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Prognostic significance of telomerase activity in neuroblastoma like tumours

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Introduction. Telomerase is an enzyme responsible for maintaining the length of telomeres – end structures of chromosomes indispensable for the proper functioning and stabilization of genetic material of eucaryotic cells. The presence of activated telomerase in neoplastic cells prevents telomere shortening and is responsible for cell immortality.
The aim of the study was to evaluate telomerase activity in neuroblastoma-like tumours and to estimate the influence of telomerase activity on patient outcome. Correlations between telomerase activity and other prognostic factors were also investigated.
Patients. 33 tissue samples obtained from 30 patients aged between 1 and 75 months (19 boys and 11 girls) were analysed. Neuroblastoma was diagnosed in 24 patients (80%), ganglioneuroblastoma in 4 (13.3%) and ganglioneuroma in 2 (6.7%) patients.
Methods. Tissue samples were frozen immediately after biopsy and stored in -80°C until the analysis. All samples were checked with alkaline phosphatase reaction in order to exclude false negative results. For telomerase activity evaluation two independent methods were used: TRAP method (Telomerase Repeat Amplification Protocol; TRAP®eze Telomerase Detection Kit, Intergen Company, S7700) and ELISA method (TeloTAGGG Telomerase PCR ELISA, Roche Diagnostics, 2013789).
Results. Telomerase activity was stated in 9 tissue samples of primary tumours, and in a regional lymph node infiltrated by neoplasm cells and in a metastatic relapse in the mediastinum. Neuroblastoma with telomerase activity presented as a more advanced malignancy (larger tumours with infiltration of regional lymph nodes or disseminated disease) and more common with prognostically unfavourable MYCN amplification. Patients with telomerase activity had significantly poorer outcome, as compared to patients without telomerase activity (5-year EFS 0.61 and 0.15, respectively; p=0.01).
Conclusion. The assessment of telomerase activity before the onset of oncological treatment may aid patient stratification and the choice of optimal therapy. The influence of telomerase on clinical outcome supports the theory of its pathogenetic role in neuroblastoma, so the enzyme could be a possible target for new antitumour inhibiting drugs.

Rokownicze znaczenie aktywności telomerazy w guzach typu neuroblastoma

Wstęp. Telomeraza jest enzymem odpowiedzialnym za utrzymanie stałej długości telomerów, końcowych struktur chromosomalnych, niezbędnych do prawidłowej funkcji i stabilności materiału genetycznego komórek eukariotycznych. Obecność zaktywowanej telomerazy w komórkach nowotworowych zapobiega skracaniu telomerów, a tym samym powoduje, że komórki te stają się nieśmiertelne.
Celem pracy było zbadanie aktywności telomerazy w guzach typu neuroblastoma i próba oceny wpływu tego parametru na rokowanie w grupie chorych z tym nowotworem. Porównano ponadto zależność występowania aktywnej telomerazy z innymi czynnikami prognostycznymi.

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Pacjenci. Materiał badawczy stanowiły 33 wycinki tkanki nowotworowej, pobrane od 30 pacjentów w wieku od 1 do 75 miesięcy (19 chłopców i 11 dziewczynek). U 24 chorych (80%) rozpoznano neuroblastoma, u 4 (13,3%) ganglioneuroblastoma, u pozostałych 2 (6,7%) ganglioneuroma.
Metoda. Bezpośrednio po pobraniu materiał tkankowy zamrażano w ciekłym azocie i przechowano w stanie zamrożenia do czasu podjęcia analizy. W celu wykluczenia wyników fałszywie ujemnych, we wszystkich próbkach metodą dot blot oznaczono aktywność fosfatazy alkalicznej. Do oznaczenia aktywności telomerazy posłużono się dwoma niezależnymi metodami: procedurą TRAP (Telomerase Repeat Amplification Protocol; TRAP®eze Telomerase Detection Kit, Intergen Company, S7700) oraz metodą ELISA (TeloTAGGG Telomerase PCR ELISA, Roche Diagnostics, 2013789).
Wyniki. Aktywność telomerazy stwierdzono w materiale pochodzącym z guza pierwotnego u 9 pacjentów, a także w nacieczonym nowotworowo węźle chłonnym oraz we wznowie przerzutowej choroby w śródpiersiu. W przypadku guzów z dodatnią aktywnością telomerazy proces chorobowy był bardziej zaawansowany (guzy dużych rozmiarów, częstsze zajęcie węzłów chłonnych, częściej stadium IV), a także częściej obserwowano obecność niekorzystnej prognostycznie amplifikacji onkogenu MYCN. Pacjenci z dodatnią aktywnością telomerazy cechowali się także znamiennie gorszym rokowaniem, w porównaniu do pacjentów telomerazonegatywnych (5-letnie EFS odpowiednio 0,61 i 0,15; p=0,01).
Wnioski. Oznaczenie aktywności telomerazy przed rozpoczęciem leczenia przeciwnowotworowego może pomóc w stratyfikacji prognostycznej chorych i wyborze najlepszej metody terapeutycznej. Wpływ telomerazy na rokowanie świadczy o udziale tego enzymu w patogenezie neuroblastoma i dlatego sugeruje się poszukiwanie nowych leków przeciwnowotworowych, hamujących aktywność tego enzymu.

