Gene amplification in PNETs/medulloblastomas: mapping of a novel amplified gene within the *MYCN* amplicon

Michael C Frühwald, M Sue O'Dorisio, Laura J Rush, Jill L Reiter, Dominic J Smiraglia, Gail Wenger, Joseph F Costello, Peter S White, Ralf Krahe, Garrett M Brodeur, Christoph Plass

Division of Pediatric Hematology and Oncology, The Ohio State University and the Comprehensive Cancer Center, Columbus, Ohio 43210, USA
M C Früwald
M S O'Dorisio*

Division of Human Cancer Genetics, The Ohio State University and the Comprehensive Cancer Center, Columbus, Ohio 43210, USA
M C Früwald
L J Rush
D J Smiraglia
R Krahe
C Plass

Department of Neuroscience, The Ohio State University and the Comprehensive Cancer Center, Columbus, Ohio 43210, USA
M C Früwald

Department of Veterinary Biosciences, The Ohio State University and the Comprehensive Cancer Center, Columbus, Ohio 43210, USA
L J Rush

Department of Biochemistry and Molecular Biology, Mayo Clinic, Rochester, Minnesota 55905, USA
J L Reiter

Department of Pathology, The Ohio State University and the Comprehensive Cancer Center, Columbus, Ohio 43210, USA
G Wenger

University of California, San Francisco, CA 94115-0128, USA
J F Costello

Division of Oncology, The Children's Hospital of Philadelphia and the University of Pennsylvania, Philadelphia, Pennsylvania 19104-4318, USA
P S White
G M Brodeur

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Abstract

***Objectives*—The pathological entity of primitive neuroectodermal tumour/ medulloblastoma (PNET/MB) comprises a very heterogeneous group of neoplasms on a clinical as well as on a molecular level. We evaluated the importance of DNA amplification in medulloblastomas and other primitive neuroectodermal tumours (PNETs) of the CNS.**

***Method*—Restriction landmark genomic scanning (RLGS), a method that allows the detection of low level amplification, was used. RLGS provides direct access to DNA sequences circumventing positional cloning efforts. Furthermore, we analysed several samples by CGH.**

***Design*—Twenty primary medulloblastomas, five supratentorial PNETs, and five medulloblastoma cell lines were studied.**

***Results*—Although our analysis confirms that gene amplification is generally a rare event in childhood PNET/MB, we found a total of 17 DNA fragments that were amplified in seven different tumours. Cloning and sequencing of several of these fragments confirmed the previous finding of *MYC* amplification in the cell line D341 Med and identified novel DNA sequences amplified in PNET/MB. We describe for the first time amplification of the novel gene, *NAG,* in a subset of PNET/MB. Despite genomic amplification, *NAG* was not overexpressed in the tumours studied. We have determined that *NAG* maps less than 50 kb 5' of *DDX1* and approximately 400 kb telomeric of *MYCN* on chromosome 2p24.**

***Conclusion*—We found a similar but slightly higher frequency of amplification than previously reported. We present several DNA fragments that may belong to the CpG islands of novel genes amplified in a small subset of PNET/MB. As an example we describe for the first time the amplification of *NAG* in the *MYCN* amplicon in PNET/MB.**

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Recent studies have attempted a molecular staging of childhood PNET/MB. Loss of heterozygosity (LOH) for chromosome 17p is present in up to 50% of medulloblastomas (infratentorial PNETs), while it was not observed in supratentorial PNETs.[5 6] In addition, there is an association between LOH for 17p and metastatic disease and a strong correlation between *MYC* amplification and poor prognosis in patients with PNET/MB.[7] The amplification of proto-oncogenes such as *MYC* has been used as a marker for advanced stage disease in other childhood tumours, especially neuroblastoma. A direct correlation between copy number in the proto-oncogene *MYCN* and clinical outcome is used to assign patients with neuroblastomas to therapeutic groups.[8]

Gene amplification appears to be an uncommon event in childhood PNET/MB. Fuller *et al*[9] pooled data from five independent studies and found that amplification of the genes for *MYC*, *MYCN*, and the epidermal growth factor receptor (*EGFR*) together was present in only 10% of medulloblastomas. DNA amplification often results in increased expression of otherwise tightly controlled genes. However, alternative mechanisms that augment gene expression exist.[10–12] Amplification of certain genes was originally described as a mechanism to prevent drug toxicity; it is now clear that gene amplification plays an important role in the progression of neoplasias owing to overexpression of growth promoting oncogenes.[12] The amplicon size in tumour cells is usually much bigger than the transcriptional unit of a single oncogene and includes several coamplified genes, a situation which complicates the identification of the actual target gene. *MYCN* amplicon sizes range from 350 kb to more than 1 Mb.[13 14] It has therefore been suggested that genes other than *MYCN* within the associated amplicon may contribute to the oncogenic potential of this amplification.[15 16]

Previous studies have shown the utility of restriction landmark genomic scanning (RLGS) in the analysis of DNA amplification in human tumour samples[17–19] because the intensities of DNA fragments visualised on RLGS autoradiographs correlate accurately with DNA copy number. Therefore, RLGS can be used to detect even low levels of gene amplification.

In the current study we used RLGS to evaluate the importance of DNA amplification in PNET/MB of the CNS. DNA fragments showing enhanced intensity on RLGS profiles were cloned by means of a new and efficient technique.[17] Our analysis showed that gene

amplification is an underestimated event in PNET/MB. We provide the first data on amplification and chromosomal mapping of the gene *NAG*, coamplified in the *MYCN* amplicon in supratentorial PNETs and medulloblastomas.

