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Expression of the cdc25B mRNA Correlated with that of N-myc in Neuroblastoma

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Background: Neuroblastoma is one of the most common solid tumors in early childhood. Overexpression of the proto-oncogene N-myc has been reported to be correlated with more malignant course of the disease. cdc25B is reported to be a target of myc and elevated in several malignant cells and tissues.
Methods: Expression of cdc25B and N-myc messenger RNAs were evaluated by real-time reverse transcription polymerase chain reaction (RT-PCR) assay in 20 tumor samples from neuroblastoma using LightCycler. The data were analyzed with reference to clinicopathological factors. Immunohistochemistry for cdc25B was also performed.
Results: There was no significant difference in the cdc25B expression between patient groups according to age, gender and clinical stage. The cdc25B mRNA expression levels were significantly correlated with N-myc mRNA levels ($y = -0.445 + 20.577x$, $p < 0.0001$).
Conclusion: We could not establish the clinical significance to determine the cdc25B mRNA level from neuroblastoma. However, we suggest that cdc25B may play an active role as a target of N-myc in neuroblastoma, although the biological function of cdc25B in neuroblastoma remains to be clarified.

Key words: cdc25B – N-myc – neuroblastoma

INTRODUCTION

Neuroblastoma is a common but enigmatic solid tumor in children. Highly aggressive neuroblastomas often exhibit amplification of the N-myc gene and are usually found as advanced disease in patients at 1 year of age or older (1,2). In clinical neuroblastomas, naturally or chemically matured tumors express reduced levels of N-myc mRNA (3). Genomic amplification of N-myc is reported to be frequently accompanied by overexpression of N-myc mRNA (4,5). However, infantile neuroblastomas have been reported to express relatively high levels of N-myc mRNA and the clinical significance of N-myc mRNA expression is controversial (3,6–9).

cdc25s can dephosphorylate threonine 14, tyrosine 15 or both on cyclin-dependent kinases (CDKs) and activate cyclin–CDK complexes to stimulate cell proliferation. It has been suggested that cdc25A and cdc25B but not cdc25C possess oncogenic properties (10). Because the myc family may induce expression of cdc25A and cdc25B (11), it was reported that cdc25B overexpressed in non-Hodgkin's lymphoma might cooperate with c-myc oncogene (12). Recent studies showed that overexpression of cdc25B was frequent in colon cancer (13) and lung cancer (14). However, there is no report on the mRNA expression of cdc25B in clinical samples from patients with neuroblastoma.

As the target molecules of N-myc gene in neuroblastoma leave much to be investigated, we examined cdc25B and N-myc transcript in patients with neuroblastoma by means of real-time reverse transcription polymerase chain reaction (RT-PCR) analysis using LightCycler. We analyzed the data with reference to the clinicopathological factors.

PATIENTS AND METHODS

The study groups included 20 neuroblastoma patients (13 boys and seven girls) who had undergone surgery at the Department of Surgery II, Nagoya City University Medical School between 1989 and 2000. The neuroblastomas were classified according to the International Neuroblastoma Staging System. Of the 20 cases, 14 were diagnosed at <1 year of age and 14 of the 20 infantile patients were identified by a mass screening system

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Table 1. Clinocopathological data for 20 neuroblastoma patients

Factor	cdc25B/GAPDH expression		
	No. (%)	mRNA level (%)	*p*-Value
Age (years):			
<1	14 (70.0)	2.361 ± 3.502	0.4829
≥1	6 (30.0)	1.283 ± 1.540	
Gender:			
Boy	13 (65.0)	2.226 ± 3.608	0.7174
Girl	7 (35.0)	1.689 ± 1.763	
MAS or symptom:			
MAS	14 (70.0)	2.598 ± 3.442	0.2178
Symptom	6 (30.0)	0.731 ± 1.226	
Stage:			
I	6 (30.0)	1.591 ± 1.689	NS
II	7 (35.0)	3.671 ± 4.537	
III	3 (15.0)	0.855 ± 0.726	
IV	4 (20.0)	0.736 ± 1.472	
Stage:			
I + II	13 (65.0)	2.711 ± 3.556	0.1846
III + IV	7 (35.0)	0.787 ± 1.124	
DNA ploidy:			
Aneuploidy	16 (80.0)	2.344 ± 3.292	0.3822
Diploidy	4 (20.0)	0.811 ± 1.429	

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MAS, detected by mass screening.

(MS) (Table 1). All tumor samples were collected at resection and immediately frozen.

