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CD44 Expression and Modulation on Human Neuroblastoma Tumours and Cell Lines

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The human CD44 cell surface glycoprotein has been involved in a variety of functions including lymphocyte homing, extracellular cell matrix attachment and tumour metastasis. A large family of variants or isoforms, generated by alternative splicing of a single gene, has been reported to be involved in the malignant process, by conferring metastatic potential to non-metastatic cells. Neuroblastoma is a tumour characterised by an aggressive and metastatic behaviour in advanced stages, with amplification of the *MYCN* protooncogene. In this report, we show that the CD44 standard molecule was highly expressed in the majority of tumours of stages 1–3, in all stage 4s and ganglioneuromas, but only in a subset of stage 4 tumours. A lack of CD44 expression was observed in all *MYCN* amplified stage 4 tumours, thus demonstrating a highly significant inverse relationship between *MYCN* amplification and CD44 expression in neuroblastoma. In addition, the expression of 4 different CD44 isoforms was measured on all specimens and was always found to be negative. Using neuroblastoma cell lines and *MYCN* expressing transfectants, we show that CD44 expression by neuroblastoma cell lines is not directly related to *MYCN* amplification, but is associated to the stage of differentiation or lineage, and to the tumorigenic properties of the cells. In addition, CD44 expression can be upmodulated parallel to differentiation or maturation as induced by retinoic acid, bromodeoxyuridine or phorbol ester. In contrast, cytokines such as IFNγ, TNFα, or growth factors such as bFGF, SCF and TGFβ were ineffective in modulating CD44 expression.

Key words: neuroblastoma, CD44, *MYCN*, differentiation, prognostic marker
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INTRODUCTION

NEUROBLASTOMA (NB), a neural crest-derived tumour, is the second most frequent solid tumour of childhood, with still poor outcome in the majority of children over 1 year of age [1]. The tumour is extremely heterogeneous in biological and clinical behaviour, and has been assigned to different biological subtypes, on the basis of clinical parameters and the expression of several genetic and biological markers that display unique and strong prognostic value [2, 3]. They include 1p deletions [4], *MYCN* amplification [5, 6], *TRKA* expression [7], and modifications of the DNA content or ploidy [8, 9]. Very recently, the expression of the adhesion molecule CD44 by NB tumours has been likewise shown to display strong association with favourable outcome and prognostic value. Its absence significantly correlated with *MYCN* amplification [10, 11].

The cell surface glycoprotein CD44 is an integral membrane glycoprotein expressed by a large variety of haematopoietic and non-haematopoietic tissues [12]. Originally described as a homing receptor of lymphocytes, it is now also credited with functions involving cell–cell and cell–matrix adhesion [13]. The predominant form or standard form in the haematopoietic system has an approximate MW of 80–90 000, and has accordingly been denoted CD44 haematopoietic or standard form [14]. A large variety of isoforms or splice variants, generated by alternative mRNA splicing, have been described to which altered functions, namely highly metastatic properties, have been specifically attributed in a variety of tumours [15–18].

Our recent results have indicated that CD44 is expressed by all tumours of stages 1–3 and 4s, and only by approximately 50% of stages 4, corresponding to those tumours with *MYCN* amplification. In addition, two CD44 isoforms CD44v3 and v6 could not be detected on any of the tumour samples [11].

In the present study, we have extended our analysis of expression of CD44 standard and all known CD44 isoforms on a large panel of NB tumours. In addition, using *MYCN* transfectants, we have further explored the relationship between CD44 expression and *MYCN* amplification or overexpression as well as the possible role of cytokines, growth factors or differentiation agents in the modulation of CD44 expression by NB cell lines.

MATERIALS AND METHODS

Monoclonal antibodies (MAbs) and probes

MAbs recognising the standard CD44 core molecule included MAb F.10.44.2 [19], an antiCD44, kindly provided by Dr Carrel (Ludwig Institute for Cancer Research, Lausanne Branch, Switzerland), and an antiCD44 MAb purchased from Serotec. Variant-specific MAbs include 3G5 (anti-v3), 3D2 (anti-v4,5) 8G5 (anti-v6), 1E8 (anti-v8,9). We also used MAb VFF7 anti-v6, kindly provided by Dr Herrlich (Karlsruhe, Germany). Other MAbs used in this study have been described elsewhere [20].

The cDNA probe for standard CD44 was kindly provided by Dr Hong Li (Department of Neurosurgery, Lausanne, Switzerland), PNb-1 plasmid was used as *MYCN* probe [6].

