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N-*myc* Gene Copy Number in Neuroblastoma Cell Lines and Resistance to Experimental Treatment

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The N-*myc* oncogene is amplified in approximately 30% of neuroblastomas. It is well established that cases of neuroblastoma with amplified N-*myc* have markedly poorer prognosis than those in which N-*myc* copy number is not elevated. The mechanism for this association is not known but may be related to cellular resistance to radiation or cytoxic drugs. Seven human neuroblastoma cell lines were used to investigate the relationship between N-*myc* copy number or expression and sensitivity to ionising radiation and to cisplatin. N-*myc* copy number was assessed by Southern blotting and hybridisation using the p-Nb1 probe. The signal produced by DNA from the cell lines was compared with that of single copy N-*myc* from normal human placental DNA. A range of N-*myc* copy numbers from 1 to 800 was found. Expression levels of N-*myc* mRNA were compared by "dot blotting" and subsequent hybridisation to the p-Nb1 probe. Radiosensitivity was assessed by surviving fraction at 2 Gy (SF_2) following ^{60}Co gamma irradiation. Values ranged from 0.13 to 0.52. Sensitivity to cisplatin was indicated by comparison of isoeffective concentrations (concentration required to produce 1 log cell kill). These ranged from 7.5 to 13 μM. Cisplatin studies showed a correlation between N-*myc* copy number (though not expression) and resistance to this drug. If this relationship is causal it may explain why treatment fails in those patients with an elevated N-*myc* copy number. However, no correlation was found between N-*myc* copy number or expression and sensitivity to radiation. It is possible that N-*myc* amplification confers resistance to some but not all treatments used in the therapy of neuroblastoma. Further investigations along these lines may lead to the identification of agents which are most appropriate for the treatment of neuroblastoma with amplified N-*myc* gene.
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INTRODUCTION

THE N-*myc* gene, which bears extensive sequence homology to c-*myc* [1,2] is classified as a proto-oncogene because it can contribute to the process of transformation [3,4]. There is a well established link between amplification of the N-*myc* gene in neuroblastoma and poor prognosis [5]. Indeed, this represents the strongest clinical correlation between an altered specific proto-oncogene and clinical outcome.

N-*myc* amplification is observed more frequently in advanced stage disease [6] and in rapidly progressing tumours [7]. In one study [7] the progression-free survival at 18 months for patients whose tumours had 1-fold, 3–10-fold and greater than 10-fold N-*myc* amplification was 70, 30 and 5%, respectively. This suggests that the aggressiveness of some neuroblastomas is dependent not simply on the possession of extra N-*myc* genes, but is a function of the number of copies of this gene.

Gene amplification and overexpression in tumour cells is often associated with drug resistance. Examples include amplification of the dihydrofolate reductase gene involved in methotrexate resistance [8] and increased transcription of the *mdr*-1 gene, whose P-glycoprotein product (gp170) is responsible for the enhanced cellular efflux of a variety of cytotoxic agents [9]. Oncogenes activated by other mechanisms may also influence the cellular response to chemotherapeutic agents. For example, *ras*-transformed NIH-3T3 cells exhibited a markedly increased resistance to cisplatin [10], an agent used in the treatment of neuroblastoma. The observation that mutant *ras* genes also significantly decreased the radiosensitivity of transfected cells [11] suggests that *ras* activation can result in the potentiation of a mechanism which overcomes the cytotoxic effects of both therapeutic agents.

The role of oncogene activation in the development of cellular radioresistance is controversial. One study [12] showed that the expression of the c-*raf*-1 oncogene enhanced the radioresistance in a human squamous carcinoma cell line. NIH/3T3 cell transfection studies have implicated activated *ras* [11,13] and c-*raf*-1 [14] oncogenes in the acquisition of tolerance to ionising radiation. However, this phenomenon may be dependent on the recipient cell type, since mink lung epithelial cells transfected with c-*myc* or H-*ras*-1 showed no change in radiosensitivity compared to the parental cell line [15].

The basis for the correlation between N-*myc* amplification and

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poor clinical outcome is unknown. Alternative explanations are that N-*myc* amplification confers rapid tumour growth, high metastatic potential or resistance to therapy. We have investigated the latter possibility using a panel of human neuroblastoma cell lines which possessed a range of values of N-*myc* copy number and expression.