Key words: telomerase, neuroblastoma, children's neoplasms
Słowa kluczowe: telomeraza, neuroblastoma, nowotwory dziecięce

Study background

Neuroblastoma-type tumours are one of the most common malignancies in children. Their common feature is their origin – from ganglion neurons of the sympathetic system. Most common neuroblastoma localizations include the suprarenal glands and the paravertebral ganglia. Neuroblastoma-type tumours may be divided into a number of subtypes, which differ as to histology, biology and clinical features. One of these subtypes covers highly malignant, undifferentiated neuroblastomas; the ganglioneuroblastomas show some degree of maturity, while ganglioneuromas are highly differentiated, mainly benign tumours [1, 2].

In this particular group of tumours one may observe relatively common spontaneous tumour regression – especially in children below 1 year of age – and, also, idiopathic tumour maturation towards high differentation. Such varied tumour biology makes patient stratification and choice of treatment difficult. On the one hand the possibility of spontaneous regression or maturation offers a chance of successful treatment even with less intensive therapy; on the other hand inadequate oncological treatment may lead to disease progression and death. Recently a wide number of studies have been conducted, aimed at analyzing the biological features of this malignancy and at preparing a system of stratification, which would be based on prognostic factors, thus allowing to decide upon the best choice of treatment. In the course of these studies a number of biological prognostic factors have been identified (e.g. the number of copies of the MYCN oncogene, the presence of chromosome 1 short arm deletion, DNA ploidy, the presence of the tyrosine kinase receptor – TRK-A – for the neural growth factor) [3]. All these have become useful in standard diagnostics. However, we are still searching for new data which would allow for more precise prognosis. The activity of telomerase, the enzyme responsible for unlimited multiplication of malignant tumours, is one of these promising factors [4-7].

Telomerase is a ribonucleoprotein enzyme which is responsible for maintaining the length of telomeres unchanged. Telomers are highly specific chromosomal end structures which consist of short DNA repetitions (TTAGGG) and proteins. They are necessary to maintain the correct functioning and stability of the genetic material of eucaryotic cells [8] as they prevent chromosome adhesion, incorrect recombination and degradation. They also facilitate complete replication, thus preventing the loss of genes necessary for cell survival in the course of cellular DNA replication. DNA polimerase, which is responsible for replication, is incapable of complete copying of the ends of linear DNA. In healthy cells every division of the genetic material causes unavoidable shortening of chromosomes and loss of a DNA fragment. The presence of telomeres prevents the loss of important genes, however each course of replication causes the loss of repeated telomere sequences. This limits the number of divisions achieved by a single cell. They may only continue until the moment when telomeres reach critical length (approx. 3 kbp) [9, 10]. A number of cells derived from structures where regeneration is a necessity (sex cells, embryonal cells, stem cells, intestinal crypt cells, cells of the sheath of the hair, hepatocytes) are capable of unlimited division due to the presence of active telomerase, the enzyme capable of rebuilding lost telomere fragments.

The activation of telomerase is one of the most characteristic features of neoplastic transformation. Due to this phenomenon neoplastic cells achieve the ability of constant division, and thus become immortal. It has been observed that in some types of malignancy the identification of active telomerase is associated with

poorer prognosis [9-12]. Studies of telomerase activity in neuroblastomas suggested, that the enzyme plays an important part in the pathogenesis and clinical course of these tumours [4-7]. The aim of the present study was to investigate the influence of telomerase activity on the prognosis of patients with embryonal neuroblastoma. We also analysed the relations between telomerase activity and a number of clinical parameters and evaluated the possibility of applying this feature as an element of routine diagnostics.

