Journal of the Egyptian Nat. Cancer Inst., Vol. 22, No. 4, December: 191-200, 2010

Telomerase Expression as Complementary Prognostic Factor in Neuroblastoma

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ABSTRACT

Background: Neuroblastoma (NB) is an aggressive tumor of childhood with a highly heterogeneous course. Identification of standard prognostic factors has several limitations. Hence, there is an increasing demand to identify new prognostic factors and tools that help in risk stratification of patients for proper treatment.

Objective: To assess the prognostic and predictive impact of quantitative telomerase expression in NB patients.

Methods: We investigated quantitative telomerase expression by immunohistochemistry in 44 neuroblastoma patients. Results were correlated with standard prognostic factors, n-myc amplification by chromogen in situ hybridization (CISH) and patients' response to treatment and survival.

Results: High telomerase expression and n-myc expression were reported in 52.3% and 56.8%; respectively. There was a significant relation between telomerase expression and n-myc amplification. Stage 3 and 4 represented 91% of patients. Thirteen patients showed complete remission, 9 partial remission, 19 no response and 3 showed progressive disease. The median follow-up was 3 years with 80% overall and 72% progression free survival for the low teleomerase expression group; and 52.2% and 55.4% for high teleomerase expression group.

High Telomerase expression by immunohistochemistry was significantly associated with n-myc amplification and with poor response to treatment with a trend toward lower overall and progression free survival.

Conclusions: Telomerase expression by immunohistochemistry is a simple potential tool for risk stratification of NB patients. CISH can serve as a readily available alternative simple tool, compared to FISH, in identifying neuroblastoma cases with abnormal n-myc gene copy number.

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Key Words: *Telomerase expression – N-myc amplification – Prognosis – Neuroblastoma.*

INTRODUCTION

Neuroblastoma (NB) accounts for approximately 6% of all childhood cancers, with an annual incidence of 8/million children under the age of 15. It is the most commonly deadly extra-cranial solid tumor in childhood **[1]**. The clinical hallmark of NB is the heterogeneous course of the disease. In children over the age of 1 year, many cases present with extensive or disseminated disease; these tumors are aggressive, chemo-resistant, and generally incurable. The dismal outlook for this group of patients accounts for the disproportionate contribution of NB to childhood cancer mortality as it represents approximately 15% of cancer related death **[2]**. In contrast, the younger age patients who present with lower stage disease **[1,2,4s]** can spontaneously regress. Therefore, the well established clinical indicators of adverse prognosis are age >1 year at diagnosis and advanced tumor stage **[3]**. Hence, clinical risk systems based on tumor histology, degree of ganglionic differentiation and the extent of Schwannian stroma have also been developed **[3-5]**.

However, these traditional parameters do not completely ensure accurate prognostic grouping in NB. Therefore new molecular markers are still needed for assessing the individual patient's prognosis more precisely **[6]**. These

markers should help in risk stratification of patients at presentation, with the most intensive treatments being reserved for high risk cases, so that children with relatively benign tumors can be spared the adverse effects of unnecessary chemotherapy **[2]**.

N-myc amplification was significantly associated with unfavorable outcome and reduced survival rates in NB which is independent of the clinical stage **[7-9]**. Therefore, it was established as a powerful clinical marker of high risk disease and currently, n-myc is the only tumor genetic feature used as a basis for treatment stratification in NB clinical trials **[10-12]**. However the individual parameters are not sufficient to predict patients' prognosis, which is why the search for new molecular markers has vital importance **[8]**.

Eukaryotic somatic cells have finite replication potential due to progressive loss of telomeres cap with each cell division. Progressive telomere shortening is implicated in cell senescence and apoptosis. This problem is usually overcome by reactivation of telomerase enzyme. Telomerase enzyme is composed of 2 subunits: Human Telomerase RNA (hTR) and human telomerase reverse transcriptase (hTERT). The later catalytic subunit is responsible for the activity of the enzyme in elongation and maintenance of Telomere structure. High activity of the telomerase enzyme has been shown to override senescence in many human malignancies, stabilizing the telomere, and immortalizing the malignant cell population. Telomerase expression has been identified as a possible prognostic factor in NB **[13]**.

A high expression of telomerase is therefore associated with unfavorable clinical and tumor genetic features and with reduced survival probability in several studies on NB **[6,14,15]**. Some studies have shown that, in stage 4 disease, telomerase values distinguished between those tumors showing good response than those that progressed with lethal outcome **[14,15]**.

