# Telomerase Activity and Telomerase Subunits Gene Expression Patterns in Neuroblastoma: A Molecular and Immunohistochemical Study Establishing Prognostic Tools for Fresh-Frozen and Paraffin-Embedded Tissues

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*Purpose:* We have recently demonstrated that telomerase activity (TA) is an independent prognostic factor in neuroblastomas. In the present study, the prognostic impact of TA and gene expression of the three major telomerase subunits is evaluated by molecular and immunohistochemical techniques in fresh-frozen and paraffin-embedded tissues.

*Patients and Methods:* One hundred thirty-three neuroblastomas of all stages were analyzed for TA. The TA levels of 75 neuroblastoma cases were correlated with gene expression of telomerase subunits hTRT, human telomerase RNA (hTR), and telomerase protein 1 (TP1) by quantitative reverse transcriptase polymerase chain reaction (RT-PCR), using an innovative approach on the LightCycler instrument (Roche Diagnostics, Mannheim, Germany). For selected cases, the applicability of RT-PCR and immunohistochemistry for hTRT expression analysis was investigated in paraffin-embedded tissues. TA and subunit expression patterns were correlated with traditional prognostic indicators and disease outcome.

*Results:* TA was present in a total of 39 (29.3%) of 133 neuroblastomas and in 31 (29.8%) of 104 initial neuroblastomas without cytotoxic pretreatment. TA was significantly correlated with both event-free and overall survival ($P < .0001$). Furthermore, we found a significant correlation between expression levels of TA and hTRT ($P < .0001$) as well as hTR ($P < .001$). Multivariate analysis revealed only TA and tumor stage but not serum lactate dehydrogenase, *MYCN* amplification, or age at diagnosis as independent prognostic factors.

*Conclusion:* The significant correlation with clinical outcome strongly recommends that analysis of TA be incorporated into the clinical investigation of each individual neuroblastoma at the time of diagnosis. Because the mere presence or absence of TA without further quantification is sufficient basis for predicting disease outcome, the telomeric repeat amplification protocol assay could be complemented with but not replaced by analysis of hTRT or hTR expression.

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N EUROBLASTOMA represents the third most common pediatric cancer and is the most common solid extracranial neoplasm of infancy and childhood, responsible for approximately 15% of all childhood cancer deaths. The clinical hallmark of neuroblastoma is heterogeneity, which is associated with a wide variety of likelihood of tumor progression. The most significant clinical predictors of outcome are age and stage, although an assessment of the patient's prognosis solely on the basis of clinical parameters is limited because of diverse biologic tumor behavior and subsequent survival rates, even at distinct clinical stages.[1,2] Therefore, the heterogeneity of this tumor entity requires cellular and molecular markers to distinguish the different biologic characteristics.

Established molecular markers such as *MYCN* copy number and loss of heterozygosity for chromosome 1p32-36 may help in predicting poor outcome in all age and stage groups.[3-7] Recent cytogenetic data also suggest that gain of chromosome arm 17q is associated with an adverse outcome in neuroblastoma.[8,9] A promising new marker is telomerase, a multicomponent ribonucleoprotein enzyme, which has been implicated in cell immortalization and tumorigenesis in almost all human tumors, including neuroblastomas.[9-14] Recently, we have shown in multivariate analysis that telomerase activity (TA) is an independent prognostic factor in neuroblastoma, which may prove useful in conjunction with established markers for assessing the individual patient's prognosis.[15] However, it is not known whether elevated TA in neuroblastoma may simply be a marker of escape from cellular senescence or whether markedly increased activity levels may

be associated with genomic instability and an increased likelihood of further mutational events. Furthermore, to date, the frequency and location of telomerase-positive cells within neuroblastomas is not known.

Recently, the catalytic subunit hTRT (or hTERT, hEST2, TP2) of human telomerase was cloned, and detection of hTRT expression by reverse transcriptase polymerase chain reaction (RT-PCR) revealed a strong correlation with TA by the telomeric repeat amplification protocol (TRAP) assay in the majority of tumors that have been examined thus far.[16-18] Other components of the telomerase holoenzyme complex, such as human telomerase RNA (hTR) and telomerase protein 1 (TP1, TLP1, hTEP1), seem to be expressed in both normal and tumor tissues, and expression levels of these genes revealed no or only limited correlation with TA.[19-21] A more precise definition of the molecular mechanism of TA by quantitative detection of RNA and protein subunit expression may therefore determine whether TA independently predicts for aggressive clinical behavior in neuroblastoma.

In the present study, we extended our previous studies and analyzed TA in 133 neuroblastoma tumors according to stage classification using our modified and highly specific TRAP assay. Furthermore, TA levels were correlated with expression of the catalytic subunits by quantitative RT-PCR for 75 cases using an innovative approach for real-time, on-line detection of reaction kinetics on the LightCycler instrument (Roche Diagnostics, Mannheim, Germany). For selected cases, the feasibility of RT-PCR performed on RNA from formalin-fixed and paraffin-embedded tissues was analyzed for expression of the catalytic subunit hTRT by using the TITAN system (Roche Diagnostics). Finally, we report the successful in situ immunohistochemical detection of anti-hTRT antibody-reactive protein in paraffin-embedded neuroblastoma sections. For the evaluation of prognostic impact, special attention was paid to event-free survival (EFS) and overall survival (OS) and the occurrence of other molecular genetic alterations in TA-positive and -negative neuroblastomas.

## PATIENTS AND METHODS

### Patients

Primary tumor specimens from 133 patients diagnosed between 1988 and 1998 were analyzed. Clinical and follow-up data were available for all patients from the German Neuroblastoma Trial. Median age at diagnosis was 13.6 months (range, 0.2 to 180.7 months). Among the 133 neuroblastoma patients, 72 patients were male and 61 were female. Staging was performed according to the International Neuroblastoma Staging System criteria and included 32 stage 1, 13 stage 2 (2.1, two patients; 2.2, 11 patients), 23 stage 3, 41 stage 4, and 24 stage 4S cases. The primary tumor was located in the adrenal glands in 81 patients, in the abdominal sympathetic trunk in 29, in the thoracic region in 15, and the cervical region in seven. One patient had primary tumor both in the

adrenal gland and in the pelvic region. Metastatic sites in stage 4 cases were bone marrow in 35 patients, bone in 24, liver in 12, distant lymph nodes in 11, intracranial in five, and the gluteal muscle in one. In stage 4S patients, metastases were found in bone marrow in 10 patients, in the liver in 15, in the skin in two, and the epididymis in two. Patients were treated according to the German Neuroblastoma Trial NB85 (n = 10), NB90 (n = 113), NB95-S (n = 5), or NB97 (n = 5), with treatment stratification as described elsewhere.[22] Surgery was performed either at diagnosis (n = 104), after pretreatment (n = 23), later in the course of disease in 4S patients without cytotoxic treatment (n = 4), or in recurrent tumor after cytotoxic pretreatment (n = 2). Median follow-up was 5.2 years (range, 1.2 to 10.6 years). Five-year OS of all patients was $0.77 \pm 0.04$ (localized disease stages 1 to 3, $0.92 \pm 0.03$; stage 4, $0.29 \pm 0.09$; stage 4S, $0.92 \pm 0.06$). Five-year EFS was $0.69 \pm 0.04$ (localized disease, $0.88 \pm 0.04$; stage 4, $0.26 \pm 0.09$; stage 4S, $0.73 \pm 0.10$).

