Pergamon

European Journal of Cancer Vol. 31A, No. 4, pp. 545–549, 1995
Elsevier Science Ltd
Printed in Great Britain
0959–8049/95 $9.50+0.00

0959-8049(95)00027-5

Evaluation of CD44 Prognostic Value in Neuroblastoma: Comparison With the Other Prognostic Factors

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CD44 gene products are potential markers of aggressiveness in different tumour models, a result which prompted us to study clinical neuroblastoma (NB) specimens. CD44 expression was determined by immunostaining of 52 tumour samples from newly diagnosed NB with a monoclonal antibody (J173) directed against an epitope common to all CD44 isoforms. CD44 immunoreactivity was detected in 37 of the tumours (71%). CD44 was expressed in all 22 NBs with favourable prognoses (stages 1, 2 or 4S), but only 50% (15/30) of advanced NB (stages 3 and 4) ($P < 10^{-4}$), suggesting that the absence, rather than the overexpression, of CD44 is a signal of tumour aggressiveness. The cumulative progression-free survival was significantly longer in patients with CD44 positive tumours compared with patients with CD44 negative tumours ($P < 10^{-5}$). More importantly, progression-free survival was also significantly higher in CD44 positive patients within the high-risk group ($P < 0.01$). In univariate analysis, we tested the prognostic value of tumour expression of CD44 in comparison with tumour stage, age, tumour histology, and presence or absence of amplification of the *MYCN* protooncogene. All five measures had significant prognostic value. The expression of CD44 and the absence of *MYCN* amplification were the most powerful predictors of a favourable outcome. In a multivariate analysis of these measures, CD44 expression and tumour stage were the only independent prognostic factors for the prediction of patient survival. NB is the first clinical model described in which tumour aggressiveness correlates with repression rather than stimulation of CD44 expression. We recommend the use of CD44 as an additional biological marker in the initial staging of NB.

Key words: CD44 cell surface expression, immunostaining, neuroblastoma, prognostic factor
Eur J Cancer, Vol. 31A, No. 4, pp. 545–549, 1995

INTRODUCTION

THE CELL surface glycoprotein CD44 is a polymorphic molecule resulting from alternative splicing and cell lineage specific glycosylation [1]. The most prevalent isoform of CD44 is a 80–90 kDa molecule named CD44H (H standing for haematopoietic) [2]. CD44 molecules act as the principal receptor for hyarulonate [3]. In addition, they are involved in the homing process [4], cell–cell or cell–extracellular matrix interactions [5], lymphocyte activation [6, 7] and the induction of homotypic cell aggregation [8].

In different models, including non-Hodgkin's lymphoma, melanoma, carcinoma and glioma, evidence was provided that the overexpression of the haematopoietic form of CD44 or its variants is also involved in enhanced tumorigenicity and metastatic behaviour [9–18]. An analysis of CD44 messenger RNA after amplification with polymerase chain reaction has shown that malignant tissues, mostly breast or colon cancers, overproduced large alternatively spliced molecular variants of CD44; in the same study, the band pattern permitted discrimination between metastatic and non-metastatic malignant proliferations [16]. The selective advantage linked to CD44 variant expression has been confirmed for colorectal carcinogenesis [11]. In diffuse large cell B lymphoma, CD44 overexpression was correlated to prognosis [13, 14].

Neuroblastoma (NB), one of the most frequent solid tumours in childhood, is characterised by a wide range of aggressiveness; the age of the patient and the stage of the tumour at diagnosis are the two major clinical prognostic factors [19, 20]. In the typical forms of the disease, children over 1 year of age who present with advanced stage 4 NB generally have poor long-term survival, despite major intensification of the treatment. In contrast, children with localised stage 1 or stage 2 NB, and infants under 1 year of age with widespread stage 4S disease usually have a favourable prognosis. In the latter, metastases, restricted to the skin, liver and bone marrow, may mature or regress spontaneously after surgical excision of the primary tumour. Finally, children with stage 3 NB represent an heterogeneous group that can share prognostic characteristics with metastatic as well as localised NB. Although they are still the most commonly used criteria, these clinical parameters, associated with the histological differentiation, are not sufficient

