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Original Paper

Analysis of Candidate Gene Co-Amplification with *MYCN* in Neuroblastoma

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Previous studies have revealed that the *MYCN* gene spans approximately 7 kb, while the amplicon has been estimated to be 100 kb to 1 Mb long [1–3]. This implies that several other genes may be present on the *MYCN* amplicon. Such co-amplified genes could contribute to the malignant phenotype and might provide an explanation for why not all patients with *MYCN* amplification have a poor outcome. We investigated 7 neuroblastoma cell lines and 167 primary tumours for the co-amplification of candidate genes known to be present near the *MYCN* locus: ornithine decarboxylase, ribonucleotide reductase, syndecan-1 and a DEAD box protein gene, *DDX1*. We also investigated further the *pG21* expressed sequence previously reported to be co-amplified with *MYCN*. No co-amplification with the first 3 genes was found in any of the cell lines or tumour samples. *DDX1* was found to be amplified along with *MYCN* in 4/6 (67%) cell lines and 18/38 (47%) of the *MYCN* amplified tumours. No amplification of *DDX1*, *ODC1*, *RRM2* or *syndecan-1* was found in the absence of *MYCN* amplification. *DDX1* co-amplification was observed to occur mainly in stage 4 or 4S patients. With the exclusion of those with 4S disease, patients with *DDX1* co-amplification had a significantly shorter mean (±SE) disease-free interval (4.1 ± 1.4, $n = 8$ months) compared with patients with *MYCN* amplification alone (19.6 ± 4.5, $n = 17$) ($P = 0.04$, Welch's unpaired t-test). The *pG21* sequence was identical to part of the *DDX1* gene. These observations indicate that there is at least 1 other gene co-amplified with *MYCN* in a proportion of cases and that those patients with *DDX1* co-amplification tend to relapse more quickly. It also implies that the *MYCN* amplicon is of varied size and/or position relative to the *MYCN* gene. © **1997 Elsevier Science Ltd.**

Key words: neuroblastoma, *MYCN*, gene co-amplification, candidate genes, ornithine decarboxylase, ribonucleotide reductase, syndecan-1, DEAD box protein gene, *DDX1*, *pG21*
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INTRODUCTION

NEUROBLASTOMA IS the most common extracranial solid tumour of childhood, accounting for 10% of all paediatric malignancies [6]. The clinical behaviour of the tumour differs widely and ranges from spontaneous regression to differentiation to malignant progression. The search for predictive biological markers and their use to guide management has therefore been found to be especially applicable for neuroblastoma. From the prognostic factors explored to date, amplification of the *MYCN* oncogene has been found to be of particular significance. Amplification of the *MYCN* oncogene, located on Chromosome 2p24 occurs in approximately 20% of neuroblastomas overall and is associated with rapid tumour progression, advanced stages and a poor prognosis [7, 8]. However, the biological basis for this has not been elucidated and the relationship between *MYCN* amplification and outcome is not absolute, with 50% of children with

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localised (stage 2) disease having a good prognosis [9]. Also, there is no explanation for why a subset of patients without *MYC*N amplification have a poor prognosis [10].

Gene amplification is one of the ways in which oncogenes are activated from their normal state to alter the growth regulatory pathways of cells during tumorigenesis. The units of amplification (amplicons) have invariably been found to extend much further than the gene which has been selected for overexpression, with the result that flanking genes are co-amplified and consequently co-overexpressed. In neuroblastoma it is not clear whether the *MYC*N gene copy number alone is sufficient to explain the associated malignant phenotype or whether this is contributed to by the co-overexpression of other genes on the same amplicon. Previous studies have established that the *MYC*N gene spans approximately 7 kb, while the amplicon has been estimated to cover 100 kb to 1 Mb[1–3]. This opens up the possibility that several other genes may be present on the amplicon and that these may contribute to the malignant phenotype.