### Protein Preparation and Telomerase Assay

Neuroblastoma specimens were derived from snap-frozen material of primary tumor biopsy or surgical resection collected in a tumor bank and stored at $-80°C$ until use. A tumor content exceeding 70% was mandatory for all of the samples included in this study. Extractions of cellular proteins of neuroblastoma tumor specimens were prepared as previously described.[23] The extracts were rapidly frozen in liquid nitrogen and stored at $-80°C$ until further use. The protein concentrations were measured by use of the Coomassie Protein Assay Reagent (Bio-Rad Laboratories, Inc, Hercules, CA) on an enzyme-linked immunosorbent assay reader and adjusted to 2 $\mu g/\mu L$.

For in vitro detection of TA, a modified version of the TRAP assay was used, including an internal PCR-amplification control from a commercially available kit (TRAPeze telomerase detection kit; Oncor, Gaithersburg, MD), resulting in a 36–base pair product that was coamplified with telomerase-elongated products in a competitive manner. Each analysis included the telomerase-positive Ewing's tumor cell line VH64, a heat-inactivated control (telomerase-positive control incubated at 85°C for 10 minutes before reaction), and a negative control (3-[3-cholamidopropyl-dimethylammonio]-1-propane-sulfonate lysis buffer instead of sample protein). Electrophoresis and semiquantitative analysis of chromatogram peaks generated from photodetector signals were performed as described[23] on an automated laser-fluorescence sequencer (ALFexpress; Pharmacia, Freiburg, Germany). TRAP data were analyzed in blind-trial fashion without any knowledge of tumor stage, patient age, EFS, or OS.

In each tumor sample, the TRAP procedure was performed twice, and levels of TA proved to be consistent in all of the samples included in this study. To rule out the prevalence of telomerase and Taq-polymerase inhibiting factors, mixed tissue samples that contained telomerase-negative and -positive extracts were prepared. The accuracy of this semiquantitative analysis was further determined by several dilution series (1:2, 1:5, 1:10, 1:50) for both telomerase-positive and -negative samples.

### Assessment of Tissue Sample Preservation by RT-PCR of RNA Content

To minimize the probability of false-negative results with a lack of or low TA resulting from tissue degradation and necrosis, RNA derived from frozen sections and corresponding to the tumor material subjected to the TRAP procedure was amplified by RT-PCR for *GAPDH* as an indirect marker of tissue integrity. Briefly, a 297–base pair fragment of the human *GAPDH* gene was amplified with primers 5′-CACCCAT-

GGCAAATTCCATGGC-3′ and 5′-GCATTGCTGATGATCTTGAG-GCT-3′, corresponding to GenBank positions 213 to 234 and 509 to 487, respectively (GenBank accession no. M33197). Only specimens that were positive for *GAPDH* mRNA were included in the present study.

## Quantitative Real-Time RT-PCR for Expression of hTRT, hTR, and TLP-1

Total RNA was isolated from fresh-frozen tissue using the TRI-SOLV system (Biozol, Eching, Germany) in accordance with the manufacturer's protocol. RNA was treated with DNase (Eurogentec, Seraing, Belgium) and purified with the RNeasy Mini Prep Kit (Qiagen, Hilden, Germany). cDNA was synthesized from approximately 2 μg of RNA using the FirstStrand Synthesis Kit (Amersham Pharmacia, Freiburg, Germany) with dT18 primers. Relative concentrations of cDNA samples were evaluated by quantitative RT-PCR of *GAPDH* performed on the LightCycler (Roche Diagnostics) followed by analysis of the gene expression of each telomerase subunit by the same procedure. To amplify the cDNA, 5-μL aliquots of reverse-transcribed cDNA (diluted 1:5) were subjected to PCR amplification in 20 μL containing a final concentration of 2 to 3 mmol/L MgCl$_2$, 0.5 μmol/L of each primer (see below), and 2 μL of ready-to-use LightCycler DNA Master SYBRGreen I (Roche Diagnostics; 10×, containing TaqDNA polymerase, reaction buffer, dNTP mix with dUTP instead of dTTP, SYBRGreen I dye, and 10 mmol/L MgCl$_2$). For hot-start PCR, 0.16 μL/sample of TaqStart Antibody (Clontech, Heidelberg, Germany) was added to the amplification mixture before the addition of primers and template cDNA. hTRT mRNA was amplified using the primer hTRT-1 (5′-cggaagagtgtctggagcaa-3′) corresponding to GenBank positions 1785 to 1804 and hTRT-2 (5′-catggactacgtcgt-gggag-3′) corresponding to positions 1961 to 1980 (GenBank accession no. AF018167). hTR RNA was amplified using the primer hTR-1 (5′-cctaactgagaagggcgtaggc-3′) corresponding to GenBank positions 849 to 870 and hTR-2 (5′-ctagaatgaacggtggaaggcg-3′) corresponding to positions 961 to 940 (GenBank accession no. AF047386). For TP1 mRNA amplification, primer TP1-1 (5′-tcaagccaaacctgaatctgag-3′) corresponding to GenBank positions 7483 to 7504 and TP1-2 (5′-cccgagtgaatctttctacgc-3′) corresponding to positions 7746 to 7726 (GenBank accession no. U86136) were used. The reaction conditions were initial denaturation at 95°C for 2 minutes followed by 35 to 40 cycles of denaturation at 95°C for 1 second, annealing at 60°C for 5 seconds, and extension at 72°C for 6 to 8 seconds (depending on amplification length). Quantitative analysis was performed using the LightCycler Software (Roche Diagnostics) using a real-time fluorogenic detection system for a kinetic rather than end point approach as on conventional agarose or polyacrylamide gels. The generation of quantitative data was based on different PCR kinetics of samples with different levels of target gene expression. We used a relative quantification in which the expression levels of the neuroblastoma samples were compared to the data from Ewing's tumor telomerase-positive cell line VH64 in a geometric dilution series (1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64). The graph of the linear regression and calculation of the regression coefficient r (which is −1 in Fig 1) served to confirm accuracy and reproducibility of this approach. For analysis, the quantitative amounts of hTRT, hTR, and TP1 gene expressions standardized on *GAPDH* expression were grouped as follows: negative, 0; less than 1:64 dilution of positive cell line, 1; 1:64 to 1:32 dilution of positive cell line, 2; 1:32 to 1:16 dilution of positive cell line, 3; 1:16 to 1:8 dilution of positive cell line, 4; 1:8 to 1:4 dilution of positive cell line, 5; 1:4 to 1:2 dilution of positive cell line, 6; and more than 1:2 dilution