In our study, primary NB specimens were analyzed for telomerase expression by immunohistochemistry and its relation to standard clinicopathological prognostic factors (including n-myc expression) and patients' outcome (response to treatment, overall and progression free survival) to assess its possible prognostic and predictive value.

MATERIAL AND METHODS

Patients and tissue samples: This study included 44 neuroblastoma patients who were diagnosed and treated at the Pediatric Department, National Cancer Institute (NCI), Cairo University, Egypt between January 2002 and December 2006. The institutional review board of the NCI approved the study.

The clinical work-up of the patients included full local and systemic imaging survey (computerized tomography [CTs], X-rays, bone scan, MIBG scan), bone marrow aspirate and biopsy, urinary vanyl mandelic acid (VMA), serum ferritin, neurone specific enolase (NSE), serum LDH, CBC, liver and renal function tests. All patients were staged according to the International Neuroblastoma Staging System (INSS) **[15]** and classified according to the children oncology group (COG) criteria **[16]**.

Accordingly patients were treated with combined multimodality therapies in the form of chemotherapy based on different combinations per risk group (OPEC, OJEC, CADO) **[17]**, surgery, radiotherapy and cis-retinoic acid. Complete surgical resection was feasible in 12 patients (10 operations were done post neoadjuvant chemotherapy), 13 patients had incomplete surgical resection and 19 patients had inoperable disease because of locally advanced and/or metastatic disease. Consolidation treatment was given to all patients except one with stage 4s who showed complete response to chemotherapy. Radiotherapy was given for palliative purposes in only 4 cases with distant metastasis (2 cases with painful bony lesions, and 2 with brain metastasis).

Disease status was evaluated (for treatment outcome) at variable stages during therapy: After induction of chemotherapy, following surgical intervention and at the end of treatment protocols.

None of the patients received therapy prior to tissue collection. Sections from paraffin blocks for each patient were obtained. Four µ thick sections (3 sections) were cut onto positive-charged slides and three 20µ thick sections

were cut in Eppendorf tubes for subsequent RNA extraction. One of the slides was stained with hematoxylin/eosin and examined microscopically to confirm the diagnosis and to assess the neoplastic: Normal cells ratio. The other three slides were used to assess telomerase expression by immunohistochemistry (IHC) and n-myc gene amplification by CISH and FISH techniques.

Immunohistochemistry: Sections were deparaffinized in xylene, hydrated through a series of graded alcohol, washed in distilled water and 0.01 PBS (pH 7.4), immersed in 10mmol/L citrate buffer (pH 6.0) and put in a microwave for 5min at 60°C for antigen retrieval. Slides were then placed in methanol containing 3% H_2O_2 for 30min at 4°C to block endogenous peroxidase activity and incubated with rabbit serum for 10min to block non-specific antibody binding sites. The primary anti-telomerase monoclonal antibody against hTERT subunit (clone NCL-hTERT, 1:50, Novocastra) was applied at a working concentration and incubated for 2 hours at 4°C. The secondary antibody and the avidin-biotin complex (ABC) were applied to slides, diaminobenzidine (DAB) was used as a chromogen and sections were counterstained with Mayer's hematoxylin. Negative controls were obtained by replacing the primary antibody by non-immunized rabbit or mouse serum. At least 500 cells were counted at 200X magnification. A non-specific cytoplasmic immunohistochemical reaction for of anti-hHTERT antibody was present in some cases especially in ganglion cells however this was not considered positive in scoring and only nuclear staining was scored positive. Similar findings were reported in previous studies for immunohistochemical detection of hTERT in paraffin embedded neuroblastoma and prostatic tissue sections, particularly in sparse material **[6,18]**.

Telomerase expression was studied on the neoplastic cells distinct brown nuclear staining as follows: +1 if telomerase expression ≥10% and <25%; +2 if telomerase expression ≥25% and <than 50%; +3 if telomerase expression ≥50% and <75%; and +4 if telomerase expression ≥75%. High expression included +3, and +4, and low expression included +1, and +2+.

Detection of n-myc gene amplification by chromogen in situ hybridization (CISH): We assessed the co-cordance rate of CISH when compared FISH for detection of n-myc gene amplification.