Methods

PATIENTS AND CELL LINES

Twenty five PNET/MB samples were analysed (table 1). All samples were obtained through the Cooperative Human Tissue Network (CHTN, Midwestern Division) in accordance with NIH guidelines. Tumour tissue was obtained at neurosurgery and immediately frozen in liquid nitrogen. Control DNA was from normal cerebella (4 year old female, 4 day old male, 11 year old male), an adult cortex, and several peripheral blood lymphocyte (PBL) samples (newborn, 3-4 years, 8-9 years, and adult). Control tissues were derived from healthy donors or patients that had died of causes unrelated to neoplasia or neurological disease. Procedures were approved by the Children's Hospital's review board. The cell lines Daoy, D283 Med, and D341 Med were obtained from ATCC (Rockville, MD) and the lines MHH-MED-1 and MHH-PNET-5 were provided by T Pietsch (Universität Bonn, Germany). Cells were cultured under conditions previously published with the exception that human umbilical cord blood serum was used as a supplement.[20]

ISOLATION OF NUCLEIC ACIDS

High molecular weight DNA for the RLGS procedure was isolated according to our previously published protocol.[17] Plasmid DNA was isolated using a QIAGEN Plasmid Mini kit (Qiagen Inc, Santa Clara, CA) and recommended protocols. Total RNA was isolated using the TRIzol reagent (GIBCO, Grand Island, NY) according to the distributor's suggestions.

RLGS

RLGS analyses of tumours and control DNA were performed as previously described.[21] All tumour and cell line RLGS profiles were compared to control profiles from three normal cerebella, an adult cerebral cortex, and at least four peripheral blood lymphocyte (PBL) profiles from children of different age groups. RLGS profiles were analysed by overlaying tumour with normal profiles and marking differences in fragment intensity on clear acetate sheets. Fragment intensity was scored by comparing individual to surrounding single copy fragments. Amplification was estimated according to the difference in intensity. To obtain information on the chromosomal location of the fragments of interest, profiles were compared to an overlay taken from a chromosome assigned RLGS (CA-RLGS) profile created by Yoshikawa *et al*.[22]

CLONE ISOLATION AND RESTRICTION MAPPING

*Not*I/*Eco*RV clones were isolated from a human genomic library previously established in our laboratory.[23] Clones of interest were identified using a catalogue of RLGS mixing profiles. The construction and applications of this tool have recently been described.[17]

SOUTHERN ANALYSIS AND PROBES

To confirm amplification of the DNA fragments 4C20 and 4C23, genomic DNA was digested with *Eco*RV alone and in combination with *Not*I. A total of 7.5 µg DNA were separated in 0.8% agarose gels and transferred onto nylon membranes by vacuum blotting (Pharmacia). Probes for hybridisation included the 1.3 kb *Not*I/*Eco*RV clone 4C20, a 600 bp *Eco*RV/*Sac*I fragment of 4C23, and a 400 bp *Xba*I fragment from exon 2 of *MYCN*.

CHROMOSOMAL MAPPING

Human specific PCR primer pairs of clones 4C20 and 4C23 were designed from the corresponding *Not*I/*Eco*RV clones. The primers were: 4C20-F, 5'-GGC AAC AAA CAT GGT GC-3' and 4C20-R, 5'-TCC ATC AGG GAG CGA AAA GAG-3'; 4C23-1, 5'-GGG GAG CAG GTT ATC TGT CAT-3' and 4C23-2, 5'-AGTAGG CAG AAG TAC AGG GC-3'; and 4C23-3, 5'-AGA GGA CAA GGA GCA GGA CAA GC-3' and 4C23-4, 5'-GGG GAC AGA ACC AAG AAT CA-3' amplifying fragments of 115 bp, 136 bp, and 143 bp, respectively. PCR conditions were initial denaturation at 95°C for five minutes followed by 30-35 cycles of 95°C for 30 seconds, 59°C for 30 seconds, and 72°C for 30 seconds, and a final extension of 72°C for 10 minutes. Initial chromosomal assignment was performed with a mono-chromosomal human-rodent somatic cell hybrid panel as described elsewhere.[24] For regional assignment and fine mapping, the GeneBridge 4 (GB4RH),[25 26] G3,[27] and TNG4 (http://www-shgc.stanford.edu/RH/) radiation hybrid mapping panels were used.[28] Minimum lod score thresholds of 15 and 4 (as the chromosomal assignment could be specified), respectively, for linkage to markers in the framework map were used for analysis of the GB4 and G3/TNG mapping panel data as recommended in the instructions for the respective RH mapping servers (http://carbon.wi.mit.edu:8000/cgi-bin/contig/rhmapper.pl and http://www-shgc.stanford.edu/RH/rhserverformnew.html). The cytogenetic location of each clone was obtained by determining the location of the flanking framework markers in the location database map (http://www.cedar.soton.ac.uk/). Each RH mapping analysis was done in duplicate.

COMPARATIVE GENOME HYBRIDISATION

CGH was performed according to the method described by Kallioniemi *et al*.[29]

MAPPING OF NAG TO A *MYCN* YAC CONTIG

Clones for a yeast artificial chromosome (YAC) contig of the *MYCN* locus have been described previously.[30] Yeast colonies were picked from AHC plates, dissolved in 50 µl H_2O, and then heated in a thermal cycler to 94°C and 55°C, each for five minutes in three sequential steps.