of positive cell line, 7. As shown in Fig 1, the quantitative data were calculated from the kinetic curve of the PCR. For this approach, the identity and specificity of the PCR product was confirmed by melting curve analysis, which is part of the LightCycler analysis program. The specific melting point of the PCR product was correlated with its molecular weight as determined by agarose gel electrophoresis and fragment length analysis on an automated laser-fluorescence sequencer (ALFexpress; Pharmacia).

## Northern Blots for hTRT Expression

Northern blots were performed using cRNA antisense and sense probes. Probes corresponding to GenBank positions 1785 to 2199 (GenBank accession no. AF018167) were synthesized by a PCR-based in vitro transcription with a T7-RNA polymerase (Roche Diagnostics) using NTP mixes with 10 mmol/L of each NTP and 3.5 mmol/L digoxigenin-labeled UTP (for antisense probe) or 6.5 mmol/L UTP (for sense probe), respectively, to generate a product of 414 bases. Primers were 5′-taatacgactcactatagggagcggaagagtgtctggagcaa-3′ (sense, T7) and 5′-ccgtcacatccaccttgaca-3′ (antisense) for generating the antisense probe and 5′-cggaagagtgtctggagcaa-3′ (sense) and 5′-taatacgactcactat-agggagccgtcacatccaccttgaca-3′ (antisense, T7) for generating the sense probe.

RNA was isolated from 10$^8$ cells of a telomerase-positive Ewing's tumor or neuroblastoma cell line, mRNA enrichment was performed by polyA selection (Dynal, Hamburg, Germany) according to standard protocols, and RNA concentration was determined. Equal amounts of RNA were electrophoresed on 1.2% agarose-2.2 mol/L formaldehyde gels and transferred onto a nylon membrane according to standard protocols. Blots were hybridized overnight at 68°C and washed according to standard procedures. Detection of specific signals was performed by chemiluminescence with antidigoxigenin-FAB fragments AP and CSPD solution (Roche Diagnostics) and exposed to Kodak XAR film (Eastman Kodak, Rochester, NY) for at least 6 hours at room temperature. Afterwards, blots were stripped and rehybridized with a *GAPDH* probe for evaluating RNA amounts.

## One-Tube RT-PCR From Paraffin-Embedded Tissue

RT-PCR to detect the *GAPDH* and the hTRT (see primer sequences above) mRNAs were performed in separate approaches using the TITAN RT-PCR system (Roche Diagnostics), according to the manufacturer's protocol. The reaction was performed in a total volume of 50 μL containing 1 μg RNA extracted from paraffin-embedded tissue, 10 pmol of each primer, 20 units of RNase inhibitor, dNTP (0.2 mmol/L each), and the components of the kit, including MgCl$_2$-containing buffer, DTT, and the enzyme mixture (AMV RT, Taq, and Pwo DNA polymerase). RT proceeded at 50°C for 30 minutes, followed by denaturation at 94°C for 2 minutes. Amplification parameters were 35 cycles of denaturation at 94°C for 30 seconds, annealing at primer-dependent temperatures for 30 seconds, and elongation at 68°C for 60 seconds, followed by a final extension of 5 minutes at 68°C. Starting from the 11th cycle, the elongation time was increased by 5 seconds per cycle. RT-PCR products were separated by electrophoresis and visualized on 8% polyacrylamide gels by the silver-staining method.

## Antibody and Immunohistochemical Detection of hTRT Protein

As recently described,[24] a part of hTRT cDNA corresponding to hTRT nucleotides 1687 to 2042 (GenBank accession no. AF015950)
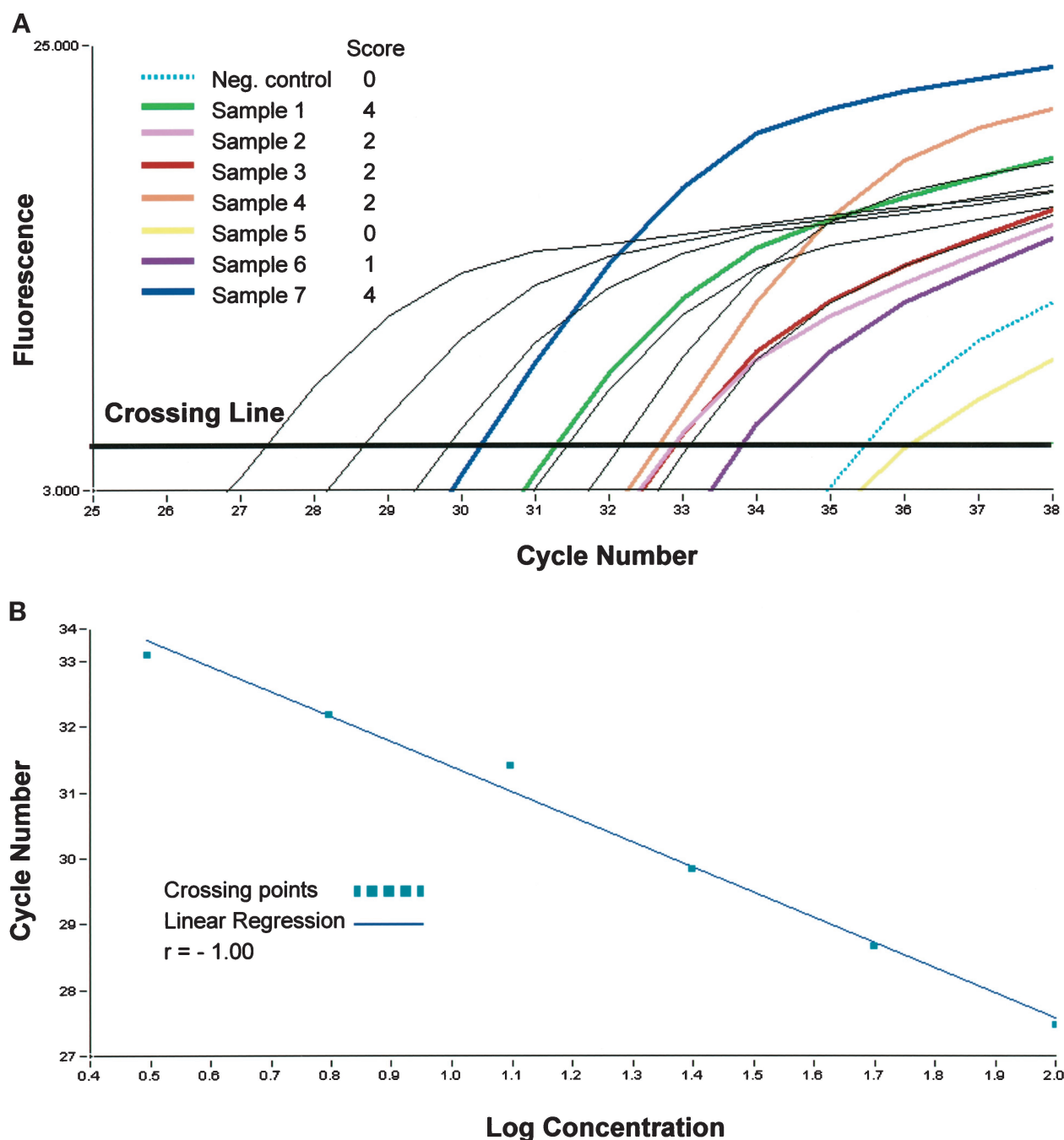
**Fig 1.** Quantitative real-time PCR of hTRT expression. Standard dilution series are shown as thin, gray curves and neuroblastoma samples with various degrees of hTRT expression as thick, colored curves. Linear regression of standard dilution series indicates accuracy and reproducibility of the analysis.