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Tumour samples and cell lines

Biopsies from primary tumours were collected at diagnosis directly from the operating room and immediately frozen in liquid nitrogen. Immunochemical analysis for the detection of CD44 standard molecule and isoforms was performed by a three-step immunophosphatase technique as previously described [21]. SK-N-SH, SK-N-BE(2) cell lines and their phenotypic variants [22] were kindly provided by Dr J. Biedler (Sloan Kettering, New York, U.S.A.). IMR-32 cell line was purchased from ATCC.

Cell lines' treatment

Cells were allowed to adhere to the bottom of the culture flask, and further treated with growth factors, bFGF (basic fibroblast growth factor) (20 ng/ml), SCF (stem cell factor) (100 ng/ml), TGFβ (transforming growth factor beta) (5 ng/ml), and cytokines IFNγ (interferon) (500 U/ml), TNFα (tumour necrosis factor) (50 U/ml) for 2–4 days in the presence of 10% fetal calf serum, before collection for the FACS analysis. Differentiation inducers, retinoic acid (RA) (5×10^{-6} M), bromodeoxyuridine (BrdU) (10 μg/ml), and phorbol ester (PMA) (10 μg/ml) were added for 8–10 days in the same conditions.

Measure of surface expression by immunofluorescence and flow cytometry

Surface expression of CD44, HLA Class 1 and CD 56 on cell lines was assessed by indirect immunofluorescence and analysed by flow cytometry using a Coulter Profile as previously described [20].

Northern blot analysis

Messenger RNA was extracted, electrophoresed and blotted as previously described [23]. The filters were successively hybridised to *MYCN* and standard CD44 specific probes. After each hybridisation, filters were washed under stringent conditions and exposed to X-rays sensitive Kodak film at −70°C for 2–48 h. The probes were stripped at 100°C for 30 min before rehybridisation.

Statistics

Statistical analysis of the immunohistochemical determinations and measurement of *MYCN* amplification data were performed using the χ^2 test of independence.

RESULTS

CD44 expression by tumour samples

Expression of CD44 standard and 4 isoforms was measured by immunohistochemical staining on a panel of 57 NB tumours, including 16 stage 1–2, 9 stage 3, 23 stage 4 and 9 stage 4s, as well as 10 cases of ganglioneuroma. *MYCN* amplification was evaluated for each case in parallel.

Results presented in Table 1 show that the majority of NB tumours, 48/57, strongly expressed the CD44 standard molecule. For stage 1–2 tumours, only one stage 2 sample did not express CD44 and was also the only one to have amplified *MYCN*. All stage 3 tumours expressed CD44, and only one was amplified. In contrast, only a subset of stage 4 tumours (15/23) expressed CD44 and *MYCN* amplification was observed only in the 8 CD44-negative cases. Strong CD44 expression was observed in all stage 4s cases, although low *MYCN* amplification (5 copies) was detected in two of them. Strong expression of CD44 was similarly measured in all the ganglioneuromas, and none of these displayed *MYCN* amplification. Figure 1 illustrates immunohistochemical staining with antiCD44 antibodies on two different stage 4 neuroblastomas, one with one *MYCN* copy (Figure 1A), another with a marked amplification of the gene (Figure 1B), and a non-amplified stage 4s tumour (Figure 1C). The tumour with *MYCN* amplification (Figure 1B) demonstrated a complete lack of staining, except for a few non-tumoral lymphoid or endothelial cells, while the two other tumours were strongly positive. Statistical analysis of the results demonstrated that association between lack of CD44 expression and *MYCN* amplification was highly significant ($P < 10^{-6}$). The expression of CD44 by primitive neuroectodermal tumours was detected only in 1/4 tumours, and no amplification of *MYCN* was observed in these samples (Table 1). Expression of four CD44 isoforms, (v3, v4,5, v6, v8,9), as measured with variant specific antibodies, could not be detected in any of the NB tumours, nor in the 10 ganglioneuroma samples.

Table 1. Immunohistochemical analysis of CD44H and isoforms expression by neuroblastoma tumours

Tumours	CD44H	CD44v	*MYCN* amplified
Neuroblastomas	48/57	0/55	12/57
Stages			
1, 2	15/16	0/15	1/16
3	9/9	0/9	1/9
4	15/23	0/23	8/23
4s	9/9	0/8	2*/9
Ganglioneuromas	10/10	0/10	0/10
PNET	1/4	nd	0/4

Results are given as number of positive samples/number samples analysed. CD44v refers to all variants tested, namely CD44v3, -v4,5, -v6 and -v8,9. * These two samples displayed a low amplification of 5 copies. PNET, primitive neuroectodermal tumour; nd, not determined.