In order to identify any genes which may be co-amplified with *MYC*N, we examined neuroblastoma cell line and primary tumour DNA samples by Southern blot hybridisation for known candidate genes which map close to the *MYC*N locus: ornithine decarboxylase, ribonucleotide reductase, syndecan-1 and a DEAD box protein gene, *DDX1*, which is part of a gene family that encodes RNA helicases. We also investigated further a cDNA clone, pG21, previously reported to be co-amplified with *MYC*N in neuroblastoma lines and primary tumours [4, 5] and found it to represent part of the *DDX1* sequence.

MATERIALS AND METHODS

Cell lines

Human neuroblastoma cell lines, SKNSH [11], IMR-32 [12] GILIN [13], NB1G [14], NB2G [15], SKNBE [16] and BE(2c) [17], with varying degrees of *MYC*N amplification, were used. NB100, a neuroepithelioma cell line [18], was also used as an unamplified control. All cell lines were maintained at 37°C at 5% CO_2 in EMEM and RPMI 1640 media (GIBCO-BRL, Grand Island, New York, U.S.A.) supplemented with 10% fetal calf serum (GIBCO), 2 mM glutamine, 100 iu/ml penicillin and 100 μg/ml streptomycin. They were all harvested during the exponential growth phase so as to minimise cell cycle dependent variation in the pattern of expression.

Tumour samples

One hundred and sixty-seven primary tumour samples were obtained through the United Kingdom Children's Cancer Study Group (UKCCSG), and transported in dry ice or at ambient temperature in culture media. Sections of tumours had been fixed and stained by conventional histopathological methods at the referring centres and diagnosed and classified according to the criteria of Shimada and associates [19].

Southern blot analysis

Total genomic DNA was extracted from cell lines and primary tumour samples with a modification of the Nucleon™ method (Scotlab, Strathclyde, Scotland). Placental, spleen or leucocyte DNAs were used as normal diploid controls and the *MYC*N amplified cell line IMR-32 used as amplified control. For Southern blot analysis, 20 μg of DNA from each sample was digested to completion with *Eco*R1 restriction endonuclease (GIBCO-BRL, Paisley, Scotland), fractionated on agarose gels and transferred to nylon membranes (Hybond-N, Amersham, U.K.) [20]. They were then sequentially hybridised with ^{32}P-labelled *MYC*N, *syndecan-1*, *ODC1* (ornithine decarboxylase), *RRM2* (ribonuclease reductase) and *DDX1* probes labelled by the random primer hexanucleotide extension method [21]. Subsequent quantitative autoradiography analysis was carried out using a Molecular Dynamics Phosphorimager (Molecular Dynamics Corp., California, U.S.A.).

Gene probes

The *MYC*N probe used was a 1.0 kb *Eco*R1/*Bam*H1 insert from the human *MYC*N cDNA clone, pNB-1. This probe is specific for exon 2 of *MYC*N [22]. The candidate gene probes were all prepared by PCR (polymerase chain reaction) amplification of part of the published genomic or cDNA sequences which were then labelled with ^{32}P. The nucleotide sequences of the PCR products were verified by the dideoxy chain termination sequencing method [23] using the Sequenase PCR product sequencing kit (Amersham Life Science, U.K.).

The pG21 sequence

The pG21 cDNA clone in the pBR 322 plasmid vector was transformed into the *E. coli* strain, INVαF′ (Invitrogen Corp., The Netherlands). Plasmid DNA was extracted by the polyethylene glycol precipitation maxiprep method and sequenced by automated and manual methods using standard Sanger dideoxy chain termination methods. The sequences obtained were aligned with the published *DDX1* sequence using the DNAstar sequence analysis software package.

RESULTS

Cell lines

Six out of seven cell lines (86%) had multiple copies of the *MYC*N gene (Figure 1a and Table 1). In 4 of the 6 (67%) *MYC*N amplified cell lines, *DDX1* amplification was also present (Figure 1b). No *DDX1* amplification was seen in the SKNSH cell line with a single copy of *MYC*N. *DDX1* mRNA expression was also analysed in the cell lines and found to show general agreement with the degree of amplification (data not shown). There was no co-amplification of *ODC*, *RRM2* or *syndecan-1* in either the *MYC*N amplified or unamplified lines (data not shown).