MATERIALS AND METHODS

Cell lines

The cell lines used were NB1-G [16], IMR-32 [17], NB2-G [18], NB100 [19], SK-N-SH [20], SK-N-BE(2)C [21] and XRNB1-G, a radioresistant subline of NB1-G [22].

Monolayer culture

All cell lines were maintained in 75-cm^2 flasks containing 20 ml of Eagle's minimal essential medium with 25 mM Hepes buffer, 10% fetal calf serum, 2 mM glutamine, penicillin/streptomycin (100 U/ml) and amphotericin B (2.5 μg/ml). All media and supplements were obtained from Gibco (Paisley, U.K.). Flasks were seeded with 5×10^5 cells and kept in an atmosphere of 5% CO_2 at 37°C.

Spheroid culture

Multicellular tumour spheroids were chosen as a model because they have many of the characteristics of avascular micrometastases [23]. These were prepared by continuous stirring of approximately 2×10^6 cells in Techne (Cambridge, U.K.) stirrer flasks for 3–4 days at 37°C, 5% CO_2. Spheroids with a diameter of about 300 μm were assayed for response to radiotherapy or chemotherapy by determination of the fraction of cells surviving treatment, as described below. It has been demonstrated previously that neuroblastoma multicellular tumour spheroids of this size present no diffusion barrier to small molecular weight drugs [24].

Several pilot studies were performed on each cell line to assess the plating efficiency and to ascertain the amount of radiation or drug required to produce 1 log cell kill before deciding on the range of radiation or drug concentrations to use. For example, NB1-G required approximately 2.5 Gy to produce 1 log cell kill. Therefore, the upper limit was set at 3.5 Gy. SK-N-BE(2)C needed 4 Gy for the same effect. Therefore, the upper limit was set at 5 Gy.

Chemotherapy

Spheroids were transferred to universal containers, medium was removed and the spheroids were resuspended in Earle's balanced salt solution (EBSS) containing cisplatin (Cyanamid, U.K.) ranging in concentration from 2.5 to 20 μM. These were incubated for 1 h at 37°C following which any unbound drug was removed by washing three times in EBSS.

Irradiation procedure

Spheroids were transferred to 25-cm^2 flasks and irradiated on a ^{60}Co unit with doses from 0.5 to 5 Gy, using a 0.8-cm perspex "build up" to ensure maximum energy deposition. The dose rate was approximately 1 Gy/min.

Estimation of cell survival

Immediately after treatment, spheroids were mechanically disaggregated by needle aspiration, and single cell suspensions were verified by microscopy. Cells were then plated into 25 cm^2 flasks. Cell numbers per flask ranged from 10^3 to 5×10^5 cells, depending on concentration of drug or dose of radiation used. Three flasks were prepared per treatment group. The cells were incubated at 37°C in an atmosphere of 5% CO_2, for 10 to 14 days until macroscopic colonies were visible. The cell lines NB1-G and XRNB1-G required "feeder layers" for colony growth. These were produced by heavily irradiating confluent NB1-G or XRNB1-G cells at 50 Gy. One million of these sterilised cells were then seeded into each experimental flask prior to incubation. Colonies were stained with a 10% (v/v) aqueous solution of Carbol Fuschin (R.A.Lamb, Middlesex, U.K.). Survival curves were constructed from colony counts using the linear quadratic model. Abortive colonies of fewer than 50 cells were disregarded.

*Estimation of N-*myc *copy number*

DNA was extracted from cell lines using proteinase K followed by phenol/chloroform [25]. Human placental DNA was used as single copy control. DNA was digested with *Eco*R1, and samples were electrophoresed through 0.8% (w/v) agarose gels [using λ/Hind III fragments (Gibco, U.K.) as molecular weight markers] and blotted [26] on to nylon filters (Amersham U.K.). These were simultaneously hybridised to radioactive p-Nb1 probe (1 kbp *Eco*R1/*Bam*H1 insert of pBR322 [1,2]) and 5′ BCR probe (2 kbp Hind III/Bgl II insert of pUC 8 [27]). The specific activity of the probes was approximately 2×10^7 counts per min per μg. The second probe was used to check the amount of DNA loaded into each well. N-*myc* copy number was assigned by comparing signals obtained by densitometric scanning of autoradiographs (Sun, U.K.).

