Y-Box-binding Protein-1 is a Potential Novel Tumour Marker for Neuroblastoma

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Abstract. *Backround: The Y-box-binding protein-1 (YB-1) is a member of a family of DNA-binding proteins and an oncogenic transcription factor that is highly expressed in cancers of the breast, lung and prostate. To date, no data are available on its role in neuroblastoma. The aim of the present study was to evaluate the YB-1 expression in neuroblastoma. Materials and Methods: A tumour tissue microarray (TMA) was constructed from 36 neuroblastoma samples which were analysed by immunohistochemistry for YB-1 expression. Results: Expression of YB-1 was detected in 35 of 37 (94.6%) neuroblastoma cases examined. Nevertheless, no correlation of YB-1 expression with survival, risk factors or stage of the disease was observed. Conclusion: As the majority of neuroblastomas express YB-1, this protein may play an important role in tumour pathogenesis. The results of this study suggest that YB-1 may serve as a novel immune marker for neuroblastoma and may be potentially useful as a therapeutic target.*

Neuroblastoma is a childhood tumour which arises from sympathetic neuroblast cells derived from the neural crest. This tumour type is known to be the most frequent solid tumour in childhood outside the central nervous system (1, 2). Although overall survival of patients suffering from this tumour type is good, the prognosis of high-risk neuroblastoma patients is still poor, despite advances in diagnosis and treatment (3). It has been found that outcome correlates strongly with clinical factors such as age, stage and chromosomal aberrations (3). With the clinical application of prognostic markers, the therapeutic approach and clinical outcome was improved in these patients (4). Therefore it can be hypothesized that even more reliable markers may play a substantial role in the improvement of the treatment and diagnosis of neuroblastoma.

The Y-box-binding protein-1 (YB-1) belongs to a family of Y-box proteins that bind DNA and RNA, and regulates gene expression through transcription and translation.

YB-1 activates genes associated with cell proliferation and cancer development, such as matrix metalloproteinase-2 (*MMP-2*), multi-drug restistance 1 (*MDR1*), epidermal growth factor receptor (*EGFR*), cyclin B1 and cyclin A (5-7). Genes associated with cell death, including the FAS cell death-associated receptor and *p53*, are repressed by YB-1. It has been shown that YB-1 is highly expressed in cancer of the breast (8), lung (9), ovary (10), bone (11), colon (12), thyroid (13), nasopharynx (14) and prostate (15) . Several reports indicate that YB-1 protein level and nuclear localisation is a prognostic marker for some types of human cancer (9, 16). To date, there are no published data regarding YB-1 expression in neuroblastoma.

The aim of this study was to evaluate the expression of YB-1 in paediatric neuroblastoma by immunohistochemistry on a tumour tissue microarray and correlate the staining pattern with clinical outcome, survival, risk factors and disease stage.

Patients and Methods

Study design and patients. For this study, samples from 37 patients with surgically resected paediatric neuroblastomas treated at the University Medical Center Hamburg-Eppendorf between November

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1999 and October 2004 were retrieved from the tissue archive. Patients were selected on the basis of availability of tissues and follow-up data. Clinical follow-up data were obtained by reviewing the hospital records, contacting patients on an outpatient basis or by phone call. Overall survival was calculated from the date of surgery to the date of death or last follow-up. None of the patients died from a cause other than neuroblastoma. All tumours were categorised into groups according to the International Neuroblastoma Staging System (INSS) (1). All data, including histological grade according to Hughes (17), age at diagnosis, *N-myc* amplification and loss of heterozygosity on chromosome 1p, were obtained from the clinical and pathological records. None of the patients had been pretreated. The study was approved by the Ethics Committee of the Chamber of Physicians in Hamburg, Germany. Written informed consent was obtained from all parents of the patients for the use of the resected samples and clinical data for research purposes.

Tissue microarray (TMA) construction. Selected paediatric neuroblastoma tissues were fixed in 4% buffered formalin and embedded in paraffin. Hematoxylin-eosin stained sections were constructed from selected primary tumour blocks to define representative tumour regions. Tissue cylinders with a diameter of 600 μm were punched out of the original donor block and arrayed on a new paraffin block using a semi automated tissue arrayer. Sections of 5 μm of the complete tissue microarray were constructed by use of the Paraffin Sectioning Aid System (Instrumentics, Hackensack, NJ, USA).