Primary tumour samples

Of the 167 tumour samples analysed, 38 were found to have *MYC*N amplification (23%) (Figure 2a). The *MYC*N copy number ranged from 4 to 70 copies, with the majority of

Table 1. *Neuroblastoma cell lines showing* MYC*N and* DDX1 *amplification status*

Cell line	*MYC*N amplification	*DDX1* amplification
SK-N-SH	–	–
NB1-G	+	+
NB2-G	+	+
IMR-32	+	+
GI-LI-N	+	+
SK-N-BE	+	–
BE(2c)	+	–

tumour samples having less than 50 copies. Of the *MYCN* amplified samples, 18/38 (47%) showed co-amplification of *DDX1* (Figure 2b). No tumour had amplification of *DDX1* without concomitant *MYCN* amplification. From analysis of 69 tumour samples, no amplification of *ODC1*, *RRM2* or *syndecan-1* was seen in either the *MYCN* amplified or unamplified tumours.

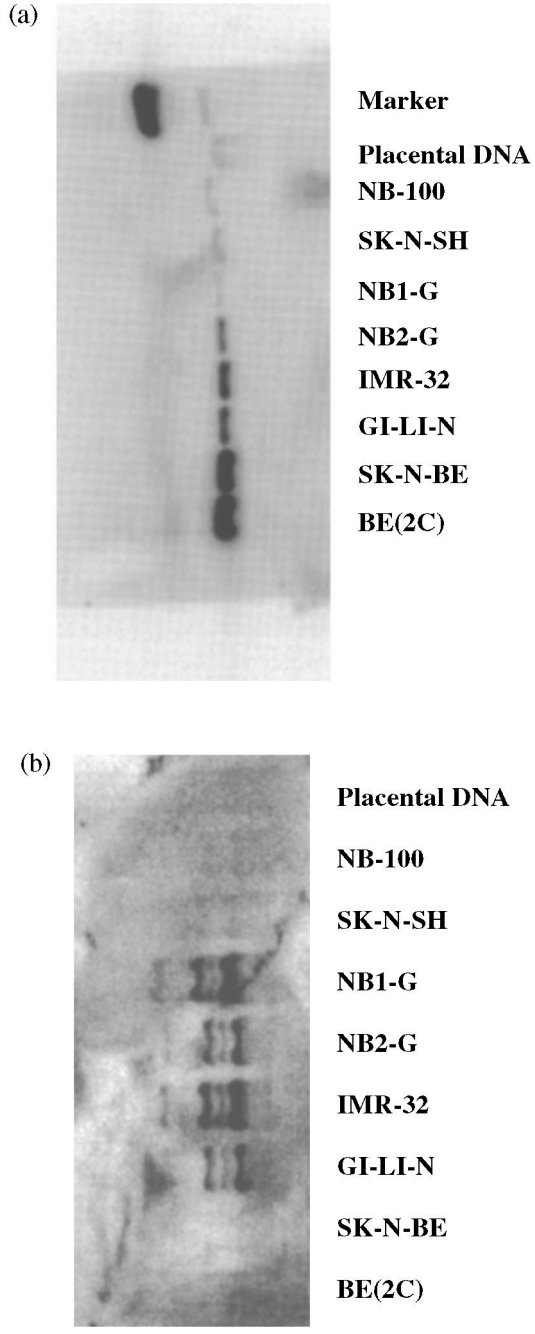

Figure 1. Southern analysis of *MYCN* and *DDX1* in neuroblastoma cell lines. (a) 20 μg each of genomic DNA from each cell line was digested with *Eco*R1 and probed for *MYCN*. *MYCN* amplification was detected in 6/7 cell lines. (b) Southern blot filter of cell lines reprobed for *DDX1* sequences. *DDX1* amplification was seen in 4/6 *MYCN* amplified lines i.e. NB1G, NB2G, IMR-32 and GILIN. No *DDX1* amplification was seen in the *MYCN* amplified lines SK-N-BE and SK-N-BE(2c).