Correspondence to:
Dr Frühwald, Westfälische Wilhelms-Universität Münster, Klinik und Poliklinik für Kinderheilkunde, Pädiatrische Hämatologie/Onkologie, Albert-Schweizer-Strasse 33, 48149 Münster, Germany, fruhwald@uni-muenster.de or Dr Plass, plass-1@medctr.osu.edu

*Present address: Department of Pediatric Hematology and Oncology, The University of Iowa, Iowa City, Iowa 52242, USA

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Table 1 Clinical characteristics and amplification data of primary tumour samples and cell lines

							Enhanced RLGS fragments																
Sample No	*Code*	*Dx*	*Gender*	*Age (y)*	*Outcome*	*CGH*	*4C20 (2)*	*4C23 (2)*	*4F62 (2)*	*3D45 (ND)*	*3C2 (ND)*	*3F6 (ND)*	*3G68 (ND)*	*3C15 (ND)*	*3C36 (ND)*	*5C10 (ND)*	*5B35 (ND)*	*4C3 (8)*	*3B35 (8)*	*4A5 (2)*	*4A23 (2)*	*3C25 (2)*	*4F53 (2)*	*3G75 (ND)*	*5E60 (ND)*
1	0107	MB	M	26	D																				
2	1014	MB	F	2	A							+													
3	9704P191	MB	M	7	A																				
4	9707P304	MB	M	3	D																				
5	9609P256	MB	M	6	D	+2p24, +3p25, +7q32-35, −9q, +10p, −10q23-ter, +12p13, +13q32, −15q26, +18, +19p13.3, −19q13.3-ter, −20p13, +21, −Xq26-ter	+	+	+																
6	9601P300	MB	M	4	A	-4q26-ter, +7q11.21,−8p23, +8q23-ter, −9p, −16q						+		+	+	+									
7	9501P002	MB	M	7	A							+					+								
8	9503P010	MB	M	9	A	+4q16, +5p15, −9q				+	+	+	+												
9	9712P611	MB	F	12	A																				
10	9410P009	MB	M	16	A							+													
11	9001Z04	MB	M	6	A																				
12	9812P5013	MB	M	14	A												+								
13	9501P050	MB	M	6	A																				
14	9611P246	MB	F	5	A																				
15	9709P601	MB	M	6	A																				
16	9708P618	MB	F	14	A																				
17	9903P901	MB	M	6	A		+					+		+											
18	9001EE01	MB	M	6	A																				
19	9905P6002	MB	M	6	A																				
20	203	MB	F	4	A								+												
21	9805P620	PN*	F	7	D		+		+									+		+	+	+	+	+	+
22	9501P003	PN	M	3	D																				
23	9602P366	PN	M	4	A																				
24	9408P004	PN	F	3	D																				
25	8910Z01	PN	M	6	?							+													
26	D341-MED																	+	+						
27	D283-MED																		+						
28	Daoy																								
29	MMH-MED-1																								
30	MMH-PNET-5																								

Dx, diagnosis; MB, medulloblastoma; PN, supratentorial PNET, A, alive; D, dead; ?, lost to follow up; + enhanced RLGS fragment.
*Secondary tumour 2 years after chemotherapy and radiation for a primary medulloblastoma.
The chromosomal location ((2), (8), or (ND), chromosome 2, 8, or assignment not possible) of RLGS fragments was determined using the CA-RLGS profile.[22]

The solution was spun down and 5 µl of the supernatant were used for PCR. To confirm the results of the PCR, 200-500 ng of YAC DNA were digested with *Eco*RI and electrophoresed on 0.8% agarose gels and then Southern blotted and hybridised with a probe derived from RT-PCR amplification of EST k6533. The primers used for this were: forward 5'-GAG GCA ACC AAA AAC ATG GT-3' and reverse 5'-TGC CTG GAA GGA CCA AAT CC-3'. The resulting PCR product was 170 bp.

SEQUENCING AND DATABASE ANALYSIS

All sequence analyses were performed in the Core Facility of the Division of Human Cancer Genetics using an ABI PRISM 377 DNA sequencer. For CG rich sequences, high annealing temperatures were used with an ABI PRISM BigDye Terminator Cycle Sequencing kit. *Not*I/*Eco*RV clones were sequenced with M13 forward and reverse oligonucleotides and by primer walking. DNA sequence files were analysed using DNAstar and Chromas software. For homology searches, sequences were submitted to the publicly available databases.

SEMIQUANTITATIVE RT-PCR

Five µg of total RNA were reverse transcribed in a volume of 20 µl for each primer, using Thermoscript (Gibco BRL, Grand Island, NY) and oligo-dT as well as random hexamers according to the manufacturer's recommendations. After termination of the transcription reaction, oligo-dT, and random hexamer primed cDNAs were combined and diluted to 80 µl. One µl of the resulting mix was used for RT-PCR experiments. Primers for the housekeeping gene, *GPI* (glucose phosphate isomerase), were *GPI*f 5'-GAC CCC CAG TTC CAG AAG CTG C-3' and *GPI*r 5'-GCA TCA CGT CCT CCG TCA CC-3' (178 bp). Primers for k6533 were k6533F 5'-AGC TTG GCC ACC TAT GAA AA-3' and k6533R 5'-AGG GAC TGG TTA AGC AGC AG-3' (210 bp). All RT-PCR primer pairs spanned introns. Forward primers were end labelled with [^{32}P]dATP using T4 polynucleotide kinase (Gibco BRL). Multiplex PCR reactions were carried out in 50 µl using 1 µl cDNA, 10 pmol of each primer (1:10 labelled/unlabelled), 1.5 mmol/l $MgCl_2$, 0.2 mmol/l each dNTP, 10 × PCR reaction buffer (Gibco BRL), 2.5% DMSO (dimethylsulphoxide), and 2.5 U Platinum *Taq* (Gibco BRL). Reactions were performed in a Perkin Elmer 9700 GeneAmp thermal cycler. Initial denaturation was 95°C for 10 minutes, followed by 21 cycles of 96°C for 30 seconds, 58°C for 30 seconds, and 72°C for one minute. Twenty one cycles were determined to be within the exponential range of amplification for both *GPI* and k6533 by ImageQuant software (Molecular Dynamics, Sunnyvale, CA). Eight µl of each reaction were run on an 8% polyacrylamide gel and bands were visualised using a phosphorimager (Molecular Dynamics). The ratio of *GPI* to k6533 products was determined for each sample using the ImageQuant software. All experiments were performed in duplicate.