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to allow a reliable prediction of disease outcome [21]. With increasing emphasis on new therapeutic strategies, the search is on for more discriminative criteria. Somatically acquired genetic abnormalities have proved potentially useful in predicting prognosis, in particular the amplification of *MYCN* oncogene in the tumour [22–27]. However, most of these markers are not routinely evaluated at diagnosis because simple and reliable techniques are not available or tumour material is not sufficient.

CD44 was thus an attractive marker for analysis. We have briefly reported that the lack of cell surface expression on NB was, together with the stage of the disease, the most powerful and independent adverse prognostic factor in a multivariate analysis [28]. We confirm these data in the present study. We analysed, in detail, the expression of CD44 on NB tumour samples representative of the different forms of the disease, and its correlation with disease stage, *MYCN* oncogene amplification, age at diagnosis and tumour cell differentiation. We confirm that this biological marker is an independent prognostic factor in NB.

PATIENTS AND METHODS

Patients and collection of the samples

Clinical NB specimens were obtained from 52 children (27 males and 25 females) with a median age of 24 months (0–172 months) between June 1985 and November 1992. Patients were classified according to international criteria [19] as follows: stage 1, tumour confined to the organ or structure of origin; stage 2, tumour extending in continuity beyond the organ or structure of origin, but not crossing the midline, possibly with homolateral involvement of regional lymph nodes; stage 3, tumour extending in continuity beyond the midline, possibly with bilateral involvement of regional lymph nodes; stage 4, large primary tumour with remote disease involving multiple sites, including bone, bone marrow, organs, soft tissues, or groups of distant lymph nodes; and stage 4S, in infants below 1 year of age with small primary tumour similar to tumour in stage 1 or 2, but with remote tumour in liver, skin or bone marrow (not bone).

Children were treated as previously described [29–31]. Patients with stage 1 disease and most patients with stage 2 or 4S disease were treated with surgery alone; a few patients with stage 2 or 4S disease received local irradiation or chemotherapy, when tumoral excision was incomplete. Patients with stage 3 or 4 disease were treated with conventional induction chemotherapy, followed by surgery and, occasionally, by additional local radiotherapy. Stage 4 patients received consolidation with megatherapy, total irradiation and autologous bone marrow transplantation, a treatment which was also used for stage 3 patients who did not respond to first line therapy or relapsed.

Tumoral 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 [32]. In a few stage 4 patients, malignant cells were obtained from highly contaminated bone marrow aspirates (more than 50% malignant cells within the mononuclear cell population). Bone marrow aspirates (14 cases) and ultrasound-guided punctures (5 cases) were harvested on heparin-free medium and purified by Ficoll separation. One half was kept for molecular analysis, whereas cytological and immunological analyses were performed on centrifuged smears, as previously described [33]. Primary tumour samples were taken surgically and divided into 3 parts, judged to be representative of the same lesion; one part was kept for histological analysis (Bouin fixation), one for molecular analysis, and the third part was frozen in isopentane for immunological analysis.

Southern blot analysis

MYCN was analysed by Southern blot technique, as previously described [25]. After extraction, DNA was digested with restriction endonuclease *Eco*R1. Ten micrograms of DNA were loaded per lane, electrophoresed through 1% agarose, transferred to nylon filters (Pall Europe Limited, Portsmouth, U.K.). Hybridisation was performed with the *MYCN* probe pNb-1 (kindly provided by J. Minna, NCI), ^{32}P-labelled by Amersham (Little Chalfont, U.K.) 'Multiprime DNA Labelling System' to a specific activity of about 10^9 cpm/μg.