Relationship to tumour stage

Co-amplification of *DDX1* with *MYCN* was found to occur predominantly in stages 4 and 4S (Table 2). Of the 10 patients with *DDX1* and *MYCN* co-amplification, 9 were stages 4/4S and one 2B. The only two 4S cases in the present series had co-amplification of *MYCN* and *DDX1* in their tumours. Of the 17 with amplification of *MYCN* only, 12 were stages 4/4S and 5 stages 1–3.

Relationship to patient response and survival

Of the 27 patients, 18 relapsed or had progressive disease (Table 2). Six out of seventeen patients with *MYCN* but not *DDX1* amplification are alive and disease free and 3/10 patients with both amplification of *MYCN* and *DDX1* are alive and disease free. Thus there was no significant difference in the proportion of survivors in these two groups. However, further analyses of those with stage 1–4 disease (excluding stage 4S cases) showed that there was a significantly shorter disease-free interval in those with amplification of the 2 genes ($P = 0.04$, Welch's unpaired t-test). The mean disease-free interval was 19.6 ± 4.5 (SE, $n = 17$) months for patients with *MYCN* amplification alone and 4.1 ± 1.4 (SE, $n = 8$) months for cases with co-amplification of *MYCN* and *DDX1*.

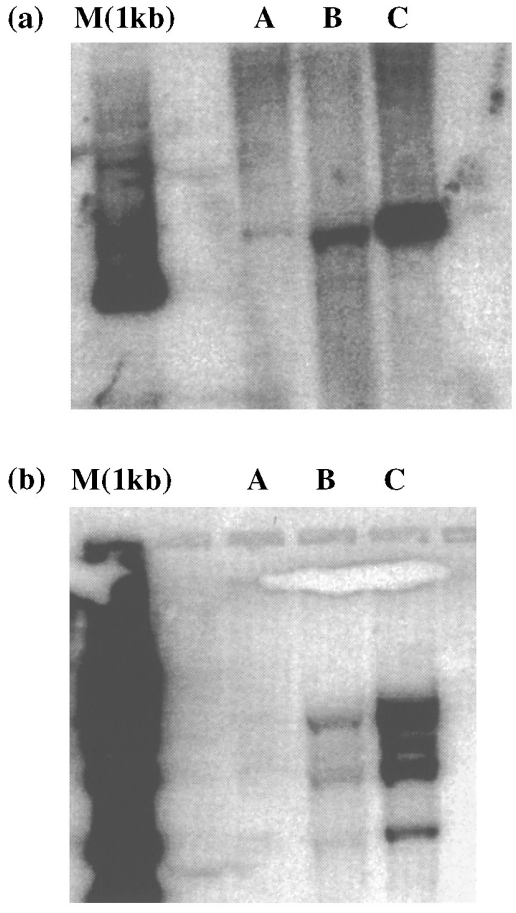

Figure 2. Southern blot analysis of tumour samples for *MYCN* and *DDX1* amplification. (a) This blot shows 1 *MYCN* amplified sample marked C. Lane A — spleen DNA (single copy control), B — IMR-32 (*MYCN* amplified control. (b) The same blot was then stripped of the *MYCN* probe and reprobed for *DDX1*. Co-amplification of *DDX1* was seen in the *MYCN* amplified tumour sample C and cell line, IMR-32.

The pG21 sequence

Kohl and associates, in a study of cell lines and tumours, reported an expressed sequence (pG21) to be amplified in 9 out of 11 neuroblastoma cell lines and 16 out of 25 tumours with amplified *MYC*N [4, 5]. However, these observations on co-amplification of the pG21 sequence have not been followed up and the cDNA sequence not determined. During the course of these studies we noted similarities between the *DDX1* gene and the pG21 cDNA clone. The transcript size detected by the pG21 probe is the same as that seen for *DDX1* and the frequency of co-amplification detected by the pG21 probe is also the same as that seen for *DDX1*. On sequencing the pG21 cDNA clone (kindly supplied by Nancy Kohl, Merck & Co. Inc., West Point, Pennsylvania, U.S.A.) it was confirmed to be the same as the published *DDX1* sequence. The relationship between the pG21 cDNA clone and the published *DDX1* sequence is shown schematically in Figure 3, in which the pG21 clone represents a portion of the *DDX1* cDNA fragment overlapping mainly with the 3′ segment of the *DDX1* coding sequence and extending to an *Eco*R1 site in the 3′ untranslated region. This is consistent with the *DDX1* probe recognising the same sized transcript on Northern blot analysis as that reported for pG21 and also with the recent mapping of *DDX1* to the same *Not*1 DNA fragment 5′ of *MYC*N [25] as that reported for pG21 [26] and further confirms the localisation of the pG21 sequence relative to the *MYC*N gene as reported by Noguchi and associates [27].

