Pergamon

European Journal of Cancer, Vol. 33, No. 12, pp. 2101–2105, 1997

Printed in Great Britain
0959-8049/97 $17.00+0.00

PII: S0959-8049(97)00236-0

Original Paper

Clinical Relevance of CD44 Cell Surface Expression and *MYCN* Gene Amplification in Neuroblastoma

V. Combaret,[1] N. Gross,[2] C. Lasset,[1] D. Frappaz,[1] C. Beretta-Brognara,[2] T. Philip,[1] D. Beck[2] and M.C. Favrot[1]

[1]Department of Tumour Biology, Centre Léon Bérard, 28 rue Laënnec, 69373 Lyon Cedex 08, France; and [2]C.H.U.V., Lausanne, Switzerland

This multicentric analysis of tumours obtained from 140 patients with neuroblastoma confirms that the lack of CD44 expression is a highly significant factor of poor prognosis and, as previously published in multivariate analysis of the four factors, i.e. *MYCN* amplification, CD44 expression, age and tumour stage, CD44 expression and tumour stage were the only independent prognostic factors of event-free survival (Combaret *et al.*, *J Clin Oncol* 1996, 14, 25–34). Furthermore, CD44 analysis affords significant prognostic discrimination in subgroups of patients with or without *MYCN* amplified tumours, both in low-stage neuroblastomas and high-grade neuroblastomas. In the subgroup of patients with low-stage neuroblastoma and the stage 4 subgroup, CD44 was the only independent prognostic factor for the prediction of event-free survival in a multivariate analysis. In conclusion, CD44 is one of the most powerful factors for predicting clinical outcome in neuroblastoma at the time of initial staging. © **1997 Elsevier Science Ltd.**

Key words: CD44-neuroblastoma, prognosis
Eur J Cancer, Vol. 33, No. 12, pp. 2101–2105, 1997

INTRODUCTION

SEVERAL CLINICAL and biological risk factors have been reported for neuroblastoma, including age at diagnosis, stage of the disease, serum ferritin and lactate dehydrogenase (LDH) levels, response of skeletal metastases to chemotherapy, tumour histology, deletion of chromosome 1p, *MYCN* amplification, DNA ploidy and trk-A expression [1–3]. However, with increasing emphasis on new therapeutic strategies, the search is on for more discriminative criteria which can predict the biological heterogeneity of neuroblastoma in certain subgroups of patients.

The cell surface glycoprotein CD44 is a polymorphic molecule resulting from alternative splicing and cell lineage-specific glycosylation. The most prevalent isoform of CD44 is an 80–90 kDa molecule termed CD44H ('H' standing for haematopoietic). CD44 acts as the principal receptor for hyaluronate and is involved in the homing process and cell–cell or cell–extracellular matrix interactions. An analysis of CD44 expression in human malignant tissues has shown that they overproduced large alternatively-spliced molecular variants of CD44, with striking differences between metastatic and non-metastatic malignant specimens [4–6]. We have shown that in neuroblastoma the lack of CD44 expression is a highly significant factor of poor prognosis [4–6], with CD44 expression and stage of the disease the only independent prognostic factors in a multivariate analysis.

In this study we examined CD44 expression in 162 samples (22 ganglioneuromas and 140 neuroblastomas) obtained from newly diagnosed patients and analysed its prognostic value.

MATERIALS AND METHODS

Specimens were obtained from 162 newly diagnosed patients treated in collaborative institutes in France (103 patients) and Switzerland (59 patients) from November 1983 to January 1996. Of these 22 were ganglioneuromas (all CD44 positive) which were excluded from the study. The rest of the report focuses on 140 neuroblastoma specimens. Neuroblastoma diagnosis and staging were performed according to INSS criteria [1]. Children were treated with the same well-standardised protocols, including high-dose chemotherapy and bone marrow transplantation for consolidation of stage 4 patients older than 1 year and relapse of stage 3 patients, as previously described [3, 7, 8].

