Pergamon

European Journal of Cancer, Vol. 33, No. 12, pp. 2098–2100, 1997

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

PII: S0959-8049(97)00211-6

Original Paper

Correlation of *MYCN* Amplification, Trk-A and CD44 Expression with Clinical Stage in 250 Patients with Neuroblastoma

K. Kramer,[1] N.-K.V. Cheung,[1] W.L. Gerald,[1] M. LaQuaglia,[1] B.H. Kushner,[1] J.-M. LeClerc,[2] L. LeSauter[3] and H.U. Saragovi[3]

[1]Department of Pediatrics and Pathology, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021, U.S.A.; [2]Department of Hematology-Oncology, St Justine Hospital, Montreal, Quebec; and [3]Department of Pharmacology and Therapeutics, McGill University, McGill Cancer Center, Montreal, Quebec, Canada

In contrast to *MYCN* amplification, expression of either trk-A or CD44 in neuroblastoma is a favourable prognostic factor and were therefore investigated in tumours from 250 patients. One hundred and eleven localised/4s (Group 1) and 139 stage 4 (Group 2) tumours were analysed. *MYCN* copy number was obtained by Southern blotting or PCR amplification and was detected in 28 stage 4 tumours. Trk-A and CD44 expression was detected by immunoperoxidase staining using murine monoclonal antibodies 5C3 and L178, respectively. Expression was scored as positive (homogeneous), mixed (heterogeneous) or non-reactive (negative). Trk-A expression was found in 95% of Group 1 tumours and 49% of Group 2 tumours. CD44 expression was found in 100% of Group 1 tumours, the majority of which had a strong homogeneous expression. Lack of CD44 expression occurred in 25% of tumours, all stage 4 neuroblastoma. Of the 28 *MYCN* amplified tumours, 27/28 (96%) were trk-A negative, and 13/28 (46%) CD44 negative. We conclude that (1) a significant percentage of stage 4 neuroblastoma express either or both trk-A and CD44, (2) the absence of CD44 expression is highly restricted to stage 4 neuroblastoma and is associated with the lack of trk-A expression, (3) trk-A negativity among CD44-positive tumours is associated with stage 4 neuroblastoma and (4) the absence of trk-A expression is highly correlated with *MYCN* amplification. © **1997 Elsevier Science Ltd.**

Key words: neuroblastoma, trk-A, neurotrophin receptor, CD44, lymphocyte homing receptor
Eur J Cancer, Vol. 33, No. 12, pp. 2098–2100, 1997

INTRODUCTION

SEVERAL PROGNOSTIC variables for childhood neuroblastoma have been described including age at diagnosis [1], *MYCN* copy number [2], deletion of the short arm of chromosome 1 [3], cellular DNA content [4], tumour histology [5], trk-A gene expression [6] and CD44 expression [7, 8]. Of these, the markers which consistently appear to have significant strong correlation with improved survival are lack of *MYCN* amplification and the expression of trk-A and CD44. We studied the prognostic impact of these three molecular variables in 250 randomly selected neuroblastoma samples in relation to stage and clinical status.

Correspondence to N.-K.V. Cheung.

MATERIALS AND METHODS

Two hundred and fifty neuroblastoma tumours were randomly selected from a tumour bank repository, based on the availability of tumour tissue and the known clinical stage and history of the tumour sample. All diagnoses of samples were confirmed by histological assessment of the tumour specimens. Patients were stratified according to stage based on the International Neuroblastoma Staging System. 111 patients had stage 1, 2, 3 or 4s disease (group 1) and 139 patients had stage 4 neuroblastoma (group 2). Overall, the mean age of

Table 1. *Comparative results of trk-A and CD44 expression*

	trk-A			CD44		
	Positive (%)	Mixed (%)	Negative (%)	Positive (%)	Mixed (%)	Negative (%)
Group 1 (Stage 1,2,3,4s)	145/250 (58)	93/250 (37)	12/250 (5)	210/250 (84)	40/250 (16)	0/250 (0)
Group 2 (Stage 4)	63/250 (25)	60/250 (24)	127/250 (51)	93/250 (37)	94/250 (38)	63/250 (25)

patients at the time of diagnosis was 35 months and 34% were less than 12 months of age.

