

## Telomerase Activity by TRAP Assay and Telomerase RNA (hTR) Expression Are Predictive of Outcome in Neuroblastoma

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**Background.** Recent studies have associated telomerase with prognostic factors and survival in neuroblastoma. **Procedure.** We examined telomerase activity by telomere repeat amplification protocol (TRAP) and expression of the RNA component of telomerase (hTR) by Northern blotting in 106 primary neuroblastoma tumors and 22 established cell lines. **Results.** Overall survival at 5 years for all 106 tumors was significantly better for patients with undetectable TRAP (75% vs. 59%;  $P = 0.03$ ) or low hTR expression (84% vs. 43%;  $P < 0.0001$ ), and especially for patients whose tumors had

both low hTR expression and undetectable TRAP (all patients, 91% vs. 54%,  $P = 0.0002$ ; for 17 stage IV-S tumors, 100% vs. 72%,  $P = 0.04$ ). Strong expression of hTR was seen in 22 cell lines from aggressive tumors, and all maintained telomere length, but 3/22 were TRAP negative. **Conclusions.** These data suggest that both hTR expression and telomerase activity via the TRAP assay should be performed concurrently to predict survival in neuroblastoma patients, particularly in stage 4-S. *Med. Pediatr. Oncol.* 35:647–650, 2000.

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### INTRODUCTION

Neuroblastoma is the most common extracranial solid tumor of infancy and childhood [1]. This embryonal tumor of the sympathetic nervous system is unique because of its biological complexity and varied clinical behavior, which ranges from spontaneous regression with no therapy to rapidly progressive disease in spite of intensive chemotherapy [1]. Because of its wide range of clinical outcome, identifying prognostic markers can increase the accuracy of risk assessment (necessary to determine therapy) and can also identify biologically relevant targets for developing new therapies.

Telomeres are specialized structures at the end of linear eukaryotic chromosomes that protect chromosome ends from double strand breaks, and are essential for chromosome stability [2]. Due to the inability of conventional DNA polymerases to fully replicate the extreme termini of linear chromosomes during DNA synthesis [2–4] telomeres progressively shorten with each cell division. To overcome this “end-replication problem”, most eukaryotic species utilize telomerase [2,5,6], a ribonucleoprotein that rebuilds telomeres, adding the specific repeating sequence, TTAGGG, via a reverse transcription reaction to the ends of chromosomes [7]. Telomerase is composed of an RNA template region (hTR) [8] and a catalytic component, the telomerase reverse transcriptase termed hTERT [9].

Telomerase activity has been reported in neuroblastoma tumors, with high activity being associated with advanced stage, MYCN amplification and poor outcome [10,11]. It has also been reported that there is a correlation between hTR expression in primary neuroblastomas, stage of disease, and patient survival [12,13]. In this study, we demonstrated that both hTR expression and telomerase activity via TRAP assay are complementary

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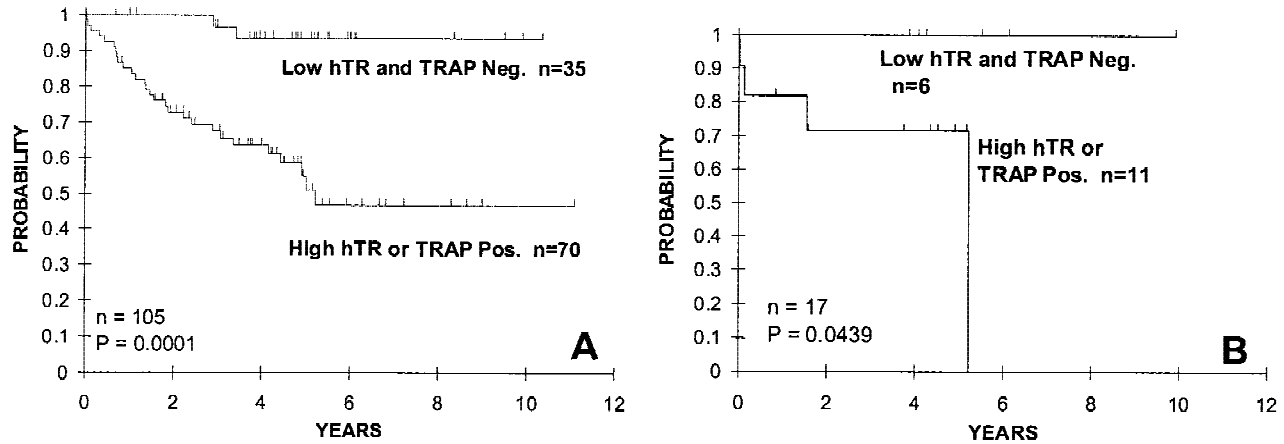
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**Fig. 1.** Kaplan-Meier analysis of overall survival for patients with low hTR and negative TRAP vs. patients with either high hTR or positive TRAP. **(A)** All 106 neuroblastoma patients studied (all stages). **(B)** 17 stage IV-S patients.

prognostic factors in neuroblastoma, and that either high hTR expression or positive TRAP activity defined a subset of stage IV-S patients with a significantly worse survival.

