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Two-Dimensional DNA Electrophoresis Identifies Novel CpG Islands Frequently Coamplified With *MYCN* in Neuroblastoma

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Background. Amplification of the oncogene MYCN in neuroblastoma has been found to correlate with aggressive tumour growth and is used as a predictor of clinical outcome. The MYCN amplicon is known to involve coamplification of extensive DNA regions. Therefore it is possible that other genes are coamplified in this amplicon and that they may play a role in the poor outcome of MYCN amplified tumours. ***Procedure.*** We have implemented an approach for the two-dimensional separation of human genomic restriction fragments to detect and isolate as yet unknown amplified sequences in the *MYCN* amplicon in neuroblastoma. Using this approach we have recently cloned a novel gene referred to as *NAG* that is frequently coamplified with *MYCN* in neuroblastoma. ***Results and Conclusions.*** We report here the identification and cloning of two ad-ditional CpG islands that are amplified in neuroblastoma. One contains a sequence that is identical to the first intron of *DDX1.* The other represents a novel CpG island that is associated with an as yet unidentified gene. We show that the novel CpG island is located in close proximity to the *MYCN* locus on chromosome 2 and is as frequently coamplified with *MYCN* in neuroblastoma as *NAG* and *DDX1.* Med. Pediatr. Oncol. 36:75–79, 2001. © 2001 Wiley-Liss, Inc.

Key words: gene amplification; neuroblastoma; *MYCN* amplicon

INTRODUCTION

Genomic amplification is a common mechanism leading to deregulated gene expression in human cancers. In neuroblastoma, *MYCN* amplification has been found to correlate with aggressive tumour growth and can serve as a predictor of clinical outcome [1,2]. There is evidence that amplification rather than up-regulation of basal expression is the main mechanism for the loss of autoregulation of *MYCN* [3] and its overexpression [4,5]. However, it is not clear how *MYCN* amplification results in poor outcome [6–8]. It is possible that coamplification of other DNA sequences plays a role in the poor outcome of *MYCN* amplified tumours.

Although the *MYCN* amplicon is known usually to involve coamplification of extensive regions of DNA, only the DEAD-box gene *DDX1,* to date, has been identified as being frequently coamplified with *MYCN* in neuroblastoma [9,10]. We have implemented an approach for the two-dimensional (2-D) separation of human genomic restriction fragments to detect novel genomic alterations in neuroblastoma cell lines and primary tumours [11,12]. The 2-D approach allows the identification and isolation of as yet unknown amplified sequences. The use of the enzyme *Not*I to digest DNA and the tagging of the fragments at the *Not*I ends allow preferential visualization and quantitative analysis of fragments containing CpG islands. Using this approach we have recently cloned a novel gene referred to as *NAG,* that is frequently coamplified with *MYCN* in neuroblastoma [13]. We report here the identification and cloning of two additional CpG islands that are amplified in neuroblastoma. One is identified as containing the first intron of *DDX1,* and the other represents a novel CpG island that is associated with an as yet unidentified gene. We show that the novel CpG island is frequently coamplified with *MYCN.*

MATERIALS AND METHODS

Cell Culture

Human neuroblastoma cell lines SKNSH [14], IMR-32 [15], KCNR [16], and NGP127 [17] have been characterized earlier. All STA-NB-3, -4, -8, -9, and -11 cell lines [18] have been established at the Children's Cancer Research Institute (Vienna, Austria) and showed the same karyotypic pattern as the tumors from which they were derived.

