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N-myc gene product expression in neuroblastoma

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SUMMARY The presence and distribution of *N-myc* gene product were studied in 13 neuroblastomas and five ganglioneuroblastomas, using immunohistochemical techniques. Nine tumours (eight neuroblastomas and one ganglioneuroblastoma of composite type) contained neuroblastoma cells with positive nuclei for *N-myc* protein. Microscopic examination showed that most of the positive neuroblastoma cells seemed to be immature, with no apparent neuronal differentiation. Nine of 11 tumours with amplified *N-myc* gene copies exhibited tumour cells with positive immunostaining for the *N-myc* gene product, while none of the seven non-*N-myc* amplified cases contained immunoreactive tumour cells. The survival of the patients positive for *N-myc* protein was significantly low compared with that of the negative ones. It is concluded that immunohistochemical staining for the *N-myc* gene product will facilitate prediction of the prognosis of patients with neuroblastoma.

The *N-myc* gene was originally identified in human neuroblastoma cell lines and was grouped as a proto-oncogene because of its partial homology to the proto-oncogene *c-myc*.[1 2] Close correlation between genomic amplification of the *N-myc* gene in neuroblastomas and the stage and aggressiveness of the tumours was first reported by Brodeur *et al*[3] and Seeger *et al*.[4] The key role of *N-myc* gene amplification in determining the biological behaviour of neuroblastomas has also been given further support.[5 6] The enhanced expression of *N-myc* has also been detected in relation to malignant progression, using Northern blot analysis or in situ hybridisation techniques.[7 8] Recently, antibodies against genetically engineered *N-myc* gene products were prepared[9–11] and these tools paved the way for retrospective immunohistochemical studies on the *N-myc* gene product.

We used immunohistochemical techniques to evaluate distribution of the *N-myc* gene product on paraffin wax sections of neuroblastomas and compared our findings with the number of *N-myc* gene copies and how this affected prediction of the prognosis of the patients.

Material and methods

Between 1979 and 1986 13 cases of neuroblastoma and five of ganglioneuroblastoma (two composite, two well differentiated, one poorly differentiated) were selected for study. In all tumours the number of copies of *N-myc* gene had been determined by Southern blot analysis, using radiolabelled *N-myc* (NB-19-21), the details of which have been reported elsewhere.[5 6]

Five micron sections of 10% formalin fixed, paraffin wax embedded material were mounted on slides coated with poly(L-lysine) for immunohistochemical study. After the tissue sections had been deparaffinised and the endogenous peroxidase activity blocked by using a short 10 minute oxidation procedure in 0·5% periodic acid, followed by treatment with a freshly prepared 0·02% sodium borohydrate for 30 minutes, the avidin-biotin peroxidase complex (ABC) method of Hsu *et al* [12] was used to determine localisation of the *N-myc* gene product. The slides were immersed in 10% normal goat serum for 30 minutes to eliminate the non-specific binding of immunoglobulins and then incubated overnight at 4°C with the affinity purified anti-human *N-myc* gene product rabbit IgG (MBL, Nagoya, Japan) diluted 1/10. The antibody was produced against the genetically engineered human *N-myc* gene product.[11] After a buffer wash 50 μg/ml of the biotinylated anti-rabbit goat immunoglobulin was applied for 30 minutes. The avidin-biotin peroxidase complex (Vector, Burlingame, California, USA) was applied for an additional 60 minutes at room temperature. The site of localisation of the peroxidase label was then identified by formation of a brown reaction product, using hydrogen peroxidase and diaminobenzidine. These sections were lightly counterstained with methyl green.

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Table *Clinical profiles and immunohistochemistry for N-myc gene product*