## MATERIALS AND METHODS

Northern blot analysis was used to detect hTR expression (defined as high or low expression) [12], and the telomeric repeat amplification protocol (TRAP) assay [14,15] was used to detect telomerase activity, defined as positive or negative activity. Patients were treated on Childrens Cancer Group studies according to their risk groups (CCG-3881 for low risk and CCG-3891 for high risk). Kaplan-Meier estimation was used to evaluate overall and event-free survival and the log-rank statistic was used to compare survival between subgroups of patients [16]. Multivariate analysis was performed using the regression method of Cox and the estimated relative risk (RR) and 95% confidence interval (CI) for each variable entered into the Cox model was summarized [17].

## RESULTS

We studied 106 untreated, primary neuroblastoma tumors. Using the TRAP assay, we found that 43 of 106 samples showed positive TRAP activity. High hTR expression by Northern blotting was seen in 43 of 106 tumors (41%). *MYCN* amplification was seen in 22 of 106 cases, and 15 of 22 (68%) *MYCN*-amplified tumors had detectable telomerase activity, whereas 14 of 22 (64%) had high hTR expression.

A fatal outcome was seen in 21 of 106 patients (20%). Positive activity by TRAP was observed in 12 of 21 (57%) tumors from fatalities, whereas 13 of 21 (62%) had high hTR expression. However, 20 out of 21 fatal

outcome cases had tumors at diagnosis with either TRAP positivity or high hTR.

We studied 17 IV-S patients, a subset of metastatic neuroblastomas that often regresses without treatment; all 17 were *MYCN* nonamplified tumors. High hTR expression was observed in five of 17 tumors (29%) and six of 17 (35%) cases had positive TRAP activity. Four patients had a fatal outcome; three of four of these fatal specimens (75%) had high hTR expression, and these same three specimens had undetectable TRAP activity. Only one of these tumors showed low hTR expression, and this same specimen had high TRAP activity. Thus, all four of the stage IV-S tumors with a fatal outcome had either a positive TRAP or high hTR.

Event-free survival and overall survival for hTR expression and TRAP activity were analyzed and as both were similar, overall survival was used throughout the study. Survival at 5 years was significantly better for all patients with low hTR (81% vs. 41%,  $P < 0.0001$ ) and for patients with stage IV-S disease (100% vs. 40%,  $P = 0.0028$ ). Overall 5-year survival was also significantly better for all patients with undetectable TRAP activity (75% vs. 59%,  $P = 0.03$ ), but positive TRAP activity did not significantly correlate with poor survival in stage IV-S patients (100% vs. 72%,  $P = 0.75$ ). As shown in Figure 1, survival at 5 years was significantly better for patients whose tumors had both low hTR expression and undetectable TRAP, vs. those patients with positive TRAP activity or high hTR expression [all patients, 91% vs. 54% ( $P = 0.0002$ ) and for the 17 stage IV-S tumors, 100% vs. 72% ( $P = 0.04$ )]. In a bivariate Cox analysis, hTR and TRAP each provided independent prognostic information, RR = 3.64 (CI = 1.82–7.31,  $P = .003$ ) for hTR and RR = 2.11 (CI = 1.11–4.15,  $P = 0.0303$ ) for TRAP. In a multivariate model including *MYCN*, hTR, and TRAP, hTR remained strongly significant (RR = 2.83, CI = 1.34–5.96,  $P = 0.0062$ ), but the evidence for

an independent action of TRAP on prognosis was reduced ( $P = 0.128$ ).

It is possible that hTR positive/TRAP negative tumors were expressing hTR without expressing telomerase activity, or alternatively, that these tumors were expressing telomerase activity that was degraded during collection or storage of the tumor specimens. We further explored these possibilities by determining hTR expression and TRAP activity in a panel of 22 continuous neuroblastoma cell lines, in which fresh, viable samples could be collected and stored under optimal conditions. Our results showed that all 22 neuroblastoma cell lines examined had high expression of hTR by Northern blot analysis, yet 3 of 22 samples (LA-N-6, CHLA-90, and SK-N-FI) showed no detectable TRAP activity. Telomere restriction fragments (TRFs) demonstrated that all cell lines studied maintained telomeres, including the three cell lines in which TRAP activity was negative. This was consistent with the data from fresh tumor samples, which showed that some aggressive neuroblastomas have high hTR expression and lack telomerase activity.

