.0001$). Also, when *hTERT* was transcribed at full length, the outcome in the stages 1 to 3 and 4S (grouped) was poorer than in stage 4 tumors without full-length transcripts, and 90% of the children with stage 4 disease and full-length hTERT relapsed within 2 years ($P < 0.0001$).

One hundred and sixteen cases with complete data sets were available for the multivariate analysis. In a model including all covariates (ie, patient age, INPC tumor grade, INSS stage, *MYCN* status, and full-length hTERT transcripts), the presence of full-length hTERT transcripts, and INPC unfavorable histology were selected, in this order, as the only independent predictors of reduced EFS (Table 3). When treatment modalities were included as covariates in the multivariate model, they were not selected as an independent predictor of EFS nor did their inclusion noticeably alter the results of the multivariate analysis.

## Discussion

There is mounting evidence indicating that, among established prognostic indicators, TA is an outstanding predictor of clinical outcome in neuroblastoma.[6,7,10,27] In light of the current endeavors to develop telomerase-inhibiting drugs, this might be of tremendous therapeutic consequence. However, TA measurements require fresh or snap-frozen tumor tissue, which is rarely available in routine pathology. We therefore searched for a simple and reliable procedure that might substitute the TRAP assay in archival material without loss of prognostic information.

A common belief is that TA depends on the presence of hTERT transcripts and many recent investigations replaced the TRAP assay by a PCR for hTERT mRNA,[17–19] which is well suitable for archival material. Nevertheless, in two recent studies the comparison of TA and hTERT transcripts unexpectedly yielded contradictory results in up to 20% of the cases examined.[7,32] The situation became even more confusing when quantitative data on hTERT transcription were correlated to the clinical outcome.[7] This apparent contradiction is nevertheless readily explained by alternate splicing of hTERT transcripts, a mechanism of telomerase regulation encountered during embryonal development.[33] In keeping with this observation, we have shown that in neuroblastoma overall hTERT expression poorly correlates with TA, which attained significant levels only when full-length hTERT transcripts were present.[27] It therefore seemed reasonable to explore the prognostic value of full-length hTERT transcripts in a clinical setting.

With this in view, we conducted a retrospective study on 137 neuroblastoma patients, and analyzed the relevance of different hTERT transcripts with regard to clinical data. To this end we developed a RT-PCR system for the selective detection of both general and full-length hTERT transcripts. The latter was accomplished by using primers that span the putative splice sites. To compensate for low-level expression, we performed an additional PCR step with a nested primer, and, additionally, all tissue blocks were checked for RNA integrity by the amplification of a ubiquitously expressed mRNA. The relatively short amplification products (200 bp for first round PCR and 128 bp for seminested PCR) allowed a reliable detection of full-length hTERT transcripts in 124 of the 137 tumors (>90%), even in paraffin-embedded specimens as old as 15 years.

We found hTERT transcripts in 45% of the cases with intact RNA. This result is in line with data from other investigators, who reported 49%[7] and 51%[32] hTERT-positive tumors. Full-length hTERT transcripts were detected in 24% of our cases, and on the basis of our previous observations,[27] these cases were presumed to possess TA. In support of this idea, we were able to demonstrate a highly significant correlation between the presence of full-length hTERT transcripts and poor outcome. This finding was further corroborated by the multivariate survival analysis, which identified full-length hTERT transcripts as the strongest independent predictor of disease outcome, followed by INPC grade.

Our results are entirely comparable to those reported in a recent study,[7] which were obtained by means of the TRAP assay on fresh material. This supports our idea that the identification of full-length hTERT transcripts may adequately replace TA measurements whenever fresh tumor tissue is unavailable. The aforementioned study[7] nevertheless reported TA in 29% of the samples examined by TRAP assay. This discrepancy may merely be attributable to the investigation of different patient cohorts. Considering that our assay is sensitive enough to detect at least five copies of full-length hTERT transcripts, and owing to the use of a stringent positive control (an RNA with a longer sequence than the product under investigation), we expect a negligible rate of false-negative results although we cannot rule out that a few cases with less than five hTERT copies might elicit low levels of TA. If so, a quantitative assessment of hTERT expression levels by real-time PCR, which is nonetheless delicate and sometimes impossible on archival specimens, might further strengthen prognostic assessments.