was fused with the glutathione S-transferase gene for the production of fused recombinant protein in *Escherichia coli*. The hTRT recombinant protein was used as an antigen to immunize rabbits. The resulting antirecombinant protein antibody named EST1.0 was purified by affinity chromatography and used for the immunohistochemical detection of hTRT protein in routinely formalin-fixed and paraffin-embedded tissues. The omission of the primary antibody served as a negative control ,and paraffin-embedded sections of the telomerase- and hTRT-positive Ewing's tumor cell line VH64 served as a positive control. The specificity of hTRT immunostaining had been determined previously by preabsorption of the antibody with an excess of specific antigen.

Tumor cells with distinct red nuclear staining were regarded as hTRT-positive. Cell counts were made at ×400 magnification using a 10 × 10 eyepiece grid on a Leitz microscope (Leitz, Wetzlar, Germany) in at least five neighboring viewing fields (yielding 500 to 1,000 cells). The hTRT labeling index was determined as the percentage of positive cells among the total number of cells counted in each case and scored as follows: less than 1%, negative; 1% to 10%, low; 10% to 50%, intermediate; more than 50%, high.

## MYCN Gene Amplification Analysis

The distinction between either amplified or unamplified MYCN copy numbers was achieved by Southern blot analysis with hybridization of EcoRI-digested tumor DNA to a MYCN probe (pNB-1) as described elsewhere,[25] and fluorescence in situ hybridization by means of methods previously described.[26] The mean number of MYCN copies were determined by simultaneous cohybridization with a chromosome 2–specific alpha-satellite probe, allowing the direct enumeration of the number of copies of chromosome 2 relative to MYCN. MYCN copy number was defined as amplified if more than three copies of the MYCN gene were present.[27,28]

## Deletion Analysis of Chromosome 1p36

High-molecular-weight DNA was isolated from matched microdissected tumor and blood samples and used as a template for PCR amplification, with specific sets of oligonucleotides at the minisatellite or microsatellite regions within 1p31-36 (DIS76, DIS214, DIS234, DIS255, DIS200, DIS203, DIS236) as described elsewhere.[29,30]

## Statistical Analysis

Kaplan-Meier[31] estimates for EFS were calculated using SPSS statistical software (Version 7.0; SPSS, Inc, Chicago, IL) and compared using the log-rank test. For multivariate analysis, Cox's proportional hazards regression model was used at the level of alpha = 5%.[32] Recurrence, progression of disease, and death were counted as events. Death resulting from therapy complications was not counted as an event but was censored for survival and EFS analysis. Fisher's exact test or the $\chi^2$ test was used where appropriate to examine a potential association between dichotomous study variables of interest.

## RESULTS

A total of 133 neuroblastomas of all stages were analyzed in blind-trial fashion by a modified nonradioactive and semiquantitative TRAP assay. TA was present in 39 tumor tissues (29%), including four (17%) of 24 stage 4S neuroblastomas, 25 (61%) of 41 stage 4 neuroblastomas, eight (35%) of 23 stage 3 neuroblastomas, 0 (0%) of 13 stage 2 neuroblastomas, and two (6%) of 32 stage 1 neuroblastomas. Of the 104 tumors resected initially without cytotoxic pretreatment, 31 (29%) showed TA. After cytotoxic pretreatment (either initially or after tumor recurrence), TA was present in eight (28%) of 29 tumors. For 128 of 133 tumors included in this study, MYCN analysis was available; relevant MYCN amplification with more than three copies[27]

**Table 1. Comprehensive Data of 133 Neuroblastomas**

| | No. of Patients | 5-Year OS | P | 5-Year EFS | P |
|---|---|---|---|---|---|
| All samples | | | | | |
| TA-positive | 39 | 0.34 ± 0.09 | | 0.25 ± 0.08 | |
| TA-negative | 94 | 0.93 ± 0.03 | < .0001 | 0.86 ± 0.04 | < .0001 |
| Initial* samples | | | | | |
| TA-positive | 31 | 0.42 ± 0.10 | | 0.33 ± 0.10 | |
| TA-negative | 73 | 0.97 ± 0.02 | < .0001 | 0.92 ± 0.03 | < .0001 |
| Stage 1, 2, 3 | | | | | |
| TA-positive | 10 | 0.67 ± 0.16 | | 0.56 ± 0.17 | |
| TA-negative | 58 | 0.97 ± 0.02 | .0001 | 0.93 ± 0.03 | .0001 |
| Stage 4S | | | | | |
| TA-positive | 4 | 0.50 ± 0.25 | | 0.25 ± 0.22 | |
| TA-negative | 20 | 1.00 ± 0.00 | .0007 | 0.83 ± 0.10 | .0031 |
| Stage 4 | | | | | |
| TA-positive | 25 | 0.09 ± 0.08 | | 0.07 ± 0.07 | |
| TA-negative | 16 | 0.61 ± 0.17 | .0043 | 0.55 ± 0.14 | .0024 |
| MYCN | | | | | |
| > 3 copies | 16* | 0.54 ± 0.14 | | 0.48 ± 0.14 | |
| ≤ 3 copies | 86* | 0.87 ± 0.04 | .0013 | 0.80 ± 0.05 | .0036 |
| LOH 1p36 | | | | | |
| Yes | 5* | 0.60 ± 0.22 | | 0.60 ± 0.22 | |
| No | 25* | 0.83 ± 0.08 | .34 | 0.80 ± 0.08 | .41 |
| Age | | | | | |
| > 1 year | 70 | 0.64 ± 0.06 | | 0.58 ± 0.06 | |
| ≤ 1 year | 63 | 0.90 ± 0.04 | .0004 | 0.80 ± 0.05 | .0056 |

Abbreviation: LOH, loss of heterozygosity.
*Initial samples, without cytotoxic pretreatment.

could be observed in 21 (16%) of 128 samples. Data on loss of heterozygosity analysis of chromosome arm 1p were available for a subset of tumors (Table 1).

