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Estimation of prognostic value of CD44 expression in neuroblastic tumours in children

Katarzyna Taran[1,2], Józef Kobos[2], Anna Sitkiewicz[3], Stanisław Sporny[1]

[1]Department of Pathology, Medical University of Łódź, Poland; [2]Department of Pathology of the Age of Development, Konopnicka Memorial Hospital, Medical University of Łódź, Poland; [3]Department of Surgery, Konopnicka Memorial Hospital, Medical University of Łódź, Poland

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Abstract

Adversities observed in treatment of children with neoplastic disease based on new diagnostic markers and new prognostic factors. Both of them allow prognosis to be established for a single patient. The aim of our study was to examine the expression of CD44 adhesion molecule in different histologic types in a neuroblastoma group of tumours (35 cases of neuroblastoma from current files and archives) and to estimate the possible prognostic value of CD44 expression by comparison with widely accepted prognostic markers and chosen histoclinical parameters (9 cases of neuroblastoma with follow-up data). We did not find a statistically significant correlation between CD44 expression and histologic type of the tumour. However, we found that all relapses appeared among patients with tumours with the strongest CD44 expression, and that in none of the investigated tumours without relapses was strong CD44 expression ever observed. We noticed CD44 expression in 88.88% of examined tissue samples which underwent statistical analysis and we found the strongest CD44 expression in tumours situated in the retroperitoneal space. Results of log-rank test and Kaplan-Meier estimation showed that a correlation between CD44 expression and survival time was close to a statistically significant value (p=0.065). We conclude that lack of a clear statistically significant correlation between CD44 expression and histoclinical parameters and currently known prognostic factors in our study is due to the presence of many CD44 isoforms, which cannot be distinguished with commercially used antibodies, but they may play a different role in pathogenesis and spread of neuroblastoma.

Key words: *neuroblastoma, CD44, prognostic factors, survival time.*

Introduction

Neuroblastoma determines 11% of all tumours in children, and is predominantly a tumour of early childhood (25-50% among infants) with two thirds of cases presenting in children younger than 5 years. Neuroblastoma is also the most frequent solid tumour outside the central nervous system. These tumours originate in the adrenal medulla or paraspinal sites where sympathetic nervous system tissue is present, e.g. in the mediastinum, the pelvis and the neck. The most interesting feature of neuroblastoma is its autonomous or called out with chemotherapy maturation of tumour cells and the phenomenon of sponta-

Communicating author:

Katarzyna Taran, MD, PhD, Department of Pathology, Medical University of Łódź, Czechosłowacka 8/10, 92-216 Łódź, Poland, tel.: +48 42 675 76 35, Email: dr.taran.patho@gmail.com

neous regression. The degree of maturity of tumour cells has become the basis of histologic classifications [20,30] and one of the main prognostic factors in this group of tumours. Favourable or unfavourable prognoses are defined on the basis of histologic parameters (the amount of stromal development, the degree of neuroblastic maturation, the mitosis-karyorrhexis index of neuroblastic cells) and patient age. A neuroblastoma containing many differentiating cells, termed a ganglioneuroblastoma, can have nodules of undifferentiated cells, whose histology, along with MYCN amplification, determines prognosis [26,30]. Evidence for sympathetic neuronal differentiation and the most characteristic clinical feature of neuroblastoma is the ability of secretion of catecholamines. The finding of elevated levels of serum catecholamines (e.g. dopamine and norepinephrine) or urine catecholamine metabolites, such as vanillylmandelic acid (VMA) or homovanillic acid (HVA), confirms the diagnosis of neuroblastoma. The most common clinical presentation of neuroblastoma in physical examination of the patient is an abdominal mass with clinical symptoms due to the presence of a tumour or symptoms due to bone metastases (bone pain). Because of the variety of clinical symptoms and the frequent occurrence of diagnostically difficult cases (e.g. bone metastases of unknown primary location of the tumour) the diagnosis of neuroblastoma is usually late and 75-80% of patients are qualified into stage III or IV of the disease [21,22]. The prognosis for patients with neuroblastoma is related to their age at diagnosis, clinical stage of disease, and, in patients older than 1 year, regional lymph node involvement. Other conventional prognostic factors include the site of primary tumour and tumour histology. A number of biologic variables are also taken into consideration: Shimada classification, tumour cell chromosome number, amplification of MYCN oncogene in tumour cells, unbalanced 11q loss of heterozygosity, and loss of heterozygosity for chromosome 1p [2,4-6,11,22,27]. The main molecular marker of poor prognosis is amplification of the MYCN gene, which is associated with the deletion of chromosome 1p and gain of the long arm of chromosome 17 (the latter independently predicts a poor prognosis). High-level expression of the MRP1 drug resistance gene is an independent indicator of decreased survival. Others used in determining therapy or investigated factors are: tumour cell telomere length, telomerase activity, urinary VMA and HVA and their ratio, dopamine, CD44 expression, TrkA gene expression, neuron-specific enolase level, serum lactate dehydrogenase level, and serum ferritin level [3,18,20,25].