*Estimation of N-*myc *expression*

Total RNA was extracted from cell lines using the RNA zol B kit (Biogenesis Ltd, U.K.), and the integrity of the RNA was assessed by electrophoresis on a 0.8% (w/v) agarose gel. Hela cells were used as a negative control since they do not express N-*myc* [28]. Poly(A) RNA was electrophoresed through a 1% (w/v) agarose/formaldehyde gel with 28S and 18S ribosomal RNA used as internal markers [29]. By northern blotting and subsequent hybridisation to the p-Nb1 probe (as above) a signal was visible at 3 kb. This corresponds to the size of N-*myc* messenger RNA. The level of expression in cell lines was compared by dot blot analysis [29] using poly(A) RNA. An expression value of 1 was assigned to SK-N-SH [30] and all other signals compared to this.

Statistical analysis

Correlations were evaluated between N-*myc* copy number or N-*myc* expression and SF_2 or cisplatin isoeffective dose (the dose of cisplatin required to produce 1 log cell kill), and also between N-*myc* copy number and N-*myc* expression. Data analysis was by Spearman's rank correlation test to assess correlation without assuming linearity. Where a positive indication of correlation was found, least squares analysis was undertaken to further elucidate the relationship.

RESULTS

*N-*myc *copy number*

Figure 1 shows an autoradiograph of cell line DNA assessed by Southern blotting. Densitometric analysis of signals revealed a range of N-*myc* copy numbers, as shown in Table 1. SK-N-BE(2)C has an unusually high copy number of 800. This was reflected by the absence of a *bcr* hybridisation signal in the SK-N-BE(2)C track.

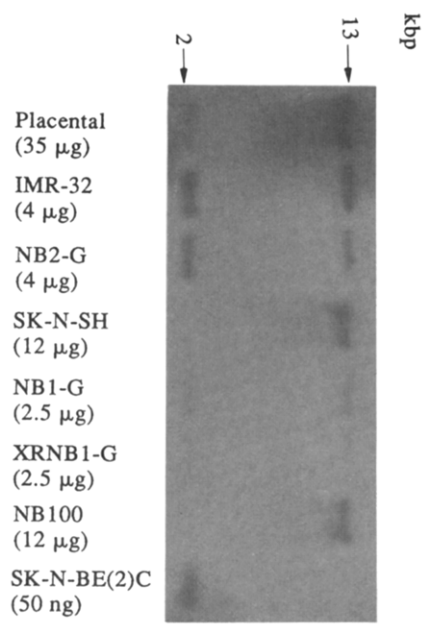

Fig. 1. Southern blot analysis of cell line DNA. DNA was digested with *Eco*R1, electrophoresed on 0.8% (w/v) agarose gels, and analysed by Southern blot. Loading was as follows: placental (35 μg), NB100 and SK-N-SH (12 μg), NB2-G and IMR-32 (4 μg), NB1-G and XRNB1-G (2.5 μg) and SK-N-BE(2)C (50 ng). Placental DNA was used as single copy control. After electrophoresis, the DNA was transferred to nylon membrane and hybridised to radioactive p-Nb1 and 5′ *bcr* probes. Specific activity of these was approximately 2 × 10^7 c.p.m./μg. The p-Nb1 probe bound to a 2 kbp fragment. The *bcr* probe (used to check loading) bound to a 13 kbp fragment.

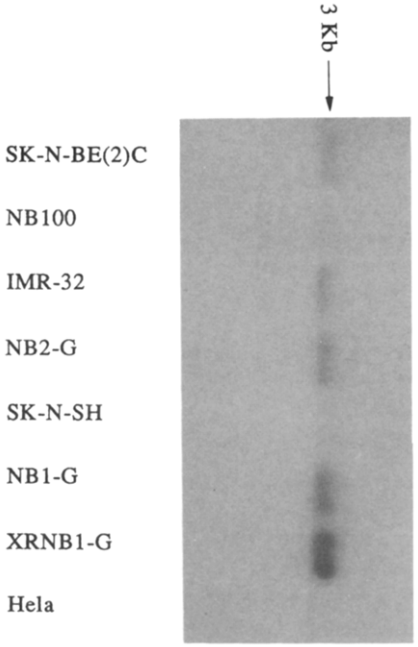

Fig. 2. Northern blot analysis of cell line RNA. 10 μg Poly(A) RNA was electrophoresed on 1% (w/v) formaldehyde/agarose gels, and analysed by northern blot. After transferring to nylon membrane, the blot was hybridised to radioactive p-Nb1 probe with a specific activity of approximately 2 × 10^7 c.p.m./μg, identifying a 3 kb poly(A) RNA species.