CISH was done according to manufacturers' recommended protocols using Digoxigenin labeled SPOT-Light n-myc DNA probe (Invitrogen, South San Francico, CA). Briefly, slides were de-paraffinized with xylene and hydrated through graded ethanol to distilled water, underwent heat pretreatment at 98°C for 15 minutes in the pretreatment buffer and washed with deionized water at RT. Digestion with pepsin was done for 5-10 minutes at 37°C followed by washing with deionized water, dehydration with graded ethanol and air-drying. The n-myc probe was added, slides were cover-slipped and sealed with rubber cement. Denaturation was performed at 94°C for 5 minutes followed by hybridization at 37°C overnight. After stringent post-hybridization wash, immuno-detection was carried out using the Invitrogen SPOT-Light TM CISH Polymer Detection Kit (California, USA), sections were counterstained with hematoxylin, dehydrated, and cover-slipped. Positive and negative controls were included in each run. The CISH results were evaluated at 200 and 400X amplifications. Cases showing >10 copies, or large clusters of the gene in >50% of the neoplastic cells were considered positive for n-myc gene amplification **[19]**.

Validation of n-myc gene amplification by FISH: Double-colored FISH was carried out on interphase nuclei for validation of CISH results using the Vysis LSI n-myc (2p24) Spectrum-Green/CEP 2 SpectrumOrange™ Probe kit (Vysis, Downers Grove, IL, USA). Ten cases positive for n-myc gene amplification by CISH and 10 cases with normal gene copy number were included in the validation study without knowing the CISH results. FISH was done as previously described **[20]**. Signals were evaluated with a fluorescent microscope (Olympus BX60 Fluorescence Microscope/Camera, Center Valley, PA, USA) containing SpectrumOrange, SpectrumGreen, and DAPI filter. At least 50 cells for each case were evaluated, without knowledge of the CISH status. The n-myc copy number was classified by the number of copies per cell according to the previously published criteria **[20]**.

RNA extraction and PCR amplification: Total RNA was extracted from collected samples

according to the described methodology of pure script RNA isolation kit (Gentra, USA) and its concentration was determined spectrophotometrically. The extracted total RNA was assessed for degradation, purity and DNA contamination by spectrophotometry and electrophoresis in 2.0% ethidium bromide-stained agarose gel. Total RNA (1.0µg) was reverse transcribed using the Superscript One-Step RT-PCR Kit with Platinum Taq (Life Technologies, Inc.) in 50-µl reaction volume at 42°C for 1 hour. This was followed by incubation at 72°C for 15min. The primer sequences used for N-myc gene (258 bp) were: The forward primer (C3F2) 5/-GCG AGC TGA TCC TCA AAC GA-3/and the reverse primer (STR) 5-TGG TCC CTG AGC GTG AGA AA-3/. The PCR cycling conditions were as follows: Denaturation at 96°C for 4min, followed by 30 cycles: 95°C for 55sec, 58°C for 1min and 72°C for 90sec with a final extension step at 72°C for 10min. The B-globin gene (520 bp) was chosen as an endogenous expression RT-PCR standard that represents a single copy number reference. Positive (IMR 32 neuroblastoma cell line which express n-myc gene amplification) and a negative PCR control were included in each cycle. Ten µl of RT-PCR products were resolved in 2.0% agarose gel and positive cases were repeated twice on two successive days **[21]**.

Statistical analysis: Data was analyzed using the SPSS statistical package version 15. Numerical data were expressed as, median and range. Qualitative data were expressed as frequency and percentage. Chi-square test (Fisher's exact test) was used to examine the relation between qualitative variables. Survival analysis was done using the Kaplan Meier method. Comparison between two survival curves was done using Log rank test was done for univariate analysis affecting both overall and progression free survival and response to therapy; respectively. *p* value ≤0.05 was considered significant.

RESULTS

Twenty four patients were males and 20 were females (ratio 1.2:1). The age ranged from 4 months to 10 years (median 3 years). The majority of cases were of advanced stage: 31 (70%) presented with stage 4, 9 (20.5%) with stage 3, 2 (4.5%) with stage 2 and another 2 (4.5%) with stage 4s. Clinicopathological features of the patients were collected by retrospective charts review (Table 1).

The median follow-up duration was 3 years (range 1.1-6.2 years). High telomerase expression (+3 and +4) was reported in 23 (52.3%) patients (Fig. 1) and this high expression was significantly associated with a high risk group (p=0.002), poor response to treatment (p=0.001), unfavorable Shimada (p=0.001), increased expression of VMA (p=0.010) and NSE (p=0.003) (Table 2). None of the 23 patients with increased telomerase expression achieved complete remission (CR), 4 (17.4%) patients achieved partial remission (PR), 18 (78.3%) showed no response (NR) and a single patient (4.3%) showed disease progression (DP). In contrast, out of the 21 patients with low telomerase expression (+1 and +2), 13 (61.9%) attained CR, 5 (23.8%) PR, 1 (4.8%) NR and 2 (9.5%) DP.