Immunhistochemical staining and evaluation of YB-1 expression. For immunhistochemical YB-1 staining, 5 μm-thick TMA sections were placed on precoated slides with 3-triethoxysilylpropylamine (Merck, Darmstadt, Germany), deparaffinised in xylol, for 1 hour and washed in series of decreasing alcohol concentrations. Antigen retrieval of the deparaffinised tissue sections was performed in a microwave for 5 min in TEC-buffer (0.05 M Tris-HCl, 0.05 M EDTA, 0.02 M Na-Citrate, pH 7.8) followed by peroxidase blocking. The sections were incubated with the polyclonal primary antibody YB-1 (1:25; Cell Signaling Technology®, Danvers, MA, USA). A biotinylated goat anti-rabbit antibody (Vector, Grünberg, Germany) was used as secondary antibody for 20 min. DAB statining reaction lasted 10 min. Slides were counterstained with haematoyxlin. Finally, slides were covered with coverslips with aqueous mounting medium (Aquatex®; Merck, Darmstadt, Germany). Negative controls for the immunhistochemical procedures consisted of substitution of the primary antibody with nonimmune rabbit IgG (Dako, Glostrub, Denmark). Immunostaining was scored by two independent investigators. Specimens were considered immunopositive for YB-1 when >20% of the tumour cells showed clear evidence of immunostaining.

Statistical analysis. Statistical analysis was performed using the SPSS statistical software package for Windows (version 13.0; SPSS Inc., Chicago, IL, USA). A value of $p<0.05$ was regarded as significant.

Results

Characteristics of the patients. A total of 37 surgically resected paediatric neuroblastoma specimens were included in this study. Out of these, 12 (32.4%) patients were younger than one year and 25 (67.2%) were older than one year at the time of diagnosis. Characteristics of the patients are summarised in Table I. *N-myc* was amplified in 3 (8.3%) cases and loss of heterozygosity (LOH) at chromosome 1p was detected in 5 patients (17%). Median survival time of all patients included in the survival analysis (n=37) was 78 months.

Table I. *Clinical, pathological, immunhistochemical and molecular characteristics of the neuroblastoma tumours in this study.*

Variable	Patients, n (%)	YB-1 positive tumours, n (%)
Total	37	35 (95%)
Age		
≤1 year	12 (32%)	12 (100%)
>1 year	25 (68%)	23 (66%)
INSS stage		
1	19 (53%)	17 (90%)
2	5 (14%)	5 (100%)
3	7 (19%)	7 (100%)
4	3 (8%)	3 (100%)
4s	2 (6%)	2 (100%)
Hughes grade		
1a/b	13 (37%)	11 (85%)
2	10 (29%)	10 (100%)
3	12 (34%)	12 (100%)
N-myc		
Amplification	3 (8%)	3 (100%)
No amplification	33 (92%)	31 (94%)
LOH 1p		
Yes	5 (17%)	5 (100%)
No	25 (83%)	25 (100%)

INSS: International Neuroblastoma Staging System; N-myc: N-myc gene amplification; LOH p1, loss of heterozygosity on chromosome 1p.

Overexpression of YB-1 in neuroblastoma. Overexpression of YB-1 was seen in 35 out of 37 (94.6%) neuroblastoma tumours. Of the 35 positive tumours, 17 showed a weak, and 18 a strong expression of YB-1.

Staining for YB-1 was observed in all tumour grades and stages according to INSS (Table I). All cases with *N-myc* amplification and LOH at 1p showed an overexpression of YB-1. The clinicopathological characteristics and their association with the YB-1 expression level are presented in Table I. Expression of nuclear YB-1 was detected in 25 of 37 patients (67.6%). Figure 1 shows representative examples for nuclear and cytoplasmatic staining of YB-1 in neuroblastomas.