Modulation of CD44 expression by NB cell lines

We have previously shown that CD44 expression by NB cell lines is variable, apparently independent of *MYCN* amplification, and that a particularly high expression is observed on the adherent, epithelial-like or S-type cell lines [11]. As shown in Table 2, some cell lines do not express detectable surface CD44 molecules (IMR-32), whereas other cell lines that have been cloned into morphologically distinct variants [21, 24], are very heterogeneous in terms of CD44 expression. As measured by surface immunofluorescence and flow cytometry, the *MYCN* non-amplified SK-N-SH and *MYCN* amplified SK-N-BE parental and variant cell lines were shown to display different patterns of CD44H expression (Table 2). A low to undetectable CD44 expression was observed on the SK-N-SH and SK-N-BE neuronal variants SH-SY5Y and BE(2)M17 respectively, which are characterised by low or absent HLA Class I expression [10], while a particularly high expression, over 90% positive cells with high MFI, was observed on the epithelial-like variant SH-EP, known to express high levels of HLA Class I. The BE(2)C intermediate variant expressed CD44 levels comparable with the parental cell line. CD44 expression on variant cell lines therefore appears to parallel that of HLA Class I antigen.

In order to further investigate the relationship between the expression of CD44 and the differentiation lineage in NB, we treated the SK-N-SH and SK-N-BE cell lines with substances

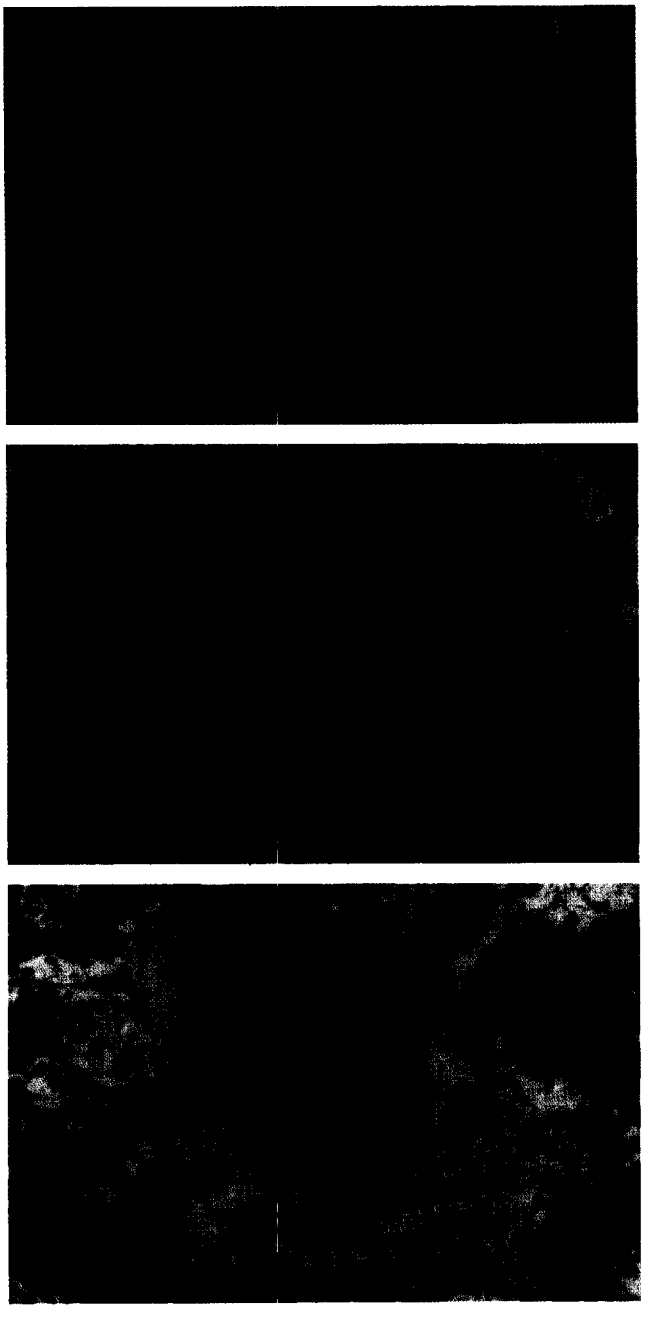

Figure 1. Immunohistochemical detection of CD44 standard molecules expression on neuroblastomas: A: stage 4, *MYCN* non-amplified tumour, B: stage 4 *MYCN* amplified (>100 copies) tumour, C: stage 4s tumour, no amplification. Magnification × 400. Pink staining, CD44 positive.