N-myc gene amplification was reported in 25 (56.8%) patients by CISH compared to 19 (43.2%) by RT-PCR (concordance 85%). This could be attributed to the lower sensitivity of the PCR amplification especially in small tissue samples with high normal: Tumor ratio or to the difficulty in obtaining a good quality RNA from paraffin-embedded tissues (PET). On the other hand, the concordance between CISH and FISH results was 99% since all the CISH positive cases used in the validation study **[10]** were also positive by FISH whereas 9 CISH negative cases were also negative by FISH and one case was FISH positive (Fig. 2).

As shown in (Table 3) n-myc gene amplification was significantly associated with a high risk (p=0.004) and poor response to treatment (p=0.001). Out of the 25 cases with n-myc gene amplification, 2 (8%) showed CR and 23 (92%) did not [6 patients showed PR, 14 showed NR and 3 showed DP]. On the other hand, in the group of patients with non amplified n-myc gene, 11 (57.9%) entered into CR and 8 (42.1%) did not.

Considering the risk group, there was a significant relation between patients' response to treatment and the risk group (p=0.003) since 4 out of the 30 HR patients (13.3%) attained CR to the given therapy compared to 26 (86.7%) who did not (8 showed PR, 16 showed NR and 2 PD). On the other hand, 7 out of the 12

(58.3%) patients with IR showed CR compared to 5 (41.7%) who did not (1 showed PR, 3 NR and 1 PD).

Survival analysis: A trend was noticed toward improved survival (Overall survival [OS] and progression free survival [PFS]) with low compared to high telomerase expression (Fig. 3 and Table 4); although this did not reach statistical significance, where the cumulative OS and PFS at 5 years were 80.2% and 72.2% respectively for the IR (Intermediate Risk) group compared to 52.9% and 55.4% for the (High Risk) HR group.

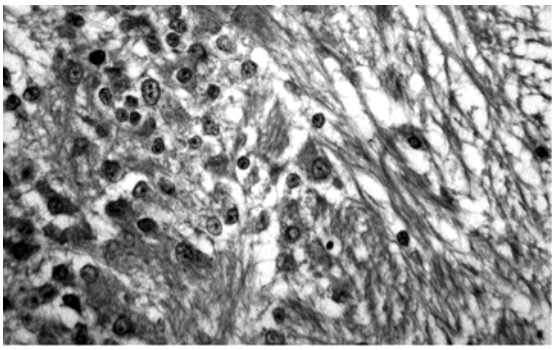

Fig. (1A): A case of ganglioneuroblastoma with absent nuclear telomerase immunoreactivity (x450), (telomerase monoclonal immunostain, DAB chromagen and Hematoxylin as counter stain).

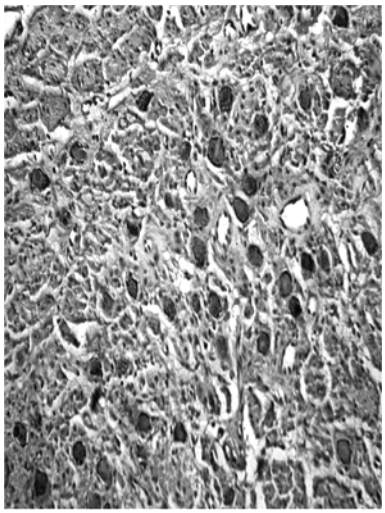

Fig. (1B): A case of ganglioneuroblastoma with mild telomerase nuclear immunoreactivity. Scattered large tumor ganglionic cells are positive for the enzyme. A cytoplasmic reaction for the enzyme is also encountered (x200), (telomerase monoclonal immunostain, DAB chromagen and Hematoxylin as counter stain).

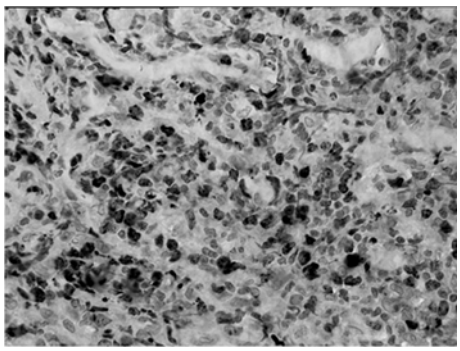

Fig. (1C): A case of neuroblastoma; unfavorable histology, showing an intermediate nuclear positive immunoreactivity for telomerase enzyme. (x400), (telomerase monoclonal immunostain, DAB chromagen and Hematoxylin as counter stain).