On the other hand, we cannot fully exclude false-positive results as normal lymphocytes are known to possess full-length hTERT transcripts even though they do not display TA unless they are stimulated for clonal expansion.[34] However, we observed an admixture of lymphocytes in less than 3% of several hundred neuroblastic tumors we had the opportunity to review, most of which were ganglioneuroblastomas or ganglioneuromas (our unpublished data). Because we failed to detect full-

length hTERT transcripts in these subgroups we premise that the percentage of lymphocytes is too low to bias the analysis. There is also evidence indicating that TA may be negatively regulated by yet unidentified factors even in the presence of full-length hTERT transcripts,[27] which again might be a source of false-positive results in the present analysis. The close congruence in the survival analysis of our data and those obtained by TA measurement[7] nevertheless implies that the percentage of inaccurately categorized cases is of little importance in larger cohorts.

The relevance of our approach is also underscored by the quandaries evinced by quantitation of hTERT transcripts. Indeed, in the previously quoted study on neuroblastoma,[7] tumors with low hTERT levels, for instance, had a worse 5-year EFS than those with intermediate to high hTERT levels, and tumors with minimal hTERT levels did better than those without *hTERT* transcription. These somewhat confounding observations imply that the biological significance of full-length hTERT transcripts overrides that of the quantitative expression of all hTERT splice variants.

The inclination of *hTERT* transcription to occur in *MYCN*-amplified tumors, which was more pronounced in the group with general hTERT transcripts ($P = 0.014$, Wilcoxon test), is well in line with previous reports.[7,10,32] As the *hTERT* promoter contains a MYCN-binding site, overexpression of MYCN is likely to arouse *hTERT* transcription. Accordingly, TA was reported to be present in the majority of *MYCN*-amplified neuroblastomas. In light of our present data, however, alternate splicing of hTERT transcripts does not seem to be triggered by MYCN but rather reflects a heterogeneity in the group of *MYCN*-amplified tumors. This is illustrated by the fact that the demonstration of full-length hTERT transcripts is able to further refine subgroups in neuroblastomas with and without *MYCN* amplification.

Another interesting angle is that, in patients with full-length hTERT transcripts, the median age at diagnosis was 32 months in contrast to 13 months in the group with predominantly spliced transcripts. In accordance with our previous study,[27] these data bridge with the neuroblastoma development scheme proposed by Hiyama and colleagues.[6] It seems that the embryonal mechanism of TA regulation may remain active for a limited lapse of time during the postnatal period. A persistence of this mechanism is likely to identify tumors that could be considered as embryological remnants with good prognosis. Sustained TA, on the other hand, would disclose a propensity to fatal disease progression.

In conclusion, our data indicate that the presence of full-length hTERT transcripts in neuroblastoma is a strong predictor of disease outcome. The fact that the presence of full-length hTERT transcripts was unrelated to some clinical and histological parameters in our study implies that, even beyond the results of the multivariate survival analysis, full-length hTERT may be a truly independent prognostic factor. Our data further suggest a potential biological equivalence between TA and full-length hTERT transcripts. In this way, this new criterion not only appears to overrule established prognostic factors but also might prove useful for the selection of patients who might stand to profit from future anti-telomerase therapy designs. Compared with the highly specific TRAP assay, a great advantage of hTERT transcript analysis is its applicability to both fresh and formalin-fixed tumor tissue, making it an easy tool for the clinical oncologist. Further studies are nevertheless needed to clarify the relationship between hTERT transcript analysis and TA regarding tumor prognosis.

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