In *MYCN* analysis, restriction enzyme digested tumour DNAs were compared in the same agarose gels (two-copy intensity) with lymphocyte DNA and with the known *MYCN* amplified DNA of a NB cell line (SKNBE:100 copy intensity). The number of amplified gene copies was measured by serial dilution of DNA to obtain a hybridisation signal of two-copy intensity (e.g., a 100-fold amplification is indicated when a 1 : 100 dilution achieves two-copy intensity).

Monoclonal antibodies (MAb)

J173 MAb directed against a determinant common to all isoforms of the CD44 molecule [34] was purchased from Immunotech (Luminy, France). AntiCD45 and antiCD56 MAbs used to quantify lymphocytes/monocytes and NB cells, respectively, were purchased from Dakopatts (Copenhagen, Denmark).

Detection of cell surface CD44 expression by immunostaining

Immunochemical staining was performed using an indirect three-stage immunoenzymatic procedure with alkaline phosphatase as previously described [35]. Briefly, air-dried slides (cryostat sections or cytocentrifuged smears) were fixed for 5 min with acetone at 4°C, incubated for 60 min with MAbs at appropriate dilution, then for 30 min with enzyme-conjugated rabbit antimouse immunoglobulins (Dakopatts) and for 30 min with enzyme-conjugated swine antirabbit immunoglobulins (Dakopatts). Washes were done with Tris buffer. The final step consisted of a 15 min incubation with Naphtol-As-Mx phosphate, dimethylformamide, levamisole and fast red (Sigma, St Louis, Missouri, U.S.A.). Slides were counterstained with haematoxylin, mounted permanently with glycerin and evaluated under an optical microscope.

Cytological or histological analyses were performed in parallel on each specimen by standard techniques. Tumours were classified as typical NB, ganglioneuroblastoma or ganglioneuroma, as previously described [35].

Statistical analysis

Statistical analysis was performed using the chi-square test. Progression-free survival was calculated according to the method of Kaplan and Meier, using the date of first progression or of last follow-up (when no progression occurred) as end points [36]. Curves were compared using the log rank test [37]. Multivariate analysis of survival was performed using the Cox model. All analyses were performed with the GMDP programme (1L and 2L procedures).

RESULTS

52 clinical NB specimens obtained at diagnosis were analysed using J173 MAb, which recognises a common determinant to all CD44 isoforms. Positive immunostaining was observed on 37 samples (71%) (Table 1).

Table 1.

Stage	Number of patients	CD44 positive		CD44 negative	
		MYCN positive	*MYCN* negative	*MYCN* positive	*MYCN* negative
1–2	13	0	13	0	0
4S	9	0	9	0	0
3	6	0	5	1	0
4	24	0	10	10	4
Total	52	0	37	11	4

As shown in Table 1, CD44 was present on all 22 samples from low-risk stage 1, 2 or 4S NB, but was only present on 15 of 30 specimens from high-risk stage 3 or stage 4 NB ($P = 0.0001$, $\chi^2 = 15.5$). The significant correlation of CD44 expression with the stage of the disease strongly suggests that it might help predict clinical outcome. We thus compared the survival of patients depending on whether tumours expressed or did not express CD44 antigen. As shown in Figure 1a, progression-free survival was longer in patients with CD44 positive tumours than in patients with CD44 negative tumours (log rank = 22.4; $P < 10^{-5}$). More importantly, within the group of 30 high-risk patients with stage 3 or 4 NB, progression-free survival also correlated with the expression of CD44 (log rank = 5.23; $P < 0.01$) (Figure 1b).

The cell surface expression of CD44 on tumour samples was paralleled with the presence or absence of *MYCN* amplification. As shown on Table 1, none of the 11 samples with *MYCN* amplification expressed the CD44 protein, whereas 37 of 41 samples without *MYCN* amplification expressed CD44 ($P < 10^{-5}$).