Case No	*Age*	*Sex*	*Primary site*	*Stage*	*Histology*	*No of N-myc copies*	*N-myc gene product*	*Follow up*
1	11y	M	Adrenal gland	III	Neuroblastoma	130	+ + +	Died 7 m
2	2y 9m	F	Adrenal gland	III	Neuroblastoma	113	+ +	Died 6 m
3	4y	M	Adrenal gland	IVA	Neuroblastoma	112	+ + +	Died 4 m
4	1y 7m	F	Adrenal gland	IVA	Neuroblastoma	92	+ +	Died 9 m
5	1y 9m	M	Adrenal gland	II	Neuroblastoma	71	+ +	Died 2·5 y
6	2y 6m	F	Adrenal gland	IVA	Neuroblastoma	37	−	Died 5 m
7	1y 1m	M	Adrenal gland	III	Neuroblastoma	37	+ +	Died 4 m
8	1y 3m	F	Adrenal gland	IVA	Neuroblastoma	36	+	Died 8 m
9	1y 9m	F	Adrenal gland	III	Ganglioneuroblastoma, composite	32	+	Died 1·7 y
10	1y	F	Adrenal gland	IVS	Neuroblastoma	8	+	Alive 3·7 y
11	5y 8m	F	Adrenal gland	IVA	Ganglioneuroblastoma, composite	6	−	Alive 4·5 y
12	7m	F	Retroperitoneum	IVS	Ganglioneuroblastoma, poorly differentiated	1	−	Alive 3·6 y
13	3y	F	Mediastinum	II	Ganglioneuroblastoma, well differentiated	1	−	Alive 3·5 y
14	9y	F	Pelvic cavity	III	Ganglioneuroblastoma, well differentiated	1	−	Alive 3·1 y
15	7m	F	Mediastinum	II	Neuroblastoma	1	−	Alive 2·2 y
16	4m	M	Retroperitoneum	III	Neuroblastoma	1	−	Alive 2·2 y
17	6y	M	Adrenal gland	IVA	Neuroblastoma	1	−	Alive 1·8 y
18	6m	M	Adrenal gland	I	Neuroblastoma	1	−	Alive 1·6 y

Abbreviations: Prevalence of positive tumour cells: + + +, almost all; + +, a moderate number; +, a few; −, none.

We examined the kidneys, adrenal glands, and sympathetic ganglia from three patients with neuroblastoma as controls. The *N-myc* gene product did not stain any of the following: nephrons; parenchymal cells of the adrenal cortex; ganglion cells and stromal elements such as vascular endothelial cells; smooth muscle cells; fibroblasts; and Schwann cells.

Results

The staining results for *N-myc* gene product, together with the clinical data and the number of *N-myc* gene copies are given in the table. In nine of the 18 tumour cells were positive for the *N-myc* gene product. Of these nine, two were positive in almost all of the tumour cells (fig 1) and four contained a moderate number of positive cells (fig 2). In the remaining three positive cells were rare. Brown benzidine products in the immunoperoxidase reaction for *N-myc* gene product were observed only in the nuclei of the neuroblastoma cells. Almost all of the positive neuroblastoma cells were immature neuroblasts, and no ganglion cells and few differentiated neuroblastic cells with nuclear enlargement and cytoplasmic eosinophilia and enlargement with distinct cell border[13] were immunoreactive to the *N-myc* gene product. Except for one ganglioneuroblastoma of a composite type with a few positive immature

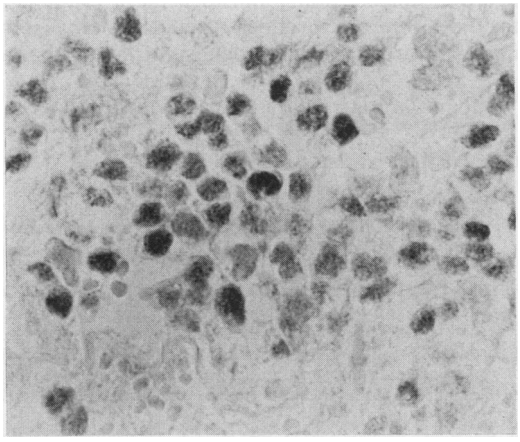

Fig 1 *Almost all neuroblastoma cells are positive for N-myc protein. Immunoreaction product is confined to the nuclei of those tumour cells. (ABC technique.)*

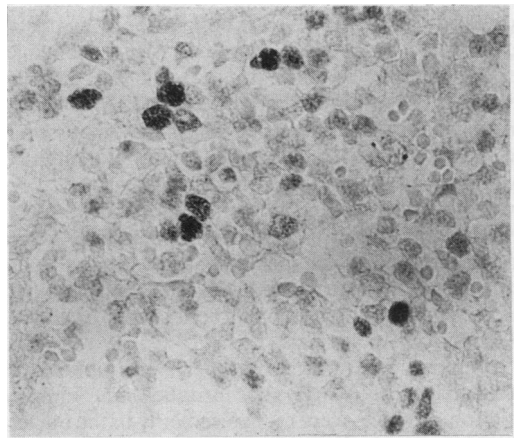

Fig 2 *Scattered neuroblastoma cells strongly positive for N-myc protein. (ABC technique.)*

neuroblasts, tumours in the positive cases were classic neuroblastomas.

