J Clin Pathol 1989;**42**:1148–1152

N-*myc* amplification in neuroblastomas: histopathological, DNA ploidy, and clinical variables

B R OPPEDAL, O ØIEN,* T JAHNSEN,* P BRANDTZAEG

*From the Laboratory for Immunohistochemistry and Immunopathology (LIIPAT) and *Laboratory for Gene Technology, Institute of Pathology, University of Oslo, The National Hospital, Oslo, Norway*

SUMMARY The association between tumour N-*myc* amplification, DNA ploidy, and various prognostic factors (patient age, tumour stage at diagnosis, primary site and histopathological differentiation) was studied in 18 children who had neuroblastoma, ganglioneuroblastoma, or ganglioneuroma. Amplification of genomic N-*myc* was observed in six patients who had been treated with chemotherapy before surgery (one with stage III and five with stage IV). All these tumours were located in the adrenal or upper retroperitoneum; five were neuroblastomas and one was a ganglioneuroblastoma. Three of them were aneuploid and three diploid. The degree of N-*myc* amplification varied from 20 to 1500 copies without relation to ploidy. All patients with tumour N-*myc* amplification died of their tumour. Amplification was always associated with poor prognosis, independent of tumour cell ploidy. Four patients without such amplification also died: three had diploid tumours, the fourth was aneuploid.

It is suggested that genomic N-*myc* amplification takes place mainly in adrenal and retroperitoneal neuroblastomas and can be a feature of tumours with and without histological signs of differentiation. The precise role of N-*myc* in the pathogenesis of neuroblastoma remains unclear.

In 1983 Schwab *et al* identified N-*myc* as an amplified gene with partial homology to *myc* cellular oncogene in cell lines of human neuroblastomas and in a neuroblastoma tumour.[1] The N-*myc* oncogene was subsequently localised to human chromosome 2 (p23–24) by in situ hybridisation. In neuroblastoma cells amplified genomic N-*myc* was localised to homogenous staining regions on different chromosomes and to double minute bodies.[2]

Amplification of genomic N-*myc* has been reported to be associated with rapid tumour progression and poor prognosis in patients with neuroblastoma.[3 4] The number of N-*myc* copies has been proposed as a valuable prognostic factor, suggesting that genomic amplification of N-*myc* may have a key role in determining the aggressiveness of neuroblastoma cells and thus their malignant phenotype.[3]

The aim of this study was to examine known prognostic factors (patient's age, clinical stage at diagnosis, primary site, histopathological differentiation, and DNA ploidy) and to compare these data with the degree of N-*myc* gene amplification in the actual neuroblastoma.

Material and methods

Tumour samples were obtained from 18 patients under the age of 15 with neuroblastoma, ganglioneuroblastoma, and ganglioneuroma, all diagnosed and treated in the same institution. The tissue specimens were collected at operation, either initially (stage I to III tumours) or after chemotherapy (stage III and IV tumours). In one case tumour samples were available on three different occasions: the stage IV primary retroperitoneal tumour (removed after chemotherapy); the local recurrence one year later (also after chemotherapy); and one of multiple abdominal metastases to the omentum (removed palliatively after irradiation one month later).

The malignant tissue was immediately brought to the laboratory and either frozen in liquid nitrogen for storage (−70°C) or fixed in ethanol and embedded in paraffin wax at 56°C according to the method of Sainte Marie.[5] Additional material from the same specimens was routinely fixed in 4% buffered formaldehyde and embedded in paraffin wax for conventional histopathological examination.

CLINICAL VARIABLES

Prognostic factors like patient's age and tumour stage[6] at diagnosis were recorded in addition to primary site and clinical outcome. No patient was lost to follow up (minimum two years). The stage III and stage IV patients were treated with combination chemotherapy—vincristine, cyclophosphamide, Cis-

Accepted for publication 29 June 1989

platinum and etoposide (OPEC)—before major surgery. Two of the stage IV patients also underwent autologous bone marrow transplantation preceded by marrow purging. Patients with stage I ganglioneuroma or ganglioneuroblastoma did not receive chemotherapy after primary surgery.