N-myc *expression*

Northern blot examination of RNA isolated from neuroblastoma cell lines showed that the labelled p-Nb1 probe hybridised to a 3 kb mRNA (Figure 2). Dot blot analysis (Figure 3), demonstrated that cell lines SK-N-SH and NB100 expressed at base line levels. The strongest signal was from XRNB1-G. By arbitrarily assigning a value of 1 to the degree of expression in the SK-N-SH line, the expression levels of other cell lines are indicated in Table 1.

Table 1. *N*-myc *copy number, N*-myc *expression, SF_2 values and isoeffective dose of cisplatin (μM) for the seven cell lines*

Cell line	N-*myc* copy number	N-*myc* expression	SF_2	Isoeffective dose of cisplatin (μM)
SK-N-SH	1	1	0.13	7.5
NB100	1	1	0.44	8.5
NB1-G	20	7	0.17	7.5
XRNB1-G	20	10	0.23	11
IMR-32	30	3	0.25	10
NB2-G	30	3	0.24	11
SK-N-BE(2)C	800	2	0.52	13

Copy number and expression of N-*myc* were evaluated using the techniques of Southern and northern blotting. SF_2 and iso-effective dose were derived from the survival curves.

Radiosensitivity

Survival curves (Figure 4) were constructed from the results of at least three separate experiments. In each experiment, the mean of three surviving fractions was determined per dose. Radiosensitivity was assigned a value corresponding to the surviving fraction at 2 Gy (SF_2). This figure was derived from the survival curve. This parameter is of clinical relevance as 2 Gy is a typical therapeutic fraction size dose. It has been demonstrated that there is a correlation between SF_2 and the level of clinical response of various tumour types [31,32]. SF_2 values of the cell lines assayed in our study ranged from 0.13 to 0.52 (Table 1). From published data, the average value for the SF_2 of neuroblastoma cell lines is approximately 0.2, whereas the relatively radioresistant melanomas, osteosarcomas and glioblastomas average 0.5 [32,33]. Therefore, NB100 and SK-N-BE(2)C, which have SF_2 values of 0.44 and 0.52, respectively, indicate unusually high radioresistance for neuroblastomas. Compared to cells derived from many other tumour types, neuroblastoma cell lines are typically radiosensitive [34]. This *in vitro* sensitivity reflects, and may explain, the response of neuroblastoma tumours to radiotherapy. Of the cell lines presented, Table 1 shows that five of these displayed this characteristic radiosensitivity, while two of the lines [NB100 and SKNBE(2)C] were markedly more resistant to ionising radiation. The cells were irradiated as spheroids, and the presence of hypoxia could conceivably account for the anomalously high radioresistance. However, the survival curves for these cells, when irradiated as monolayers, were not significantly more sensitive (data not shown), making it unlikely that hypoxia was affecting our results. Although cell lines derived from one tumour type may have a mean SF_2 that is characteristic of their tumour of origin, it is common to find considerable variation about this mean [35], and the results presented here confirm this.