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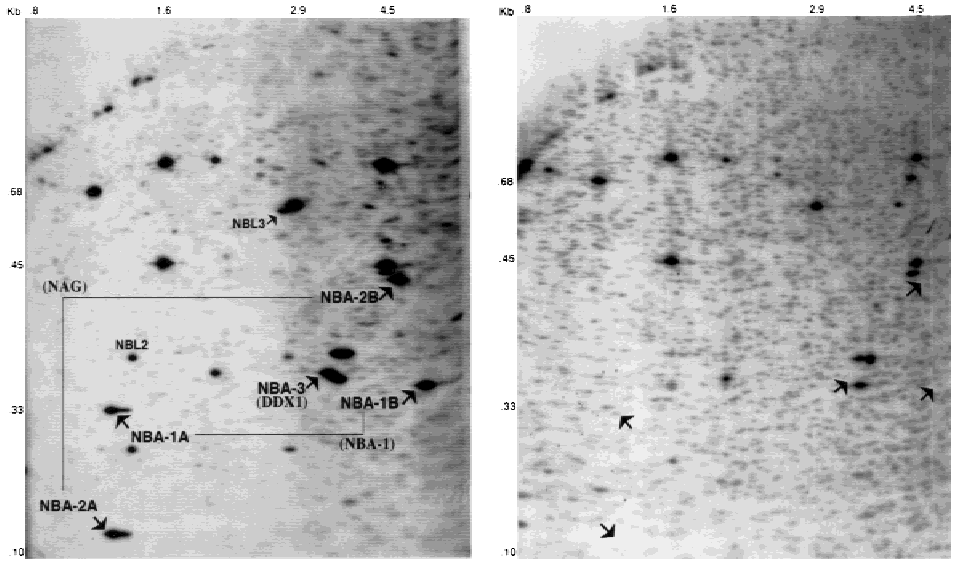

Fig. 1. Images of 2-D gels for neuroblastoma cell line STA-NB-11 **(left)** and peripheral blood lymphocytes from the patient from whose tumor the cell line was derived **(right).** Large arrows point to spots with increased intensity owing to the fragments being amplified. Thin lines join pairs of spots whose fragments share the same *Not*I restriction site. The names for genes are shown in parentheses. Small arrows point to fragments from tandemly repeated DNA that is often demethylated in neuroblastomas [11,23].