Material

Telomerase activity was investigated in 33 tissue samples obtained in the course of diagnostic biopsy or tumour resection from 30 patients (19 boys, 11 girls) aged from 1 to 75 months (median age: 15.5 months) with neuroblastoma-like tumour. In all cases the tissue samples underwent histological verification by another pathologist in order to confirm the initial diagnosis and to decide, whether the obtained tissue sample is representative for the tumour and may be used for further analysis. In the case of 19 patients the material was obtained before the onset of treatment (63.3%) and in the remaining 11 patients (36.7%) the samples were obtained during chemotherapy. In the case of one child we also obtained an infiltrated lymph node, in another – a malignant lesion present in the neighbouring suprarenal gland and in yet another – telomerase activity was analysed in a tissue sample obtained from the metastatic relapse found in mediastinum.

In 24 patients (80%) neuroblastoma, in 4 (13.3%) – ganglioneuroblastoma and in the remaining 2 (6.7%) – ganglioneuroma were diagnosed. In 26 patients (86.7%) the primary tumour was located in the suprarenal gland, in 3 (10%) in the mediastinum and in 1 child (3.3%) within the structures of the neck. Regional lymph node involvement was observed in 14 patients (46.7%). At the time of diagnosis distant metastases were present in 17 patients (56.7%) – usually to the liver (6 pts.), bone marrow (5 pts.) and bones (4 pts.). Median follow-up time for the entire group was 26.5 months, median follow-up time for patients surviving till this day was 29 months.

Methods

Specimens

The tissue samples were preserved in liquid nitrogen (-170° Centigrade) directly after obtaining. Samples from 27 patients were kept at –80° Centigrade, from the remaining 3 patients at –20° Centigrade until analysis. Before defrosting the samples were placed into teflon jars with carbide-volframe beads (B. Braun Biotech International; Melsungen) and homogenized in a microdismembrator (2000 r.p.m. over 30 seconds). In order to avoid telomerase inactivation all further processing took place "on ice" in 0° Centigrade. The homogenized samples were dissolved in the CHAPS buffer (supplied with the TRAPeze kit®) at a ratio of 25 µl per 20-50 µg of tissue adding an RNA-ase inhibitor (Gibco Brl. 40 U/µl) at a ratio of 5 µl per 1 ml of CHAPS buffer. After 30 min. incubation "on ice" the samples were centrifuged (12000 r.p.m. at 4° for 20 minutes). 25 µg of the supernatant were taken for analysis of protein content, the remaining part was frozen in liquid nitrogen and kept at -80° Centigrade until further analysis.

Alcaline phosphatase activity

In order to avoid false negative results alcalic phosphatase activity was determined in all samples by the dot-blot method. 1µl of the analysed tissue sample (CHAPS buffer lysate) was placed on a nylon membrane (Nylon Membrane, Roche) and blocked with a 1% solution of powdered milk dissolved in maleinian acid buffer (pH 7.5; 30 min), rinsed twice on a shaker in maleinian acid buffer (pH 7.5) with an addition of 0.1% Tween solution (10 min. each time). Then the membrane was balanced in a solution containing 100 mM TRIS base and 50 mM $MgCl_2$ for every 500 ml of buffer. Next we added 500 µl of CSPD "ready to use" (Roche, 1755633) and incubated in the dark for 5 min. Thus prepared membrane was incubated for 20 min. in 37° Centigrade in a radiographic cassette. In order to obtain the results we exposed the chemiluminescence film (Hyperfilm™ ECL™ igh Performance Chemiluminescence Film, Amersham Pharmacia Biotech) for 2-5 minutes, and then exposed it.