Materials and methods

We selected for our study 35 formalin-fixed and paraffin-embedded neuroblastoma tissue sections from the files of the Department of Pathology of the Age of Development and Department of Pathology, Konopnicka Memorial Hospital, Medical University of Łódź. From these tissues samples paraffin blocks about the thickness 3-4 micrometres were prepared and stained with haematoxylin and eosin (HE) and they were used for immunohistochemical research with use of CD44 (H-CAM) Clone F10-44-2 (Novocastra) and with immunoperoxidase reaction according to Hsu (EnVision+ System, Peroxidase – DAB (DAKO). For the purpose of our study all of the previously diagnosed tumours became reclassified according to current criteria for this group (based on the International Neuroblastoma Pathology Classification). Estimation of the expression of investigated protein was examined with a computer image analysis system (Multi Scan Base v. 8.08 – Computer Scanning System, Ltd.). All examined microscopic pictures (Nikon Microphot FXA) were transferred to the computer by camera (CC2OP).

In immunohistochemical research we accepted cytoplasmic type of reaction – the brown colour of the cytoplasm of neoplastic cells – and we rated CD44 expression as: weak (less than 10% of positive tumour cells), of intermediate degree (from 10% to 60%), or strong (more than 60%). The expression of investigated protein was compared with recognized prognostic factors (histologic type, stage of disease, tumour location and age of the patients) and with chosen histoclinical features (sex, the presence of metastases or relapses, performed treatment and survival time). For the analysis we used the statistical package SYSTAT for Windows (Version 5.03, SYSTAT, Inc, Evaston, Illinois, USA, license no.: DA021594). Survival analysis was made with use of the package "Survival" (Version 1.0 Inc, Evaston, Illinois, USA, license no.: DA061688). Mean values and standard deviation were calculated. Non-parametric tests of Mann-Whitney and Kruskall-Wallis analysis, and chi2 test were used. For all tests $p<0.05$ was accepted. The total survival time was counted from the day of the diagnosis to the day of death of the patient or to the last day of observation,

and in our investigations we used the Kaplan-Meier method and the log-rank test.

Results

Results of histologic examination in the neuroblastoma group of tumours.

Tissue samples from 35 tumours were examined. Most of the investigated tumours were diagnosed as neuroblastoma poorly differentiated (31-88.58%). We diagnosed three differentiating neuroblastomas (8.57%) and one stroma-rich ganglioneuroblastoma (2.86%).

Results of immunohistochemical research of the CD44 adhesive molecule in neuroblastoma group of tumours and their statistical analysis.

We found six immunohistochemical reactions doubtful because of technical matters (the oldest cases). Within the remaining 29 samples a positive CD44 reaction was found in 19 (65.52%) and was rated as: strong – 10 cases (34.48%), of intermediate degree – 8 cases (27.5%), and weak – 1 case (3.45%). There was no statistically significant correlation between CD44 expression (Figs. 1 and 2) and histologic type of tumour.

Estimation of prognostic value of CD44 expression in correlation with prognostic factors and chosen histoclinical features in neuroblastoma group of tumours.