### Correlation of TRAP and Survival for All Stages Combined

The correlation of TA status determined by the TRAP assay with the clinical data of 133 patients included in the German Neuroblastoma Trial was established under simple-blind study conditions to prevent any subjective impact on the estimation of TA levels. Clinical outcome was significantly correlated, with the presence of TA distinguishing between TA-positive (without further discrimination of low- and high-grade TA) and -negative tumors. For all tumors (n = 133), 5-year EFS for 94 patients with telomerase-negative tumors was 0.86 ± 0.04 versus 0.25 ± 0.08 for 39 patients with telomerase-positive tumors at $P <$ .0001. Five-year OS for patients with telomerase-negative tumors was 0.93 ± 0.03 versus 0.34 ± 0.09 for telomerase-positive tumors at $P <$ .0001. For initial samples (n = 104), 5-year EFS for 73 patients with telomerase-negative tumors was 0.92 ± 0.03 versus 0.33 ± 0.10 for 31 patients with telomerase-positive tumors at $P <$ .0001 (Fig 2A). Five-year OS for patients with telomerase-negative tumors was 0.97 ± 0.02 versus 0.42 ± 0.10 for patients with telomerase-positive tumors at $P <$ .0001 (Fig 2B). Numbers for EFS and OS for localized disease (stages 1, 2, and 3), stage 4, and stage 4S are listed in Table 1.

### Comparison of TA With MYCN Status

MYCN status was available for a total of 128 patients. The tumors of 107 patients had MYCN copy numbers ≤ three. Patients with TA-positive tumors and MYCN copy numbers ≤ three in their tumors (n = 22) taken before cytotoxic treatment were found to have a 5-year EFS of 0.31 ± 0.11, whereas those with TA-negative tumors (n = 85) reached a 5-year EFS rate of 0.87 ± 0.04 ($P <$ .0001).

### Comparison of TA in TRAP Assay With Gene Expression of Telomerase Components

From 75 neuroblastomas, frozen sections were made for extraction of both protein and RNA. For analysis, quantitative amounts of hTRT, hTR, and TP1 gene expressions standardized on GAPDH expression were grouped on a score system ranging from 0 to 7 (see Patients and Methods, under Quantitative Real-Time RT-PCR for Expression of hTRT, hTR, and TLP-1). On quantitative RT-PCR analysis on the LightCycler instrument, hTRT expression was found in 37 (49%) of 75 neuroblastomas. For correlating hTRT expression with TA in the TRAP assay, hTRT expression levels of each neuroblastoma sample were compared with
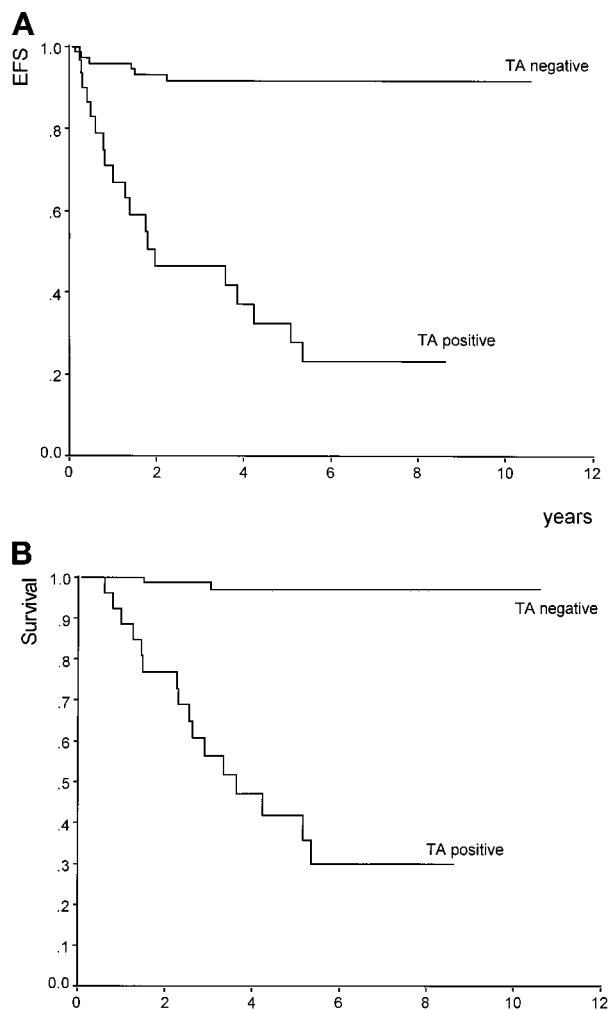


Fig 2. (A) Five-year EFS in 104 all-stage neuroblastomas without cytotoxic pretreatment (TA-negative, n = 73; TA-positive, n = 31; $P <$ .0001). (B) Five-year OS in 104 all-stage neuroblastomas without cytotoxic pretreatment (TA-negative, n = 73; TA-positive, n = 31; $P <$ .0001)

hTRT expression of a dilution series (see Patients and Methods, under Quantitative Real-Time RT-PCR for Expression of hTRT, hTR, and TLP-1) of a telomerase-positive (TRAP-positive) Ewing's tumor cell line (Fig 1). Twenty-two (29.3%) of 75 neuroblastomas were positive for both TA and hTRT mRNA, and 37 (49.3%) of 75 were negative for both TA and hTRT mRNA. One neuroblastoma (1.3%) was only positive for TA but not for hTRT mRNA, and 15 neuroblastomas (20%) were only positive for hTRT mRNA, but not for TA. Among the 22 neuroblastomas that were positive for both TA and hTRT mRNA, there was a significant statistical correlation ($P <$ .001) between quantitative expression levels of TA and hTRT mRNA. That is, 10 samples with intermediate or high TA levels (score of 2