Evaluation of protein content

We estimated the standard curve using the standard BSA solution (bovine serum albumin – 1 mg/ml supplied with the kit). In order to estimate the standard curve we used the following standard solutions: 50 µl BSA, 40 µl BSA + 10 µl H_2O, 30 µl BSA + 20 µl H_2O, 20 µl BSA + 30 µl H_2O, 10 µl BSA + 40 µl H_2O and 50 µl H_2O. The analysed samples were prepared in two concentrations – 1:5 and 1:10. We added 1 ml of a solution obtained by adding 20 µl of 4% $CuSO_4$ (Sigma, C2284) per 1 ml of BCA (Bicinchoninic Acid Solution; Sigma, B9643) and incubated in a water bath in 37° Centigrade for 30 min. Using the standard solutions we prepared the standard curve. Next the absorbancy of the analysed samples at wave length λ=562 nm was measured and protein content was calculated.

Evaluation of telomerase activity

In order to obtain reliable results we estimated telomerase activity in each sample using two different methods – TRAP (Telomerase Repeat Amplification Protocol) with the TRAPeze® Telomerase Detection Kit (Intergen Company, S7700) and the ELISA method with the TeloTAGGG Telomerase PCR ELISA PLUS (Roche, 2013789).

The TRAP method

2 µl of previously prepared samples containing 0.25 µg/µl and 0.025 µg/µl protein dissolved in the CHAPS buffer were put into free RNA-ase vials. Simultaneously we prepared positive (1 µl TSR8 supplied with the kit + 1 µl dH_2O) and negative (2 µl CHAPS buffer) controls. Then we added 48 µl of the "Master Mix" solution to each vial ("Master Mix" solution was prepared with the following substances supplied with the kit: 10xTRAP Reaction Buffer – 5.0 µl, TS Primer – 1.0 µl, 50x dNTP Mix – 1.0 µl, TRAP Primer Mix – 1.0 µl, *Taq*-Polymerase – 0.4 µl (2U), dH_2O – 39.6 µl). We performed the PCR (Polymerase Chain Reaction) using 33 and 35 amplification cycles. The PCR was performed according to the following scheme: incubation in 30° Centigrade for 30 min; next incubation in 90° Centigrade for 2 mins, next – 33 or 35 cycles, each including 30 s. 57° Centigrade + 30 s. 94° Centigrade. When the PCR was complete we added dye to each sample (0.25% Bromophenol Blue/0.25% Xylene cyanol in 50% glycerol/50mM EDTA) and placed it on non-denaturating gel (6% Long Rager Gel). The results were obtained with the aid of a sequencer – DNA Sequencer Model 4000 (MWG-Biotech, Ebersberg) – read-out conditions: 35 mA, 31W, approx. 1000V, 50° Centigrade; read-out time: approx. 40 min. Samples were considered positive if at least three strias were discerned, beginning with the stria for 50 bp DNA and, additionally, a 36 bp stria which providing the internal control of PCR correctness. Samples were considered negative if only the 36 bp stria was present. If the 36 bp stria was

absent the sample was considered as non-interpratable and the analysis was repeated until a conclusive result was obtained.

The ELISA method

In this method we used the TeloTAGGG Telomerase PCR ELISA PLUS kit. 2 μl of each sample were added to 30 μl of the reagent, which consisted of: biotynylated telomerase substrate P1-TS, the P2 binding primer, nucleotides, DNA *Taq*-polymerase and 5 μl 0.001 amol/μl standard DNA 216 bp of length (substrates supplies with the kit). Each analysis contained a positive control, obtained aforehand by adding 1 μl 0.001 amol/μl DNA the sequence of which was identical with the telomerase product which consisted of 8 repetitions of telomere sequences (TS8) and with a negative control obtained by adding 1 μl of the CHAPS buffer to the reagent. Apart from this a separate negative control was prepared for each sample by heating the sample to 94° Centigrade for 20 min. Nuclease-free water was added to all samples up to 50 μl and the samples were incubated for 30 min. at 37° Centigrade and then inactivated by heating to 94° Centigrade over 5 min. Then 31 cycles of the PCR were performed (one cycle consisted of 94° Centigrade for 30 seconds, 50° Centigrade for 30 seconds and 72° Centigrade for 90 seconds). The last stage of the PCR was incubation in 72° Centigrade for 10 minutes. Then 2.5 μl of the PCR products were added to 10 μl of the denaturating reagent and incubated at room temperature for 10 minutes. After incubation we added 100 μl of hybrydising buffer to each sample and incubated it for 2 hours in 37° Centigrade. After this time all the samples were placed on a streptavidine-coated MTP (microtiter plate), covered with self-adhesive film and incubated at the same temperature for one more hour. Afer incubation the solution was decanted and each fossa was carefully rinsed with the rinsing solution. Next 100 μl of policlonal sheep antibody conjugated with horseradish peroxidase (Anti-DIG-HRP) was added to each fossa, covered with self-adhesive film and incubated for another 30 min. at room temperature on a shaker (300/min). Then the solution was decanted and each fossa was carefully rinsed with the rinsing solution. In the next stage 100 μl of the TMB reagent (supplied with the kit) was added to each fossa and incubated on the shaker (200/min) for 20 minutes. As the last step 100 μl of the reaction-inhibiting reagent (supplied with the kit) was added and absorbance (A) was read for each sample at a wave length of 450 nm, with the use of a reference wave length of 590 nm using the ELISA 340 ATTC reader (SLT Labinstruments, Crailsheim). The negative control was considered valid, if the difference in absorbance ($A_{450nm} - A_{590nm}$) was less than 0.1. The sample was considered telomerase-positive if the difference in absorbance (sample absorbance minus absorbance of the thermically inactivated sample) was twice as high as background absorbance. The results of the ELISA test are presented in Table I.