Among 11 with amplified *N-myc* gene copies, nine had tumour cells positive for the *N-myc* protein. The remaining two were stage IVA neuroblastoma and stage IVA ganglioneuroblastoma of the composite type, respectively, both with no positive cells. These two tumours were composed of more differentiated neuroblastic cells than the other amplified ones. In contrast, there were no immunoreactive tumour cells in the seven non-amplified cases.

Tumour cells positive for *N-myc* protein were not observed in any of the four patients under 1 year of age, but such cells were present in nine (64%) of 14 patients over 1 year of age. Of nine with *N-myc* positive tumour cells in the tumour, all but one died within one year after surgery. The one exception was a patient with a stage IVS tumour; the child was still alive 44 months after surgery. Of the nine with an *N-myc* protein negative tumour, one died and tumour recurred in the other. The remaining seven were alive at the time of writing with no evidence of disease, with a follow up ranging from 19 to 54 months. There was a significant difference in the survival curves (generalised Wilcoxon test) between patients with an *N-myc* protein positive neuroblastoma and those with an *N-myc* protein negative tumour ($p < 0.01$).

Discussion

In 1986 Ikegaki *et al* identified and characterised the *N-myc* gene product in human neuroblastoma cells, using monoclonal antibodies with defined specificities. These were prepared against a bacterially expressed fusion protein containing a portion of the *N-myc* sequence.[10] The relative level of the polypeptides was roughly proportional to the level of *N-myc* transcripts present in a panel of neuroblastoma cell lines. We applied an affinity purified rabbit antibody against the genetically engineered human *N-myc* oncogene product prepared by Shimatake *et al* [11] to paraffin wax sections. Those authors reported that: (i) Western blot analysis showed the presence of the *N-myc* protein (63 kd) in the nuclear fraction of the neuroblastoma cells examined; (ii) immunohistochemically, the *N-myc* protein was distributed in the nuclei of neuroblastoma cells obtained from various culture cell lines, a xenograft, and a surgical specimen; (iii) the anti-*N-myc* antibody did not react with cells producing *c-myc* in the enzyme linked immunosorbent assay (ELISA), or in immunohistochemical tests.

In nine of our 18 tumours, there were tumour cells positive for the *N-myc* gene product. The immunoreaction products were recognised only in the nuclei of neuroblastoma cells, with virtually no staining in the cytoplasm of these cells or normal ganglion cells, nephrons, and vascular or other stromal elements. Almost all the positive cells seemed to be primitive neuroblasts and were not observed among neuroblastic cells showing differentiation, even in the same tumour. A close relation of *N-myc* oncogene positivity to undifferentiated phenotypes in neuroblastoma cells is compatible with the finding that in situ hybridisation to sections of neuroblastomas showed a high expression of *N-myc*, predominantly in the undifferentiated neuroblasts.[7,8] The expression of *N-myc* was found to be greatly decreased before morphological evidence of differentiation when primitive human neuroblastoma cell lines were differentiated in vitro by the addition of retinoic acid.[14,15] The *N-myc* oncogene may therefore have an important role in the oncogenicity, proliferating ability, and regulation of cell differentiation of neuroblastomas.

Increased production of *N-myc* protein seems to correlate closely with *N-myc* gene amplification and a poor prognosis. Nine of the 11 neuroblastic tumours with the amplified *N-myc* gene copies contained *N-myc* protein positive tumour cells, while none of the seven non-amplified cases showed immunostaining for *N-myc*. Because the *N-myc* gene product has been reported to have a short half life (30–40 minutes),[10] the negativity for the *N-myc* protein in the two cases with amplified *N-myc* gene copies may have been the result of a loss of intranuclear *N-myc* protein by the prolonged lapse of time until fixation or delayed permeation of fixatives. Even though there may be some loss of the antigen, patients with an *N-myc* immunoreactive neuroblastoma had a significantly poorer prognosis than did those with a neuroblastoma negative for *N-myc* protein. These results suggest that amplification and consequent increased transcripts of the *N-myc* oncogene may be related to the aggressiveness of neuroblastomas. Immunohistochemical examination for the *N-myc* gene product in neuroblastomas is thus useful for predicting the prognosis of these patients.

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