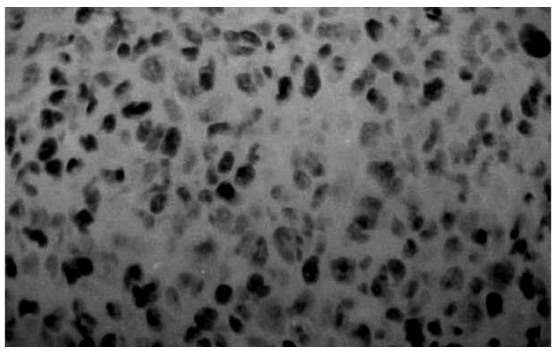

Fig. (1D): A case of neuroblastoma; unfavorable histology, showing a high nuclear positive immunoreactivity for telomerase enzyme. (x450), (telomerase monoclonal immunostain, DAB chromagen and Hematoxylin as counter stain).

Fig. (1): A case of NB with high (+3) telomerase (hTRT) expression showing brown, nuclear immunostaining in more than 90% of the cells, (B) A case of NB with moderate (+2) telomerase expression showing brown, nuclear immunostaining in 45% of the cells, (C) A case of NB with mild (+1) telomerase expression showing brown, nuclear immunostaining in 15% of the cells, (D) A case of NB negative for telomerase (hTRT) expression.

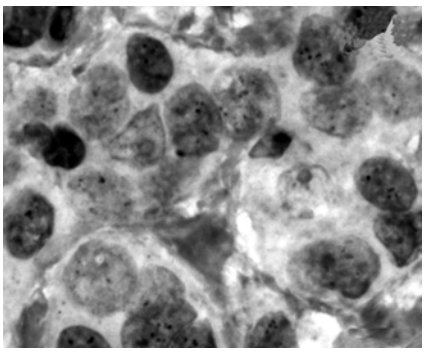

Fig. (2A): A case of primary neuroblastoma showing normal n-myc gene copy number by CISH.

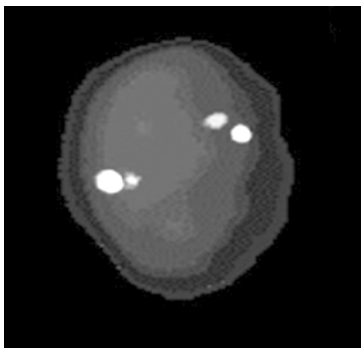

Fig. (2B): A case of primary neuroblastoma showing normal n-myc gene copy number by CISH and FISH.

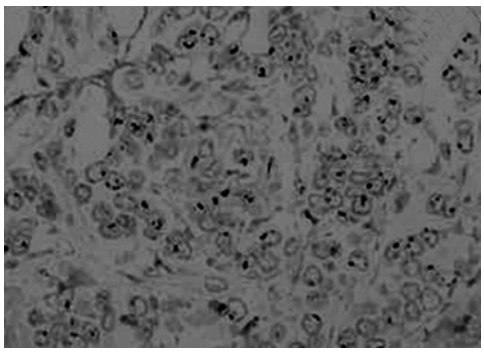

Fig. (2C): A case of primary neuroblastoma showing n-myc gene amplification by CISH appearing as brown clusters in the nuclei of almost all neoplastic cells whereas the nuclei of the tumor cells are visualized by hematoxylin counterstain.

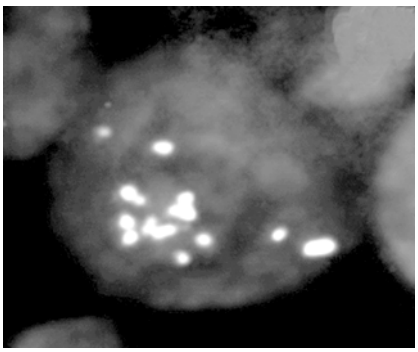

Fig. (2D): A case of primary neuroblastoma showing n-myc gene amplification by CISH appearing as brown clusters in the nuclei of almost all neoplastic cells whereas the nuclei of the tumor cells are visualized by hematoxylin counterstain and the same case showing n-myc gene amplification by FISH where n-myc is seen as green dots, while the normal control signal (centromeric probe for chromosome 2) is seen as red dots. The nuclei of the tumor cells are visualized by DAPI counter-stain.

Fig. (2): A case of primary neuroblastoma showing (A) normal n-myc gene copy number by CISH and by (B) FISH. (C) A case of primary neuroblastoma showing n-myc gene amplification by CISH appearing as brown clusters in the nuclei of almost all neoplastic cells whereas the nuclei of the tumor cells are visualized by hematoxylin counterstain and (D) the same case showing n-myc gene amplification by FISH where n-myc is seen as green dots, while the normal control signal (centromeric probe for chromosome 2) is seen as red dots. The nuclei of the tumor cells are visualized by DAPI counter-stain.

