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## Original Paper

# Telomerase Expression in Primary Neuroblastomas

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**Maintenance of chromosomal telomeres is necessary for continued cell growth, and this is carried out in germline tissues by telomerase. In contrast to most somatic tissues, many tumours have telomerase activity. The RNA component of human telomerase (hTR) was measured by Northern analyses of 150 primary untreated neuroblastomas and compared with clinical stage at diagnosis. hTR expression > 33 (relative to cell line control = 100) was seen in 41% of all tumours and the frequency of hTR > 33 increased with stage of disease. Expression of hTR may be involved in progression of neuroblastoma.**


**Key words: neuroblastoma, telomerase, tumour progression**
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## INTRODUCTION

NEUROBLASTOMAS SHOWS a spectrum of behaviours from spontaneous regression of even widespread tumours to progression in spite of intensive chemoradiotherapy [1]. Stage of disease and age of the patient at diagnosis are associated with clinical outcome [2] and a variety of the tumour's biological properties have been shown to correlate with aggressive behaviour in neuroblastoma including histopathology [3], genomic amplification for the *MYCN* proto-oncogene [4], tumour DNA ploidy [5], lack of expression of the CD44 antigen [6] or for the trk-A neurotrophin receptor [7, 8], allelic loss (LOH) of chromosome 1p [9] and expression of the multidrug-resistance-associated protein MRP [10].

Specialised structures which protect the ends of chromosomes called telomeres are maintained by the enzyme telomerase, due to the inability of routine synthetic enzymes to replicate the ends of linear DNA [11]. Telomerase is a ribonucleoprotein that synthesises the TTAGGG telomeric repeats, prevents cell senescence by maintaining telomeres and is expressed in germline but not in most normal somatic cells [12]. Telomerase activity has been detected in 90% of primary tumours but not in normal somatic tissues and it appears to be necessary for immortalisation of malignant cells [13]. Telomerase activity has been detected in the majority of neuroblastomas, with high activity being associated with advanced stage, *MYCN* amplification and/or 1P LOH and poor outcome [14]. Low telomerase activity has been associated with favourable prognostic features and clinical outcome, and has been observed in three stage IV-S tumours that spontaneously regressed.

The RNA template component of human telomerase (hTR) has been cloned [15]. We determined hTR expression in primary neuroblastomas and showed that it relates to stage of disease.

## PATIENTS AND METHODS

*Patients and tumour specimens*

Diagnosis and staging (using the Evans staging system) were performed at the patient's institution according to standard pathological and clinical criteria [1]. Appropriate informed consent procedures were followed.

Primary untreated neuroblastomas were obtained surgically at diagnosis and snap-frozen. Without thawing, specimens were divided so that one portion was stored in Tissue-Tek O.C.T. embedding compound for histopathological

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examination and others were allocated for DNA and RNA extraction or telomerase repeat amplification protocol (TRAP) assay (see below); all were maintained frozen at −70°C. One hundred and fifty specimens were chosen from the CCG tumour bank for study so that each clinical stage was represented.

*RNA isolation and Northern blot analysis*

Total RNA was prepared [16] and 20 μg were electrophoresed in a 1% agarose–formaldehyde denaturing gel using 1xMOPS buffer (200 mM *N*-morpholinopropanesulphonic acid, 50 mM sodium acetate, 10 mM EDTA, pH 7.0). Following transfer to nylon filters in 20xSSC by Northern blotting [17], [$^{32}$P]-labelled DNA probes were hybridised in 500 mM $Na_2HPO_4/NaH_3PO_4$ (pH = 7.2), 1 mM EDTA, 1% BSA, 7% SDS and 30% formamide at 65°C. Probes used were hTR, a full-length probe for human telomerase [15], and human β-actin [18]. After hybridisation, filters were washed using deionised, distilled and DEPC (diethyl pyrocarbonate) treated water. The hybridisation signal was assessed quantitatively using a BIO-RAD Phosophorimager. Each blot contained a standard RNA sample prepared from the SMS-LHN human neuroblastoma cell line [19]. For each tumour, the hTR signal was normalised to the β-actin signal, determined from the same blot that was stripped and subjected to repeat hybridisation. For each blot, the value for SMS-LHN was set to 100, and all samples in a given blot were expressed relative to that value, designated here the 'hTR value'.

*Determination of telomerase enzyme activity*

The telomeric repeat amplification protocol (TRAP) was used to assess telomerase activity [13]. Crude cytoplasmic extract from tumour tissues was prepared using a detergent-based lysis procedure and then reacted so that telomerase in the sample adds TTAGGG repeats on to a substrate oligonucleotide and the extended product is amplified by polymerase chain reaction (PCR). To compare hTR expression of neuroblastomas with telomerase activity, tumour samples from 32 representative tumours were divided, a portion utilised to extract RNA and a portion homogenised and telomerase activity determined by the TRAP assay.

*Statistical analyses*

Tests of hypotheses for tabular data were performed using Student's *t*-test, with all *P* values reported for two-tailed alternatives. A cutpoint analysis was performed in which different levels of hTR expression were compared to stage; based on this preliminary analysis with the first 60 tumours, values of 0–33 were defined as no or low expression and > 33 was defined as positive expression and these cut points were then applied prospectively to the remaining 90 tumours. Correlations were examined using Pearson's coefficient.