Telomerase activity

All the patients were divided into three groups depending upon the telomerase activity determined in the tissue samples obtained from the tumour. The groups covered patients with high telomerase activity, low telomerase activity and no telomerase activity. The group with high telomerase activity consisted of patients in whom this phenomenon was observed both in the 0.25 μg/μl and 0.025 μg/μl. The group with low telomerase activity consisted of patients in whom this phenomenon was observed only in the 0.25 μg/μl sample. The group with no telomerase activity consisted of patients in whom no such activity was observed in any of the samples.

Statistical analysis

Statistical analysis was performed using the *Statistica® 97Pl* for *Windows®* software. The differences in the frequency of parameter appearance between the analysed groups were evaluated with the Fisher's exact test. The event-free survival (EFS) curve was estimated according to the Kaplan-Meier method [14] beginning with the day of therapy onset to the day

Table I. ELISA test results – values of absorbance measured at wave length of 450 nm reduced by the value of background absorbance measured at wave length of 590 nm

Patient	Protein concentration 0.25 μg/μl			Protein concentration 0.025 μg/μl		
	Negative control	Analyzed sample	Telomerase activity	Negative control	Analyzed sample	Telomerase activity
1	0.035	0.030	none	0.040	0.023	none
2	0.067	0.062	none	0.056	0.055	none
3	0.078	0.061	none	0.062	0.058	none
4	0.059	0.055	none	0.078	0.060	none
5	0.027	0.047	none	0.024	0.035	none
6	0.085	1.787	present	0.081	1.690	present
7	0.055	1.352	present	0.021	1.282	present
8	0.065	0.154	present	0.061	0.073	none
9	0.078	0.062	none	0.059	0.057	none
10	0.047	0.041	none	0.044	0.043	none
11	0.034	0.062	none	0.028	0.030	none
12	0.058	0.037	none	0.025	0.032	none
13	0.057	0.370	present	0.610	0.136	present
14	0.043	0.441	present	0.052	1.560	present
15	0.028	0.709	present	0.031	0.404	present
16	0.037	0.030	none	0.046	0.026	none
17	0.069	0.077	none	0.055	0.056	none
18	0.098	1.424	present	0.058	0.490	present
19	0.049	0.462	present	0.079	1.090	present
20	0.102	0.085	none	0.062	0.068	none
21	0.069	0.058	none	0.053	0.053	none
22	0.048	0.336	present	0.031	0.188	present
23	0.078	0.069	none	0.054	0.058	none
24	0.027	0.037	none	0.033	0.035	none
25	0.057	0.062	none	0.063	0.065	none
26	0.050	0.148	present	0.046	0.066	none
27	0.026	0.836	present	0.031	0.561	present

of failure or to the day of the most recent follow-up. Failure was defined as disease progression, recurrence or patient death (regardless of the cause). Differences between survival curves were verified with the log-rank test [15]. Statistical analysis was performed at the confidence level of $\alpha=0.05$. The significance level was assumed to be $p<0.05$.

