Prognostic Impact of Activated Leucocyte Cell Adhesion Molecule (ALCAM/CD166) in Infantile Neuroblastoma

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Abstract. *Background/Aim: Activated leukocyte cell adhesion molecule (ALCAM/CD166) as a member of the 'immunoglobulin superfamily' is known to be involved in cancer cell proliferation and migration. The aim of this study was to investigate the expression of ALCAM in neuroblastoma tissues. Materials and Methods: ALCAM expression was analyzed in primary neuroblastoma specimens by immunohistochemistry on microarray sections. Histopathological and clinical data were correlated with ALCAM expression and survival analysis was performed. Results: Sixty-six children were included in the study. Strong expression of ALCAM was detected in 52 (79%) of the samples. Weak expression was significantly correlated with the International Neuroblastoma Staging System (INSS) stage (p=0.024) and positive n-MYC amplification (p=0.019). Recurrence-free survival (RFS) and overall survival (OS) were significantly shorter if ALCAM was expressed weakly (p=0.032 and p=0.001). Conclusion: Weak ALCAM expression was significantly correlated with established markers for poor prognosis, as well as shorter RFS and OS. ALCAM might be considered as a prognostic marker for infantile neuroblastoma.*

Neuroblastoma is known as the most frequent solid tumour in childhood outside the central nervous system arising from neuroblast cells (1). Although overall survival of children suffering from this type of tumour is good, the prognosis of children with high-risk neuroblastoma is quite poor (2). Several clinical factors such as age, stage and chromosomal aberrations, have been found to be prognosticly relevant (3, 4). However, valid markers to guide the treatment of children, especially with high-risk disease, are still missing.

Cell adhesion molecules play an important role in the development and metastatic process of malignant tumours. Recently, the activated leucocyte cell adhesion molecule (ALCAM/CD166) has been identified as a member of this molecular family (5). ALCAM is a highly conserved, 110 kDa multidomain transmembrane type 1 glycoprotein of the 'immunoglobulin superfamily' that mediates homotypic and heterotypic interactions between tumour cells and the extracellular matrix (6, 7). It plays an important role in different biological activities, for example in neurogenesis and haematopoiesis, and is a participant of the immune response (5, 8). Several studies have reported on its potential as a prognostic marker for different tumour entities, such as melanoma, pancreatic and ampullary adenocarcinoma, as well as colorectal, gynaecological and neuroendocrine carcinomas (9-16)

The aim of this study was to examine the expression of ALCAM in infantile neuroblastoma using a tumour microarray (TMA) in correlation with clinical and histopathological data to investigate its prognostic value for tumour recurrence and overall survival (OS) in affected children.

Patients and Methods

Patients and clinical data. The Ethics Committee of the Chamber of Physicians in Hamburg, Germany, approved the study. Written informed consent was obtained from all parents for the analysis of resected specimens for research purposes. Sixty-six surgically resected neuroblastoma, treated at the University Medical Center Hamburg Eppendorf, Germany, between November 1999 and October 2004, were retrospectively included in the study without

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any pre-selection. Corresponding data, including survival and relapse, as well as sex, age at diagnosis (≤/>1 year), stage (International Neuroblastoma Staging System (INSS); 1, 2, 3, 4, 4s), histological grade according to Hughes (1a/1b, 2, 3), *n-MYC* amplification and loss of heterozygosity of chromosome 1p (LOH1p) were obtained from the clinical and pathological records.

Tissue microarray construction. Selected paediatric neuroblastoma tissues were fixed in 4% buffered formalin and embedded in paraffin as described by Reichelt *et al*. (17). Haematoxylin-eosin stained sections were cut from selected primary tumour blocks with representative tumour regions. Tissue cylinders with a diameter of 600 μm were punched out of the original donor block and transferred on a new paraffin block using a semi-automated tissue arrayer. Subsequently, 5-μm sections of the complete TMA were constructed using the Paraffin Sectioning Aid System (Instrumentics, Hackensack, NJ, USA).