Correspondence to M.C. Favrot.

Tumour specimens were obtained at diagnosis by surgical biopsy or excision of the primary tumour in stage 1, 2 and 4S disease, or by ultrasound-guided puncture of the primary tumour in stage 3 and 4 disease [9]. In a few stage 4 patients, malignant cells were obtained from highly contaminated bone marrow aspirates. Handling and storage of the specimens, as well as cytohistological analysis, were performed as previously described [10].

MYCN was analysed by Southern blot technique with the *MYCN* probe pNb-1 (kindly provided by J. Minna, National Cancer Institute, U.S.A.), as previously described [11]. The presence of more than two copies of *MYCN* in all samples was considered as amplification.

CD44 cell surface expression was detected by immunostaining using an indirect three-stage immunoenzymatic procedure with alkaline phosphatase as previously described [10]. Briefly, air-dried slides (cryostat sections or cytocentrifuged smears) were fixed for 5 min with acetone at 4°C and incubated for 60 min, with monoclonal antibodies (MAbs) at appropriate dilutions, then for 30 min with enzyme-conjugated rabbit anti-mouse immunoglobulins and for 30 min with enzyme-conjugated swine anti-rabbit immunoglobulins. Washes were performed with Tris buffer. The final step consisted of a 15 min incubation with Naphtol-As-Mx phosphate, dimethylformamide, levamisole and Rast Red (Sigma, St. Louis, Missouri, U.S.A.). Slides were counterstained with haematoxylin, mounted permanently with glycerin and evaluated under an optical microscope. Three slides were performed for the analysis of each sample: CD44 was detected with MAbs J173 (Immunotech, Luminy, France) or F 10.44.2 (kindly provided by Dr S. Carrel, Ludwig Institute for Cancer Research, Lausanne Branch, Switzerland) directed against epitopes in the CD44 constant region [12, 13]. Lymphocytes and monocytes were quantified by immunostaining with anti-CD45 panleucocyte MAb and neuroblastoma cells with anti-CD56; anti-CD45, anti-CD56 and all enzyme-conjugated immunoglobins were purchased from Dakopatts (Copenhagen, Denmark). In the interpretation of results of bone marrow cytocentrifuged smears or ultrasound-guided puncture, tumour cells were distinguished from haematopoietic cells according to cytological features, and the analysis of malignant cells focused on those which formed typical clumps on the smear. The samples were classified as positive for CD44 expression when greater than 10% of tumour cells showed positive reactivity with anti-CD44 MAb. However, in almost all positive samples, staining was strong and homogeneous in the whole malignant population.

Statistical analyses were performed according to the procedure of the BMDP package (BMDP Statistical Software, Los Angeles, California, U.S.A.).

Statistical analyses were performed using the χ^2 test. Event-free survival was calculated according to the method of Kaplan and Meier. End points were the date of the first event, i.e. progression or death and the date of the last follow-up evaluation when no event occurred [14]. Curves were compared using the log-rank test. Multivariate analysis was performed using the Cox proportional hazards model [15].

RESULTS

Correlation between CD44 cell surface expression, MYC*N amplification, age and stage of the disease (Table* 1*)*

CD44 expression was detected in 108/140 neuroblastoma tumour samples. CD44 expression was strongly related to the stage of disease: CD44 was expressed in 82 of 87 (94%) low-grade diseases (stages 1, 2, 3 and 4S), but only in 26 of 53 (49%) stage 4 specimens ($P < 0.00001$; $\chi^2 = 35.63$). Furthermore, CD44 expression strongly correlated with patient's age, as it was expressed on 61 of 66 tumours from infants less than 1 year of age, but only in 47 of 74 tumours from older children ($P < 0.0001$; $\chi^2 = 14.94$). *MYCN* gene amplification was present in 32 tumour specimens and was strongly associated with a lack of CD44 expression. Indeed, of 32 CD44-negative specimens, 26 (81%) were *MYCN* amplified and only 6 (19%) were non-amplified. In contrast, of 108 CD44-positive specimens, 102 (94%) showed no *MYCN* amplification and 6 (6%) were amplified ($P < 0.00001$; $\chi^2 = 75.97$). Within the 6 *MYCN* amplified and CD44-positive specimens, 3 were stage 4S neuroblastoma with only 5 *MYCN* copies, one was stage 3 and two were stage 4 neuroblastoma, with 20–50 *MYCN* copies.