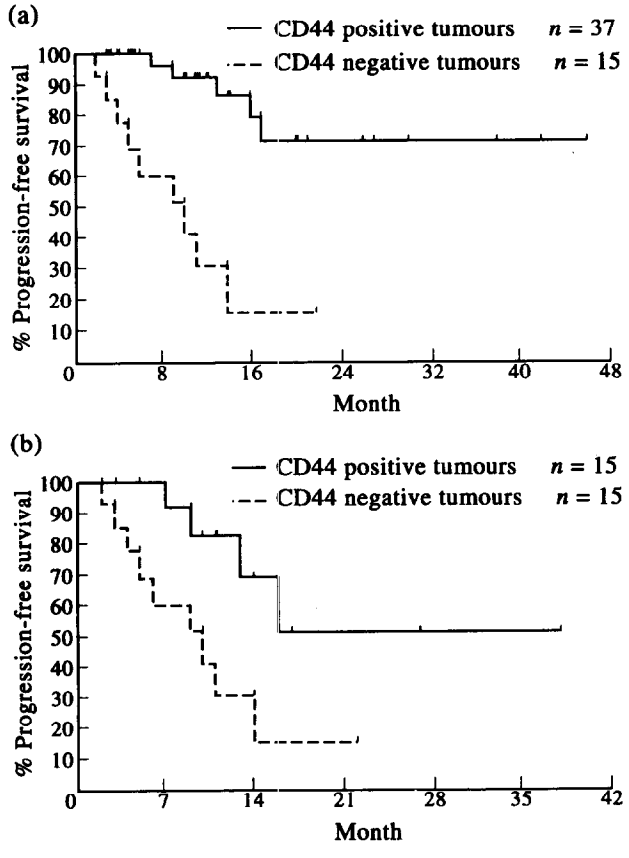

Figure 1. (a) Analysis of progression-free survival according to CD44 expression in patients with neuroblastoma. (b) Analysis of progression-free survival according to CD44 expression within the group of high-risk patients (stages 3 and 4).

In this cohort of 52 patients, 2 of 21 infants versus 13 of 31 children over 1 year of age had CD44 negative tumours ($P < 0.02$). Of the 52 tumours, 41 were undifferentiated NB, whereas 11 were classified as differentiated NB (ganglioneuroblastoma or ganglioneuroma). CD44 expression was observed on the 11 differentiated tumours, but only on 26 of 41 undifferentiated specimens ($P < 0.05$). In univariate analyses, we tested the prognostic value of CD44 expression in comparison with tumour stage, age, tumour histology, and presence or absence of *MYCN* protooncogene amplification. All five parameters had significant prognostic value (Table 2). CD44 expression and the absence of *MYCN* amplification were the most powerful predictors of a favourable clinical outcome. In a multivariate analysis of these parameters, CD44 expression and tumour stage were the only independent prognostic factors for the prediction of patient survival (Table 2).

DISCUSSION

CD44 is a new prognostic marker in NB. The very significant correlation of CD44 expression with disease stage, *MYCN* amplification, tumour histology and age at diagnosis explains how this marker may be predictive of clinical outcome as shown in this group of 52 patients. Of greater importance is the identification of a subgroup of patients in disease stages with different prognoses. In this respect, CD44 expression predicted progression-free survival in the group of 30 high-risk patients with stage 3 and 4 disease. In multivariate analyses, including parameters tested above, CD44 expression and tumour stage were the only independent prognostic factors for the prediction of patient survival. Recently, Nakagawara and associates identified the level of *TRK* mRNA expressed in NB tumours as a new biological marker for prognosis in this disease [38]. However, when the outcome was adjusted to the effect of *MYCN*, *TRK* mRNA expression remained significant only in patients without *MYCN* amplification; in contrast, CD44 expression was significant in the whole series of patients described above. CD44 may thus prove to be one of the most powerful biological factors to predict clinical outcome. Unlike the analysis of genetic abnormalities, the analysis of CD44 expression is a sensitive and rapid technique which requires minimum amounts of tumour material, and can be easily standardised for laboratory routine.