Immunohistochemical staining for ALCAM. The CD166 staining protocol for paraffin-embedded tissue was optimized in an extensive multistep procedure on various benign and malignant tissues to establish a selective staining with lowest background signals. All freshly cut TMA sections were analysed on the same day in a single experiment. CD166 expression was detected using a primary mouse monoclonal antibody (clone: MOG/07, dilution 1:450; Novocastra, Newcastle upon Tyne, UK) after boiling the sections in an autoclave in citrate buffer, pH 7.8. The Envision system (DAKO, Glostrup, Denmark) was used to visualize the immunostaining. The staining intensity and the fraction of positive tumour cells were scored for each tissue spot, as described recently (21, 23). In brief, staining intensity (0, 1+, 2+, 3+) and the fraction of positive tumour cells were scored for each tissue spot. A final score was calculated from these two parameters according to the following parameters: Negative scores had staining intensity of 0, weak scores were classified as staining intensity of 1+ in ≤70% of tumour cells or 2+ in ≤30% of tumour cells; Moderate scores had staining intensity of 1+ in >70% of tumour cells, staining intensity of 2+ in >30% and ≤70% of tumour cells or staining intensity of 3+ in ≤30% of tumour cells; Strong scores had staining intensity of 2+ in >70% of tumour cells or staining intensity of 3+ in >30% of tumour cells; Weak scores were defined as weak ALCAM expression, whereas moderate and strong scores were defined as strong ALCAM expression. Only membranous staining was evaluated as cytoplasmic staining always corresponded to stronger membranous staining. Immunohistochemical analysis of the sections was performed by two independent investigators (RW and MT) blinded to the patients' identity or clinical status. In discrepant cases, a pathologist reviewed the cases to reach a consensus.

Statistical analysis. SPSS Statistics for Windows (Version 20, SPSS Inc., IBM, Armonk, NY, USA) was used for statistical analysis. Interdependence between expression of ALCAM (strong *versus* weak) and the clinical data was calculated using Chi-squared and Fisher's exact tests and displayed in cross tables. Kaplan-Meier survival curves were analysed using the log-rank test. Univariate and multivariate analyses were performed for prognostic factors for overall survival using the Cox regression model. All tests were two-sided. *p*-Values less than 0.05 were considered to be statistically significant.

Table I. *Clinical, pathologic, molecular and immunohistochemical characteristics*.

Variable	Number of patients	ALCAM expression		*p*-Value
		Weak	Strong	
Age				
≤1 year	22 (33%)	18 (35%)	4 (29%)	0.759
>1 year	44 (67%)	34 (65%)	10 (71%)	
Gender				
female	27 (42%)	20 (40%)	7 (50%)	0.551
male	37 (58%)	30 (60%)	7 (50%)	
INSS				
1	26 (41%)	21 (42%)	5 (36%)	0.024
2	15 (23%)	15 (30%)	0 (0%)	
3	9 (14%)	7 (14%)	2 (14%)	
4	9 (14%)	4 (8%)	5 (36%)	
4s	5 (8%)	3 (6%)	2 (14%)	
Hughes grade				
1a/b	23 (36%)	18 (36%)	5 (36%)	0.769
2	18 (28%)	15 (30%)	3 (21%)	
3	23 (36%)	17 (34%)	6 (43%)	
n-MYC amplification				
positive	9 (14%)	4 (8%)	5 (36%)	0.019
negative	55 (86%)	46 (92%)	9 (64%)	
LOH1p detection				
positive	10 (19%)	7 (15%)	3 (43%)	0.114
negative	43 (81%)	39 (85%)	4 (57%)	

INSS: International Neuroblastoma Staging System; ALCAM, activated leucocyte cell adhesion molecule; Data are presented in cross-tables. *p*-Values were determined using two-sided Fisher's exact and Chi-squared tests.

Results

Patients' characteristics. A total of 66 children (27 female, 37 male) suffering from neuroblastoma were included in this study (Table I). In 37 (58%) patients, the tumour was located adrenal. Median follow-up time of all children for survival analysis was 72 month. Median age at diagnosis was 30 months. Twenty-two children (33%) were younger than one year at diagnosis. *N-MYC* amplification was positive in nine cases (14%). LOH1p was observed in ten children (19%).

Expression of ALCAM in neuroblastoma tissue. Immunohistochemistry showed a predominant membranous expression of the ALCAM molecule with a heterogeneous staining pattern inside the cancerous lesions (Figure 1). Strong expression of ALCAM was detected in 52 (79%) of 66 neuroblastoma tumours. Weak ALCAM expression was significantly associated with the INSS stage (p=0.024) and a positive *n-MYC* amplification (p=0.019) but not with sex, age, Hughes grade or positive detection of LOH1p (Table I). Survival analysis revealed a significant shorter cumulative recurrence-free survival (RFS) (p=0.032) and OS (p=0.001) in children with weak ALCAM expression (Figure 2).