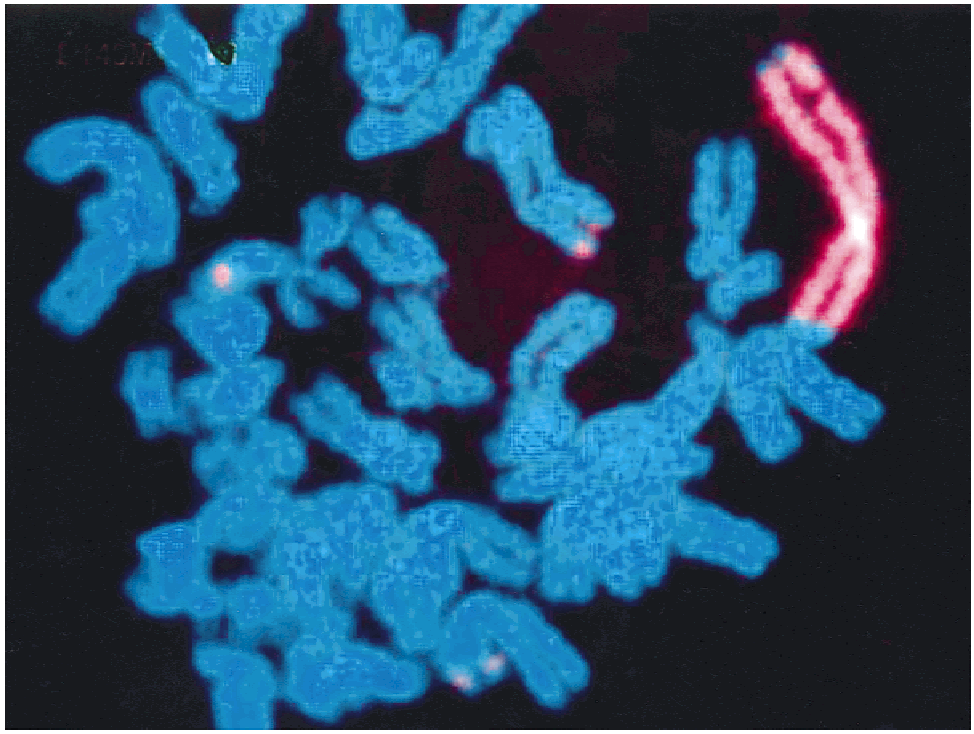

Fig. 2. Fluorescence in situ hybridisation of the CpG island *NBA-1* on metaphase chromosomes of neuroblastoma cell line NGP127. The BAC clone containing CpG island *NBA-1* was labeled with biotin and visualized with TRITC-labeled antibodies. Chromosomes were counterstained with DAPI.

2-D Gel Analysis

A detailed description of the experimental conditions can be found in [11]. Briefly, genomic DNA was digested with *Not*I and *Eco*RV restriction enzymes, and the *Not*I derived 5′ protruding ends were ^{32}P-labeled. After first-dimensional separation of the fragments in agarose disc gels, a second digestion was done in situ with *Dpn*II. The resulting fragments were separated perpendicularly in a polyacrylamide gel. 2-D gels were dried and exposed to PhosphoImager plates (Molecular Dynamics, Sunnyvale, CA). Digital images were obtained after scanning of the PhosphoImager plates.

Cloning of the Amplified DNA Fragments

DNA fragments were cloned from preparative gels as previously described [11]. Briefly, both radiolabeled and

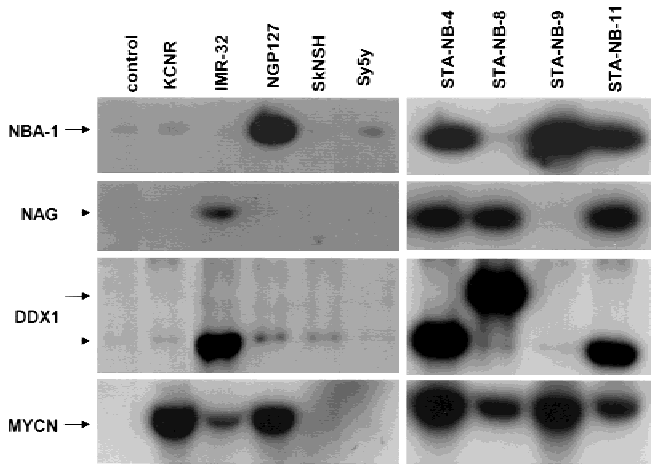

Fig. 3. Southern blot detection of *MYCN, DDX1, NAG,* and *NBA-1* amplification in neuroblastoma cell lines. Genomic DNA of nine cell lines and of peripheral blood lymphocytes of a healthy donor (control) was digested with *Not*I and *Eco*RV, the enzymes for the first-dimensional separation in the 2-D analysis. The filter was hybridized sequentially with a 0.8 kb fragment of the *MYCN* probe pNb-1 and with the cloned *Not*I/*Dpn*II fragment *NBA-3, NBA-2A,* and *NBA-1A* as probe for *DDX1, NAG,* and the CpG island *NBA-1,* respectively. The *DDX1* probe detects two different *Not*I/*Eco*RV fragments of 3.4 kb and 6.0 kb, which may represent a polymorphism for one of the restriction sites.