HISTOPATHOLOGICAL GRADING AND IMMUNOHISTOCHEMICAL STAINING

Routine sections stained with haematoxylin, azophloxine, and saffron were used for histological tumour grading, essentially as described by Beckwith.[7] The diagnosis was verified by immunohistochemical techniques with a monoclonal antibody (UJ13A, ascitic fluid 1/1000) to neuroblastoma cells.[8] A three-step fluorescence technique was applied on sections (4 μm) of tissue fixed in ethanol and embedded in paraffin wax, as described previously.[9]

DNA FLOW CYTOMETRIC EXAMINATION

Tumour tissue was prepared for flow cytometry from fresh frozen tissue (14 specimens) or from ethanol-fixed, paraffin wax embedded material (six specimens) according to the method of Hedley,[10] with minor modifications.[11] Briefly, representative paraffin wax sections (100 μm) were dewaxed in xylene, rehydrated in graded ethanol, mechanically disrupted, digested in pepsin and filtered to obtain single cell suspensions. The fresh frozen tissue was minced and digested in pepsin before being incubated in DNase-free RNase and being stained with ethidium bromide (10 μg/ml; Calbiochem, California, USA). Emission measurements were performed in an ICP 11 Flow cytometer (PHYWE AG, Göttingen, West Germany). Cells from the suspensions were additionally stained with Papanicolaou and Orange G EA 36 for light microscopical examination and differential counting to ensure that tumour cells were measured. The tumour cell DNA histograms were divided into diploid and aneuploid (hyperdiploid or near triploid) groups.[11]

DNA EXTRACTION AND EVALUATION OF GENOMIC N-*myc* AMPLIFICATION

Fresh frozen tumour tissue (−70°C) was used in only two cases. The method of Goelz *et al*[12] was adapted with some modifications for extraction of DNA from the ethanol fixed and paraffin wax embedded specimens. Malignant tissue was cut out neatly from the paraffin wax blocks, and remaining paraffin wax was removed by heating the tissue to 56°C followed by incubation in xylol at room temperature overnight. Finely minced tissue (about 50 mg from each specimen) was suspended in 2% sodium dodecyl sulfate (SDS) and 1 mg/ml proteinase K. DNA was extracted with phenol-chloroform and precipitated by adding cold ethanol.

This modified Goelz technique was also used on formalin fixed and parffin wax embedded specimens of neuroblastoma. Initially we tested the quality of the DNA obained from normal tonsils fixed either in formalin or ethanol, with or without paraffin wax embedding and compared it with the quality obtained from fresh frozen tissue (−70°C).

The quality of the extracted DNA was analysed by electrophoresis in 0·7% agarose gels (5 μg DNA a lane) which were stained with ethidium bromide. N-*myc* amplification was tested in slot-blots (5 μg DNA a slot). After overnight hybridisation with radiolabelled human N-*myc* proto-oncogene probe (Amersham; Buckinghamshire, England), the filters were washed and exposed to Amersham Hyperfilm MP for four to five days at −70°C. Determination of the number of N-*myc* copies was carried out by stepwise 10-fold dilution of the DNA and densitometric evaluation of the signals in relation to that of the germ line (single copy per haploid genom, 5 μg DNA per slot). Five of the tumours with amplified N-*myc* were additionally analysed by Southern blots. Ten μg DNA from each tumour was digested with restriction endonucleases (*PstI* and *EcoRI*) and separated in an agarose gel before it was transferred to nylon membranes (Biotrans, ICN) and hybridised with radiolabelled human N-*myc* probes.

The Wilcoxon non-parametric test and Pearson's correlation analysis were used for statistical evaluation. A difference of $p \leqslant 0{\cdot}05$ was regarded as significant.

Results

The clinical findings are given in table 1. Notably, only four of the patients in this study were below the age of 1½ years.

Ten patients died from tumour recurrence, all within two years; these included the two who had had autologous bone marrow transplantation (cases 12 and 15). Eight patients are still alive and in complete remission at the time of writing; one of them (case 5) who underwent surgery for a dumb-bell thoracic tumour, had an intraspinal recurrence five years later but has remained well for two years following treatment.

HISTOPATHOLOGICAL GRADING AND IMMUNOHISTOCHEMICAL STAINING

Nine patients had undifferentiated neuroblastomas (⩽5% differentiating elements—that is, cells, fibrils, and fibres), eight had ganglioneuroblastomas (5% to 50% differentiating elements), and one patient had a ganglioneuroma consisting of differentiated elements alone (table 1).