We examined neuroblastoma tissue sections from nine children – seven boys (77.77%) and 2 girls (22.22%), F:M=0.3:1. The youngest patient was 6 months old, the oldest patient was 7.5 years old (mean: 38 months, standard deviation: 29.51; median: 33). Most analyzed cases – 8 (89%) – were diagnosed among children above one year of age. Staging of the diseases was found as: III – 3 patients, and IV – 6 patients; there were no recognized patients with I or II, or IVS. In most cases – 7 (77.77%) – tumours were situated in the retroperitoneal space. The presence of metastases (all of them in bone marrow) was recognized in 6 patients (66.66%). In case № 4 surgery was performed because of life-threatening symptoms; the patient died one day after surgical intervention. Preoperative chemotherapy and surgery were performed in nine of the patients (100%), the removal of lymph nodes in five (55.55%), and postoperative chemotherapy in eight (88.88%). The time of observation was from 2 to 69 months (mean: 38.11; standard deviation: 21.91; median: 33). Five children (55.55%) died because of neoplastic disease. Survival time in those cases was from 2 to 33 months (mean: 22; standard deviation: 12.29; median: 27). Recurrences were recognized in five patients (55.55%) - from 1 to 3 months from the time of the end of treatment (mean: 1.75, standard deviation: 0.96, median: 1.5). Four children (44.44%) live without symptoms of neoplastic disease. Clinical characterization of the patients is presented in Table I.

In immunohistochemical research on the neuroblastoma group of tumours (the 9 cases described above) we observed CD44 expression in 8 cases (88.88%) and we found it as: strong – 3 cases (33.33%), of intermediate degree – 4 cases (44.44%), and weak – 1 case (11.11%). In males (7 cases) we no-

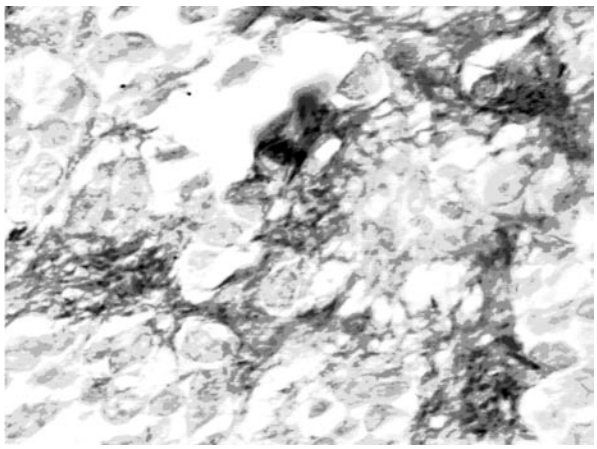

Fig. 1. Strong CD44 expression in poorly differentiated neuroblastoma cells × 600

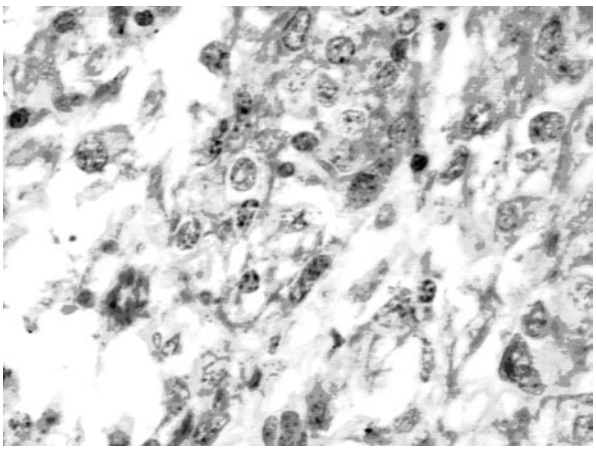

Fig. 2. Intermediate degree CD44 expression in poorly differentiated neuroblastoma cells × 400

Table I. Age, sex, stage of disease, tumour location, performed treatment and clinical course in the examined neuroblastoma group of tumours

No	Age	Sex	L	S	Q1	Su	LN	Q2	M	R	RT	D	TT
1	32	m	r	IV	Y	Y	Y	Y	Y	Y	2	Y	33
2	6	f	r	IV	Y	Y	Y	Y	Y	N	-	N	50
3	60	m	s	III	Y	Y	N	Y	N	N	-	N	69
4	72	f	r	III	Y	Y	N	Y	N	N	-	Y	2
5	21	m	r	IV	Y	Y	Y	Y	Y	N	-	Y	27
6	23	m	r	IV	Y	Y	Y	Y	Y	Y	1	Y	19
7	20	m	r	IV	Y	Y	Y	Y	Y	N	-	N	65
8	11	m	r	IV	Y	Y	N	Y	Y	Y	3	Y	29
9	90	m	s	III	Y	Y	N	Y	N	Y	1	N	49