Table 2. CD44 expression by NB cell lines

Cell lines (phenotype)		CD44H % pos (MFI)	HLA Class I % pos (MFI)	*MYCN* amplification
IMR-32	(N)	4 (2.1)	1.8 (2.9)	+
SK-N-SH	(N/S)	37 (6.3)	28 (3.6)	−
SH-SY5Y	(N)	11 (4)	8.8 (4)	−
SH-EP	(S)	96 (50.2)	88 (4.7)	−
SH-IN	(I)	31.9 (6.1)	65 (4.7)	−
SK-N-BE(2)	(N/I)	22 (10)	46 (8.7)	+
BE(2)C	(I)	25.9 (4)	15.7 (4.7)	+
BE(2)M17	(N)	3.2 (6.7)	2 (6.8)	+

Results of flow cytometry are given as % positive cells (MFI, mean positive fluorescence intensity). Non-specific staining by the fluorescent second antibody was always less than 8% of cells with a log mean fluorescence intensity of 3–4, not exceeding 7. *MYCN* amplification was scored + in the presence of more than 3 copies of the *MYCN* gene. Cell phenotype is indicated for each cell line. N, neuronal; S, epithelial-like; I, intermediate.

Table 3. Modulation of CD44 expression by growth factors, cytokines and differentiation factors

	CD44 % pos (MFI)	HLA Class I % pos (MFI)	CD56 % pos (MFI)
SK-N-BE			
Untreated	23 (10)	11 (5.4)	80 (8)
IFNγ	21 (10.4)	19 (5.9)	73 (6)
TNFα	21 (8)	45 (6.4)	74 (6.3)
SCF	20 (9.8)	11 (5.5)	66 (7)
TGFβ	21.5 (7.3)	13.8 (3.7)	80.7 (5.7)
bFGF	30 (10.6)	10.8 (4)	74 (7.5)
RA	33.9 (18)	11 (6)	93 (9)
BrdU	33 (17)	44 (9)	54 (9)
PMA	28 (18)	28 (8)	84 (6)
SK-N-SH			
Untreated	25 (5.7)	24 (5)	83 (9)
IFN	22 (8)	96.7 (75.9)	93 (11)
TNF	17.2 (9.1)	41 (8)	90 (11.5)
SCF	13.6 (8.8)	13.8 (9.1)	94.3 (10.6)
bFGF	15.9 (9.3)	14.2 (10.2)	93 (11.7)
RA	85 (30)	70 (5)	76 (8.3)
BrdU	68 (14)	64 (14)	64 (8.8)
PMA	73 (17)	50 (5)	84 (7.8)

Results of flow cytometry are expressed as % positive cells (MFI). Non-specific staining (obtained with cells stained with second fluorescent antibody only) was lower than 5% [5].

known to induce NB cells to differentiate, such as RA, BrdU and PMA, and analysed a possible modulation of CD44 pattern of expression. In addition, we tested a hypothetical role of growth factors or cytokines such as IFNγ, TNFα, bFGF, SCF and TGFβ, likely to be present in the bone marrow microenvironment, in the induction or modulation of CD44 standard expression.

The surface expression of CD44, HLA Class I and CD56 have been similarly measured on untreated and treated cells by surface immunofluorescence and flow cytometry. Results presented in Table 3 show that 20–25% of untreated SK-N-BE or SK-N-SH cells constitutively express the standard CD44 molecule. No expression of any of the 4 CD44 isoforms tested was detected (data not shown). Treatment with growth factors or cytokines did not greatly modulate the expression of CD44, both at the level of % positive cells or mean fluorescence intensity (MFI). As a control, upmodulation of HLA Class I was observed in both cell lines as a result of exposure to IFNγ and TNFα.