NB is the first clinical model described in which tumour aggressiveness is correlated with repression rather than stimulation of CD44 expression as previously described for other models [9–18]. CD44 is expressed on non-neuronal derivatives

Table 2. Univariate and multivariate analysis of CD44 prognostic value

	Univariate analysis		Multivariate analysis	
	Chi-square value	*P* value	Chi-square value	*P* value
MYCN	15.48	0.0001	0.00	0.95
CD44	22.43	<0.00001	5.95	0.014
Clinical stage	11.82	0.0006	2.91	0.088
Age	4.60	0.032	0.81	0.37
Histology	7.04	0.008	1.93	0.16

of the neural crest, in particular on pheochromocytoma and melanoma, as it is expressed on astrocytes and microglia in normal brain, but not in neurons [10, 39–41]. However, the absence of the protein has been described in a few cell lines derived from small cell lung carcinoma, another tumour of neuroectodermal origin [12]. In NB, CD44 expression may reflect the origin of the cell and the stage of transformation during neural crest differentiation. It might also contribute to maturation and spontaneous regression of low grade stage 1 and 2 NB, and of metastatic stage 4S NB observed in infants under 1 year of age. The correlation between the non-expression of CD44 molecule's *MYCN* and oncogene amplification was established. The repression of CD44 gene transcription has been shown in NB cell lines [41], and these data support the hypothesis of transcriptional regulation of the CD44 gene by mycN protein, as has been described for other molecules [42].

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Acknowledgements—This work has been supported by the Association pour la Recherche sur le Cancer, the Fondation de France and the Rhône Departmental Committee of the French National League against Cancer.

Pergamon

European Journal of Cancer Vol. 31A, No. 4, pp. 549–552, 1995
Elsevier Science Ltd
Printed in Great Britain
0959–8049/95 $9.50+0.00

0959-8049(95)00061-5

Differentiation and Prognosis of Neuroblastoma in Correlation to the Expression of CD44s

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Cell–cell and cell–extracellular matrix interactions mediated by cell adhesion molecules (for example CD44) play an important role in the cascade of metastasis and the progression of human malignant tumours. The most important aim of this review was, on the basis of our results and the literature, to show the correlation between the expression of CD44s and differentiation and prognosis of neuroblastoma. Surprisingly and in contrast to most other malignant tumours, neuroblastomas exhibited an inverse correlation between CD44s expression and tumour progression. It can be stated that CD44s is a prognostic marker in neuroblastoma which correlates significantly with the grade of tumour cell differentiation, but not with clinical stage. Moreover, there exists a statistically significant correlation between *MYCN* oncogene amplification and the lack of CD44s expression.

Key words: neuroblastoma, CD44, *MYCN*
Eur J Cancer, Vol. 31A, No. 4, pp. 549–552, 1995

INTRODUCTION

CELL–CELL and cell–extracellular matrix interactions mediated by cell adhesion molecules play an important role in the cascade of metastasis and the progression of human tumours [1–4]. A qualitative and/or quantitative effect on the expression of these adhesion molecules, of which CD44 is a member, is exerted by genomic DNA alterations, such as amplifications, translocations, insertions, deletions and point mutations, but also by alterations in mRNA composition, such as alternative splicing or post-translational changes. The changed expression of certain adhesion molecules is apparently brought about by a selective process and in such a way that only a particular subpopulation of tumour cells acquires the ability to separate itself from the tumour cell cluster by cell–cell and cell–extracellular matrix interactions, invade through the basement membrane, migrate in the extracellular matrix (active locomotion), or disseminate into blood or lymphatic vessels.

For CD44, such interactions have been established *in vitro* for the first time in the vascular dissemination of melanoma and lymphoma cells, and in the migration of rat pancreas carcinoma cells in the extracellular matrix [5–7].