NORTHERN HYBRIDISATION

Hybridisation of multi-tissue northern blots, MTN-Blots, (Clonetech, Palo Alto, CA) was performed as recommended by the supplier.

Results

DETECTION OF ENHANCED DNA FRAGMENTS IN PNET/MEDULLOBLASTOMA BY RLGS

Twenty medulloblastomas and five supratentorial PNETs were analysed by RLGS for the presence of DNA amplification. RLGS profiles were created using *Not*I as the restriction landmark enzyme followed by *Eco*RV and *Hin*fI digests. Tumour samples were scored for the presence of enhanced RLGS fragments indicative of DNA amplification. A total of 19 different RLGS fragments showed a higher intensity in the tumour profiles compared to control profiles (fig 1). A summary of the tumours that exhibited enhanced fragment intensities and the putative chromosomal location of the corresponding DNA fragments as far as we could evaluate from chromosome assigned RLGS (CA-RLGS) is provided in table 1.[22]

Because *Not*I is a methylation sensitive restriction enzyme, the analysis of DNA amplification is complicated. Enhanced fragments in a tumour profile may result from either DNA amplification or hypomethylation of normally methylated *Not*I sites. Indeed, two of the 19 enhanced fragments (3F6 and 3G68) that we observed had been identified previously as repetitive elements, which exhibit variable hypomethylation.[31 32]

Equal enhanced fragment intensities on the same RLGS profile suggest that fragments belong to the same amplicon. This notion is supported by the finding that the fragments 4C20, 4C23, 4F62, 4A5, 4A23, 3C25, and 4F53 are all located on chromosome 2, as determined by comparison of RLGS profiles with the CA-RLGS profile by Yoshikawa *et al*.[22] In addition, fragments 4C20 and 4C23 detect the same sized amplified fragment on Southern analysis in tumour sample No 5 (data not shown). Tumour sample No 21 showed two groups of enhanced DNA fragments: group 1, 4C20, 4F62, 4A5, 4A23, 3C25, 4F53 presumably all located on chromosome 2 and group 2, 4C3 and 3B35 localised to chromosome 8 as described above. The DNA fragments within a group exhibited equal intensity on autoradiography, while there was a distinct difference in intensity between fragments of groups 1 and 2 suggesting their derivation from two different amplica.

Furthermore, three samples (Nos 5, 6, and 8) that had shown fragment enhancements on RLGS were analysed using CGH (table 1). Among other cytogenetic aberrations medulloblastoma sample No 5 showed an amplification of 2p24. Tumour sample No 8 showed, besides other rearrangements, gain on the short arm of chromosome 5 (5p15) a region that has been found amplified in medulloblastomas.[33] RLGS fragments 3D45 or 3C2 could be derived from this amplicon on 5p15. Sample

No 6 exhibited gains on chromosome 7q and 8q and RLGS fragments 3C15, 3C36, or 5C10 could represent these amplica.

Excluding the two demethylated repetitive element sequences described above, a total of 17 DNA fragments were found enhanced in seven out of 25 PNET/MB. One recurrent PNET (No 21) showed the maximum of eight aberrant DNA fragments, supporting the fact that genetic abnormalities, including gene amplifications, accumulate in the course of disease progression.

In summary, we have identified three tumours (Nos 5, 17, and 21) with amplification involving a region on chromosome 2p24, possibly *MYCN*, one tumour (No 8) with possible amplification of 4q16 or 5p15, and a fifth tumour (No 6) with gains on 7q11 and 8q23. Two additional tumours (Nos 7 and 12) showed enhancement of DNA fragment 5B35, which could either represent DNA amplification or hypomethylation since both tumours showed enhancement of the previously described hypomethylated fragment 3F6 (table 1). Thus a minimum of five out of 25 (20%) tumours exhibited gene amplification, confirmed by at least two different techniques (CGH and RLGS or RLGS and Southern blotting). This number is higher than the 10% published and could be as high as 28% (seven tumours with enhanced RLGS fragments out of a total of 25 analysed tumours).[9]

INCREASED FRAGMENT INTENSITY CORRESPONDS TO DNA AMPLIFICATION

To confirm the results of our RLGS analysis, the enhanced DNA fragments 4C20, 4C23, and 3B35 were cloned and sequenced.[17] Fragment 3B35 was mapped to chromosome 8 by comparison to the CA-RLGS profile and identified as part of the 5' CpG island and first exon of the *MYC* gene.[17] This fragment was enhanced only in the cell lines D283 Med and D341 Med. We estimated a 20-fold amplification in D341 Med and a five-fold amplification in the D283 Med RLGS profile. This is in accordance with the data previously published.[34 35]

Chromosomal assignment of 4C20 and 4C23 using a mono-chromosomal mapping panel verified the localisation obtained by comparison to the CA-RLGS profile to chromosome 2. When used as a probe for Southern analysis on primary medulloblastomas and supratentorial PNETs, 4C20 detected amplification of this DNA in the same samples as seen on RLGS (Nos 5, 17, and 21). Fig 2A shows as a representative example the Southern blot of tumour No 21. The same level of amplification as for 4C20 was seen for 4C23, which was changed only in tumour sample No 5 (data not shown). To refine the chromosomal location, we carried out radiation hybrid mapping with three independent RH mapping panels providing increasingly higher levels of resolution.