The same cell lines were treated for 8–10 days with RA, BrdU and PMA. Such treatments induced a neuronal (RA and PMA) or Schwann/glial (BrdU) morphological differentiation (not shown) in both cell lines. As represented in Table 3, treatment with all three substances resulted in an important increase of % positive cells and MFI for CD44 expression. The highest increase was

observed with SK-N-SH cells exposed to RA with an increase from 25 to 85% positive cells and a MFI increase from 5.7 to 30. PMA and BrdU similarly increased the CD44 expression on SK-N-SH cells up to 68% and 73% positive cells, respectively, with MFI of 14–17. An increase of HLA Class I expression as result of BrdU, RA and PMA treatment was also observed. In contrast, CD56 expression remained unchanged whatever treatment.

CD44 expression on MYCN transfected SK-N-SH clones

In a previous study, we transfected the SK-N-SH cell line with an *MYCN* expression vector, and selected stable and clonal *MYCN* expressing clones [25]. Most of these clones were shown to display an adherent, epithelial-like S-phenotype despite high *MYCN* expression, both at the level of mRNA and protein. Some of these clones (SH-400, SH-907) additionally acquired tumorigenic properties, with the ability to induce tumours in nude mice.

In order to further investigate the relation between *MYCN* and CD44 expression on NB cells, CD44 and *MYCN* mRNA levels were measured in parallel in the parental SK-N-SH cells, in the *MYCN*-expressing transfectants SH-400, SH-907 and SH-310, and in the SH-400 induced mouse tumour SH-400N. Results of Northern blot analysis are illustrated in Figure 2. The parental cell line SK-N-SH expressed low levels of CD44 and no detectable *MYCN* (lane 5). The transfectants SH-400 (lane 2), SH-310 (lane 3) and SH-907 (lane 4) expressed moderate to high levels of CD44 and high levels of exogenous *MYCN*. The nude mouse tumour, SH-400N (lane 1), still expressed high levels of exogenous *MYCN* but no detectable CD44 mRNA.

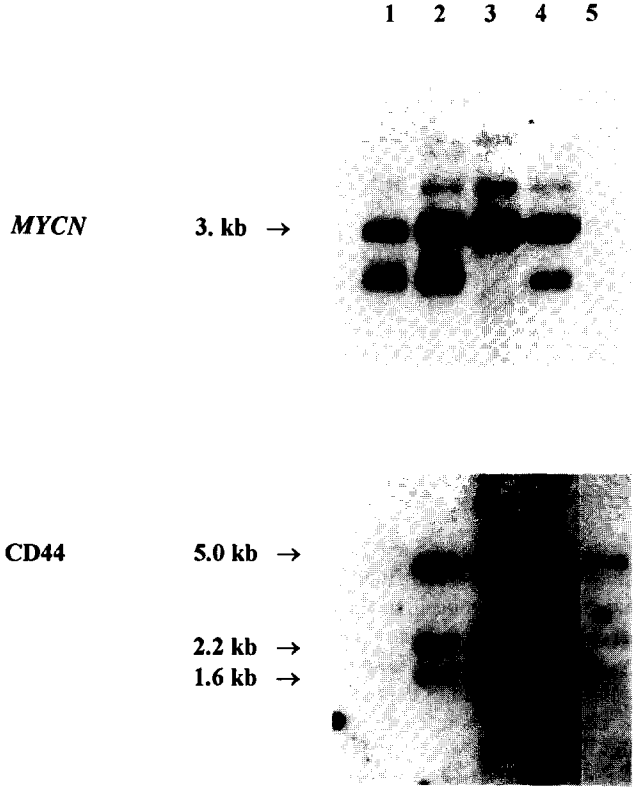

Figure 2. Northern blot analysis of *MYCN* and CD44 mRNA levels on SK-N-SH parental cells and transfectants: Lanes 1: SH-400N, 2: SH-400, 3: SH-310, 4: SH-907, 5: SK-N-SH.

DISCUSSION

In the present study, we analysed the expression of CD44 standard and isoforms on an extended panel of NB tumours, and investigated possible modulations of CD44 expression as induced by exposure of NB cells to cytokines, growth factors or differentiation inducers, or by insertion of an *MYCN* expression vector.