Table 2. Patient characteristics, MYC*N and* DDX1 *amplification status and survival data of the 27 evaluable primary neuroblastomas*

Patient	Age at diagnosis (years)	Stage	*MYC*N amplification	*DDX1* amplification	Status	FU (months)
1	1.3	3	+	–	DWD	27
2	1.0	2	+	–	ADF	14
3	0.3	4	+	–	ADF	55
4	2.8	4	+	–	ADF	55
5	3.6	4	+	–	ADF	41
6	0.5	3	+	–	DWD	5
7	1.0	4	+	–	AWD	14
8	1.8	4	+	–	AWD	24
9	1.0	1	+	–	DWD	7
10	2.0	4	+	–	DWD	9
11	0.3	4S	+	+	ADF	56
12	7.7	4	+	+	ADF	17
13	1.3	2B	+	+	AWD	8
14	0.6	4	+	+	DWD	4
15	2.2	4	+	+	AWD	10
16	1.7	4	+	+	DWD	15
17	14	4	+	+	ADF	12
18	2.2	4	+	–	DWD	0.5
19	1.5	3	+	–	ADF	10
20	0.8	4	+	+	DWD	10
21	11.4	4	+	–	DWD	8
22	1.5	4	+	–	DWD	4
23	U	4S	+	+	DWD	14
24	U	4	+	+	DWD	13
25	0.6	4	+	–	ADF	60
26	1.0	4	+	–	AWD	31
27	1.0	4	+	–	DWD	11

FU, follow-up time, or time to first relapse; ADF, alive and disease free; AWD, alive with disease; DWD, dead with disease.

DISCUSSION

The possible co-amplification of other genes with the *MYC*N gene in neuroblastoma has not been extensively explored. The *CYM*N gene, located on the antisense *MYC*N strand, has been found to be overexpressed in two neuroblastoma cell lines with amplified and overexpressed *MYC*N [28]. Treatment of these cell lines with retinoic acid caused a reduction in both *CYM*N and *MYC*N expression. It has been suggested that the close structural relationship between the 2 genes and their coregulation in tumour cell lines may indicate that *CYM*N is invariably amplified in tumours with *MYC*N amplification [28]. In another study [3] in which a detailed restriction map of the amplified core region in 33 neuroblastomas was constructed, the only CpG island found in a 130 kb region mapped by restriction endonuclease digestion was one associated with the *MYC*N gene. Hence, the conclusion from this study was that *MYC*N was the only gene in the core domain of the amplicon. However, 40% of human genes are not associated with a CpG island and so this does not exclude the possibility of other genes being co-amplified in a significant portion of *MYC*N amplified cases, especially since the *MYC*N amplicon has been reported to vary widely in size [1–3] and is generally bigger than the 130 kb core domain referred to by Hiemstra and associates [3]. The fact that neither the *ODC1*, *syndecan-1* or *RRM2* genes were amplified in either the cell lines or in the 69 tumour samples analysed in our study indicates that these genes are rarely included in the amplification unit and most probably are located at or beyond these distances from *MYC*N.