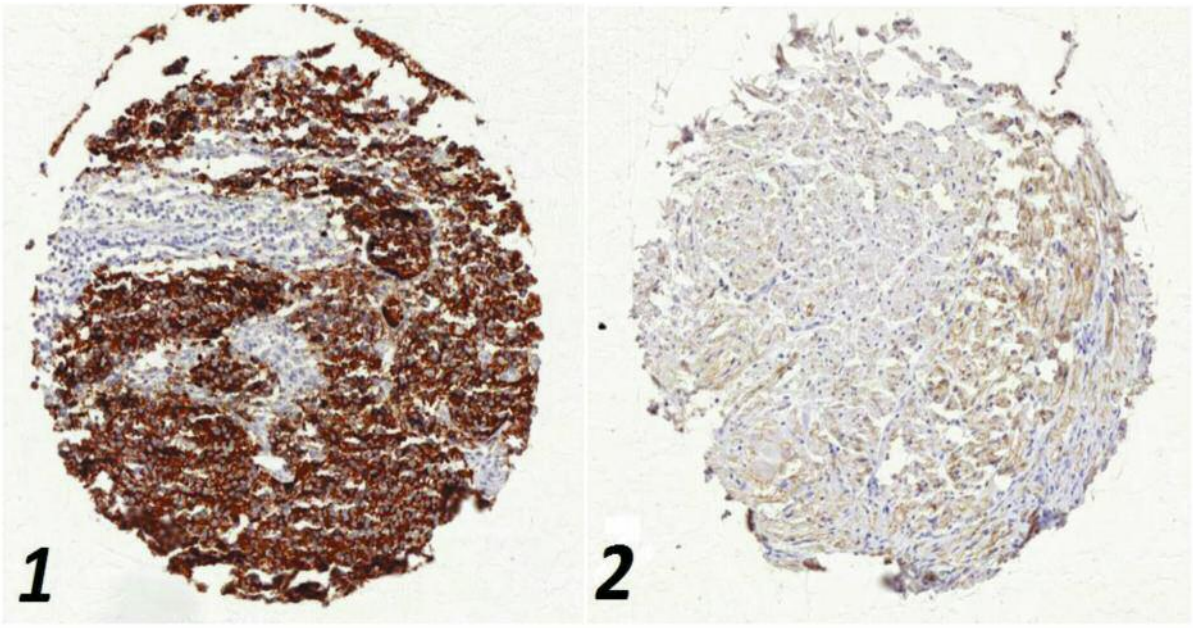

Figure 1. *Activated leucocyte cell adhesion molecule (ALCAM) expression in infantile neuroblastoma. Representative examples of immunohistochemical strongly positive (1) and weakly positive (2) staining for ALCAM/CD166 (magnification ×100).*

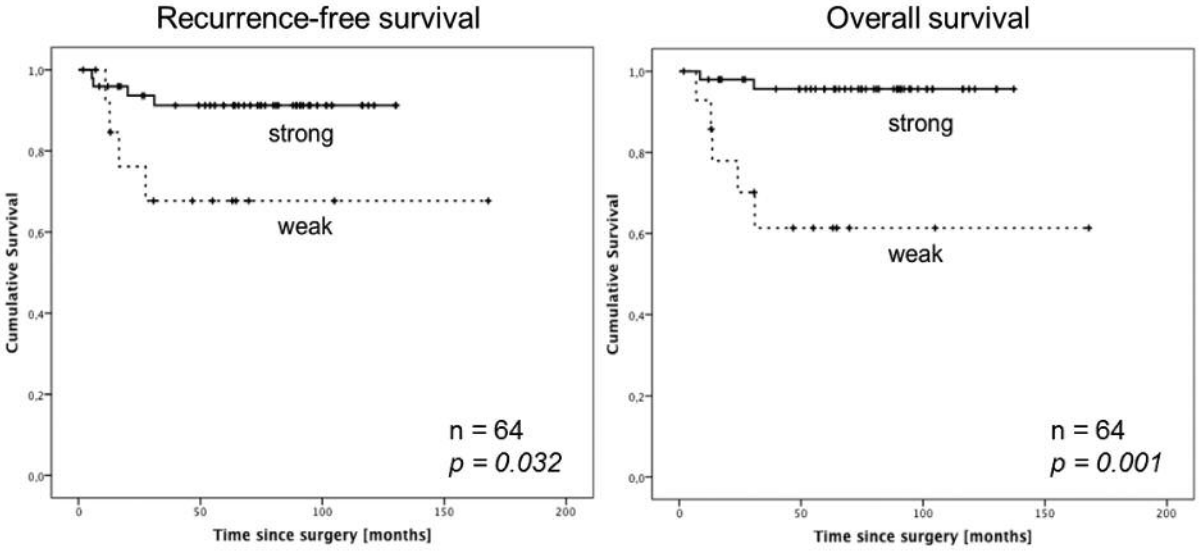

Figure 2. *Kaplan-Meier survival curves for disease-free survival (A) and overall survival (B) in the months after surgical resection. Survival was significantly shortened in the group with weak activated leucocyte cell adhesion molecule (ALCAM) expression of infantile neuroblastomas.*

Multivariate analysis. Univariate Cox regression analysis showed a significant impact of INSS stage, Hughes grade, *n-MYC* amplification, LOH1p detection and strong ALCAM expression on disease-free survival. However, when implemented in a multivariate analysis, none of these factors sustained as an independent prognosticator (data not shown).