CD44 expression and event-free survival

The prognostic value of CD44 expression was tested in a univariate analysis in comparison with tumour stage, age of the patient and presence or absence of *MYCN* amplification. All parameters had significant prognostic value. CD44 expression strongly correlated with survival. The 5-year survival rate in the group of patients with CD44-positive tumours reached 81%, versus 11% in the group with CD44-negative tumours ($\chi^2 = 68.91$, $P < 0.000001$). When survival was analysed according to the *MYCN* status of the tumour, the 5-year survival rates were 77% for patients without *MYCN* amplification and 25% for patients with *MYCN* amplification ($\chi^2 = 33.53$, $P < 0.000001$). As shown in Figure 1, the analysis of CD44 expression differentiates between two subgroups of patients with or without *MYCN*-amplified tumours. In the subgroup of patients without *MYCN* amplification, the 5-year event-free survival rate was 81% for CD44-positive tumours and 0% for CD44-negative

Table 1. *Correlation between CD44 cell surface expression, tumour stage and age*

	Total		Stage 1		Stage 2		Stage 3		Stage 4		Stage 4S	
	CD44+	CD44−	CD44+	CD44−	CD44+	CD44−	CD44+	CD44−	CD44+	CD44−	CD44+	CD44−
Age < 12 months ($n = 66$)	61	5										
MYCN no amplification	58	2	15	1	15	0	6	0	7	0	15	1
MYCN amplification	3	3	0	0	0	0	0	1	0	2	3	0
Age ≥ 12 months ($n = 74$)	47	27										
MYCN no amplification	44	4	9	0	11	0	7	0	17	4		
MYCN amplification	3	23	0	1	0	1	1	0	2	21		
Total = 140	108	32	24	2	26	1	14	1	26	27	18	1

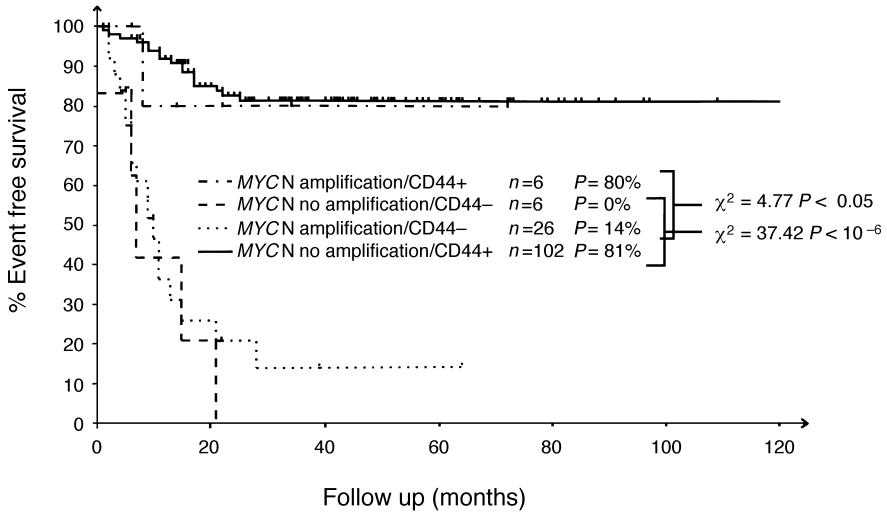

Figure 1. Event-free survival in 140 patients with neuroblastoma according to the combined analysis of CD44 expression and *MYCN* amplification.

tumours ($\chi^2 = 37.42$, $P < 0.000001$). In the population of patients with *MYCN* amplified tumours, patients with CD44-negative tumours had a 14% 5-year event-free survival rate, versus 80% for patients with CD44-positive tumours ($\chi^2 = 4.77$, $P < 0.05$).

