ORIGINAL ARTICLE

Limitations in the ability of NB84 to detect metastatic neuroblastoma cells in bone marrow

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Background: The accurate assessment of metastases is an essential component of the staging process for children with neuroblastoma.

Aims: To study the sensitivity of the immunohistochemical marker neuroblastoma 84 (NB84) for the detection of bone marrow infiltrates in children with stage 4 neuroblastoma.

Methods: Primary tumour specimens, bone marrow trephine biopsy specimens and lymph node metastases, taken from children with neuroblastoma that had metastasised to bone marrow, were assessed with a panel of commonly used immunohistochemical markers for neuroblastoma. A comparison was drawn between the sensitivity of the marker NB84 for primary tumours and for bone marrow metastases.

Results: NB84 immunolabelled all pre-chemotherapy and post-chemotherapy (n = 24) paired primary tumour specimens, as well as each of a further 20, unpaired, pre-chemotherapy primary tumour specimens. It also labelled all (n = 4) lymph node metastases. Immunolabelling of bone marrow trephine biopsy specimens (21/33) was less sensitive. Of 16 primary tumour specimens with a paired bone marrow trephine biopsy specimen, all immunostained positive, whereas only 62.5% of bone marrow biopsy specimens immunostained positive for NB84. The number of bone marrow biopsy specimens immunostaining for NB84 was significantly lower than the number of paired primary tumour specimens (p = 0.041).

Conclusions: NB84 remains a useful marker for the diagnosis of neuroblastoma in primary tumour specimens, but not for neuroblastoma that has metastasised to bone marrow.

The detection of cells that have metastasised to bone marrow is a key step in the assessment of a child with neuroblastoma, both at presentation and during treatment. The presence of bone marrow metastases results in a child's disease being staged as 4 or 4s, according to the International Neuroblastoma Staging System.¹ Children >1 year presenting with stage 4 disease have a poor prognosis and consequently receive intensive, multimodality treatment with associated morbidity and sometimes mortality. Accuracy of initial staging is therefore important in the diagnosis and management of neuroblastoma.

Undifferentiated neuroblastoma can be difficult to diagnose, given its similarity to other small round blue cell tumours of childhood such as Ewing's sarcoma, rhabdomyosarcoma, Burkitt's lymphoma and lymphoblastic lymphoma. Histological and cytological assessment using haematoxylin and eosin (H&E)-stained slides is complemented by immunohistochemical techniques to improve sensitivity and specificity. A range of immunohistochemical markers is routinely used to identify neuroblastoma, the most common being neuroblastoma 84 (NB84), synaptophysin, protein gene product 9.5 (PGP9.5), neurofilament and neurone-specific enolase.

NB84, a commercially available monoclonal antibody, recognises an uncharacterised, 57-kDa molecule expressed by many normal human cell types.² It is very sensitive for identifying neuroblastoma cells in primary tumours.³ NB84 is not, however, highly specific, staining a proportion of Ewing's sarcomas, medulloblastomas and desmoplastic small round cell tumours. Other small round cell malignancies, such as rhabdomyosarcomas and lymphomas, do not seem to show reactivity.³

Our study aimed to determine the sensitivity of NB84 as an immunohistochemical marker for the detection of metastatic neuroblastoma cells in bone marrow biopsy specimens.

METHODS

Ethical approval was received from the local research ethics committee. Specimens were retrospectively identified using

the North of England Children and Young Persons Malignant Disease Registry from cases registered between 1968 and 1999.^{4,5} Bone marrow biopsy specimens were included if they contained sufficient neuroblastoma infiltrate for detection by a consultant haematologist (MMR), whether or not there was a paired primary tumour specimen. Four primary tumours had paired lymph node metastases. Table 1 describes the specimens identified.

Tumour specimens were routinely fixed in formaldehyde and embedded in paraffin wax. Bone marrow biopsy specimens were decalcified before paraffin wax embedding. Specimens received before June 2004 were immersed in 25% Gooding and Stewarts solution (33% formic acid and <10% formaldehyde) for 8 h, with continuous agitation. Specimens received after June 2004 were decalcified in a 10% formal solution (10% formic acid and 10% formaldehyde).