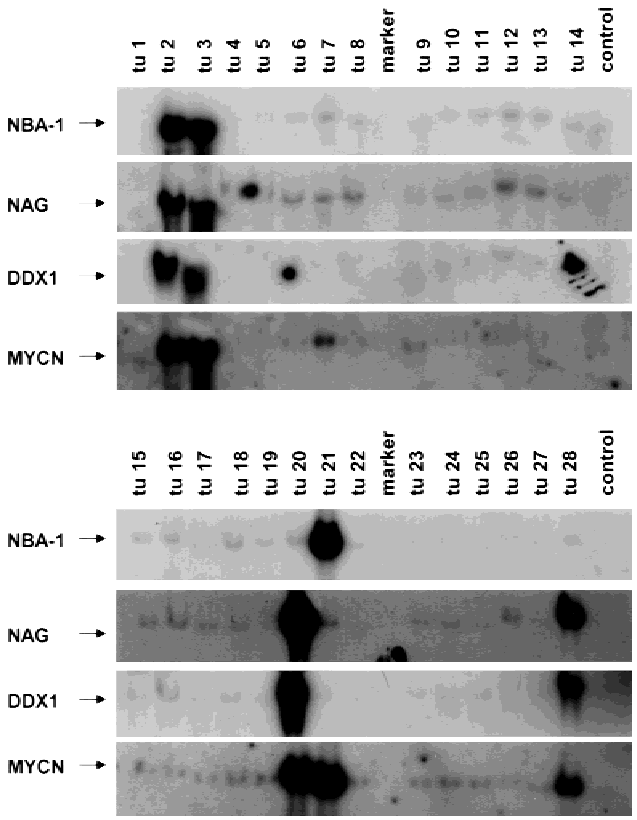

Fig. 4. Southern blot detection of *MYCN, DDX1, NAG,* and *NBA-1* amplification in neuroblastoma tumours. Tumour DNA and DNA of peripheral blood lymphocytes of a healthy donor (control) were digested with *Bam*HI. The filters were hybridized sequentially with probes as described for Figure 3.

nonradiolabeled genomic digests of cell line STA-NB-11 were loaded onto first-dimension agarose gels. After the second-dimension separation in polyacrylamide, the gels were exposed to X-ray film. The *Not*I/*Dpn*II fragments corresponding to the DNA spots of interest were recovered and ligated in a *Not*I/*Bam*HI digested pBC-vector (ClonTech, Palo Alto, CA) in the presence of DNA ligase at 16°C for 40 hr. The transformation was performed by electroporation with the Epicurian Coli XL1-Blue MRF electroporation-competent cells (Stratagene, La Jolla, CA).

RESULTS AND DISCUSSION

2-D DNA Electrophoresis Identifies CpG Islands Frequently Coamplified With *MYCN* in Neuroblastoma

We have undertaken an initial 2-D DNA electrophoresis analysis for six neuroblastoma cell lines. The 2-D patterns obtained were compared to a series of more than 200 patterns of a variety of normal tissues and other malignant and nonmalignant cell lines in order to identify multicopy fragments that are derived from amplified DNA in the neuroblastoma cell lines. Five *Not*I/*Dpn*II fragments designated *NBA-1A, -1B, 2A, -2B,* and *-3* (Fig. 1) were present in multiple copies only in neuroblastoma cell lines that contain an amplified *MYCN* gene as determined by Southern analysis. However, none of the five fragments was derived from the *MYCN* gene; the latter yields, with the enzyme combination utilized, fragments of >10 kb that are outside the first-dimension separation range.

Two fragments designated *NBA-2A* and *-2B* were recently cloned and shown to be part of a novel gene, which we have cloned and referred to as *NAG* [13]. *NAG* was shown to be frequently coamplified with *MYCN.* In this study we cloned the three other multicopy *Not*/*Dpn*II fragments *NBA-1A, -1B,* and *-3* from preparative 2-D gels as described in Materials and Methods. The cloned fragments were sequenced, and the novel sequences were compared to a known sequences from the EMBL database. Starting at the *Not*I site a stretch of 181 bp of the 290 bp fragment *NBA-3* was identical with a CpG island in the database. This sequence also matches the sequence for the first intron of *DDX1* (R. Godbout, personal communication). Fragments *NBA-1A* and *-1B* showed no significant homology with any of the known sequences in the database. Using the 253 bp fragment *NBA-1A* as a probe, we isolated a BAC clone (BAC14876). A 6.3 kb *Pst*I fragment of this BAC clone that encompassed *NBA-1A* was subcloned. Partial sequence analysis of the subclone revealed that the 287 bp fragment NBA-1B is en-