or 3 on a scale of 0 to 3) displayed intermediate or high hTRT mRNA levels (score of $\geq 4$ on a scale of 0 to 7) and, vice versa, 11 samples with low TA levels (score of 1) displayed low hTRT mRNA levels (score of 2 or 3). A single case of a stage 4 neuroblastoma after cytotoxic pretreatment revealed low TA (score of 1) and high hTRT mRNA expression (score of 5). Among the 15 samples that were positive for hTRT mRNA but negative for TA, almost all cases (n = 14) had hTRT levels that were minimal (score of 1). These minimal (score of 1) hTRT levels always had values that were significantly lower than neuroblastomas with low TA and low hTRT. Furthermore, minimal hTRT levels were found in up to 50% of analyzed tumor-free tissues, such as normal breast or normal liver (data not shown). However, there was a single case with no TA in TRAP assay that repeatedly displayed high hTRT mRNA levels by RT-PCR (score of 4). This sample was from a stage 1 neuroblastoma of a 32-month-old (age at diagnosis) child with relapse-free clinical follow-up for 46 months. Further investigations revealed no Taq polymerase inhibitors in the TRAP assay and no *MYCN* amplification in this sample. Using cRNA sense and antisense probes for Northern blots on RNA from a TA-positive Ewing's tumor and neuroblastoma cell line, the specificity of hTRT expression detected by our RT-PCR system was confirmed.

On the basis of 67 neuroblastomas without prior cytotoxic treatment, 5-year EFS was $0.85 \pm 0.06$ for 37 patients with hTRT-negative tumors (score of 0), $1.0 \pm 0.0$ for 11 patients whose tumors had minimal hTRT (score of 1), $0.38 \pm 0.17$ for nine patients whose tumors had low hTRT (score of 2 to 3), and $0.44 \pm 0.17$ for 10 patients whose tumors had intermediate to high hTRT (score of $\geq 4$) at $P < .0001$ (Fig 3A). Five-year OS was $0.93 \pm 0.05$ for 37 patients with hTRT-negative tumors (score of 0), $1.0 \pm 0.0$ for 11 patients whose tumors had minimal hTRT (score of 1), $0.73 \pm 0.17$ for nine patients whose tumors had low hTRT (score of 2 to 3), and $0.42 \pm 0.17$ for 10 patients whose tumors had intermediate to high hTRT (score of $\geq 4$) at $P < .0001$.

hTR mRNA expression at different levels was found in all but one neuroblastoma examined, and expression of hTR mRNA was also significantly correlated with TA ($P < .001$). On the basis of 66 neuroblastomas without prior cytotoxic treatment, 5-year EFS was $0.93 \pm 0.05$ for 34 patients whose tumors had negative (n = 1) or low hTR expression (n = 33), $0.54 \pm 0.11$ for 24 patients whose tumors had intermediate hTR, and $0.54 \pm 0.20$ for eight patients whose tumors had high hTR at $P = .0005$ (Fig 3B). Five-year OS was $0.96 \pm 0.04$ for 34 patients whose tumors had negative or low hTR, $0.75 \pm 0.10$ for 24 patients whose
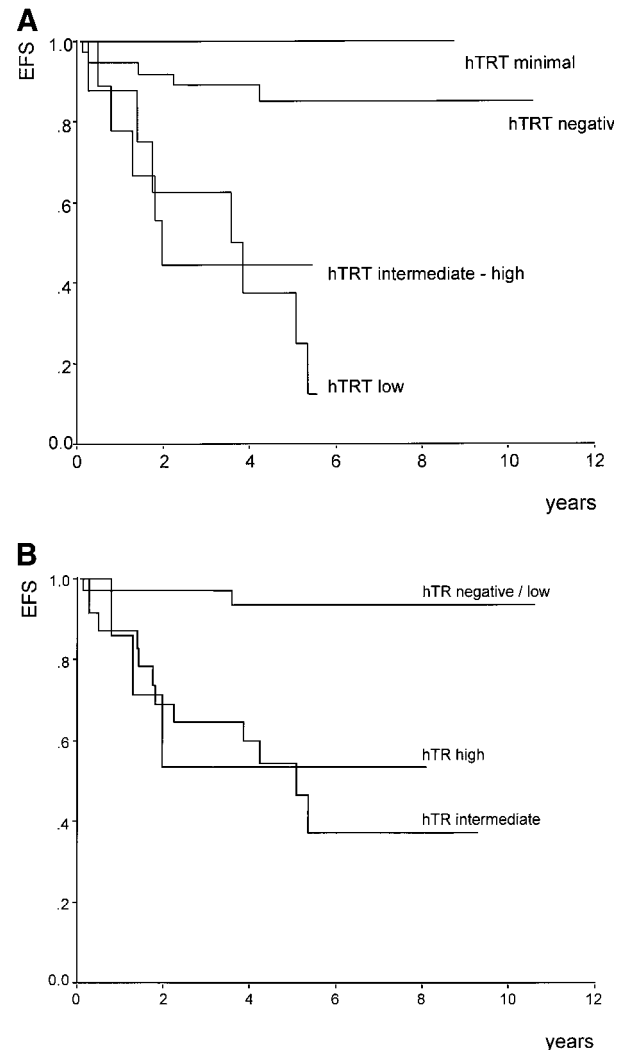


**Fig 3.** (A) Five-year EFS in 67 all-stage neuroblastomas without cytotoxic pretreatment (hTRT-negative, n = 37; hTRT-minimal, n = 11; hTRT-low, n = 9; hTRT-intermediate to -high, n = 10; $P < .0001$). (B) Five-year EFS in 66 all-stage neuroblastomas without cytotoxic pretreatment (hTR-negative/low, n = 34; hTR-intermediate, n = 24; hTR-high, n = 8; $P = .0005$)

tumors had intermediate hTR, and $0.54 \pm 0.20$ for eight patients whose tumors had high hTR at $P = .0012$.

TP1 mRNA expression was found in 90% of the samples, but there was no correlation between TA and TP1 expression levels. High TP1 mRNA levels were also found in two thirds of the TA-negative neuroblastomas, and at the same time, low TP1 mRNA levels were also associated with some TA-positive neuroblastomas with high TA levels.