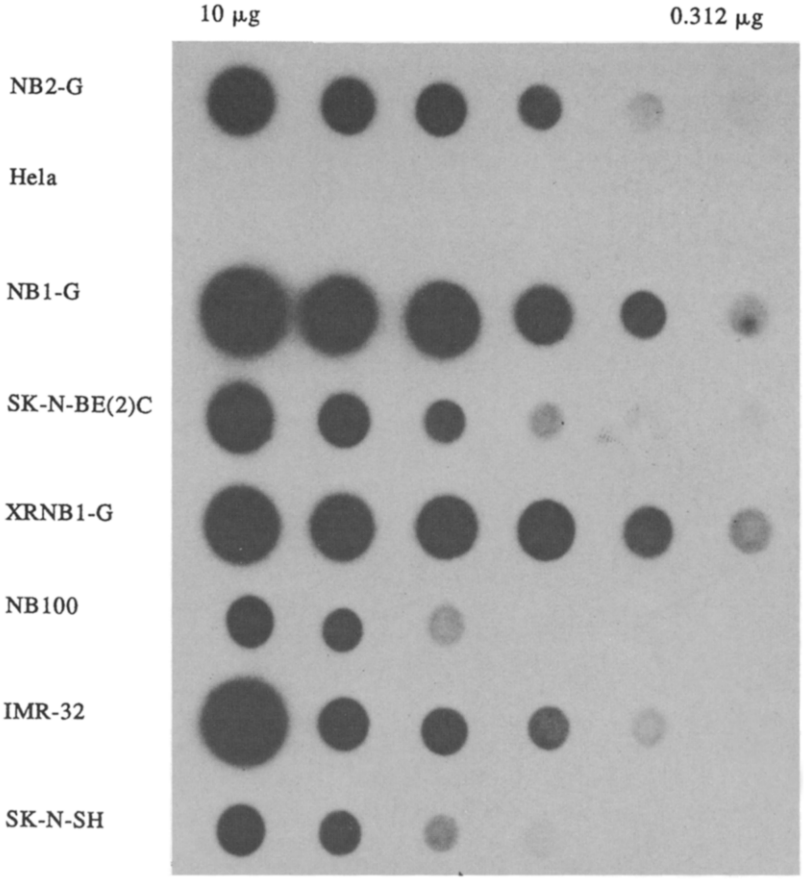

Fig. 3. Dot blot analysis of cell line RNA. Poly(A) RNA was denatured in formaldehyde at 65°C for 5 min and 2-fold serial dilutions were adjusted to 20 × SSC and applied to nylon. The blot was then hybridised as above.

Chemosensitivity

Survival curves (Figure 5) were constructed as for radiosensitivity, i.e. triplicate determinations of at least three separate experiments. Response to chemotherapy was estimated by comparison of isoeffective dose (IED): the concentration (μM) of cisplatin required to produce one log cell kill. This ranged from 7.5 μM for NB1-G to 13 μM for SK-N-BE(2)C (Table 1).

*Correlations between N-*myc *data and resistance to therapy*

The relationships between N-*myc* copy number or expression and resistance to radiotherapy or chemotherapy are summarised in Table 2. A significant relationship was demonstrated between N-*myc* copy number and cisplatin isoeffective dose. In order to accommodate the wide range of N-*myc* amplification values, the relationship between the $\log_{10}$ (N-*myc* copy number) and cisplatin isoeffective dose was also examined (Table 2). The correlation between these was slightly greater (Figure 6).

No correlation was found between N-*myc* expression and cisplatin resistance; nor between copy number or expression and response to radiotherapy. Furthermore, there was no relationship found between copy number and expression.

DISCUSSION

These studies have demonstrated an association between N-*myc* copy number and cisplatin resistance. In particular, the relationship between isoeffective dose of cisplatin and the logarithm of N-*myc* copy number showed a significant correlation. This represents the first demonstration of a possible explanation for treatment failure in neuroblastoma patients with amplified N-*myc*. Whether this is due to N-*myc*-enhanced DNA repair following cisplatin-induced cross-link damage or due to decreased cisplatin uptake remains to be investigated. Neither N-*myc* amplification nor its expression bore a relationship to the survival of cultured human neuroblastoma cells following their treatment with ionising radiation. There was no relation between N-*myc* expression and response to cisplatin treatment.

Multiple copies of certain genes or their enhanced expression are frequently associated with resistance to cytotoxic drugs in tumour cells. Studies on *mdr*-1 expression in neuroblastoma patients have shown that enhanced expression was a result of previous chemotherapy, but was not related to N-*myc* copy number [36]. However, in a study of 35 patients an inverse relationship between expression of the *mdr*-1 and N-*myc* genes was demonstrated [37].

There have been very few investigations into N-*myc* copy number and expression before and after chemotherapy in neuroblastoma patients. Those studies which have been carried out indicate an increase in N-*myc* expression after treatment, but no consistency in alteration of N-*myc* copy number [38–40]. These were not extensive studies and only involved a few patient samples. One large study [41] involving 60 patients, showed no variation in N-*myc* copy number throughout the course of treatment.

In our present study, we chose cisplatin because it is one of the most common drugs used in the treatment of neuroblastoma, either as a single agent or as part of a polychemotherapeutic regime. Although it does not participate in the multidrug resistance phenomenon, its effectiveness as a cytotoxic agent is often reduced by the development of drug-resistant tumour cells. Mechanisms of resistance to cisplatin are not fully understood, but perhaps enhanced DNA repair is involved [42,43].