Results

The two methods applied for the evaluation of telomerase activity showed a highly significant, positive correlation of the results ($R=0.8$; $p<0.0001$). All the analysed tissue samples presented positive reactions for alkaline phosphatase activity (data not shown), however in the case of 3 samples stored at -20° Centigrade additional RNA isolation and division revealed significant RNA degradation (data not shown). These samples did not reveal telomerase activation, however, due to the RNA degradation they were excluded from further analysis as doubtful.

Results obtained from the remaining 27 patients (27 samples obtained from the primary tumours and those additionally obtained from 3 patients from this group, i.e. one metastatic lymph node, one sample obtained from a tumour found in the neighbouring suprarenal gland and one sample obtained from a metastatic lesion in the mediastinum) are presented in Table II (including detailed clinical data). Altogether, telomerase activity was observed in the material obtained from the primary tumour of 11 patients (high in 9 patients and low in 2 patients). Positive telomerase activity were also found in the sample from the metastatic lymph node and in the material obtained from recurred case. Tumours with high telomerase activity were, in general, larger, i.e. over 10 cm in diameter ($p=0.03$). Here it is necessary to add that in all stage IVs cases the primary tumours were less than 10 cm in diameter. Moreover, MYCN oncogene amplification was more common in the case of tumours with high telomerase activity, as well as were regional lymph node involvement and stage IV (the differences were on the verge of significance: $0.1 > p > 0.05$; see Table III). Also, in this group of patients, treatment failure was more often noticed ($p=0.03$).

Table II. Patient characteristics

Patient	Age (gender)	Stage[a)]	Histology[b)]	Therapy prior to biopsy	N Status	Tumour size (cm)	Amp *MYCN*	Telomerase activity	Patient state
1	58 (m)	1	GN mature	none	N0	2.5x2x1.5	-	none	CR
2	41 (f)	2	GNB	none	N0	4x4x4	-	none	CR
3	10 (m)	2	NBL d	none	N0	6x5.5x3	-	none	CR
4	2 (f)	2	NBL d	none	N0	5x4.5x3	n.d.	none	CR
5	13 (f)	2	NBL d	none	N0	5.5x4.5x4	-	none	CR
6	9 (f)	2	NBL ud	none	N+	5x4x4	-	high	LR, 2. CR
6*	9 (f)	2	NBL ud	none	N+	-	-	high	LR, 2. CR
7	71 (f)	3	GNB	none	N+	19x14x6.5	-	high	CR
8	72 (m)	3	GNB	yes	N+	5x4x4	n.d.	low	CR
9	49 (m)	3	NBL d	yes	N+	huge (>10 cm)	-	none	MR, RC
9**	49 (m)	-	NBL d	yes	N+	-	n.d.	high	MR, RC
10	7 (m)	3	NBL ud	yes	N+	huge (>10 cm)	-	none	LR, 2. CR
11	12 (m)	3	NBL pd	yes	N0	6.2x3.7x3	-	none	SM, death
12	12 (m)	3	NBL ud	none	N0	n.d.	-	none	CR
13	44 (m)	4	GN maturing	yes	N+	7x7x6	-	high	CR
14	11 (m)	4	GNB	yes	N+	6.1x4.8x4	+	high	LR, death
15	75 (m)	4	NBL d	yes	N0	10x7x5	-	high	LR, death
16	3 (f)	4	NBL ud	none	N+	6x5x5	-	none	CR
17	28 (f)	4	NBL ud	yes	N+	9x7.5x5.5	+	none	MR, death
18	21 (f)	4	NBL d	none	N+	10x8.5x8	+	high	MxR, death
19	51 (m)	4	NBL ud	yes	N+	11x8x6	n.d.	high	MR, death
20	48 (m)	4	NBL d	yes	N+	3x3x2	n.d.	none	MR, death
21	54 (m)	4	NBL d	yes	N+	9.2x8.4x5	n.d.	none	PR, death
22	18 (m)	4	NBL pd	none	N+	huge (>10 cm)	-	high	LR, death
23	1 (m)	4	NBL ud	none	N0	7x6x3	-	none	PR
23***	1 (m)	4	NBL ud	none	N0	1x1x1	-	none	PR
24	4 (m)	4s	NBL d	none	N0	7x7x6	-	none	CR
25	1 (m)	4s	NBL d	yes	N0	6.1x5.5x5	-	none	CR
26	6 (m)	4s	NBL ud	yes	N0	5x4x3.5	n.d.	low	CR
27	7 (f)	4s	NBL ud	none	N0	6.5x4.5x4	+	high	MR, death