### hTRT mRNA Expression in Paraffin-Embedded Tissues

hTRT expression by RT-PCR from fresh-frozen and paraffin-embedded tissues was compared in 16 selected
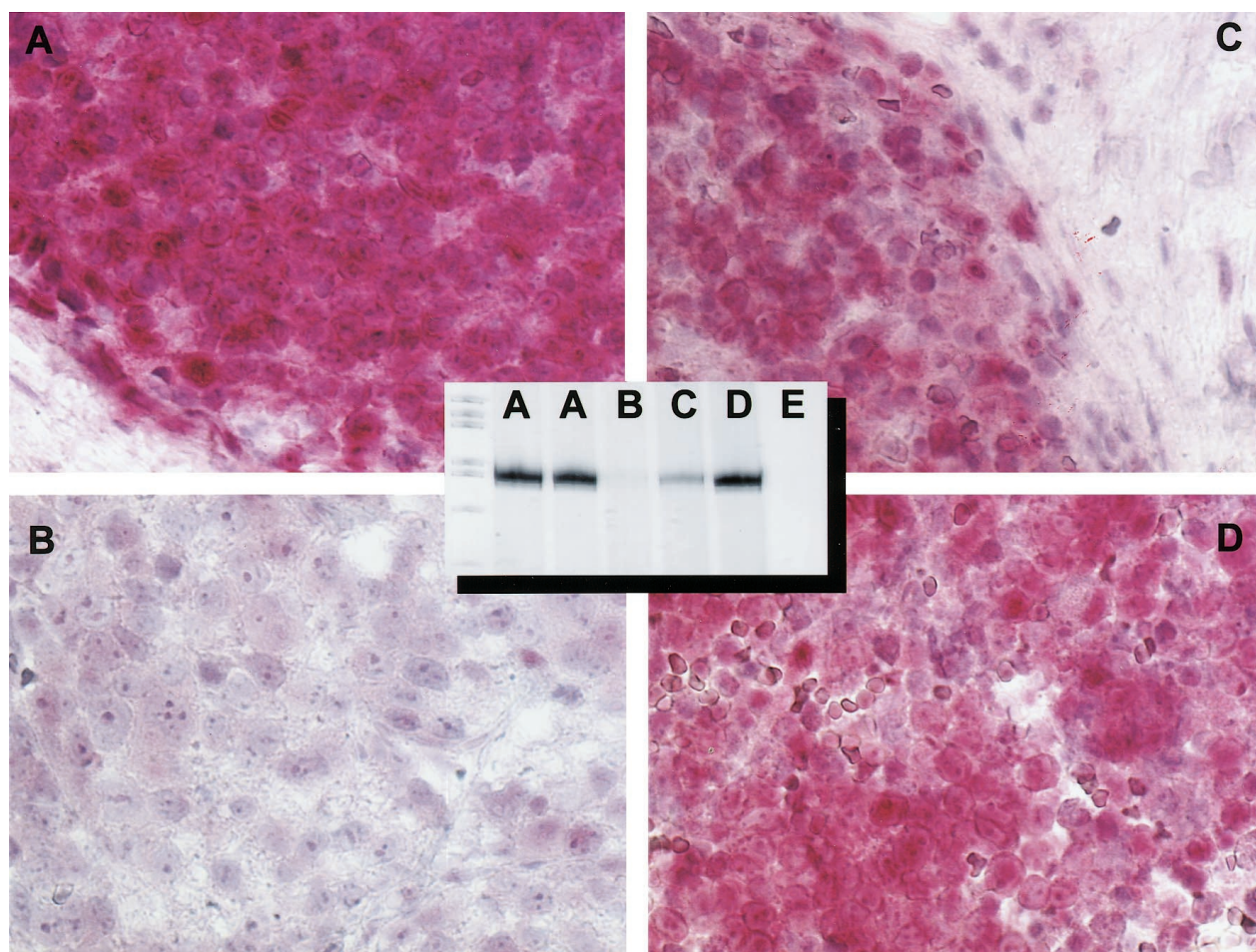
Fig 4.  Detection of hTRT gene and protein expression in paraffin-embedded neuroblastomas (NB). (A) Positive control (RNA from [left] fresh-frozen and [right] paraffin-embedded tissue); (B) stage 1 NB, negative by RT-PCR and immunohistochemistry; (C) stage 4 NB, intermediate hTRT expression; (D) stage 3 NB, high hTRT expression; (E) RT-PCR negative control.

cases from the archives. For assessing tissue sample preservation, RNA from these 16 formalin-fixed and paraffin-embedded neuroblastoma tissues was initially amplified by RT-PCR (TITAN system) for the *GAPDH* gene. In 11 of 16 cases, suitable RNA for RT-PCR was available. These 11 cases included five stage 4 neuroblastomas (with intermediate or high TA and hTRT mRNA expression in corresponding fresh-frozen tissue), three stage 3 neuroblastomas (with intermediate or high TA and hTRT mRNA expression in corresponding fresh-frozen tissue), and three stage 1 or 2 neuroblastoma (with neither TA nor hTRT mRNA expression in corresponding fresh-frozen tissue). As shown in Fig 4, for three of 11 cases, hTRT mRNA expression was detectable in two cases with distinct expression levels, whereas one case was completely negative. Comparison of hTRT mRNA expression from the corresponding fresh-

frozen tissues revealed similar expression patterns as with the paraffin-embedded tissue. hTRT mRNA expression in these two cases was correlated with fatal outcome, whereas the hTRT mRNA negative stage 1 neuroblastoma case is relapse- and disease-free after 54 months of follow-up.

### Immunohistochemical Detection of hTRT Protein in Fresh-Frozen and Paraffin-Embedded Neuroblastoma Tissue Sections

hTRT protein expression could be detected in both fresh-frozen and paraffin-embedded neuroblastoma tissues of 19 selected cases using the EST1.0 antibody. Tumor cell nuclei of the neuroblastoma cells were strongly positive to the specific antibody (Fig 4) but not to preabsorbed antibody. Seven neuroblastomas that were negative for TA and hTRT mRNA expression showed no or less than 1% nuclear

**Table 2.** Cox Regression Model for Multivariate Analysis (n = 122)

| Covariate | $_e\beta$ | P |
|---|---|---|
| TA, negative versus positive | 3.32 | .0024 |
| Tumor stage, 1,2,3,4S v 4 | 2.67 | .0275 |
| Serum LDH, normal versus increased* | 2.24 | .116 |
| MYCN copy number, ≤ 3 v > 3 | 1.68 | .21 |
| Age at diagnosis, continuous | 0.9993 | .91 |

*Serum LDH was considered increased at levels greater than 400 U/L for children ≤ 1 year of age and greater than 300 U/L for children older than 1 year (according to criteria of the German Neuroblastoma Trial).

staining with the EST1.0 antibody. Five neuroblastomas with low TA and hTRT mRNA expression revealed 1% to 10% nuclear staining, and four samples with intermediate TA and hTRT mRNA expression showed 10% to 50% nuclear staining. Three neuroblastomas with high TA and hTRT mRNA expression displayed specific nuclear staining for anti-hTRT antibody in more than 50% of the cells. As seen in Fig 4, stromal cells of the surrounding tissue such as fibroblasts, vascular endothelial cells, and smooth muscle cells were not positive for hTRT staining.