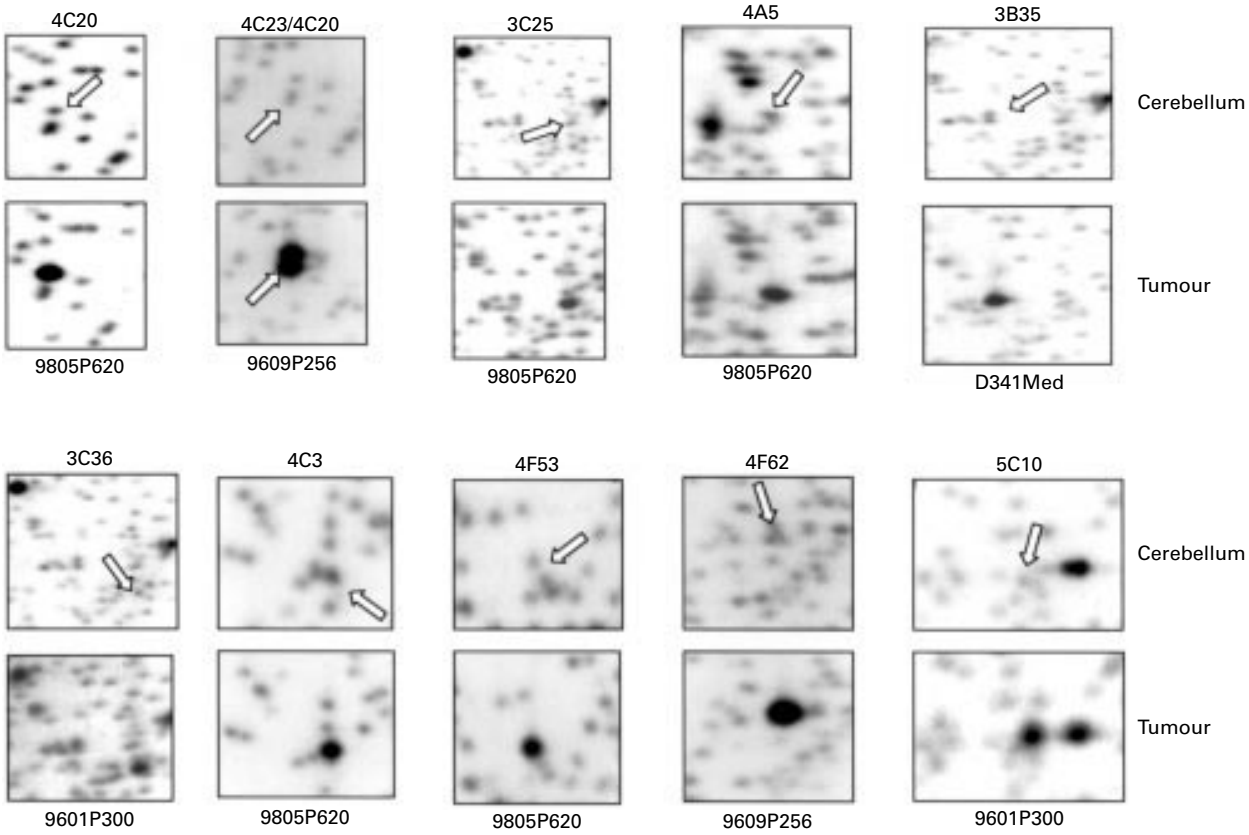

Figure 1 Detection of enhanced DNA fragments in PNET/MB on RLGS profiles. Shown is a comparison between DNA fragments, which are enhanced on RLGS profiles from PNET/MB, with single copy fragments in normal cerebellum profiles. Open arrows point to single copy fragments in the normal cerebellum profile (top rows) that are amplified in the tumours (bottom rows). The arrow points to the enhanced RLGS fragment 4C23 of tumour sample No 5 (9609P256). The number on top of each pair of RLGS profile sections is the RLGS DNA fragment identifier. The number on the bottom of each pair identifies the tumour samples. All normal RLGS profile sections in this figure were taken from the cerebellum profile of a 4 year old male who had died from a cardiomyopathy.