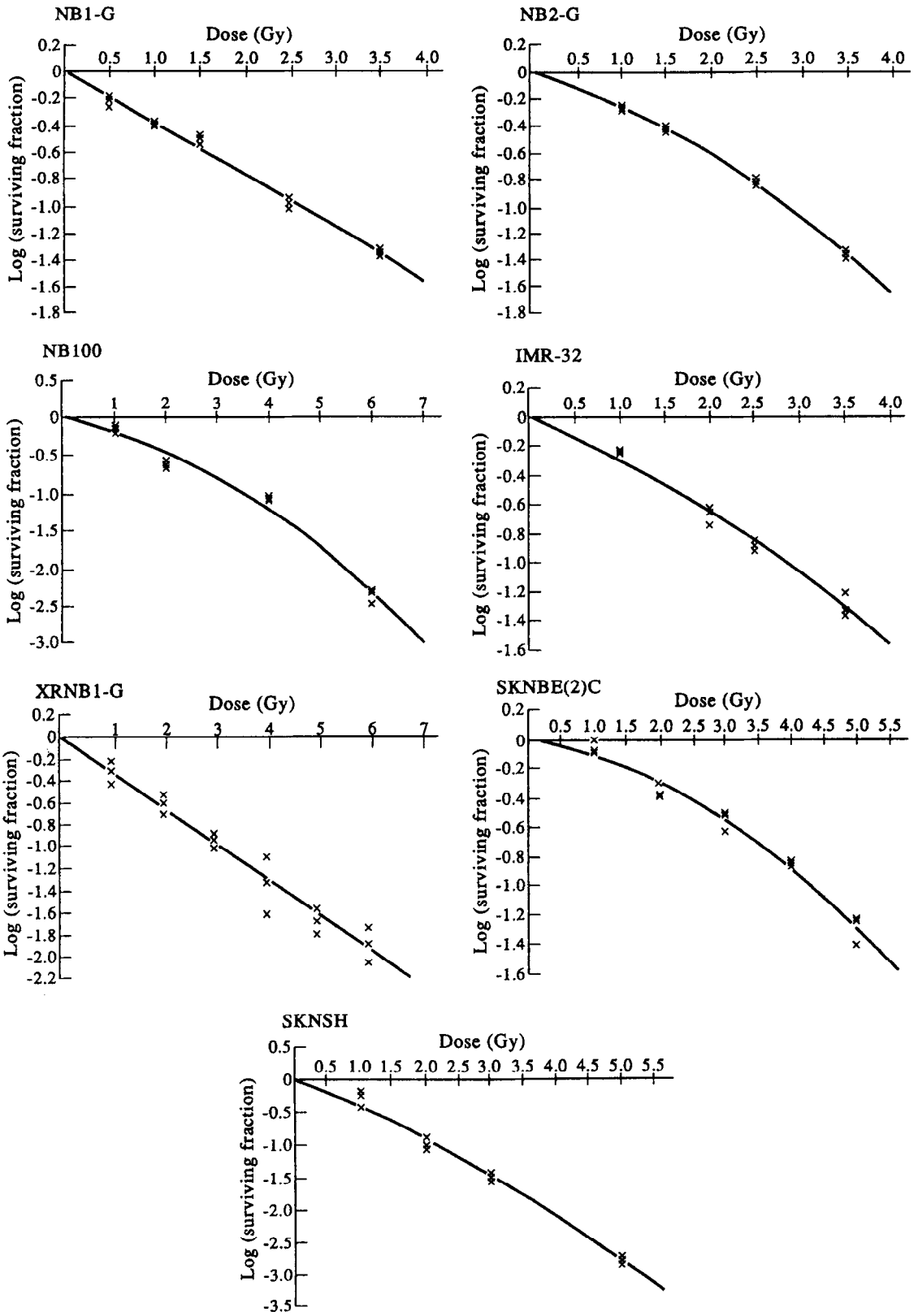

Fig. 4. Radiation survival curves for cell lines. Each plot shows the log of surviving fraction against dose (Gy). Three separate estimates were used for the surviving fraction at each dose.