All neoplasms had tumour elements reacting with

Table 1 *Clinicopathological features and molecular biology data*

Case No/Sex	Histopathology*	At diagnosis: Tumour stage†	At diagnosis: Age (in years)	At diagnosis: Primary site	Tumour cell ploidy	N-myc amplification‡	Outcome
1/f	Ganglioneuroma	I	8 2/12	Retroperitoneal, extra adrenal	Diploid	—	No evidence of tumour
2/f	Ganglioneuroblastoma	I	4 2/12	Retroperitoneal, extra adrenal	Aneuploid	—	No evidence of tumour
3/f	Ganglioneuroblastoma	II	8 3/12	Retroperitoneal, extra adrenal	Diploid	—	No evidence of tumour
4/m	Ganglioneuroblastoma	III	1	Retroperitoneal, extra adrenal	Aneuploid	—	No evidence of tumour
5/m	Ganglioneuroblastoma	III	1 11/12	Mediastinal	Diploid	—	No evidence of tumour[a]
6/m	Neuroblastoma	III	3/12	Retroperitoneal, extra adrenal	Diploid	—	No evidence of tumour
7/m	Neuroblastoma	III	1 7/12	Adrenal	Diploid	1500	Dead of tumour
8/m	Neuroblastoma	III	5 11/12	Mediastinal	Aneuploid	—	No evidence of tumour[b]
9/f	Ganglioneuroblastoma	IV	6/12	Adrenal	Diploid	20	Dead of tumour
10/m	Ganglioneuroblastoma	IV	1 6/12	Retroperitoneal, extra adrenal	Diploid	—	No evidence of tumour
11/f	Ganglioneuroblastoma	IV	4 4/12	Retriperitoneal	Diploid	—	Dead of tumour
12/m	Ganglioneuroblastoma	IV	9 6/12	Retroperitoneal, extra adrenal	Aneuploid	—	Dead of tumour[c]
13/m	Neuroblastoma	IV	2 4/12	Retroperitoneal, extra adrenal	Diploid	460	Dead of tumour
14/f	Neuroblastoma	IV	4 8/12	Adrenal	Aneuploid	30	Dead of tumour
15/m	Neuroblastoma	IV	2 2/12	Retroperitoneal, extra adrenal	Aneuploid	40	Dead of tumour[c]
16/f	Neuroblastoma	IV	2 9/12	Retroperitoneal, extra adrenal	Diploid	—	Dead of tumour
17/f	Neuroblastoma	IV	2 10/12	Adrenal	Aneuploid	1050	Dead of tumour
18/m	Neuroblastoma	IV	6 2/12	Unknown, primary not found	Diploid	—	Dead of tumour

*Histopathological grading according to Beckwith.[7]
†Tumour stage at diagnosis according to Evans.[6]
‡Number of genomic N-*myc* copies.
[a]recurrence 5 years after primary treatment, without evidence of disease two years after recurrence; [b]living with renal failure; [c]transplanted with autologous bone marrow.

the UJ13A antibody; staining was seen in undifferentiated cells with a characteristic "hairy" surface and in interstitial fibrils and fibres.

DNA FLOW CYTOMETRIC EXAMINATION

The mean number of cell nuclei obtained for flow cytometry was 20 980 (range, 10 050–64 760). By differential counting of smears of dispersed cells stained with Papanicolaou and Orange G, the samples were found to contain 88%–95% neoplastic cells. The remaining cells were lymphocytes and fibroblasts.

All histograms were readable with respect to DNA profile. Eleven of the neoplasms were typically diploid, the remaining seven aneuploid with a hyperdiploid (near triploid) DNA index between 1·4 and 1·7 (table 1). The tumour material from case 15 obtained on three different occasions, showed a shift from aneuploid (primary) to diploid (recurrence and a subsequent metastasis).