*Table* 1. *Distribution of patients studied for hTR expression by stage, and the relationship of stage to hTR expression. Mean hTR value was calculated from the Northern blots (as described in Materials and Methods). Also shown are the percentage of tumours with 'positive' expression (hTR value* > 33*)*

| Stage | No. of patients | Mean hTR | %hTR > 33 |
|---|---|---|---|
| I | 14 | 17 | 7 |
| II | 39 | 23 | 18 |
| IV-S | 33 | 28 | 30 |
| III | 21 | 36 | 38 |
| IV | 43 | 60 | 80 |
| All stages | 150 | 36 | 40 |

## RESULTS

A Northern blot of telomerase RNA (hTR) expression in representative tumours from each stage is shown in Figure 1, with hTR expressed as a 0.45 kb transcript. Quantitation of hTR expression for 150 primary neuroblastoma tumours demonstrated that the average level of hTR expression increased with stage of disease (Table 1). Average hTR values were 17 for stage I, 23 for stage II, 28 for stage IV-S, 36 for stage III and 60 for stage IV, relative to a cell line control present on each blot (the SMS-LHN cell line, which defined hTR = 100). A level of hTR expression > 33 was considered significant hTR expression, and the percentage of tumours by stage expressing hTR > 33 increased with stage of disease (Table 1). This cutpoint for hTR expression was defined for the first 60 tumours and then applied prospectively to all subsequent analyses.

We compared hTR expression to telomerase activity determined by the TRAP assay for 32 tumours. Although some tumours with high hTR expression showed high telomerase activity, there were tumours expressing high and intermediate hTR levels that showed very low or non-detectable telomerase activity, and overall correlation between telomerase activity and hTR expression showed an *R* value of 0.71 by Pearson's coefficient.

## DISCUSSION

Telomerase is necessary for maintaining chromosomal telomeres, and while it is not expressed in most somatic cells, it appears to be 're-activated' in most tumours [13]. Other investigators have shown that telomerase activity has been detected in 94% of neuroblastomas examined; of 79 patients studied, 16 had high telomerase activity and tended to have bad prognostic features (*MYCN* amplification in 11, high stage in 13) and a poor clinical outcome [14]. Of 63 patients with low or negative telomerase activity, none showed *MYCN* amplification, 42 were stage I/II, 7 were stage IV-S, 18 were stage III/IV and only 2 of the 63 patients had fatal disease [14].

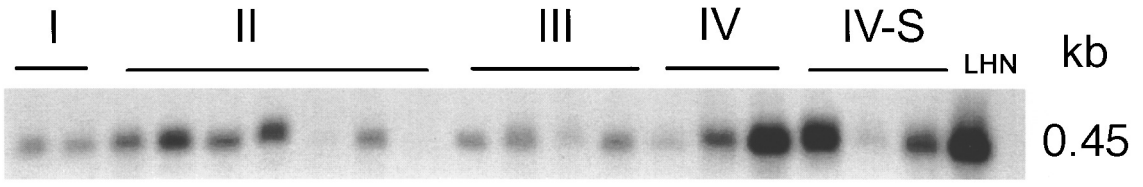


**Figure 1. Northern blot showing expression of hTR (a 0.45 kb transcript) for tumours from each stage of disease. As this blot is a small number of tumours compare with the entire 150 used to determine values for hTR expression, relative levels of expression between stages are not comparable to Table 1.**

Thus, neuroblastomas which fail to express telomerase may have limited capability for sustained, immortal growth.

In spite of the sensitive nature of the TRAP assay for telomerase activity, as in any enzymatic assay, a major concern in using such an assay is that tumour tissue could be subjected to various conditions that could artificially destroy enzyme activity prior to freezing of the tissue. The ability to assess the quality of RNA isolated by examining expression of 'housekeeping' genes overcomes some of the potential problems inherent in studying enzyme activity. As the RNA template portion of human telomerase has recently been cloned [15], we undertook a study of hTR expression in neuroblastoma.

We showed that the RNA component of human telomerase is expressed in primary neuroblastomas and that it increased with stage of disease (hTR was 17 for Stage I to 60 for Stage IV). Similarly, the percentage of tumours expressing a significant level of hTR increased with stage, from 7% in stage I to 80% of stage IV tumours.

While there was a correlation between telomerase activity and hTR levels ($R = 0.71$), some tumours with $hTR > 33$ showed little or no telomerase activity by TRAP. Only one of the tumours examined that had high TRAP activity showed low hTR expression. It is not clear why hTR expression does not correlate strongly with telomerase activity. Other investigators have shown a moderate correlation between telomerase activity and hTR expression in cell lines and tumour tissues [20]. Possible explanations include degradation of telomerase activity (but not degradation of RNA) during tumour tissue collection, a dissociation between hTR RNA expression and expression of other components of the telomerase complex. These explanations are very plausible given the multicomponent nature of telomerase that is sensitive to RNAses, proteases and heat treatment.

The increase in amount of hTR RNA with stage of disease suggests that expression of hTR is associated with tumour progression in neuroblastoma. As telomerase activity may be necessary for sustained growth of malignant cells [12, 13], and as the RNA component of telomerase appears to be a necessary component of human telomerase [15], neuroblastomas which fail to express hTR may have limited growth potential. It has been hypothesised that low telomerase activity in some stage IV-S tumours leads to telomere shortening and eventual spontaneous regression [14]. Our data show that high hTR expression is associated with advanced stage neuroblastomas, while most stage I and II and many stage IV-S tumours have low hTR expression. Thus, a lack of hTR expression could be a factor that limits the growth potential of some neuroblastoma, while high hTR expression may contribute to aggressive tumour behaviour by allowing unlimited cell proliferation.

The ability to quantitate readily hTR RNA in neuroblastoma tissues, the ability to control for negative results by showing that RNA is intact using other cDNA probes and the relationship between hTR expression and stage of disease suggests that hTR expression should be a useful prognostic marker in neuroblastoma. We are conducting further studies on the potential of hTR as a prognostic marker and we are currently determining the relationship of hTR expression to clinical outcome.


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