Specimens were stained with H&E and immunolabelled as detailed later.

Table 1 Combinations of specimens studied for each of 61 cases

No of cases (n)	Primary NB pre-therapy	Primary NB post-therapy	Bone marrow biopsy	Lymph node metastases
24	✓	✓		
16	✓		✓	
17			✓	
4	✓			✓

NB, neuroblastoma.

Abbreviations: H&E, haematoxylin and eosin; NB84, neuroblastoma 84; PGP9.5, protein gene product 9.5

Figure 1 Neuroblastoma (NB84) immunostaining of a paired neuroblastoma primary tumour and bone marrow metastasis. (A) Haematoxylin and eosin (H&E) staining of neuroblastoma infiltrating the bone marrow. (B) NB84 immunostaining of bone marrow showing absence of immunostaining. (C) H&E staining of paired primary tumour. (D) NB84 immunostaining of paired primary tumour.

Immunohistochemical study

Immunohistochemical analysis was carried out on the tumour and bone marrow specimens. Table 2 lists the pretreatments and antibodies used for the analysis. The process was carried out by BioGenex i6000 (BioGenex Laboratories, San Ramon, California, USA) for synaptophysin and PGP9.5. Other antibodies were applied manually. Dilutions for each of the antibodies were determined according to the manufacturer's recommendations. In brief, after pretreatment with 0.1% trypsin for 10 min or microwave pressure cooking in a standard citrate buffer for 4 min, slides were incubated with primary antibodies for 30 min. A biotinylated goat anti-mouse secondary antibody was used. Streptavidin–biotin peroxidase was used as the developing system and diaminobenzidine as the chromogen.

Statistical analysis

Statistical analysis was carried out using SPSS V.11 software. A McNemar's test was carried out to identify a difference in NB84 sensitivity between primary tumours and matched bone marrow metastases. A probability of $p < 0.05$ was considered to be significant.

RESULTS

Each of the 44 primary, pre-chemotherapy neuroblastoma specimens immunostained positive for NB84. Of the 24 pre-chemotherapy specimens that had paired pre- and

post-chemotherapy primary neuroblastoma available, all 48 showed NB84 immunostaining.

Of the 33 bone marrow trephine biopsy specimens, 21 (63.6%) were positive for NB84. All biopsy specimens showed neuroblastoma infiltrates when reviewed by MMR. Of the 12 cases that did not show bone marrow NB84 immunostaining, six primary tumours were undifferentiated neuroblastoma, five were differentiating neuroblastoma and one was a ganglioneuroblastoma.

Of the 33 bone marrow trephine biopsy specimens, 16 had a paired primary tumour specimen (table 3). Although all the primary tumour specimens showed NB84, only 10 (62.5%) of the paired bone marrow trephine biopsy specimens also stained positive (fig 1). A significant difference was observed between the number of primary tumour specimens and the number of paired bone marrow biopsy specimens immunostaining positive for NB84 ($p = 0.041$). For comparison, Bcl-2 and Ki-67 immunostaining in primary tumour and bone marrow specimens was also examined. No significant difference was seen in immunostaining between primary tumour and bone marrow trephine biopsy specimens using either Bcl-2 or Ki-67, $p = 0.480$ and $p = 0.683$, respectively (table 3). This confirms that immunostaining of bone marrow does occur with other antibodies.

Two bone marrow biopsy specimens heavily infiltrated with neuroblastoma, which had not shown NB84 immunostaining, but whose paired primary tumour had, were stained further with PGP9.5 and synaptophysin. Both showed positive immunostaining for these markers. Four primary

Table 2 Summary of antibodies

Antibody	Pretreatment	Type	Dilution	Manufacturer
NB84	Trypsin*	Monoclonal	1:100	NovoCastra (Newcastle, UK)
Ki-67	Pressure cooking/citrate†	Monoclonal	1:100	NovoCastra
Bcl-2	Pressure cooking/citrate†	Monoclonal	1:100	NovoCastra
PGP9.5	Pressure cooking/citrate†	Polyclonal	1:150	Dako (Glostrup, Denmark)
Synaptophysin	NP	Polyclonal	1:50	Dako

NB, neuroblastoma; NP, no pretreatment; PGP, protein gene product.