Nuclear YB-1 expression is not associated with outcome in neuroblastoma. Follow-up data of all 37 patients included in this study were collected. For survival analysis, two groups were created: the first group consisted of neuroblastomas characterised by strong YB-1 expression, and the second

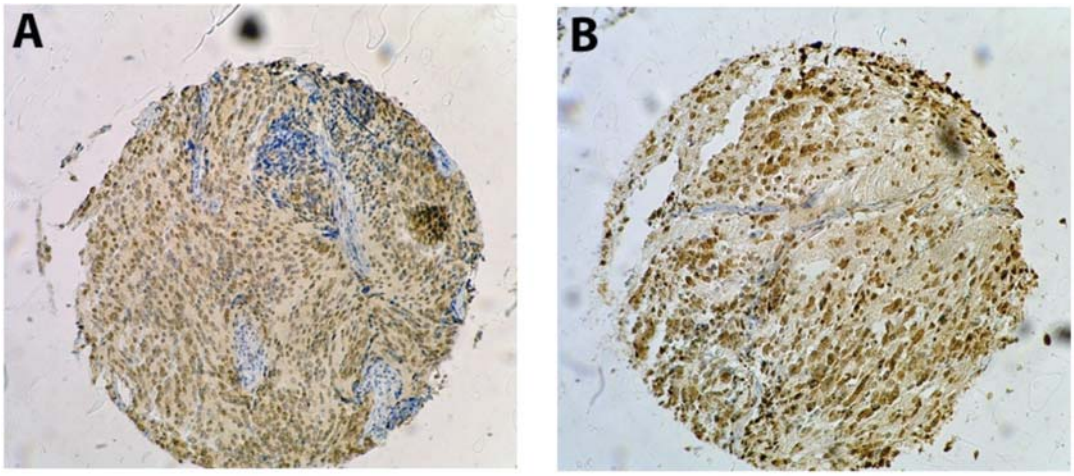

Figure 1. *YB-1 expression in neuroblastoma. Representative examples of immunohistochemical cytoplasmatic (A) and nuclear (B) staining for YB-1 (magnification ×100).*

group consisted of neuroblastomas with weak or no YB-1 expression. Comparison of the overall and event-free survival between these two groups did not reveal a significant correlation (data not shown).

Furthermore, there was no significant correlation between the expression of nuclear YB-1 and age, histological grade, tumour stage and overall or event-free survival (data not shown).

Discussion

YB-1 is the prototypic member of a family of DNA-binding proteins that contain a highly conserved cold-shock domain and interact with defined residues (Y-boxes) within the promoter regions of various genes, especially growth-related genes (18). This 50 kDa multifunctional protein is expressed in various types of cancer. The level of YB-1 expression has been shown to be predictive of poor outcome in patients with breast cancer (16). Resistance to chemotoxic effects may also be predicted, as target genes include multidrug resistance genes (such as P-glycoprotein and *MRP2*) (8,19). Overexpression of YB-1 may therefore be a suitable indicator for aggressiveness of human malignant disease growth. For example, increased YB-1 levels were detected in breast cancer (8), osteosarcoma (11), colorectal carcinoma (12), thyroid neoplasms (13), ovarian cancer (20) and prostate cancer (15). But to date, no data regarding the role of YB-1 in neuroblastoma are available.

Since YB-1 is a clinically important molecule that may be linked causally to the development of different tumour entities, the aim of this study was to examine YB-1 expression in neuroblastoma.

Therefore, the expression of YB-1 in 37 cases of paediatric neuroblastoma was investigated. Overexpression of YB-1 was detected in 94.6% of cases diagnosed as neuroblastomas. Besides this, all cases with *N-myc* amplification and LOH at 1p exhibited YB-1 overexpression. In previous reports, expression of YB-1 was often asociated with poor prognosis and metastasis formation (10, 11). In this study, using the tissue array and the collected clinical data, no correlation of YB-1 expression with survival, risk factors or stage of disease was apparent.

Nuclear YB-1 expression was detected in 25 out of 37 patients (67.6%). Although, there was no significant correlation between the expression of nuclear YB-1 and survival, it is remarkable that over half of the neuroblastomas in this study showed a nuclear staining pattern. A nuclear YB-1 expression is associated with poor prognosis according to different studies (8-10). Nuclear localisation of YB-1 is required for its transcriptional regulation of different genes. Previous studies showed that nuclear YB-1 is associated with drug resistance in cancer (21) and P-glycoprotein expression in breast cancer (8). Fujita *et al*. (22) reported that an increase in P-glycoprotein expression observed in patients treated with paclitaxel was paralleled by strong nuclear YB-1 expression.

The limitation of the present study was the low number of tumour specimens analysed. The high level of tissue expression in neuroblastoma cells may relate to a function of this molecule during neuronal cell development. Lu *et al*. (23) reported the involvement of YB-1 in brain development. Since the data in this study showed high expression levels of this marker in neuroblastoma, YB-1 may also be a potential target for novel therapeutic approaches.

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