## DISCUSSION

It has previously been shown that the RNA component of human telomerase (hTR) is expressed in primary neuroblastomas, and that the level of expression increased with stage of disease [13] and correlated with clinical outcome [12]. Telomerase activity has also been shown to be a useful prognostic marker for neuroblastoma [10,11]. We undertook the present study to determine the concordance of hTR expression (by Northern blotting) and telomerase activity (by the TRAP assay) as prognostic markers for neuroblastoma, especially in stage IV-S tumors.

Our results for detection of telomerase activity by TRAP in neuroblastoma differ from data originally reported for neuroblastoma [10]. We observed that tumors with high telomerase activity did not always show high-risk features and negative/undetectable TRAP activity was found in aggressive tumors, even in those cases that led to a fatal outcome for the patient. Our results are consistent with observations of another group [11] as we found that most of the tumor samples were TRAP negative. *MYCN* amplified tumors more frequently showed positive TRAP activity or high hTR expression, yet all three were independent prognostic variables. Analysis of both telomerase activities via TRAP and hTR expression seems to provide a more powerful discriminator of prognosis than did analysis of only hTR or TRAP, particularly in stage IV-S patients.

The levels of expression of the RNA component of human telomerase (hTR) did not always parallel the levels of telomerase activity in either the neuroblastoma tumor samples or cell lines. Other investigators have

shown that expression of the RNA component of telomerase does not tightly correlate with enzyme activity [18–20] raising the possibility that in some tumors, and in neuroblastoma in particular, regulation of the various components of telomerase may be complex and involve different molecular mechanisms. Although the possibility of degradation of the telomerase enzyme during tumor tissue collection exists, our observation of TRAP negativity and telomere maintenance in 3 of 22 cell lines, in which the cells were collected under ideal conditions, indicates that true TRAP-negative, aggressive neuroblastomas occur. We have recently shown that unlike all other neuroblastoma cell lines examined, those same three cell lines have virtually undetectable hTERT RNA expression (Choi et al., submitted). Therefore, it is likely that at least some telomerase-negative neuroblastomas have alternative (non-TRAP detectable) mechanisms for telomere maintenance.

There is increasing evidence that indicates that a telomerase-independent pathway for telomere length maintenance exists [21,22]. In our study, all three telomerase-negative cell lines (SK-N-FI, CHLA-90, and LA-N-6) had very long telomeres (approximately 23 Kb), a characteristic seen in ALT (Alternative Lengthening of Telomeres) cell lines by other authors [21–25]. This suggests that these cell lines have been able to overcome telomere shortening through a telomerase-independent mechanism. The nature of this alternative mechanism is currently unknown in vertebrates, although it has been speculated that ALT is due to nonreciprocal recombination between telomere repeats, a mechanism that has been reported for telomerase-deleted mutant yeast strains [26].

High expression of hTR predicts an aggressive phenotype in neuroblastoma independent of *MYCN* amplification (Reynolds et al., submitted). Telomerase activity via the TRAP assay is being employed in a variety of tumors as an indicator of malignant cells or as a prognostic marker [27–31], and has been correlated with clinical outcome in neuroblastomas [3,8]. Our results demonstrate that due to the independence of hTR and telomerase activity by TRAP, both should be performed concurrently to predict survival in neuroblastoma.

## REFERENCES

1. Reynolds CP, Seeger RC. Neuroblastoma. In: Haskell CM, editor. Cancer treatment. Philadelphia, PA: W.B. Saunders; 2000.
2. Blackburn EH. Structure and function of telomeres. *Nature* 1991; 350:569–573.
3. Greider CW. Telomerase is processive. *Mol Cell Biol* 1991;11: 4572–4580.
4. Harley CB. Telomere loss: Mitotic clock or genetic time bomb? *Mutat Res* 1991;256:271–282.
5. Harley CB, Futcher AB, Greider CW. Telomeres shorten during ageing of human fibroblasts. *Nature* 1990;345:458–460.