* – lymph node specimen, ** – relapse specimen, *** – specimen from tumour in neighbouring suprarenal gland, age – given in months, m – male gender, f – female gender, a) – classification acc. to Evans et al. [13], b) – classification acc. to Shimada et al. [1], GN – ganglioneuroma, GNB – ganglioneuroblastoma, NBL – neuroblastoma: ud – undifferentiated, pd – poorly differentiated, d – differentiating, Status N – regional nodal status, N0 – regional lymph nodes negative, N+ – regional lymph nodes involved, Amp *MYCN* – amplification of *MYCN* oncogen, CR – complete remission, 2. CR – secondary complete remission, PR – partial remission, LR – Local relapse, MR – metastatic relapse, MxR – mixed relapse, PR – progression, SM – secondary malignancy (acute lymphoblastic leukaemia), n.d. – no data

Table III. Telomerase activity according to the analyzed clinical, histological and genetic prognostic parameters

Parameter	Telomerase activity		*p*
	Negative	Positive	value[1]
Patient age: Mean: 26.9 months			
Median: 15.5 months			
- < 1 year	8 (44.4%)	3 (33.3%)	NS
- ≥ 1 year	10 (55.6%)	6 (66.7%)	
Tumour size*:			
- < 10 cm	15 (88.2%)	4 (44.4%)	0.03
- ≥ 10 cm	2 (11.8%)	5 (55.6%)	
Regional lymph node involvement:			
- yes	7 (38.9%)	7 (77.8%)	0.07
- no	11 (61.1%)	2 (22.2%)	
Stage:			
- Stage I, II, III, IVs	13 (72.2%)	3 (33.3%)	0.06
- Stage IV	5 (27.8%)	6 (66.7%)	
*MYCN*** oncogene amplification:			
- yes	1 (7.7%)	4 (57.1%)	0.1
- no	12 (92.3%)	3 (42.9%)	
Histology a):			
- favourable	12 (66.7%)	6 (66.7%)	NS
- unfavourable	6 (33.3%)	3 (33.3%)	
Death:			
- yes	4 (22.2%)	7 (77.8%)	0.03
- no	14 (77.8%)	2 (22.2%)	

* – no data regarding tumour size in one patient, ** – basing on accessible data, a) – classification acc. to Shimada et al. [1],
1– based on Fisher's exact test, NS – statistically non-significant

An analysis of the estimated event-free survival (EFS) in the analysed group of children with neuroblastoma has shown that patients with none or low telomerase activity of the tumours presented with a significantly better prognosis as compared to patients with high telomerase activity. 5-year EFS was 0.61 and 0.15, respectively; p=0.01 (Figure 1). The other significant prognostic factors in the included individuals were as follows: the size of the primary tumour (<10 cm vs. >10 cm: 5-year EFS 0.62 vs. 0.14, respectively; p<0.05), regional lymph node involvement (N0 vs. N+: 5-year EFS 0.77 vs. 0.22, respectively; p=0.02), clinical stage (I, II, III and IVs vs. IV: 5-year EFS 0.65 vs. 0.15, respectively; p<0.05) and MYCN oncogene amplification (amp MYCN- vs. amp MYCN +; 5-year EFS 0.57 vs. 0, respectively; p=0.03).

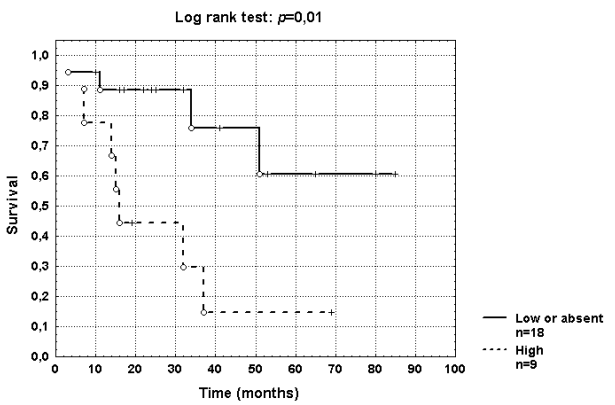

Figure 1. Event-free survival in relation to the level of telomerase activity within the tumour cells