Trk-A and CD44 protein expression were determined by immunohistochemical analysis on 8 μm frozen tumour sections using murine monoclonal antibody 5C3, previously shown to be specific for human p140 trk-A [9] and monoclonal antibody L178 (Becton-Dickenson, San Jose, California, U.S.A.), directed against an epitope common to all CD44 isoforms. Tumours were graded as positive (>90% of cells immunoreactive), mixed (10–90% of cells immunoreactive) or negative (<10% of cells immunoreactive). *MYCN* copy number was obtained by Southern blotting [10] or the polymerase chain reaction [11].

RESULTS

Of the tumours in group 1 patients, 95% demonstrated trk-A protein expression, and 100% demonstrated CD44 protein expression, the majority of which had strong, homogeneous reactivity. Among tumours in group 2 patients, trk-A expression was found in 49% and CD44 expression in 75%. Whereas a lack of trk-A expression was only found in 5% of favourable stage neuroblastoma, 51% of stage 4 patients were trk-A negative. Lack of CD44 expression was exclusively demonstrated in patients with stage 4 disease (Table 1). Ninety-five per cent of group 1 tumours were positive for both trk-A and CD44 versus 45% of group 2 tumours. Lack of both trk-A and CD44 expression was found in 21% of group 2 tumours. No patient with a favourable clinical stage was negative for both markers (Table 2).

In this series, 28 samples had an amplified *MYCN* copy number, all in patients with stage 4 disease. Trk-A protein detection was absent in 27 of 28 tumours (96%). CD44 expression was absent in 13 *MYCN* amplified tumours (46%). By univariate analysis, stage, age, single copy *MYCN* and positive trk-A and CD44 protein expression were all highly significant in predicting survival using Kaplan–Meier estimates. In multivariate analysis using the Cox proportional hazards regression model, however, stage and *MYCN* copy number were the most significant in predicting survival.

DISCUSSION

Numerous prognostic biological markers have been used to predict whether neuroblastoma behaves as a benign or locally invasive tumour or as its aggressive, metastatic, lethal counterpart. This challenge, in part, is owed to the extreme differences in treatment regimens imparted on the localised resected tumour (often no further therapy) versus the aggressive treatment approach for stage 4 disease. We assessed the correlation of trk-A and CD44 protein expression and *MYCN* copy number with clinical stage in a large random selection of neuroblastoma tumours from patients with known detailed clinical histories.

The expression of CD44 and trk-A strongly correlated with localised/stage 4s neuroblastoma, where 100% and 95% are immunoreactive with specific MAbs, respectively. Lack of CD44 expression was exclusively found in stage 4 tumours. Lack of trk-A expression similarly strongly correlated with stage 4 neuroblastoma and in addition was observed in 96% of *MYCN* amplified tumours. Lack of CD44 expression was strongly associated with the concurrent lack of trk-A expression. Given the close association of these markers with stage, the prognostic significance of each is expected. From our analysis, however, stage and the absence of *MYCN* amplification remain the most predictive determinant of prolonged survival. It is likely, therefore, that for the subset of patients with stage 4 disease who appear to have favourable biological markers (i.e single copy *MYCN*, positive trk-A and CD44 expression), other biological parameters dictate the aggresive clinical behaviour. For such patients, further evaluation of other prognostic variables such as 1p chromosomal deletion, multidrug resistant proteins, and the effects of treatment and treatment intensity should be considered.

Table 2. *Correlation of both trk-A and CD44 with clinical stage*

CD44 Trk-A	Positive Positive	Positive Negative	Negative Positive	Negative Negative
Group 1 (Stage 1,2,3,4s)	238/250 (95%)	12/250 (5%)	0/250 (0%)	0/250 (0%)
Group 2 (Stage 4)	112/250 (45%)	77/250 (31%)	8/250 (3%)	53/250 (21%)

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