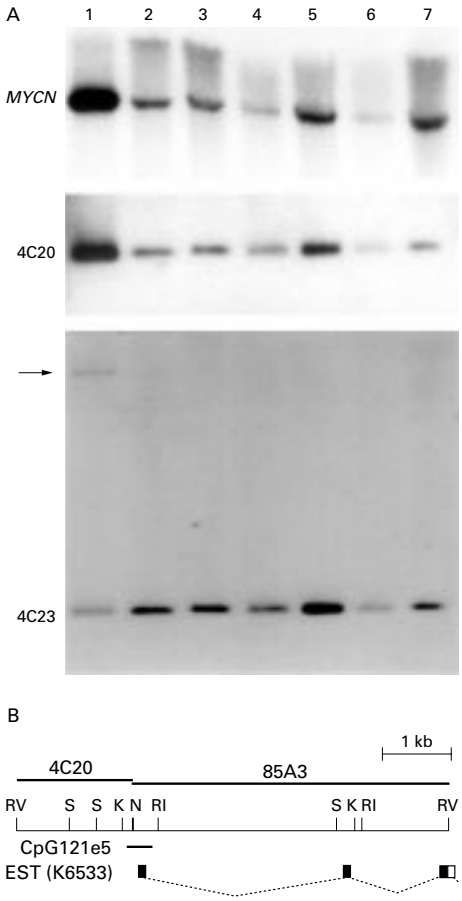

Figure 2 Analysis of NAG amplification in PNET/MB. (A) Representative Southern hybridisation showing coamplification of NAG (4C20) with MYCN in the supratentorial PNET No 21; 7.5 µg of DNA were digested with NotI/EcoRV electrophoresed and hybridised with probes for MYCN, 4C20 (5' CpG island of NAG) and 4C23 as described in Materials and methods. Lanes 1 and 2 represent the supratentorial PNETs No 21 (lane 1) and No 23 (lane 2), lanes 3-6 are the medulloblastomas No 15 (lane 3), No 3 (lane 4), No 18 (lane 5), and No 1 (lane 6). Lane 7 corresponds to normal PBL DNA from a newborn infant. While MYCN and 4C20 are amplified in tumour No 21 (lane 1), no amplification of 4C23 was detected in this sample. The closed arrow points to an additional band in sample No 21 possibly because of a rearrangement at this locus. (B) Restriction map of 4C20 and the adjacent NotI/EcoRV clone 85A3. Shown is an alignment of the restriction map of clone 4C20 and the adjacent 3' NotI/EcoRV clone 85A3 with the sequence of the CpG island clone 121e5 and EST k6533. While 4C20 represents part of the 5' CpG island of the novel NAG gene, EST k6533 represents the first three putative exons of that gene.

Both clones mapped to the identical location between markers D2S131 and D2S312, a region on 2p24.3 coinciding with the *MYCN* region.[36] Southern blot experiments showed that all tumours with *NAG* amplification also had amplification of *MYCN*. This suggested that both amplified fragments are part of the *MYCN* amplicon. Interestingly, *MYCN* was shown previously to be amplified in medulloblastomas only rarely.[37]

4C20 REPRESENTS A NOVEL GENE WITHIN THE *MYCN* AMPLICON

While 4C23 showed no homology to any known sequences in the database, 4C20 showed a high homology (100% and 97%) over 40 bp to two CpG island clones previously submitted to GenBank (clone cpg172a11 Accession No Z54901 and clone cpg121e5 Accession No Z54487) (fig 2B). These two clones had 52 bp homology with an EST derived from fetal heart (k6533, Accession No AA247962). EST clone k6533 was identified recently as part of *NAG* (neuroblastoma amplified gene), which is amplified and overexpressed in a subset of neuroblastomas with *MYCN* amplification.[16] Using a 170 bp fragment from this EST as a probe for Southern hybridisation, we detected amplification of the same sized fragment as clone 4C20, indicating the homology of both fragments. In addition we obtained the *Not*I/*Eco*RV fragment 3' of 4C20 from our *Not*I-*Eco*RV library by filter hybridisation using the PCR product from EST k6533 as a probe. Sequence analysis of this clone, 85A3, and k6533 showed that these clones overlapped by 144 bp. Nucleotides 1-52 of k6533 are located immediately 3' of the *Not*I site, while bp 53-105 and 106-144 are located approximately 2.8 kb and 5 kb downstream of the *Not*I site respectively (fig 2B).

To determine the tissue distribution and the approximate transcript size of *NAG*, we used the k6533 probe to hybridise multi-tissue northern blots. Our results suggest a *NAG* transcript size of 7 kb, which is widely expressed in normal tissues, with highest levels in heart (H) and skeletal muscle (Sm) followed by brain (Br), pancreas (Pa), placenta (Pl), and kidney (Ki). Little or no expression was observed in liver (Li), small intestine, and thymus (fig 3A, data for small intestine and thymus not shown).

Expression of *NAG* was tested in two medulloblastoma samples (Nos 5 and 8). Since the amounts of RNA were insufficient for northern hybridisation a semiquantitative RT-PCR assay was used. Expression levels of *NAG* were compared to those of the housekeeping gene *GPI*. RNA from normal cerebella, PBL, adult brain, and five medulloblastoma cell lines were used as controls (fig 3B). Intensities of RT-PCR products were scanned using a phosphorimager. Relative levels of *NAG* expression (compared to *GPI*) were determined for each sample (fig 3C). The relative expression in the cell lines ranged from 0.58 (lane 6 = MHH-PNET-5) to 1.56 (lane 4 = MHH-MED-1) indicating the variability of this experiment. Relative expression levels in the patient samples ranged from 0.54 (lane 13 = normal cerebellum 9609P325) to 1.36 (lane 9 = MB No 8). Only the adult brain sample (lane 3) showed a relatively low ratio, indicating either a reduced expression of *NAG* in normal adult cerebral cortex compared to childhood cerebellum or an increased *GPI* expression. Tumour No 5, which showed the highest amplification levels of *NAG* by RLGS and by Southern analysis, did not show overexpression of the gene.

NAG MAPS 400 KB TELOMERIC TO *MYCN*

Since *NAG* mapped to chromosome 2p24 and was coamplified with *MYCN* in PNET/MB samples, we hypothesised that *NAG* might be in close physical proximity to *MYCN*. To test