L – tumour location; r – retroperitoneal space; s – mediastinum; S – stage; Q1 – preoperative chemotherapy; Su – surgery; LN – lymph node removal; Q2 – postsurgical chemotherapy; M – the presence of metastases; R – the presence of relapses; TR – time of relapse (number of months after the end of treatment); D – death of the patient; TT – observation time (number of months); m – male; f – female; Y – yes; N – no.

ticed all kinds of expression types: strong – 2 cases, of intermediate degree – 3 cases, weak – 1 case, and negative reaction (lack of expression) – 1 case. In females (2 cases) we exclusively found strong expression or expression of intermediate degree. In tissue sections originating from patients with stage IV of the disease (6 cases) we observed: strong expression – 2 cases, expression of intermediate degree – 3 cases, and weak – 1 case. In stage III (3 cases) we noticed: negative reaction (lack of expression) – 1 case, expression of intermediate degree – 1 case, and strong – 1 case. In all tumours situated in the retroperitoneal space (7 cases) we found: strong expression – 3 cases, expression of intermediate degree – 3 cases, and weak – 1 case. In tumours located in the mediastinum (2 cases) we observed CD44 expression of average degree or lack of expression. Among tumours with the presence of metastases (6 cases) we always observed CD44 expression: strong – 2 cases, expression of intermediate degree – 3 cases, and weak – 1 case. Among tumours without metastases (3 cases) we noticed strong expression, expression of intermediate degree, and lack of CD44 expression. Relapses were diagnosed in 4 patients. All recurrences appeared in patients with tumours with strong (2 cases) or of intermediate degree of CD44 expression (2 cases). In none of the investigated tumours without relapses was strong CD44 expression found. We observed only expression of average degree – 2 cases, weak – 2 cases, and lack of expression – 1 case. Results of log-rank test and Kaplan-Meier estimation showed a negative influence of the expression of CD44 adhesive molecule on survival time of the patients close to a statistically significant value ($p = 0.065$). There was no statistically significant correlation between CD44 expression and age, sex, stage, tumour location or the presence of metastases or recurrences. In our study on remaining histoclinical features we found the following statistically significant correlations: between the stage of the disease and the location of the tumour, and between location of the tumour and the presence of metastases. All patients in stage IV of disease (6 cases) suffered from tumour located in the retroperitoneal space. In all cases of tumours without metastases (2 cases) the tumours were situated in the mediastinum. The correlation between location of the tumour and death of the patient was close to a statistically significant value, $p=0.073$. All fatal cases (5 cases) appeared also among patients with tumours located in the retroperitoneal space. Essential statistically important correlations between histoclinical features in the neuroblastoma group of tumours are shown in Table II.

Discussion

Neoplastic disease is diagnosed in 1300 children in Poland every year. Most of the patients can be cured or permanent remission can be achieved thanks to performed treatment. However, despite progress observed in the area of paediatric oncology, malignancies are still the second cause of death among children above one year of age. The neoplastic process in children is very dynamic in its clinical

Table II. Essential correlations between histoclinical features

Feature I	Feature II	p	Pearson Chi^2	Spearman Rho
stage of disease	tumour location	p=0.023	5.143	0.756
stage of disease	presence of metastases	p=0.003	9.000	1.000
presence of metastases	tumour location	p=0.023	5.143	0.756
tumour location	death	p=0.073	3.214	0.598