DDX1 is a member of the DEAD box protein family of genes which encode RNA helicases. *DDX1* was found to be co-amplified with the *MYC*N gene in the neuroblastoma cell line IMR-32 and two retinoblastoma cell lines [29]. This gene, also known as the HuDBP-RB (Human Dead Box protein identified in RB cells), has been mapped to Ch 2p24 [29]. The DEAD box protein family consists of at least 30 putative RNA helicases characterised by 8 conserved motifs including the D(asp)-E(Glu)-A(Ala)-D(Asp) box [30, 31]. The DEAD box proteins are involved in alteration of RNA secondary structure and have been implicated in the regulation of translation initiation and RNA splicing. Some members of the family are differentially expressed during embryogenesis, cellular growth and division [31]. One of the members of this family is the *RCK* gene. It has been found that rck/p54 protein expression is elevated in malignant transformed cells, such as neuroblastoma, which arise from tissues which normally show only low or no expression of this protein [32]. Because *DDX1* can also modify RNA secondary structure, amplification and consequent overexpression of

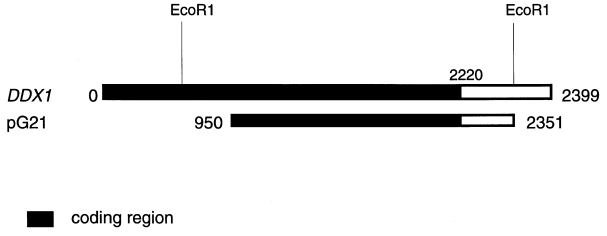

Figure 3. The relationship of the pG21 cDNA clone to the published *DDX1* sequence. The pG21 sequence was found to form part of the *DDX1* sequence, encompassing most of the coding region, extending from bases 950 to 2351.

this gene could result in significantly altered levels of mRNAs and proteins in the cell.

In the present study, *MYCN* amplification was seen in 23% of cases, in keeping with previously published data [7, 8]. Concomitant *DDX1* amplification was seen in 47% of the *MYCN* amplified samples. This compares with a recent study of 32 neuroblastomas by Squire and associates in which *DDX1* co-amplification with *MYCN* was seen in 7 out of 13 tumours (54%) [33]. In the present study, *DDX1* amplification was always associated with increased *DDX1* mRNA levels in the cell lines (data not shown). *DDX1* expression in tumour samples was not examined. In our earlier report of 16 cases with *MYCN* amplification, in which 6 had amplification of *DDX1*, we observed a general trend towards a poor prognosis in those cases with both *MYCN* and *DDX1* amplification compared to those with *MYCN* amplification alone [24]. With the number of samples increased to 27, the trend towards a significantly reduced mean disease-free interval for the subgroup of cases with co-amplification of *MYCN* and *DDX1* was maintained. This extends and strengthens data from recent studies by Squire and associates [33] and our own earlier report. Since then, 3 other studies have been published showing *DDX1* co-amplification with *MYCN* in neuroblastoma [25, 27, 34]. However, none of these papers have mentioned a significant difference in outcome between the two groups or related the observations to the stage and grade of the tumours.

The fact that there are other genes on the amplicon raises the question as to whether *MYCN* is the sole driving force after all, or whether there is a contribution by other expressed genes on the amplicon such as *DDX1*. Since *MYCN* is always present on the amplicon, it is hard to argue against the selection pressure being primarily for increased *MYCN* expression. However, this does not exclude the possibility that other genes such as *DDX1* may contribute to tumour progression or response to therapy, when co-amplified and overexpressed. This is supported by the shorter disease-free interval seen in cases showing *DDX1* co-amplification with *MYCN* compared with that seen in cases of *MYCN* amplification alone. Also there is a higher incidence of *DDX1* and *MYCN* co-amplification in stage 4 tumours, compared with stages 1–3, suggesting that *DDX1* may play a role in distant spread of the tumour.

The fact that co-amplification occurs in two retinoblastoma cell lines [29] as well as in neuroblastoma lines and tumours indicates that it may play a role in neuroectodermally derived tumours. Whereas *MYCN* regulates gene expression at the transcriptional level, this may be complemented by the action of *DDX1* on mRNA stability or other aspects of post-transcriptional control, such as translation initiation. Further studies are required to elucidate the exact role that *DDX1* plays in tumorigenesis in general and the specific contribution of *DDX1* in neuroblastoma outcome and progression. The possibility also remains that other genes in the vicinity of *MYCN* remain to be discovered.

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