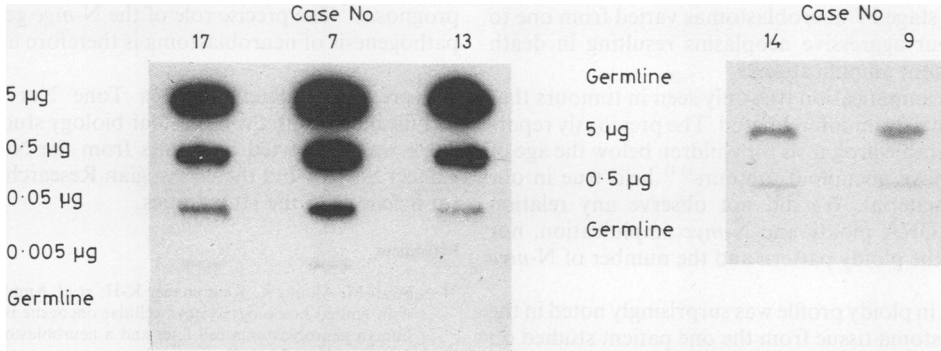
GENOMIC N-*myc* AMPLIFICATION

Using the Goelz method[12] on human tonsils, we tested DNA obtained after different tissue processing methods. Analytical electrophoresis in agarose gels showed that the DNA quality of ethanol fixed and paraffin wax embedded material was just as good as that of fresh frozen tissue; formalin fixation resulted in fragmented DNA of poor quality, depending mainly on the fixation time. If the tissue had been left in formalin for more than one day, no distinct DNA strand appeared on ethanol precipitation but only a faint blurring which represented small DNA fragments. We

Table 2 *Sequential variations in DNA ploidy and N-myc amplification in tumour tissue from case 15 with stage IV neuroblastoma*

Tumour	Age (in years)	Tumour site	Tumour cell ploidy	N-myc amplification*
Primary	2 2/12	Retroperitoneum	Aneuploid	40
Recurrence	3 1/12	Local recurrence	Diploid	1560
Metastasis	3 2/12	Ommentum majus	Diploid	40

*Number of genomic N-*myc* copies.



Figs 1a and b *Slot blot analysis of DNA (5 µg, serially diluted) obtained from five neuroblastomas.*

did not obtain satisfactory blots when we applied the Goelz method on formalin fixed and paraffin wax embedded neuroblastoma tissue either.

The results reported below were therefore based on ethanol fixed and paraffin wax embedded, or fresh frozen (two samples) material. The slot blot hybridisations were done three times with similar results. The specificity of the slot-blots was confirmed by Southern blotting. *PstI*- and *EcoRI*-digested tumour and germline DNA were compared. The tumour DNA showed identical but amplified bands. Several other bands were also seen in the tumour blots.

N-*myc* amplification was seen in six of the 18 tumours. With dilutions of DNA and densitometric scanning the amplification was estimated to range from 20 to 1560 copies (table 1 and figs 1a, 1b, and 2).

Tumour tissue obtained on three different occasions from the same patient (case 15) showed varying N-*myc* amplification: about 40 copies in the primary retroperitoneal tumour, removed after chemotherapy; about 1560 copies in the recurrence removed one year later, also after chemotherapy; and about 40 copies in the abdominal metastasis removed after radiation to relieve obstruction symptoms (fig 2, table 2).

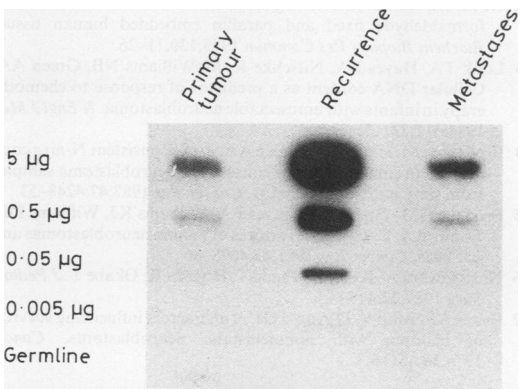


Fig 2 *Slot blot analysis of DNA (5 µg, serially diluted) obtained from neuroblastoma tumours from one patient on three different occasions.*

Genomic N-*myc* amplification and tumour cell ploidy were independent variables (Pearson's correlation coefficient near to 0). Our data showed better prognosis ($p \leqslant 0{\cdot}05$) for patients with differentiated tumours ($p = 0{\cdot}05$), low tumour stage at diagnosis ($p = 0{\cdot}001$), and no N-*myc* amplification ($p = 0{\cdot}01$). The results should, however, be interpreted with caution as the numbers were small.