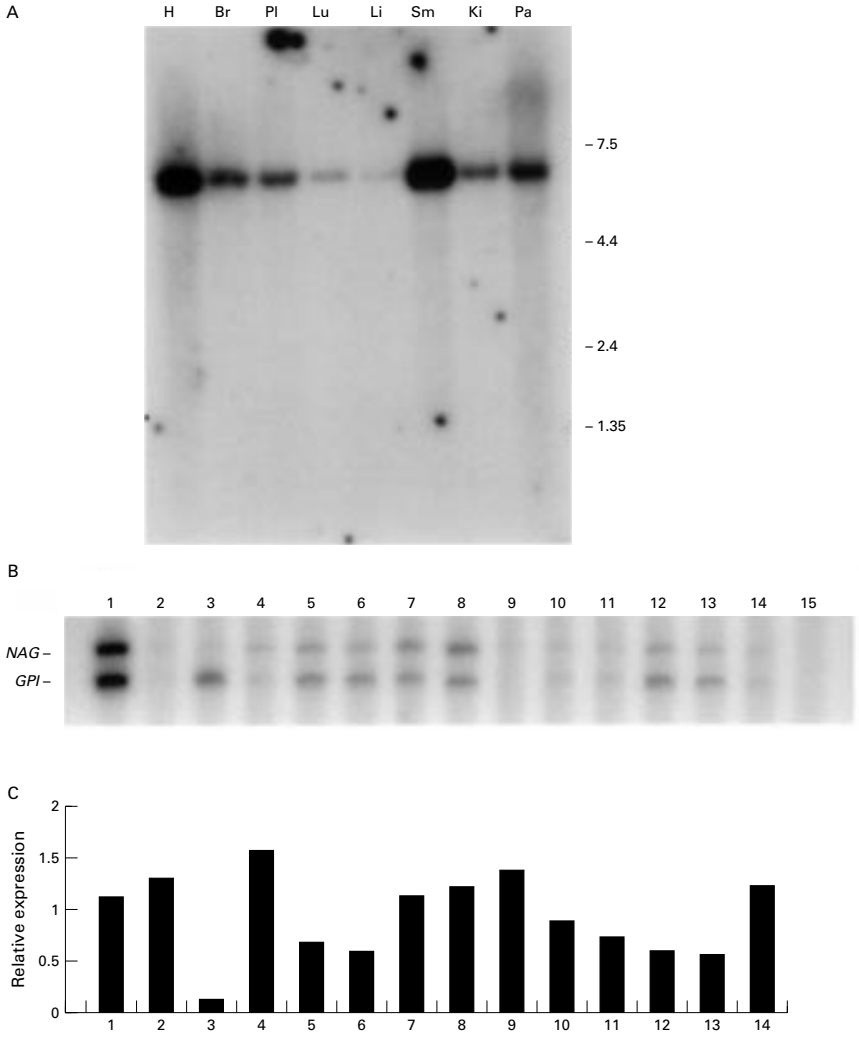

Figure 3 Expression analysis of NAG in medulloblastomas. (A) Multiple tissue northern blot hybridised with a probe derived from EST k6533, located 5' in the NAG gene. Hybridisation of k6533 to poly(A)+ generated RNA shows a near ubiquitous expression of NAG. Highest expression levels are seen in heart (H) and skeletal muscle (Sm) followed by pancreas (Pa), brain (Br), placenta (Pl), and kidney (Ki). The transcript size is about 7 kb. (B) Autoradiography of RT-PCR products of NAG (210 bp) and GPI (178 bp) using RNA from control samples: PBL (lane 2), adult brain (lane 3), normal cerebella 9703P309 (lane 10), 9609P322 (lane 11), 9609P315 (lane 12), 9609P325 (lane 13), and 9704P318 (lane 14); medulloblastoma cell lines: MHH-MED-1 (lane 4), Daoy (lane 5), MHH-PNET-5 (lane 6), D341 Med (lane 7), D283 Med (lane 8); as well as medulloblastoma samples No 21 (lane 1) and No 8 (lane 9). Lane 15 is the negative control. (C) Relative expression of NAG compared to GPI in medulloblastoma and control samples based on intensity levels of the respective RT-PCR products. The order of sample numbers is as in (B).

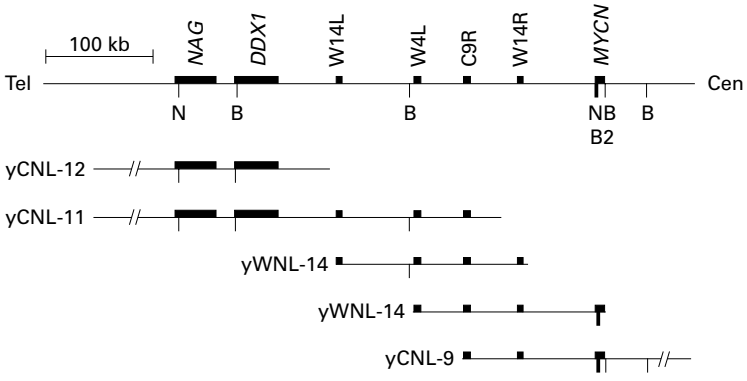

Figure 4 YAC map of the normal MYCN locus in 2p24. Physical map of MYCN, DDX1, and NAG. The restriction and probe maps are based on a previous study.[30] NotI (N) and BssHII (B) restriction sites are shown as vertical lines. The probes W14L, W4L, C9R, and W14R were derived from the left end (5') of YACs yWNL-14 and yWNL-4 and from the right end (3') of yWNL-4 and yCNL-9, respectively. TEL, telomeric; CEN, centromeric.

this hypothesis we localised *NAG* on a YAC contig spanning the *MYCN* locus.[13]

Four *Not*I sites are present in the contig; these sites are located within the CpG island of *MYCN*, 400 kb telomeric (5' of *MYCN*), 600 kb and 750 kb centromeric of *MYCN*. We mapped EST k6533 by PCR and by Southern hybridisation to YACs yCNL-11 and yCNL-12 suggesting that *NAG* is associated with the telomeric *Not*I site approximately 400 kb 5' of *MYCN* (fig 4).