It is important to establish whether the clinical association between elevated copies of the N-*myc* gene and poor prognosis is due to overproduction of N-*myc* protein, or whether N-*myc* amplification reflects the operation of some other oncogenic mechanism(s), such as the amplification of other putative oncogenes, or oncogene-activating translocations. Several workers [28, 30, 44] have found that the level of expression is not necessarily reflected in the degree of amplification of N-*myc*. This observation is supported by the present study (see Table 1). In a study of 33 neuroblastomas, Nisen and colleagues [28]

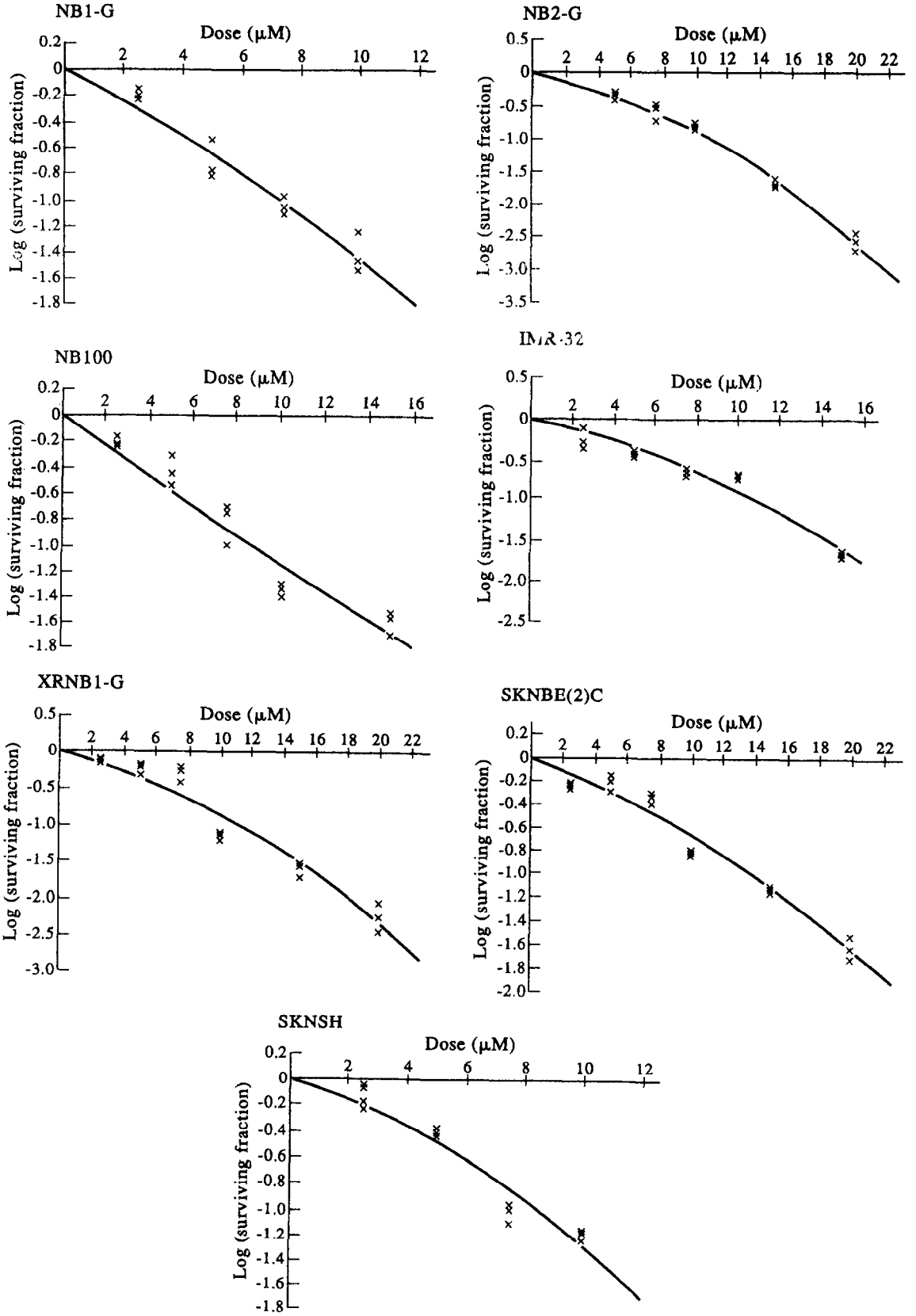

Fig. 5. Chemotherapy survival curves for cell lines. Each plot shows the log of surviving fraction against concentration of cisplatin (μM). Three separate estimates were used for the surviving fraction at each dose.