6. Harley CB, Kim NW, Prowse KR, et al. Telomerase, cell immortality, and cancer. *Cold Spring Harbor Symp Quant Biol* 1994;59:307–315.
7. Greider CW. Telomerase activity, cell proliferation, and cancer. *Proc Natl Acad Sci USA* 1998;95:90–92.
8. Feng J, Funk WD, Wang SS, et al. The RNA component of human telomerase. *Science* 1995;269:1236–1241.
9. Nakamura TM, Morin GB, Chapman KB, et al. Telomerase catalytic subunit homologs from fission yeast and human. *Science* 1997;277:955–959.
10. Hiyama E, Hiyama K, Yokoyama T, et al. Correlating telomerase activity levels with human neuroblastoma outcomes. *Nature Med* 1995;1:249–255.
11. Poremba C, Willenbring H, Hero B, et al. Telomerase activity distinguishes between neuroblastomas with good and poor prognosis. *Ann Oncol* 1999;10:715–721.
12. Reynolds CP, Zuo JJ, Hong CM, et al. Telomerase RNA expression in neuroblastoma correlates with high stage and clinical outcome. *Proc Am Assoc Cancer Res (GENERIC)* 1996;37:199–199.
13. Reynolds CP, Zuo JJ, Kim NW, et al. Telomerase expression in primary neuroblastomas. *Eur J Cancer* 1997;33:1929–1931.
14. Kim NW, Piatyszek MA, Prowse KR, et al. Specific association of human telomerase activity with immortal cells and cancer. *Science* 1994;266:2011–2015.
15. Kim NW, Wu F. Advances in quantification and characterization of telomerase activity by the telomeric repeat amplification protocol (TRAP). *Nucl Acids Res* 1997;25:2595–2597.
16. Kaplan E, Meier P. Nonparametric estimation from incomplete observations. *J Am Stat Assoc* 1958;53:457–481.
17. Cox DR. Regression models and life tables. *J R Stat Soc* 1972;34:187–220.
18. Blasco MA, Rizen M, Greider CW, et al. Differential regulation of telomerase activity and telomerase RNA during multi-stage tumorigenesis. *Nature Genet* 1996;12:200–204.
19. Ponte P, Gunning P, Blau H, et al. Human actin genes are single copy for alpha-skeletal and alpha-cardiac actin but multicopy for beta- and gamma-cytoskeletal genes: 3' untranslated regions are isotype specific but are conserved in evolution. *Mol Cell Biol* 1983;3:1783–1791.
20. Shimada H, Chatten J, Newton WAJ, et al. Histopathologic prognostic factors in neuroblastic tumors: Definition of subtypes of ganglioneuroblastoma and an age-linked classification of neuroblastomas. *J Natl Cancer Inst* 1984;73:405–416.
21. Bryan TM, Englezou A, Dalla-Pozza L, et al. Evidence for an alternative mechanism for maintaining telomere length in human tumors and tumor-derived cell lines. *Nature Med* 1997;3:1271–1274.
22. Bryan TM, Marusic L, Bacchetti S, et al. The telomere lengthening mechanism in telomerase-negative immortal human cells does not involve the telomerase RNA subunit. *Human Mol Genet* 1997;6:921–926.
23. Bryan TM, Englezou A, Dunham MA, et al. Telomere length dynamics in telomerase-positive immortal human cell populations. *Exp Cell Res* 1998;239:370–378.
24. Bryan TM, Englezou A, Gupta J, et al. Telomere elongation in immortal human cells without detectable telomerase activity. *EMBO J* 1995;14:4240–4248.
25. Bryan TM, Reddel RR. Telomere dynamics and telomerase activity in in vitro immortalised human cells. *Eur J Cancer* 1997;33:767–773.
26. Lundblad V, Blackburn EH. An alternative pathway for yeast telomere maintenance rescues est1-senescence. *Cell* 1993;73:347–360.
27. Clark GM, Osborne CK, Levitt D, et al. Telomerase activity and survival of patients with node-positive breast cancer. *J Natl Cancer Inst* 1997;89:1874–1881.
28. Langford LA, Piatyszek MA, Xu R, et al. Telomerase activity in ordinary meningiomas predicts poor outcome. *Human Pathol* 1997;28:416–420.
29. Mehle C, Piatyszek MA, Ljungberg B, et al. Telomerase activity in human renal cell carcinoma. *Oncogene* 1996;13:161–166.
30. Nakatani K, Yoshimi N, Mori H, et al. The significant role of telomerase activity in human brain tumors. *Cancer* 1997;80:471–476.
31. Yahata N, Ohyashiki K, Ohyashiki JH, et al. Telomerase activity in lung cancer cells obtained from bronchial washings. *J Natl Cancer Inst* 1998;90:684–690.