The prognostic value of these two biological variables were analysed both in low-stage neuroblastoma and high-grade neuroblastoma. Within the group of low-stage neuroblastoma (stage 1, 2, 3 and 4S) the 5-year event-free survival rate was 92% for CD44-positive tumours versus 0% for CD44-negative tumours ($\chi^2 = 36$, $P < 0.000001$). The 5-year survival rate was 92% for patients without *MYCN* amplified low-stage tumours versus 51% for patients with *MYCN* amplified tumours ($\chi^2 = 12.05$, $P < 0.001$). The combined analyses of CD44 expression and *MYCN* amplification showed that, within the cohort of low-stage neuroblastoma patients, the presence or absence of CD44 clearly predicted outcome ($\chi^2 = 4.64$, $P < 0.05$ with *MYCN* amplification and $\chi^2 = 14.01$, $P < 10^{-3}$ without *MYCN* amplification, respectively) (Figure 2). Of note, in the subgroup of patients with CD44-positive and *MYCN*-non amplified tumours, 5 patients relapsed, but are alive after appropriate treatment (more than 5 years follow-up for three and 6 months for two).

Within the group of stage 4 neuroblastoma, the prognosis of patients according to CD44 was significantly different, with an event-free survival rate of 42% in CD44-positive tumours and only 12% in CD44-negative tumours ($\chi^2 = 8.44$, $P < 0.01$). When *MYCN* amplification was analysed, the 5-year survival rate was 34% for patients without amplification and 17% for those with amplification ($\chi^2 = 3.50$, $P = 0.06$). The combined analyses of CD44 expression and *MYCN* amplification showed that, within the cohort of patients without *MYCN* amplification, the presence or absence of CD44 clearly predicted outcome ($\chi^2 = 5.52$, $P < 0.05$) (Figure 3).

Multivariate analysis of event-free survival (Table 2)

The effects of biological and clinical parameters were analysed in a multivariate analysis. In the total population, CD44 expression and tumour stage were the only independent prognostic factors for identification of high-risk patients. The multivariate analysis was performed individually on the

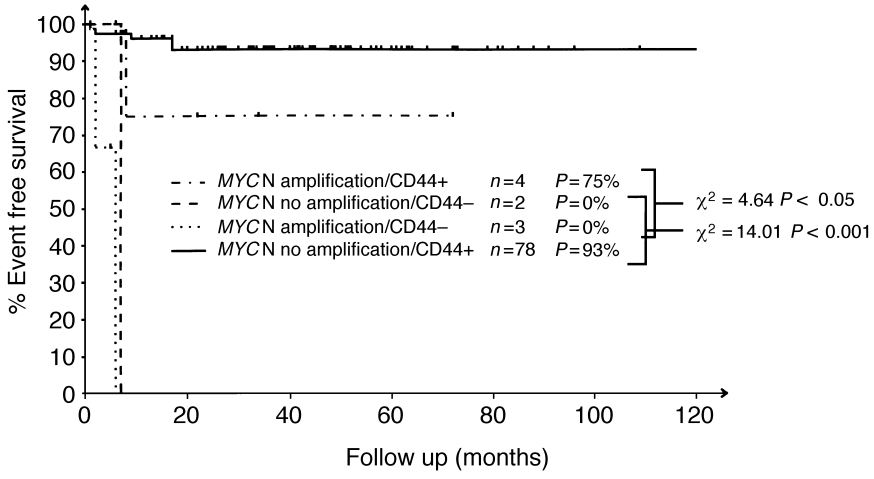

Figure 2. Event-free survival in 87 patients with low-stage neuroblastoma (stage 1, 2, 3, 4S) according to the combined analysis of CD44 expression and *MYCN* amplification.