Our results demonstrate that high expression of the CD44 standard molecule can be detected on a majority of NB tumours. However, a subset of tumours displays no CD44 expression. All CD44-negative samples, one stage 2 and 8 stage 4, were *MYCN* amplified and only one *MYCN*-amplified sample, a stage 3, expressed CD44. The highly significant relationship between lack of CD44 expression and *MYCN* amplification is thus confirmed and strengthened in this cohort of patients. The positive CD44 expression by two stage 4s with low *MYCN* amplification is interesting, and might reveal that the level of *MYCN* amplification is critical in the relationship with CD44 expression. An analysis of the clinical relevance of CD44 expression and *MYCN* amplification in a multicentric study is currently being performed but lack of CD44 expression has already been shown to be one of the most valuable prognosis factors for poor outcome [10, 11]. Whereas in other tumour models, overexpression of CD44 standard molecule and particular isoforms has been suspected to play a role in tumour progression and dissemination [15–18, 26, 27], the presence of CD44 on NB cells, in contrast, might confer them decreased tumorigenic and metastatic properties. In addition, the lack of detectable expression of CD44 isoforms (v3, v4,5, v6 and v8,9) on CD44 standard-positive NB of all stages and ganglioneuromas is consistent with the absence of a major role for these molecules in the tumour biology of neuroblastoma [11].

Since highly malignant NB cells preferentially disseminate to the bone marrow, we investigated possible modulations of CD44 expression by different growth factors or cytokines, some of which are associated with the bone marrow microenvironment and are mitogenic for NB cells [28, 29]. Our results showed that IFNγ and TNFα were unable to change expression of CD44 on two different NB cell lines, but did enhance HLA Class I expression. Growth factors SCF, bFGF, TGFβ similarly did not modify the pattern of CD44 expression. Thus, the presence of these growth factors and cytokines does not appear to influence the expression of CD44 by NB cells, and consequently are unlikely to be related to the site-specific metastasic properties of NB cells.

In contrast, differentiation factors, such as RA, PMA or BrdU, which are known to induce a neuronal or Schwannian-type differentiation in SK-N-BE and SK-N-SH cell lines, respectively [22, 24], were able to markedly increase CD44 expression. Both the percentage of positive cells and the density of cell surface molecules were increased. These results further demonstrate that expression of CD44 by NB cells is linked to differentiation as already reported [11, 30]. It might also reflect their developmental origin, as suggested by the pattern of CD44 expression observed on other tumours of similar origin, such as PNET tumours, and cell lines, as well as the pattern of CD44 expression in developing neural crest cells [11, 31]. Moreover, such results have important therapeutic consequences, since induction of CD44 expression, parallel to treatment-associated tumour maturation and differentiation, might contribute to tumour regression.

The relationship between CD44 expression and *MYCN* amplification was further studied using NB cell lines and recently

described stable transfectants, obtained after insertion of an *MYCN* expression vector into the SK-N-SH cells [25]. Absence of CD44 expression secondary to decreased activity of the CD44 promoter on some NB cell lines has been described [32]. Our previous results suggested that overexpression of a *MYCN* transcription factor could be involved in the regulation of CD44 expression [25].

The *MYCN* expressing transfectants (SH-400, SH-907, SH-310) had been shown to display a Schwann/glial morphological phenotype with high HLA class I expression, similar to the S-type variant SH-EP. Moderate to high CD44 expression was detected on these cells, showing that, as for other amplified cell lines with a mixed or S-type morphology (SK-N-BE, CA-2-E), CD44 expression did not appear to be directly related to amplification and overexpression of *MYCN*, but rather to the morphological cell type and lineage. These transfectants display tumorigenic properties and, unlike the parental cell line, induce tumours in nude mice, giving rise to SH-400N and SH-907N cell lines. Our results show that the transfectant SH-400 expressed both CD44 and *MYCN*, while the tumorigenic clone SH-400N expressed *MYCN* but not CD44 mRNA transcripts.

Our interpretation of these results is that a highly tumorigenic, *MYCN* expressing and CD44 negative clone, SH-400N, has been selected by growth in the nude mice. This result confirms that low or absence of CD44 expression in NB cell lines is not necessarily directly related to *MYCN* amplification and expression, but suggests that lack of CD44 expression might be related to the tumorigenic property, which was induced by insertion of *MYCN* expression in the SK-N-SH cell line. Whether the absence of CD44 with or without *MYCN* amplification is a requirement for growth in nude mice or for dissemination is now being investigated.

In conclusion, lack of CD44 expression in NB tumours is observed mostly in advanced stages, is significantly associated with *MYCN* amplification, and represents a highly valuable poor prognosis marker. Experiments performed with cell lines and transfectants have shown that CD44 expression is not directly associated with *MYCN* overexpression in these cells, but could be related to their stage and type of differentiation and tumorigenic properties.

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