Discussion

We used RLGS to scan the genomes of medulloblastomas and supratentorial PNETs for DNA amplification. Our data indicate a slightly higher frequency of DNA amplification, but in general confirm the findings of other investigators that DNA amplification is a rare event in these tumours.[9 34 35 38] Even though we detected

enhanced DNA fragments in up to seven of 25 tumours, there is very little overlap of amplified DNA sequences between tumours and some fragments are uniquely enhanced in only one tumour. This supports the notion that very few genes are consistently amplified in PNET/MB. The DNA fragments 4C20, 4F62, 3C15, and 5B35 were found enhanced in more than one tumour and may thus represent more commonly amplified genes. For example, 4C20 was amplified in three of the 25 analysed tumours and corresponds to the 5' CpG island of *NAG*. Our finding of a higher frequency in single locus amplifications compared to published data has to be validated by the characterisation of all amplified DNA sequences or genes detected by RLGS.

The gene that is thought to be the most commonly amplified gene in medulloblastomas, *MYC*,[7] was not amplified in any of the primary tumours when studied by RLGS. Southern analysis of eight of these tumours did not detect any *MYC* amplification. This finding allows only two interpretations: (1) *MYC* amplification is present in our samples, but does not involve the 5' CpG islands and therefore does not cause an enhancement of fragment 3B35, (2) the extent of *MYC* amplification in medulloblastomas has been overestimated and may need re-evaluation in a large number of tumours. The DNA fragment 4C3, amplified in tumour No 21, is located on chromosome 8 and also amplified in D341 Med, a cell line with a characteristic *MYC* amplification. It is possible that No 21 shows amplification of *MYC* in addition to amplification of *MYCN* and that 4C3 represents a gene within the *MYC* amplicon or part of the *MYC* gene itself.

The utility of the two dimensional gel electrophoresis method RLGS in the detection of known and novel amplified DNA sequences has been reported.[18 19 39] RLGS mixing gels allow for direct access to clones and DNA sequences.[17] The majority of *Not*I restriction sites are located in CpG islands located mainly in the 5' region of genes.[40] Thus, RLGS profiles can be viewed as a display of CpG island or gene sequences. Support for this assumption is derived from our cloning of several known genes from RLGS profiles like *MYC*, the oestrogen receptor, *WIT-1*, *CDK6*, and others.[17 21 39–41] Even though some of the enhanced CpG island fragments detected in the current study may map to the same amplicon (that is, 4C20, 4C23, 4F62, 4A5, 4A23, 3C25, and 4F53 on chromosome 2), it is very likely that each fragment is associated with a different, possibly novel gene.

For example, CGH analysis of sample No 8 showed gains on chromosomes 4q16 and 5p15. RLGS analysis of the corresponding tumour detected two enhanced fragments, which may correspond to an amplicon recently described on chromosome 5p15.3.[33] Furthermore, the study by Reardon *et al*[33] describes an additional amplicon on 11q22.3, which may well be represented by one or more of the enhanced fragments of our study, which have not been cloned at this time. To date no candidate oncogene has been described on 11q22.3 or 5p15.

The EST clone k6533, identified in our study, is part of a previously described gene, *NAG*, or neuroblastoma amplified gene.[16] *NAG* was identified in a two dimensional gel electrophoresis analysis of neuroblastomas using a slightly different approach when compared to ours. While our enzyme combination is *Not*I/*Eco*RV/*Hin*dIII, Wimmer *et al*[16] used *Not*I/*Eco*RV/*Dpn*II. The DNA fragments therefore have different sizes and migrate to different locations on the profile. The fragment 4C20 is 355 bp and 4C23 is slightly smaller, while NBA-2B of Wimmer *et al*[16] is 455 bp in size and NBA-2A is 139 bp. While the homology between NBA-2B and 4C20 is certain owing to the homology to the associated CpG island, we cannot be sure that 4C23 corresponds to NBA-2A. Secondly, these authors found a cDNA for the *NAG* gene of 4.5 kb, while our multi-tissue northern analysis shows a transcript size of approximately 7 kb (fig 3A). One explanation for the discrepancy in transcript size is that our probe (located in the 5' end of *NAG*) detects an alternative transcript of *NAG* that was missed by using a probe located closer to the 3' end.

NAG was previously localised telomeric to *MYCN* by double colour FISH.[16] We have determined the exact location of *NAG* on a YAC contig of the *MYCN* locus on chromosome 2p24. *NAG* maps to the telomeric *Not*I site in the contig approximately 400 kb 5' of the *Not*I site associated with *MYCN*. Since we did not detect any *NAG* overexpression in the tested PNET/MB, the gene is most likely a coamplified gene in the *MYCN* amplicon. Several other genes have been reported to be coamplified with *MYCN* including ornithine decarboxylase *(ODC)*, ribonucleotide reductase *(RRM2)*, syndecan-1 *(SDC)*, propiomelanocortin (*POMC*), and DEAD box protein (*DDX1*).[42] Of these, only *DDX1* has been shown to be coamplified with *MYCN* in more than a few tumours.

Neither *DDX1* nor *NAG* have been found amplified in the absence of *MYCN* amplification. Kuroda *et al*[30] mapped *DDX1* 340 kb 5' of *MYCN* and therefore approximately 60 kb 3' of the *NAG* locus. Neither gene lies within the 130 kb core domain for *MYCN* amplification, defined in neuroblastomas.[43] Furthermore, Reiter and Brodeur[44] have shown that *MYCN* is the only consistently amplified and overexpressed gene in neuroblastomas.

In conclusion, we evaluated the frequency of gene amplifications in medulloblastomas and other PNETs of the CNS. The gene *NAG*, previously found amplified and overexpressed in neuroblastomas, is also amplified in a subset of PNET/MB but always coamplified with *MYCN*. Therefore we suggest renaming it as "*N-MYC* coamplified gene" to stress the importance of *MYCN*.

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