RT-PCR Assays for cdc25B and N-myc

Total RNA was extracted from clinical samples using an Isogen kit (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. RNA concentration was determined spectrophotometrically and adjusted to 200 ng/ml. Total RNA was extracted from neuroblastoma cell lines, IMR32 and CHP 134. This RNA was used as a positive control. RNA (1 µg) was reverse transcribed using Superscript II enzyme (Gibco BRL, Gaithersburg, MD) with 0.5 mg of oligo$(dT)_{12–16}$ (Amersham Pharmacia Biotech, Piscataway, NJ). The reaction mixture was incubated at 42°C for 50 min followed by incubation at 72°C for 15 min. To ensure the fidelity of mRNA extraction and reverse transcription, all samples were subjected to PCR amplification with oligonucleotide primers specific for the constitutively expressed gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and normalized. All PCR reactions were performed using a LightCycler-FirstStart DNA Master SYBR Green I kit (Roche Molecular Biochemicals, Mannheim, Germany). The primer sequences for cdc25B gene were forward primer 5-TCTCATCTGAGCGTGGGC-3 and reverse primer 5-CTTCAGGCCTCGAAAGGC-3. The cycling conditions were as follows: initial denaturation at 95°C for 10 min, followed by 60 cycles at 94°C for 15 s, 57°C for 5 s and 72°C for 14 s. The primer sequences for N-myc gene (240 bp) were forward primer 5-GACCACAAGGCCCTCAGTAC-3 and reverse primer 5-GTGGATGGGAAGGCATCGTT-3. The cycling conditions were as follows: initial denaturation at 95°C for 10 min, followed by 60 cycles at 94°C for 15 s, 57°C for 5 s and 72°C for 11 s.

Immunohistochemistry

Mouse anti-human cdc25B mAb was obtained from Transduction Laboratories (Lexington, KY). Tissue sections (4 µm thick) were deparaffined in xylene, rehydrated and stained with hematoxylin. Endogenous peroxidase was blocked with 0.3% H_2O_2. After blocking with Block Ace Solution (Dako Japan, Kyoto, Japan), the slides were incubated with the primary antibody overnight at 4°C. The dilution of cdc25B antibody was 1:200. An Envision Kit and DAB substrate were used to visualize the antibody binding and the sections were counterstained with methyl green.

Statistical Methods

Statistical analysis was done using the Stat-View software package (Abacus Concepts). Student's *t*-test was used to evaluate the significance of the expression in paired groups. Differences among the means of the stage in the patients with neuroblastomas were examined using Fisher's PLSD method. Correlation between the mRNA levels of cdc25B and N-myc was evaluated by Spearman's rank test. The overall survival of neuroblastoma patients was examined by the Kaplan–Meier method. It was considered significant when the *p*-value was <0.05.

RESULTS

Immunohistochemistry

The staining for cdc25B was weak in most of the neuroblastoma samples. In one case with strong staining, the localization of cdc25B was cytoplasmic (Fig. 1).

cdc25B and N-myc Expression

Using quantitative RT-PCR, all tumor samples were shown to have N-myc transcript and the N-myc/GAPDH mRNA level was 0.120 ± 0.116 (mean ± standard deviation). There was no significant correlation between the N-myc mRNA expression and the clinicopathological features such as the patient's age, gender and clinical stage (data not shown). Of 20 neuroblastomas studied, 17 had cdc25B transcript and the cdc25B/GAPDH mRNA level was 2.032 ± 3.047. The level was higher than the cdc25B/GAPDH mRNA level from normal adult ganglion tissue (0.035 ± 0.016), but the difference was not significant. The relationship between the gene expression of cdc25B

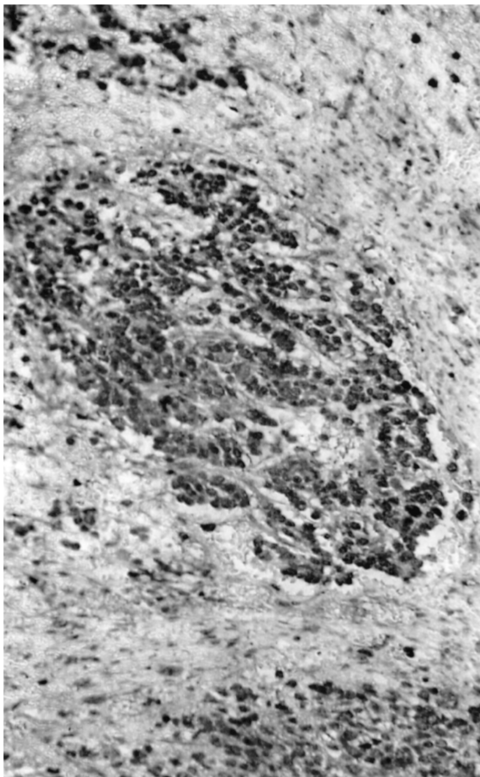

Figure 1. cdc25B protein expression in neuroblastoma tissue section by immunohistochemistry.