Discussion

The telomere-telomerase conception has enhanced our understanding of carcinogenesis. Activated telomerase allows to rebuild telomeres (which ordinarily shorten in the course of DNA replication), thus allowing the cells to undergo unlimited division [8]. In many patients with neuroblastoma both a decrease in the average telomerase length and the presence of activated telomerase were observed [4-7, 16, 17]. Hiyama et al. have presented a hypothesis which explains the role of telomerase in neuroblastomas [18]. According to their conception originally the malignant cells do not contain active telomerase and in the course of their multiplication the telomeres become significantly shorter. Those tumours in which telomerase is not activated are in fact those, in the case of which we observe regression – probably arising from apoptosis which is activated when the telomeres are significantly shortened. However, if any of the malignant cells during this critical period of time undergo telomerase reactivation, the active enzyme stabilizes the telomeres and thus the multiplying cells become immortal. The cellular clone equipped with activated telomerase begins to dominate, and the disease takes on an aggressive course. There are also some interesting

speculations regarding tumours, in which long telomeres are observed and in which telomerase activity is present, but is relatively low [5, 18]. In these patients good treatment results are quite common. These tumours most likely originate from poorly differentiated embryonal cells, in which active telomerase is present from the very beginning, as a residue of the fetal period. It is, therefore, assumed that in these tumours better prognosis is associated with a higher sensitivity to signals stimulating the maturation of neurons [18].

Our studies have confirmed the significant influence of telomerase activity on the disease course of neuroblastomas. In patients in whom we observed high telomerase activity within malignant cells the disease was more advanced (larger primary tumours, stage IV more common, nodal infiltration) and the patients had poorer prognosis. It would, therefore, be most advisable to include this parameter into the standard diagnostics of children with neuroblastoma – especially because the prognostic factors analyzed at the moment fail to differentiate patients according to prognosis. Further studies should be performed on a larger group of neuroblastoma patients, especially in stage IVs (children < 1 year of age with the presence of liver, bone marrow or skin metastases). Although in a majority of these children we observe idiopathic tumour regression, yet in some patients the disease takes on a very aggressive course and leads to treatment failure [19]. It has been reported, that in this group of patients the evaluation of telomerase activity allows to differentiate children in whom one may expect idiopathic tumour regression, from children in whom the disease will be aggressive and who, therefore, need intensive treatment [20]. Evaluation of telomerase activity may also be very useful for the identification of micrometastases and residual disease [21]. On the other hand, intensive research on substances which may block telomerase will certainly help to develop new, effective antitumours drugs, which probable would be characterized by relatively milder side-effects. The results of such studies appear to be highly promising [22].

It is necessary to remember, that the prognostic value of telomerase activity depends on the precision of the analysis. The laboratory conditions must be very stable in order to consider the results reliable. Because of its RNA component, which is necessary for the correct functioning of the enzyme, telomerase is very sensitive to RNA-ases and undergoes fast inactivation at room temperature. Therefore, the tissue sample must be placed in liquid nitrogen directly after sampling and stored at -80° Centigrade. It appears that the temperature of ordinary freezers (-20° Centigrade) is too high, and results obtained after storage in such conditions must be considered doubtful. Cytostatics, which cause tumour necrosis, and therefore reduce the number of cells in which telomerase remains active, also could affect the results of analysis. In addition some cytostatics specifically block telomerase (e.g. platin derivatives) [23]. Therefore chemotherapy applied before obtaining tumour samples for analysis may also render the interpretation of the results difficult. In order to unify the results it is necessary to evaluate telomerase activity in tissue samples obtained before the onset of therapy. If telomerase activity is present in tissue samples obtained after the onset of cytostatic treatment the result must be considered as a very grave prognostic factor. It is proof of extremely high proliferative ability, and thus of high aggressiveness of the tumour. If, however, tissue samples obtained after the administration of cytostatics appear to possess no telomerase activity it cannot be considered as proof that the tumour was, originally, lacking in active telomerase. The negative result may have been brought on by the treatment itself and could be not a biological feature of the tumour [7].

To summarise, we may state that the analysis of telomerase activity is a valuable method for the stratification of patients with neuroblastoma. Its introduction into the routine diagnostics undertaken in these malignancies will certainly allow to pronounce the risk for each patient and thus allow to choose the optimal treatment method.

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