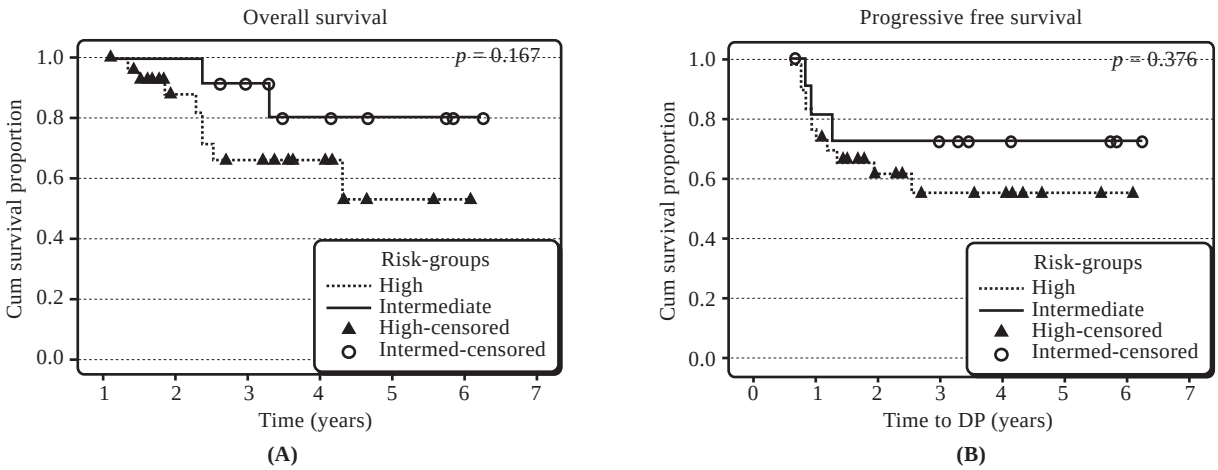

Fig. (3): Kaplan and Meier curves of the (a) overall survival and (b) progression free survival in low and high telomerase activity groups.

Table (1): Clinicopathological features of the 44 neuroblastoma patients under study.

Item	No (44)	%
Age		
≤365 day	17	38.6
>365 day	27	61.4
Sex		
Male	24	54.5
Female	20	45.5
Stage		
Stage 2	2	4.5
Stage 3	9	20.5
Stage 4	31	70.4
Stage 4s	2	4.5
Shimeda		
Favorable	23	52.3
Unfavorable	21	47.7
N-myc amplification		
Negative	19	43.2
Positive	25	56.8
Telomerase expression		
Low	21	47.7
High	23	52.3
Risk group		
Low	2	4.5
Intermediate	12	27.3
High	30	68.2
Response		
Complete response	13	29.5
No complete response	31	70.5
Survival status		
Alive	34	77.2
Dead	10	22.7

Table (2): Relation between telomerase expression and the clinico-patological parameters of the studied patients.

Item	Telomerase expression		*p* value
	High	Low	
Age			
≤365 days (n=17)	3 (17.6%)	14 (82.4)	
>365 days (n=27)	20 (74.1%)	7 (25.9%)	<0.001
*Stage**			
Stage 3 (n=9)	3 (33.3%)	6 (66.7%)	
Stage 4 (n=31)	20 (64.5%)	11 (35.5%)	<0.090
VMA (median 84.0)			
Below median (n=21)	7 (33.3%)	14 (66.7%)	
Above median (n=23)	16 (69.6%)	7 (30.4%)	<0.010
Ferritin (median 145.0)			
Below median (n=20)	8 (40%)	12 (60%)	
Above median (n=24)	15 (62.5%)	9 (37.5%)	<0.130
NSE (median 161.5)			
Below median (n=21)	6 (28.6%)	15 (71.4%)	
Above median (n=23	17 (73.9%)	6 (26.1%)	<0.003
Shimada			
Unfavorable (n=21)	18 (85.7%)	3 (14.3%)	
Favorable (n=23)	5 (21.7%)	18 (78.3%)	<0.001
*Risk-group***			
High (n=30)	21 (70%)	9 (30%)	
Intermediate (n=12)	2 (16.7%)	10 (83.3%)	<0.002
N-myc			
Non amplified (n=19)	5 (26.3%)	14 (73.7%)	
Amplified (n=25)	18 (72%)	7 (28%)	<0.003
Response			
CR (n=13)	0 (0%)	13 (61.9%)	
No CR (n=31)	23 (100%)	8 (38.1%)	<0.001

* : Stage 2 and 4s were excluded (small number).
** : Low risk group patients (n=2) were excluded.
NSE : Neuron specific enolase.
VMA : Vanillyl mandelic acid.