*Table 2. Relationship between N-*myc *data and resistance to therapy*

Relationship	*Spearman's statistic	*P*	Least squares analysis(*r*)	*P*
Copy no. versus SF_2	0.532	0.219	—	—
Expression versus SF_2	−0.255	0.582	—	—
Copy no. versus IED	0.766	0.044	0.708	<0.05
Log_{10}(copy no.) versus IED	0.766	0.044	0.806	<0.05
Expression versus IED	0.222	0.632	—	—
Copy no. versus expression	0.318	0.487	—	—

*See Materials and Methods for explanation. Data analysis was by Spearman's rank correlation test to assess correlation without assuming linearity. Where a positive indication of correlation was found, least squares analysis was performed to further analyse the relationship.

showed that N-*myc* amplification was related to poor clinical outcome whereas expression was of no prognostic significance. Clearly, further evaluation of the significance of N-*myc* overexpression is required.

Although the function of the N-*myc* protein is unknown in either neoplastic or normal tissues, it has been suggested that it may regulate gene expression during embryonic development since transcription factors contain regions homologous to N-*myc* [45, 46]. Rosolen and colleagues [47] used synthetic anti-sense oligonucleotides to specifically inhibit N-*myc* mRNA without affecting c-*myc* protein production, and showed a resulting decrease in growth in the CHP100 cell line (neuroepithelioma: single copy N-*myc*). A recent study showed an increase in the half life of the N-*myc* protein in a neuroblastoma cell line established from a patient with single copy N-*myc*, but rapidly progressive disease [48]. They suggested that since N-*myc* protein is thought to have a regulatory role, extension of its half-life may be important in the control of growth in those neuroblastomas lacking N-*myc* amplification, but which nonetheless rapidly proliferate. Recent studies suggest that N-*myc* expression, determined by immunohistochemical staining for the N-*myc* protein, was related to unfavourable prognosis [49, 50] and correlated with N-*myc* amplification [49]. The possibility of an association between expression and prognosis is an unresolved issue. In common with the majority of investigations, we could establish no such correlation. However, it is conceivable that expression levels, as assessed by mRNA assay, do not reliably reflect the true level of N-*myc* protein product.

In established cell lines, the majority of amplified N-*myc* DNA is found in homogeneously staining regions (HSR). These units can be as large as chromosome 1, and various estimates have shown that even 30 or so copies of N-*myc* may only constitute 0.1% of an HSR [51,52]. Therefore, it would seem likely that other important genes reside in an HSR. These putative genes have yet to be defined. It is possible that cisplatin resistance could be the result of expression of unknown genes which are co-amplified with N-*myc*.

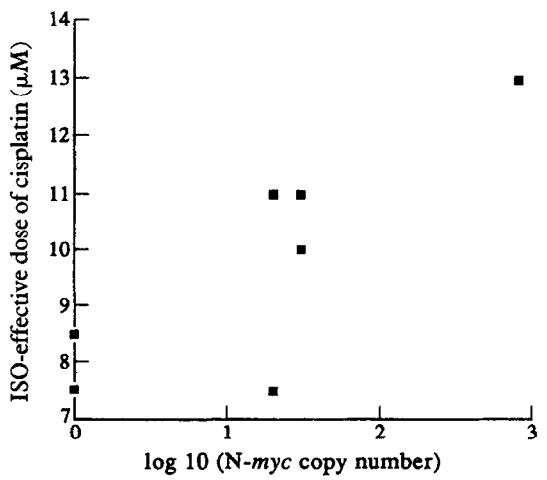

Fig. 6. Relationship between log_{10} (N-*myc* copy number) and iso-effective dose of cisplatin. Plot of iso-effective dose of cisplatin (μM dose required to produce 1 log cell kill) versus log_{10} (N-*myc* copy number) for the seven cell lines. Each point represents the result of at least three separate experiments for each cell line. Iso-effective dose was derived from survival curves.

CONCLUSIONS

We set out to investigate the relationship between N-*myc* amplification/expression and resistance to radiotherapy and chemotherapy in neuroblastomas. The results suggest a correlation between N-*myc* copy number and resistance to cisplatin. This could have important clinical relevance since cisplatin is one of the main treatments for neuroblastoma.

It is important to discover whether the statistical association between N-*myc* copy number and cisplatin resistance is causal and if so, whether N-*myc* amplification confers resistance to other drugs (e.g. etoposide, vincristine) which are useful in the treatment of neuroblastoma. It is possible that such studies could lead to new strategies for the treatment of neuroblastoma patients with amplified N-*myc* gene.

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