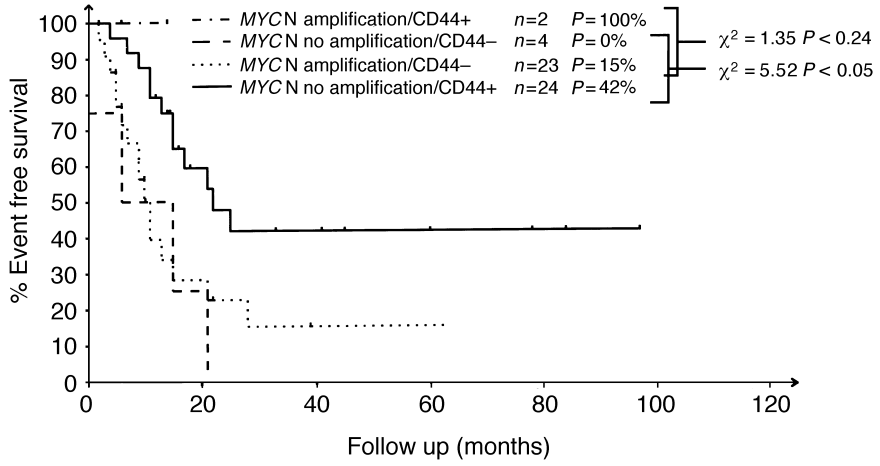

Figure 3. Event-free survival in 53 patients with stage 4 neuroblastoma according to the combined analysis of CD44 expression and *MYCN* amplification.

Table 2. Multivariate analysis of survival according to clinical and biologic variables

Variable	Total population $n = 140$		Stage 4 neuroblastoma $n = 53$		Low-stage neuroblastoma (stage 1, 2, 3, 4S) $n = 87$	
	χ^2	P	χ^2	P	χ^2	P
CD44 expression	11.8	< 0.001	7.11	< 0.01	15.79	0.0001
Tumour stage	10.7	0.001				
MYCN amplification	0.52	0.47	0.83	0.36	1.80	0.18
Age $\leq$ 12 months	0.01	0.90	0	0.93	0.72	0.40

low-stage neuroblastoma subgroup (stages 1, 2, 3 and 4S) and the stage 4 subgroup. In each subgroup, CD44 was the only significant factor for the prediction of event-free survival.

DISCUSSION

Results obtained in this multicentric cohort of 140 patients with neuroblastoma confirm our previously published data [4–6]. In multivariate analysis including the stage of the disease, the age at diagnosis, the presence or absence of *MYCN* amplification and CD44 expression, disease stage and CD44 expression are the only independent prognostic markers. The analysis of CD44 defines prognostic subgroups of patients with or without *MYCN*-amplified tumours. Indeed, CD44 was the only independent prognosis factor both in low-stage disease and in high-grade stage 4 neuroblastoma. The lack of CD44 expression in metastatic neuroblastoma patients without *MYCN* amplification allows a subgroup of patients who are likely to fail maximal therapy to be identified, who might thus be candidates for new therapeutic approaches. Low-stage neuroblastoma patients whose tumours did not express CD44 protein had 0% event-free survival, independently of *MYCN* tumour status. Finally, for patients with tumours displaying less than 10 *MYCN* copies, CD44 expression might indicate a favourable clinical evolution of the disease, as observed in 3 stage 4S patients whose tumours showed only five *MYCN* copies. Unlike the analysis of other biological abnormalities, the analysis of CD44 expression is a sensitive and rapid technique that requires a minimum amount of tumour material and can be easily standardised for routine laboratory use. The immunocytological staining of specimens on centrifuged smears allows individual examination of tumour cells, which permits the analysis of different samples, such as bone marrow aspirates or ultrasound-guided punctures of the primary tumour, that contain only small quantities of tumour cells.

In conclusion, CD44 may thus prove to be one of the most powerful biological factors in the prediction of clinical outcome, although we cannot yet clearly explain the function of this molecule in neuroblastoma. We recommend the use of CD44 as an additional biological marker in the initial staging of neuroblastoma.