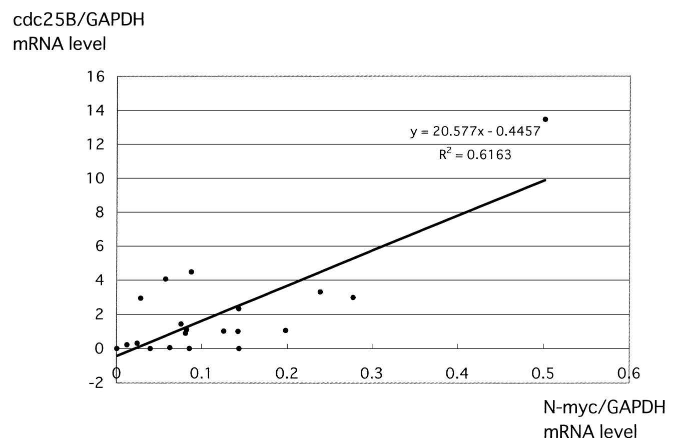

Figure 2. Correlation of the mRNA levels between N-myc and cdc25B.

in neuroblastoma and the patients' clinicopathological factors is shown in Table 1. There was no significant difference in the cdc25B expression between patient groups according to age and gender. There was no significant difference in the cdc25B expression among tumors with different clinical stage. There was a positive correlation between the mRNA expression levels of cdc25B and N-myc genes ($y = -0.445 + 20.577x$, $p < 0.0001$, Fig. 2).

SURVIVAL

Eleven of 13 (84.6%) patients with cdc25B/GAPDH < 2.032 were alive and six of seven (85.7%) patients with cdc25B/GAPDH > 2.032 were alive. There was no difference in survival between the neuroblastoma patients with high and those with low cdc25B expression.

DISCUSSION

In the present study, cdc25B mRNA expression was analyzed for the first time in clinical samples of neuroblastoma. cdc25B mRNA was expressed in 17 of 20 neuroblastoma samples studied. We have shown that the cdc25B mRNA level was correlated with N-myc mRNA level in neuroblastoma. It has been shown previously that the human cdc25B gene is overexpressed in non-Hodgkin's lymphoma and correlated with c-myc gene expression (11). Unexpectedly, we found that there was no significant difference in the cdc25B expression among tumors with different clinical stages in neuroblastomas. This is in apparent contradiction with previous reports showing that an increase of tissue cdc25B expression in colorectal cancer correlated with more advances clinical stages (12).

Recent study showed cdc25s including cdc25A and cdc25B were direct transcriptional targets of c-myc (14). The myc family of proto-oncogenes, comprising c-myc, N-myc and L-myc, has a homology with each other and is involved in some of the most important cellular processes, such as cell proliferation, differentiation, apoptosis and neoplastic phenomena. N-myc is overexpressed in a restricted set of malignancies, predominantly in neuroblastomas and related tumors (1,2). It has been reported that N-myc mRNA expression of the tumor from neuroblastoma in mass screening groups was generally higher than that in clinical symptom groups (15). Recently, a function of N-myc not only related to cell proliferation or retention of the undifferentiated phenotype was documented in neuroblastoma. When ectopic expression of N-myc was enforced in neuroblastoma cells, apoptosis was induced when cells were treated with IFN-γ or with a cytotoxic drug (16,17). It was suggested that tumors with N-myc amplification acquire treatment resistance not by overexpression of N-myc but by additional dysfunction in apoptosis signaling pathways (17). These findings may suggest that enhanced expression of N-myc could function as a death signal in concert with other biological factors in neuroblastomas.

Many tumors found by screening regress or mature spontaneously by a mechanism probably involving apoptosis (18). It is an interesting possibility that expression of cdc25B in these tumors is related to this process. Although the biological function of cdc25B in neuroblastoma remains to be clarified, further studies will demonstrate that the level of cdc25B mRNA expression in neuroblastoma may be a factor related to cell proliferation or cell death or both. We could not establish the clinical significance to determine the cdc25B mRNA level from neuroblastoma in this paper. However, we suggest that

cdc25B may play an active role as a target of N-myc in neuroblastoma.

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