Discussion

Our results suggest that there is a relation between poor prognosis and genomic N-*myc* amplification in neuroblastomas, as previously reported by Seeger *et al.*[3] All six patients with amplified N-*myc* oncogene had clinically advanced (stage III and IV) and aggressive tumours which were fatal. Seeger *et al* reported a three to 300-fold genomic N-*myc* amplification in their study of untreated primary neuroblastomas.[3] Our results should be interpreted with some caution as many of the patients had already received some treatment. Based on comparison with the data of Seeger *et al*,[3] however, treatment did not seem to reduce the incidence of tumours with N-*myc* amplification. In their material N-*myc* amplification was seen in 32 of 60 (53%) stage III and IV neuroblastomas, which is similar to the six of 13 (46%) observed in our pre-treated material.

Our material included more than 60% of all neuroblastomas diagnosed and treated in Norway over four years, and no patient was lost to follow up. We tried to expand the material by extracting DNA from old formalin fixed and paraffin wax embedded tissue blocks of neuroblastoma. In contrast to Tsuda *et al*,[4] we did, however, not obtain DNA of sufficiently good quality for molecular biology studies. The available number of patients was therefore limited. Seeger *et al* proposed that genomic N-*myc* amplification might be crucial in determining the aggressiveness of neuroblastomas[3]; they found an inverse relation between the number of N-*myc* gene copies and progression-free survival.[3] In our study the number of

copies in stage IV neuroblastomas varied from one to 1500. Four aggressive neoplasms resulting in death were without amplification.

N-*myc* amplification was only seen in tumours that were primarily undifferentiated. The previously reported favourable prognosis for children below the age of 1½ who have aneuploid tumours[11 13] held true in our limited material. We did not observe any relation between DNA ploidy and N-*myc* amplification, nor between the ploidy pattern and the number of N-*myc* copies.

A shift in ploidy profile was surprisingly noted in the neuroblastoma tissue from the one patient studied on three occasions: the initial retroperitoneal tumour (stage IV) was aneuploid with a 40-fold N-*myc* amplification; a local recurrence appeared eight months later and was removed after repeated high dose chemotherapy, and this tumour was a diploid undifferentiated neuroblastoma with 1560-fold N-*myc* amplification. One of the subsequent multiple metastases was found to be diploid with a 40-fold N-*myc* amplification. Histologically, the neoplasm was predominantly undifferentiated at all times. Discrepancy in amplification might have been due to tumour heterogeneity, with a small subset of tumour cells with genomic amplification present in the primary tumour being the dominant clone during the first recurrence. After its removal the patient soon presented with abdominal and pulmonary metastases and died within six months, suggesting more aggressive behaviour in the diploid recurrence with high N-*myc* amplification.

This shift in copy numbers of the N-*myc* gene in a tumour on various occasions contrasts with the findings of other reports[4 14] but agrees with the cytogenetic studies by Brodeur *et al*[15]: they found either double minute bodies or homogeneous staining regions in some subpopulations of two neuroblastoma cell lines. Most studies on N-*myc* amplification or expression have been done on DNA or RNA extracted from cells in suspension. The blotting techniques provide average values and may not show subpopulations. In situ hybridisation on neuroblastoma tumour sections and cell line cytospin slides might therefore be of considerable interest.

In conclusion, we found that N-*myc* amplification is a common but not obligatory feature of aggressive, stage III and IV, mainly undifferentiated neuroblastomas, unrelated to a specific DNA ploidy pattern. For the individual patient, therefore, lack of N-*myc* amplification does not exclude poor prognosis. As previously noted by Nakagwara,[16] such amplification was seen only in neuroblastomas presenting in the adrenals and in the retroperitoneum (suprarenal region). The biological importance of this association is obscure, but a retroperitoneal or adrenal primary site is known to harbour tumours with a poor prognosis.[17] The precise role of the N-*myc* gene in the pathogenesis of neuroblastoma is therefore unknown.

We are most grateful to Ms Tone Narvesen for technical help with the molecular biology studies. This work was supported by grants from the Norwegian Cancer Society and the Norwegian Research Council for Science and the Humanities.

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Requests for reprints to: Borghild Roald Oppedal, Department of Pathology, Ullevål Hospital, N-0407 Oslo 4, Norway.