Table (3): The correlation between n-myc gene status and the clinico-patological parameters of the studied patients.

Item	N-myc		*p* value
	Non amplified	Amplified	
Age			
≤365 days (n=17)	9 (52.9%)	8 (47.1%)	
>365 days (n=27)	10 (37%)	17 (63%)	<0.300
*Stage**			
Stage 3 (n=9)	5 (55.6%)	4 (44.4%)	
Stage 4 (n=31)	11 (35.5%)	20 (64.5%)	<0.200
VMA (median 84.0)			
Below median (n=21)	12 (57.1%)	9 (42.9%)	
Above median (n=23)	7 (30.4%)	16 (69.6%)	<0.740
Ferritin (median 145.0)			
Below median (n=20)	9 (45%)	11 (55%)	
Above median (n=24)	10 (41.7%)	14 (58.3)	<0.820
NSE (median 161.5)			
Below median (n=21)	12 (57.1%)	9 (42.9%)	
Above median (n=23)	7 (30.4%)	16 (69.6%)	<0.070
Shimada			
Unfavorable (n=21)	6 (28.6%)	15 (71.4%)	
Favorable (n=23)	13 (56.5%)	10 (43.5%)	<0.620
*Risk group***			
High risk (n=30)	8 (26.7%)	22 (73.3%)	
Intermediate risk (n=12)	9 (75%)	3 (25%)	<0.004
Telomerase			
Low (n=21)	14 (66.7%)	7 (33.3%)	
High (n=23)	5 (21.7%)	18 (78.3%)	<0.003
Response			
CR (n=13)	11 (57.9%)	2 (8%)	
No CR (n=31)	8 (42.1%)	23 (92%)	<0.001

* : Stage 2 and 4s were excluded (small number). NSE : Neuron specific enolase.
**: Low risk group patients (n=2) were excluded. VMA : Vanillyl mandelic acid.

Table (4): The relation between survival rates (progression free and overall survival), telomerase expression and n-myc gene status for each risk group.

Item	IR (N=12)					HR (N=30)				
	No.	OS (80.2%)	"*p* "	PFS (72.7%)	"*p* "	No.	OS (52.9%)	"*p* "	PFS (55.4%)	"*p* "
Telomerase expression										
High	2	50%	*	50%	*	21	54.1%	<0.23	56.4%	<0.330
low	10	85.7%		77.7%		9	57.1%		64.8%	
N-myc gene status										
Amplified	3	0%	*	48.4%	*	22	48.6	<0.82	48.4%	<0.290
Non-amplified	9	100%		75%		8	72.9%		75%	

IR : Intermediate risk. OS : Overall survival. TA : Telomerase activity.
HR : High risk. PFS : Progression free survival. * : Technically non computable "*p*" value.

DISCUSSION

Neuroblastoma is the most common malignant disease of infancy and a deadly extracranial tumor in childhood. It has served as a model for risk stratification system based on both clinical and tumor biologic data. Therefore, identifying markers of increased risk of recurrence and treatment failure in the low, intermediate and even the high risk groups is of utmost importance **[2]**. There is increasing evidence that analysis of n-myc agene amplification alone does not ensure completely accurate prognostic grouping which is also true for other traditional molecular and clinical markers **[22]**. Telomerase expression has emerged in the last few years as an unfavorable prognostic marker in some solid tumors, which is associated with poor clinical response and reduced survival rates **[23]**.

The results of the present study confirm the predictive value of high telomerase expression and n-myc gene amplification in the intermediate and high risk groups of NB patients since this high expression was significantly associated with worse outcome. Similar results were previously reported **[6,9-11,14]**. We also reported a highly significant correlation between these two markers which is in agreement with Maitra et al. **[24]**. Our results confirm the findings of Mac et al. **[25]** who demonstrated that the hTERT promotor of the telomerase contains binding sites for the transcription factor of n-myc.

The data presented in the current study, with its long median follow-up, suggests that telomerase expression by immunohistochemistry is a predictor factor for treatment response in NB patients since patients with high telomerase expression revealed a significantly worse response to treatment compared to those with low telomerase expression. Moreover, a high telomerase expression by immunohistochemistry was significantly associated with poor prognostic factors including a high risk group, an unfavorable Shimada, increased expression of VMA and NSE as well as n-myc gene amplification (Table 3).