*Trypsin: 0.1% trypsin for 10 min.

†Pressure cooking in a standard citrate buffer for 4 min.

Table 3 Staining pattern of primary tumour and bone marrow pairs

Case	Primary tumour			Bone marrow biopsy specimens		
	NB84	Bcl-2	Ki-67	NB84	Bcl-2	Ki-67
1	+	+	—	+	+	+
2	+	+	—	—	+	—
3	+	+	+	+	+	+
4	+	+	+	—	+	+
5	+	—	+	—	—	+
6	+	+	+	+	+	+
7	+	+	—	+	+	+
8	+	—	+	+	+	—
9	+	+	+	+	+	+
10	+	—	+	+	+	—
11	+	+	+	+	+	+
12	+	+	+	—	+	+
13	+	+	+	—	+	+
14	+	+	—	+	+	+
15	+	+	+	—	+	+
16	+	+	—	+	+	+
Total positive	100%	81.3%	68.8%	62.5%	93.8%	81.3%

NB, neuroblastoma.

tumours with matched lymph node metastases were immunostained for NB84. All eight specimens showed NB84 staining, indicating that NB84 was positive in other, non-bone marrow, metastatic sites.

DISCUSSION

The detection of metastases to bone marrow is an important step in the accurate staging of neuroblastoma. Our study aimed to assess the sensitivity of the monoclonal antibody NB84 in identifying bone marrow metastases in children with neuroblastoma.

Previous reports have shown that NB84 is a highly sensitive marker for primary neuroblastoma tumours.^{2,3} Our study again showed this high sensitivity in primary neuroblastoma biopsy specimens, a finding that persisted after chemotherapy.

NB84 immunostaining in bone marrow trephine biopsy specimens was significantly less sensitive, identifying only 63.6% of tumours that had metastasised to bone marrow, by H&E analysis. This makes NB84 a less attractive immunohistochemical marker for identifying neuroblastoma in bone marrow than it has proved to be in primary tumour specimens. The reason for this difference is unclear. It is possible that bone marrow metastases do not immunostain with NB84 because they do not express the epitope against which it is reactive. This may be because it is the less well-differentiated tumour cells that tend to metastasise. However, a spectrum of histology from undifferentiated neuroblastoma to ganglioneuroblastoma was seen in the group of NB84-negative bone marrow trephine specimens. Our study also showed NB84 positivity in a small number of lymph node metastases. In addition, markers seen on differentiating and poorly differentiated neuroblastoma—namely, PGP9.5, synaptophysin, Ki-67 and Bcl-2—stained strongly on a small number of bone marrow biopsy specimens known to contain neuroblastoma cells. PGP9.5 has previously been seen on peripheral blood and bone marrow metastases, identifying minimal metastatic disease in patients with localised disease.⁶

If de-differentiation is not responsible for the lack of NB84 staining, then perhaps there is a factor specific to bone marrow metastases, which down regulates the production of the protein against which NB84 is directed. Given that the identity of the target protein is unknown, it is difficult to discount this possibility.

Finally, it is possible that the processes associated with preparing bone marrow biopsy specimens for immunostaining are responsible for the lack of immunostaining. Perhaps

the most likely candidate for this is decalcification, as this is not required for preparation of any other tissue specimens. Further work is required to determine whether decalcification or another step during immunostaining affects NB84 immunolabelling.

Our study has shown the significantly lower sensitivity of NB84 to neuroblastoma cells in bone marrow biopsy specimens compared with primary tumour. Whatever the cause for this finding, it suggests that NB84 has a limited role in the detection of neuroblastoma metastases to bone marrow. Although NB84 may increase the overall sensitivity of a panel of immunohistochemical stains for detection of primary neuroblastoma, it does not seem to offer such a benefit in the detection of bone marrow metastases.

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