1. Brodeur GM, Pritchard J, Berthold F, *et al.* Revisions of the international criteria for neuroblastoma diagnosis, staging, and response to treatment. *J Clin Oncol* 1993, **11**, 1466-1477.
2. Brodeur GM, Azar C, Brother M, *et al.* Neuroblastoma: Effect of genetic factors on prognosis and treatment. *Cancer* 1992, **70**, 1685–1694.
3. Philip T, Zucker JM, Bernard JL, *et al.* Improved survival at 2 and 5 years in the LMCE1 unselected group of 72 children with stage IV neuroblastoma older than 1 year of age at diagnosis: is cure possible in a small subgroup. *J Clin Oncol* 1991, **9**, 1037–1044.
4. Favrot MC, Combaret V, Lasset C. CD44: a new prognostic marker for neuroblastoma. *N Engl J Med* 1993, **329**, 1965.
5. Gross N, Beretta C, Peruisseau G, *et al.* CD44H expression by human neuroblastoma cells: relation to MYCN amplification and lineage differentiation. *Cancer Res* 1994, **54**, 4238–4242.
6. Combaret V, Gross N, Lasset C, *et al.* Clinical relevance of CD44 cell surface expression and *MYCN* gene amplification in a multicentric analysis of 121 pediatric neuroblastomas. *J Clin Oncol* 1996, **14**, 25–34.
7. Philip T, Bernard JL, Zucker JM, *et al.* High dose chemotherapy with bone marrow transplantation as consolidation treatment in neuroblastoma: an unselected group of stage IV patients over one year of age. *J Clin Oncol* 1989, **5**, 266–271.

8. Zucker JM, Philip T, Bernard JL, *et al.* Single or double consolidation treatment according to remission status after initial therapy in metastatic neuroblastoma: first results of LMCE3 study in 40 patients. In Evans AE, D'Angio GJ, Knudson AG Jr, Seeder RC, eds. *Advances in Neuroblastoma Research*. New-York, Wiley-Liss Inc., 1991, 543–551.
9. Thiesse P, Kaemmerlen P, Bouffet E, *et al.* Percutaneous fine needle guided biopsies of the primary tumour in extensive neuroblastoma. In Evans AE, D'Angio GJ, Knudson AG Jr, Seeder RC, eds. *Advances in Neuroblastoma Research*. New-York, Wiley-Liss Inc., 1991, 503–507.
10. Favrot MC, Combaret V, Goillot E, *et al.* Expression of leucocyte adhesion molecules on 66 clinical neuroblastoma specimens. *Int J Cancer* 1991, **48**, 502–510.
11. Combaret V, Wang Q, Favrot MC, *et al.* Clinical value of N-myc oncogene amplification in 52 patients with neuroblastoma included in recent therapeutic protocols. *Eur J Cancer Clin Oncol* 1989, **24**, 1607–1612.
12. Pesando JM, Hoffman P, Abed M. Antibody-induced antigenic modulation is antigen dependent: characterization of 22 proteins on a malignant human B cell line. *J Immunol* 1986, **137**, 3689–3695.
13. Dalchau R, Kirkley R, Fabre JW. Monoclonal antibody to a human brain-granulocyte T lymphocyte antigen probably homologous to the W3/13 antigen of the rat. *Eur J Immunol* 1980, **10**, 745–749.
14. Kaplan ES, Meier P. Non parametric estimations from incomplete observation. *Ann Stat Assoc* 1958, **53**, 457–481.
15. Mantel H, Haenzel W. Statistical aspects of the analysis of data from retrospective studies of disease. *J Natl Cancer Inst* 1959, **22**, 719–748.

Acknowledgements—This work was supported by grants from the French Association for Cancer Research, the Fondation de France, the Rhône Committee of the French National League against Cancer and the Swiss National Scientific Foundation (grant no. 32-26 340-89).