The prognostic relevance of telomerase, namely TE (Telomerase Expression) and telomere length variation has been previously addressed by several groups, who demonstrated a correlation between poor outcome and high TE **[6,13,14,23,26]**. Isobe et al. **[27]** found that all the unfavorable NB samples in their series were positive for hTERT by PCR compared to 29% only of the favorable cases. Consequently, they concluded that hTERT mRNA might be used as a potential prognostic marker for NB. Similarly, Binz et al., **[28]** demonstrated that, the unfavorable NB cases are characterized not only by n-myc gene amplification, but also by high TE. They proved in their study that telomestatin, G quadriplex interactive agent, limits the cellular lifespan of NB cells through disruption of telomerase maintenance. Moreover, Struetker et al. **[26]** demonstrated that undetectable or low TE was detected more often in younger patients (age <1 year) in correlation with absence of adverse prognostic factors, whereas high TE was identified in children older than 1 year and was associated with poor prognostic markers such as n-myc gene amplification, 1p deletion, TRK-A expression and advanced disease stage. The results of the present study are comparable to previously mentioned reports as a telomerase protein over expression was significantly correlated with

poor response to treatment and poor prognostic parameters **[26].**

To the best of our knowledge, only few studies have assessed telomerase protein expression in NB **[13,14]**. Assessment of Telomerase protein expression by immunohistochemical techniques is simple, rapid and could be practiced in most pathology labs. It also permits correlation with morphology to assure the expression of the protein in the neoplastic cells which is not possible by PCR or FISH techniques. It also avoids the false negative results, caused by the neutralizing effect of normal cells in the specimen, or false positive results, caused by the contamination, of the PCR, which is not usually present in the pathology labs. Moreover, assessment of TE requires fresh tumor samples that are not always available in daily clinical practice.

A trend was noticed toward improved survival (Overall survival [OS] and progression free survival [PFS]) with low compared to high telomerase expression and survival rates, clinical risk group and neither telomerase expression nor n-myc gene amplification (Fig. 3 and Table 4); these findings did not reach statistical significance.

Although our results contradicts with some previously published data **[6,13,23]**. This could be attributed to the relatively small number of cases in the present study, which hampered survival analyses in the IR group.

Our data regarding the significant relation between Telomerase expression and n-myc gene amplification and patients' response to treatment are consistent with previous reports in this context **[29,30]**. In the study of Brodeur et al. **[29]** n-myc amplification was found in 50% of stage 3 and 4 tumors but in none of stage 1 and 2 tumors. Multiple studies **[9,10,14,30]** subsequently confirmed this association with progressive disease, and showed that n-myc amplification correlates with a greatly increased risk of poor response to therapy and fatal outcome. In the study of Seeger et al. **[10]** the adverse associations of n-myc amplification was linked to treatment failure in early stage disease, whereas normal n-myc status was associated with continuing remission in stage 3 and 4 tumors. N-myc amplification is thus firmly implicated in the malignant aggressiveness of NB tumors and a powerful clinical marker for high risk disease **[2].**

Recently, chromogenic in situ hybridization (CISH) emerged as an alternative technique for assessing gene amplification status to overcome FISH limitations using a peroxidase-based chromogenic reaction and a bright-field microscopy. It uses DNA probes generated by Subtracted probe Technology (SPT), which remove repetitive DNA sequences and therefore, the final probe is very specific, and the need for blocking non-specific hybridization with cot-1 DNA in traditional FISH probes is eliminated **[6,14]**. The practicality of FISH is limited by the need for fluorescence microscopy equipment and technical expertise. CISH provides a simple alternative to FISH; CISH utilizes a chromogen that can be visualized by light microscopy, permitting interpretation while simultaneously examining the tissue histology and correlating CISH results with cytoarchitectural details in the examined cancer sections.

In the present study, we evaluated the utility of CISH in detecting n-myc gene copy number by comparing n-myc CISH with FISH results in a cohort of patients known to have a high prevalence of n-myc mutations. We demonstrated that CISH can reliably be used to detect n-myc gene copy number changes and we propose that CISH can serve as an easy and readily available tool for identifying neuroblastoma cases with abnormal n-myc gene copy number.

We conclude that quantitative telomerase expression by immnohistochemistry is a potential prognostic marker for intermediate and high risk NB patients, which is highly predictive of poor treatment response. It is commonly associated with n-myc gene amplification. Further prospective studies with larger patient samples and longer observation periods are required to better evaluate the correlation of TE with patients' survival.

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