Discussion

The aim of our study was to investigate the expression of ALCAM in infantile neuroblastoma tissues in a representative cohort including all INSS stages. In the majority of samples (79%), ALCAM was strongly expressed;

weak expression was significantly correlated with the prognostic markers INSS stage and positive *n-MYC* amplification but not with sex, age at diagnosis, Hughes grade and LOH1p expression. Moreover, RFS and OS were shorter for weak ALCAM expression.

Up until now, ALCAM expression has been analysed in adult tumours of different entities at varying localisations (cytoplasmic and/or membranous) with divergent prognostic effects (18). Ishigami *et al*., who recorded both cytoplasmic and membranous staining intensity of CD166 in gastric cancer, found that strong ALCAM expression contributed to poor outcome (19). This correlation was also reported by Kahlert *et al*., who analyzed the expression of ALCAM in pancreatic cancer (20). In breast and ovarian carcinomas, however, increased cytoplasmic and decreased membranous expression of ALCAM was associated with a worse prognosis (21, 22). Discrepant statistical and morphological results were extensively discussed for CD166 expression in pancreas cancer (23), concluding that varying immunohistochemistry protocols with different grades of sensitivity and specificity may lead to significant discrepancies in staining patterns. For this reason, in this study, a highly standardized and comprehensive protocol for immmunohistochemical analysis of tissue microarray was applied (24).

Only one group has analyzed ALCAM expression in 23 neuroblastoma tumours by immunoperoxidase technique applying a semi quantitative grading system based on different percentage thresholds of positive tumour cell structures (25). In this preselected cohort, including only patients with INSS stage 1 and 2 without *n-MYC* amplification, a strong expression of ALCAM at the membrane of the neuroblast body or low levels in the neuropil area were associated with relapse.

In our study, only membranous immunostaining was analyzed because ALCAM expression always predominated at the cell membrane. The intensity of cytoplasmic staining was related to the intensity of the membrane staining and did not appear in the absence of membrane staining. Furthermore, no differences between expression of ALCAM in the membrane and in the neuropil area were noticed.

Interestingly, the observed ALCAM expression parallels the expression of the neuronal cell adhesion molecule L1 (L1-CAM/CD171) in neuroblastoma tissue as formerly reported by our group (26). L1-CAM is a membrane glycoprotein, which also belongs to the 'immunoglobulin superfamily'. In contrast to most adult tumours, L1-CAM expression rather indicated a more favourable clinical outcome in high-risk children. It was stated that L1-CAM may be a marker for developing neuronal cells to identify more mature stages of neuroblastic cells and is associated with less aggressive tumour cell behaviour. Thus, ALCAM overexpression may also indicate a more favourable outcome. On the contrary, weak expression of the adhesion molecules ALCAM and L1-CAM in infantile neuroblastoma tumours may ease the detachment of tumour cells, thus causing a more aggressive tumour progression correlated with a shortened RFS and OS, as already hypothesised for ALCAM in breast cancer patients (27). However, the molecular mechanisms and interactions of ALCAM deserve further investigation for a better understanding of its role in tumour proliferation and metastatic invasion.

Looking ahead from our results, the expression level of ALCAM in infantile neuroblastoma could serve as a diagnostic and prognostic marker, especially because it has been demonstrated that ALCAM can also be detected in serum after cleavage from the cell surface by metalloproteases in different tumours (14, 22, 28). Therefore, future studies should focus on the detection of serum ALCAM levels in neuroblastoma patients to test its potential as a biomarker in this easy-accessible compartment. Obviously, the detection of one single marker may not provide sufficient prognostic information but the combination of a panel of biomarkers might allow an improved prediction and monitoring of therapy for children suffering from neuroblastoma.

Conclusion

In this study, a strong expression of ALCAM was found in most neuroblastoma tissues. The survival analysis revealed a significant shorter RFS and OS in children with a weak ALCAM expression, suggesting a prognostic impact of ALCAM in this type of solid childhood tumours. Further studies are needed to verify the potential of ALCAM expression levels as a promising predictive and therapy-monitoring tool in infantile neuroblastoma.

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