course, and a wide variety of clinical symptoms is observed. Unfortunately in microscopic examination of tissue specimens taken from the tumour pathologists usually observe only a monotonous population of small, round, poorly-differentiated cells. Separation of the group of small round cell tumours of childhood confirms the presence of difficulties in the routine histologic examination, and the need for new markers which allow the final diagnosis to be made correctly [1]. In most neuroblastoma cases diagnosis is made due to clinical examination, laboratory tests (catecholamines and levels of their metabolites), and the histological examinations performed on the post-operative specimen which confirms the initial diagnosis. The estimation of various immunohistochemical markers (e.g. NB84, neurospecific enolase or neurofilaments) is helpful in differential diagnosis in the case of difficulties in histologic examination of the routine (haematoxylin and eosin) stained tissue slides. Among all research new molecular biology techniques seem to be very useful in diagnostic procedures also. Identification of some genetic abnormalities typical for the type of neoplasm (e.g. WT1;EWS gene for desmoplastic small round cell tumours) or even typical for the tumour subtype (e.g. PAX3; FKHR gene for alveolar rhabdomyosarcoma) allows the final diagnosis to be made and proper treatment performed. In neuroblastoma examination molecular research (amplification of NMYC gene) has become an element of routine diagnostic procedures, with an influence on the prognosis and on treatment. However, to determine the prognosis for a particular patient with neuroblastoma is still a problem of the greatest importance [3,12]. Estimation of numerous previously recognized prognostic markers (stage, tumour location, age, concentration of biochemical markers in the serum of the blood – ferritins, NSE, LDH, the relation of concentration of dopamine to norepinephrine in the urine and the amplification of MYCN oncogene), and many other factors of unknown value (1p deletion, loss of heterozygosity within 1p, the presence of TrkA protooncogene and of the n-ras gene, DNA ploidy, the expression of CD44, bcl-2, HNK-1, IGF-1, PDGF, NGF-R, APO (CD95), the ganglioside GD2, the presence of vasoactive intestinal peptide), have so far not lead to the creation of a reliable system for estimating the degree of risk of progression of neoplastic disease and the probability of death of patients [4,7-10,13,14,19,20]. It was proved that adhesive molecules, in which CD44 is included, play an important role in progression of neoplastic disease due to: release of single neoplastic cells from primary tumour (cadherin E), penetration of neoplastic cells to the vessels (integrin), binding of neoplasmatic cells to endothelium (selectins) and aggregation of tumour cells with platelets (integrins and immunoglobulins). In spite of intensive research the CD44 molecule is comparatively little recognized. High expression of this molecule was observed on the surface of skin, cervix, endometrium, stomach, colon and of prostate cancer cells. It was described in the literature that the presence of the CD44 molecule allows neoplastic cells to metastasize. However, results of other research have proved that not increase but decrease of CD44 expression appeared as an unfavourable prognostic factor in prostate and bladder cancers [10,15,16,29]. In our opinion the lack of a clear statistically significant correlation described in literature results demands an attentive interpretation. It was a surprise that in our research on neuroblastomas, which according to the literature should be very dynamic and very aggressive in their clinical behaviour, we observed CD44 expression in only 65.52% of tumour tissue samples. However, among patients in stage III and IV of the disease who underwent statistical analysis we found CD44 expression in 88.88% of cases and we noticed that the strongest CD44 expression was observed in tumours situated in the retroperitoneal space, and that all relapses appeared in patients with tumours with the strongest CD44 expression. Among none of the investigated tumours without relapses was strong CD44 expression ever

found. Results of log-rank test and the Kaplan-Meier method showed that the influence of CD44 adhesive molecule expression on survival time of the patients appeared close to a statistically significant value and in metastasizing tumours we always observed CD44 expression. Also, all the recurrences appeared among patients with high CD44 expression in tumour cells. Unfortunately there was no significant correlation between CD44 expression and any of the examined histoclinical features, nor with commonly used neuroblastoma prognostic markers. This discrepancy may appear due to extremely complicated mechanisms of CD44 expression. The process of alternative RNA splicing and modifications of the product of the CD44 gene after translation lead to the formation of numerous CD44 isoforms (about thirty are described so far). These particles may have different adhesive properties and probably different, even opposed influence on the progression of the neoplastic process. According to our knowledge it has already been proved that expression of one of the CD44 variants named CD44v6 was connected with the progression of neoplastic disease [17,23,24,29]. We conclude that the results of our study complete some elements of the knowledge of the process of dissemination and progression of neuroblastoma and that a final estimation of the prognostic value of CD44 expression in this group of tumours and explanation of doubts will be possible when commercially produced antibodies against many CD44 isoforms are available. Separate research on all of the CD44 isoforms may show properties of this adhesive molecule in a new light. It is necessary to note that results of investigation of the prognostic value of other potential prognostic factors in the neuroblastoma group of tumours, e.g. TrkA protooncogene or 1p region abnormalities, are also under discussion. Even the commonly known prognostic value of MYCN amplification is still discussed – in contrast to MYCN gene amplification, the degree of expression of the MYCN gene in the tumour does not predict prognosis [7]. The presence of essential differences in current opinion on the prognostic value of examined biological factors leads to the conclusion that neuroblastoma complex is a very heterogeneous group of entities, similar in their morphological features, but with sometimes surprising clinical course. We believe that some new entities will become separated from this group in future, just as neuroblastoma among children younger than 1 year of age has already been excluded.

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