compassed in the BAC subclone and that fragments *NBA-1A* and *-1B* contained (one at its 5′ end and the other at its 3′ end) the same *Not*I restriction site and will thus be referred to together as *NBA-1*. Approximately 1,023 bp of the BAC subclone sequence surrounding the *Not*I site revealed the characteristics of a CpG island with a C and G content of 65% of the nucleotides and a CpG/GpC ratio of 0.61. Thus it is likely that NBA-1 represents a novel CpG island that is associated with an as yet unidentified gene. Using the BAC clone as probe, we performed fluorescence in situ hybridization (FISH) on metaphases of neuroblastoma cell line NGP127 (Fig. 2), which exhibits amplification of the NBA-1 fragment. Bright signals are observed at the chromosome bands 2p23–24 confirming the localization of *NBA-1* in close vicinity to the locus of the *MYCN* gene. Furthermore, hybridization signals in the HSR integrated at chromosome band 4p16 confirmed the coamplification of *NBA-1* with *MYCN* in the HSR of this cell line [19].

Comparison of the Frequency of Coamplification of *DDX1, NAG,* and fragment *NBA-1* With *MYCN*

Because *NBA-1* was found to be amplified in three of the four *MYCN*-amplified neuroblastoma cell lines in our initial analysis, it was likely that this CpG island is frequently encompassed in the *MYCN* amplicon in neuroblastoma as *DDX1* and *NAG*. To compare the frequency of coamplification of *DDX1*, *NAG*, and the newly cloned CpG island with *MYCN*, we utilized for Southern analysis the cloned *Not*I/*Dpn*II fragments *NBA-3* and *NBA-2A*, and *NBA-1A* as probes for *DDX1*, *NAG*, and the newly cloned CpG island, respectively (Figs. 3, 4). A total of 10 neuroblastoma cell lines and a total of 65 neuroblastoma tumor samples were analyzed. Amplification of either of the three fragments was found only in neuroblastomas that were also amplified for *MYCN*. In two of the eight *MYCN*-amplified cell lines (STA-NB-4 and STA-NB-11) and in five of thirteen *MYCN*-amplified tumors, all three fragments were amplified. In three cell lines (IMR-32, STA-NB-3, STA-NB-8) and in four tumours, both *DDX1* and *NAG* were coamplified with *MYCN*. Cell lines NGP-127 and STA-NB-9 and the remaining four *MYCN*-amplified tumors showed coamplification of only the *NBA-3* fragment with *MYCN*. Thus we found that *DDX1* and *NAG* were coamplfied in five of eight (62%) cell lines and in nine of thirteen (70%) of the tumors, and there were no cases in which one was amplified without the other. The *NBA-1* CpG island was coamplified with *MYCN* in four of eight (50%) of the cell lines and nine of thirteen (70%) of the tumours. Cell line KCNR but none of the tumors showed amplification of only the *MYCN* gene.

The *MYCN* amplicon contains two *Not*I sites located less then 50 kb from each other, within a 500 kb region at the 5′ end of *MYCN* [20]. It is likely that the CpG islands of *DDX1* and *NAG*, which were always coamplified in our series of neuroblastomas, contain the two *Not*I restriction sites. The CpG island encompassed by fragment *NBA-1* shows a partly overlapping but distinct pattern of coamplification compared to *DDX1* and *NAG*. Thus it may be presumed that *NBA-1* is located at the 3′ end of *MYCN*. According to Akiyama et al. [20], the closest *Not*I site 3′ of *MYCN* has been found at a distance of 680 kb.

Amplification of *MYCN* is a well-established prognostic factor in neuroblastoma, and ovenexpression of *MYCN* can contribute to cell transformation [21] and targeted expression of *MYCN* results in neuroblastoma in transgenic mice [22]. However, given that the *MYCN* amplicon contains additional genes including *DDX1*, *NAG*, or yet a third gene associated with the CpG island *NBA-1*, the heterogeneity in the *MYCN* amplicon may influence the biology of neuroblastoma.

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