*Multivariate Analysis*

The Cox regression model for EFS was built on data of 122 patients from whom the most covariates were available. TA (negative *v* positive), stage (1, 2, 3, 4S, *v* 4), serum lactate dehydrogenase (LDH; normal *v* increased), *MYCN* copy number (≤ three *v* > three), and age (continuous) were entered as covariates. The model revealed only TA and stage but not serum LDH, *MYCN* amplification, or age as independent prognostic factors (Table 2).

## DISCUSSION

Corroborating our previous results from two smaller studies,[9,15] TA was demonstrated to be significantly correlated with poor prognosis in neuroblastoma. In this study according to stage classification, multivariate analysis revealed only TA and stage as independent prognostic factors, whereas serum LDH, *MYCN* status, and age were not. The high accuracy with which tumors prone to unfavorable outcome can be identified makes TA a reliable prognostic tool in neuroblastoma. Although it is not proven so far that TA will not change under cytotoxic pretreatment, our data suggest that TA analysis is suitable in all neuroblastomas irrespective of their pretreatment status: comparing the number of TA-positive tumors among all neuroblastomas in this study (n = 133) versus the subset of initial samples without pretreatment (n = 104), we found no difference between these two groups (39 of 133 equals 29.3% of all neuroblastomas *v* 31 of 104 equals 29.8% of initial neuroblastomas). Above all, the significant correlation of clinical outcome with presence of TA strongly recommends

that the analysis of TA in individual tumors should be accorded a priority at least equal to that of the determination of established markers, such as *MYCN* and loss of heterozygosity of chromosome 1p, thereby facilitating risk-directed therapy.[33]

The recent cloning of the telomerase holoenzyme components hTRT, hTR, and TP1 allows a more precise definition of the molecular mechanisms of TA in neuroblastoma. hTRT has been identified as the catalytic subunit of telomerase, and expression of hTRT mRNA is usually observed at high levels in telomerase-positive cancer cell lines and malignant tumors, but not in adjacent normal tissues. Furthermore, a strong correlation was found between TA and hTRT mRNA expression in different tumor types.[20,34-36] Recently, Hiyama et al[37] found in a smaller series of neuroblastoma (35 cases) hTRT mRNA expression in all 13 tumors with high TA and in five of 23 tumors with low or undetectable TA. Using a kinetic rather than end point approach for quantification of PCR products, we could demonstrate a significant correlation between distinct levels of TA and hTRT mRNA expression for the majority of neuroblastomas in our study. Our finding that 14 neuroblastomas in this study displayed minimal expression of hTRT mRNA without detectable TA is in agreement with studies on other tumors in which it was found that hTRT mRNA expression did not always give rise to TA.[24] Recent studies by Xu et al[35] provide new insights into the regulatory control of TA at the molecular level: during differentiation of HL-60 promyelocytic leukemic cells, suppression of TA is preceded by a downregulation of hTRT mRNA, which is achieved through inhibition of its transcription. Furthermore, Xu et al showed that the onset of reduction of hTRT expression during cellular differentiation was at least in part independent of cell proliferation status, and suppression of TA was unrelated to hTR and TP1 expression in differentiation of HL-60 cells.

In the case of limited material from very small biopsy samples or formalin-fixed and paraffin-embedded tissues (for example, archived material), immunohistochemical detection of hTRT by anti-hTRT antibody-reactive protein in tissue sections may be an indicator of TA in the tumor cells. Additionally, for retrospective studies on archived material, RT-PCR to detect hTRT mRNA expression from paraffin-embedded tissues may be another option for indirect assessment of TA. To our knowledge, we have demonstrated for the first time that expression of hTRT mRNA can be determined even from paraffin-embedded tissues and that this expression seems to be correlated with hTRT gene and protein expression as well as TA from corresponding fresh-frozen tumor tissue. For this analysis, however, one should keep in mind that RT-PCR from paraffin-embedded tissue–derived RNA may not always allow accurate quan-

tification of gene expression levels, which is an important limitation of directly deducing TA levels from hTRT expression. Prospective studies with larger case numbers should be performed to further evaluate the prognostic impact of hTRT RNA or protein detection in paraffin samples.

Focusing on the other gene components of the telomerase complex, different studies showed that the human telomerase RNA component hTR and telomerase protein TP1 mRNA are broadly expressed in both malignant and tumor-free normal tissue. Previous investigations furthermore revealed no significant correlation between TA and the expression of hTR or TP1 mRNA in the vast majority of human tumors.[19-21] Other authors suggest that overexpression of hTR may be correlated with proliferative cell activity.[38] So far, there are only limited data on hTR expression compared with TA in neuroblastomas. In a small series of five neuroblastomas and one ganglioneuroma, Maitra et al[39] described a correlation between TA, *MYCN* amplification, and hTR expression determined by in situ hybridization. In our series, we found a significant correlation between hTR mRNA expression levels and TA.

Although we found a significant correlation between TA determined by the TRAP and hTRT mRNA expression by quantitative RT-PCR, our data suggest that the TRAP assay is a more powerful molecular tool that could be complemented but not be replaced by analysis of hTRT expression in the clinical setting. Whereas the mere presence or absence of TA—irrespective of activity levels—is associated with either favorable or unfavorable clinical outcome, the interpretation of hTRT expression as a prognostic marker is much more difficult. First, accurate quantification of hTRT expression levels is necessary and requires correlation to a defined standard, because, as shown above, minimal hTRT levels are associated with a favorable prognosis, whereas low, intermediate or high hTRT levels predict unfavorable outcome. Second, accurate quantification requires special and expensive equipment, such as new PCR systems for real-time detection of reaction kinetics and, furthermore, standardized reaction kits to permit the comparison of results between different laboratories.

As the molecular biology of neuroblastoma has led to a combined clinical and risk stratification, the search for new molecular markers for assessing the individual patient's prognosis is of vital importance, because there is evidence that analysis of *MYCN* amplification does not ensure completely accurate prognostic grouping,[8] which unfortunately is also true for other traditional molecular and clinical markers. This study and previous investigations by our group and others suggest that analysis of TA by the TRAP assay is a powerful additional molecular tool for distinguishing neuroblastomas with good and poor prognosis. Along with other emerging markers such as gain of chromosome arm 17q,[8] we propose that analysis of TA should be incorporated into the clinical investigation of each individual neuroblastoma case at the time of diagnosis.

## ACKNOWLEDGMENT

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