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Aberrant methylation of multiple genes in neuroblastic tumours: relationship with *MYCN* amplification and allelic status at 1p

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Abstract

Aberrant hypermethylation occurs in tumour cell CpG islands and is an important pathway for the repression of gene transcription in cancers. We investigated aberrant hypermethylation of 11 genes by methylation-specific polymerase chain reaction (PCR), after treatment of the DNA with bisulphite, and correlated the findings with *MYCN* amplification and allelic status at 1p in a series of 44 neuroblastic tumours. This tumour series includes five ganglioneuromas (G), one ganglioneuroblastoma (GN) and 38 neuroblastomas (six stage 1 tumours; five stage 2 tumours; six stage 3 cases; 19 stage 4 tumours, and two stage 4S cases). Aberrant methylation of at least one of the 11 genes studied was detected in 95% (42 of 44) of the cases. The frequencies of aberrant methylation were: 64% for thrombospondin-1 (*THBS1*); 30% for tissue inhibitor of metalloproteinase 3 (*TIMP-3*); 27% for O^6-methylguanine-DNA methyltransferase (*MGMT*); 25% for *p73*; 18% for *RB1*; 14% for death-associated protein kinase (*DAPK*), $p14^{ARF}$, $p16^{INK4a}$ and *caspase 8*, and 0% for *TP53* and glutathione S-transferase P1 (*GSTP1*). No aberrant methylation was observed in four control normal tissue samples (brain and adrenal medulla). *MYCN* amplification was found in 11 cases (all stage 4 neuroblastomas), whereas allelic loss at 1p was identified in 16 samples (13 stage 4 and two stage 3 neuroblastomas, and one ganglioneuroma). All but one case with *caspase 8* methylation also displayed *MYCN* amplification. Our results suggest that promoter hypermethylation is a frequent epigenetic event in the tumorigenesis of neuroblastic tumours, but no specific pattern of hypermethylated genes could be demonstrated.

Keywords: CpG island; Methylation; Neuroblastoma; *MYCN* amplification; 1p deletion; *Caspase 8*; *THBS1*; *MGMT*; *p73*; *RB1*

1. Introduction

According to Schwab and colleagues [1], neuroblastic tumours are ''childhood embryonal neoplasms composed of migrating neuroectodermal cells derived from the neural crest and destined for the adrenal medulla and sympathetic nervous system''. Four types of neuroblastic tumours are characterised by a distinct schwannian stroma component and they are classified as neuroblastoma, nodular or intermixed ganglioneuroblastoma, and ganglioneuroma [1]. Neuroblastoma is one of the most common paediatric solid tumours and is clinically variable since some tumours undergo spontaneous regression, while others have metastasised at presentation. Accordingly, the tumour is commonly classified by the stage of tumour development [2]. Patients with early stage (1, 2 and 3) tumours have good/intermediate overall survival chances; however, stage 4 tumours are generally characterised by an unfavourable prognosis. The disseminated form of the disease (stage 4S) involves multiple organs/tissues and has a better prognosis than regular stage 4 tumours.

The molecular pathology of neuroblastoma shows several genomic alterations including amplification of the *MYCN* oncogene, loss of heterozygosity (LOH) at 1p, 2q, 9p, 11q, 14q and 18q, and gain of genetic material at 17q (for a review see Ref. [1]). The $p16^{INK4a}$ gene

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on 9p21 and the *dcc* gene on 18q21 have been proposed as candidate tumour suppressor genes that are inactivated in neuroblastoma [3,4], and up to three distinct genes of this category involved in neuroblastoma development appear to be located on 1p35.36 [5]. *MYCN* amplification and 1p deletion are indicators of poor prognosis; since both anomalies frequently present in association (90% of cases with *MYCN* amplifcation also display 1p loss), they characterise a genetically distinct subgroup of aggressive neuroblastomas [6].

CpG islands are 0.5 to 2.0 Kb DNA regions rich in cytosine-guanine dinucleotides, present in the 5′ region of approximately half of human genes [7]. Aberrant methylation of cytosines within CpG islands is associated with loss of gene expression by repression of transcription of tumour-related genes, and this loss contributes to the pathogenesis and the progression of malignant neoplasms [7]. Examples of this process have been reported in a wide range of tumour types and cancer-related genes [8], but little information is available for neuroblastic tumours. There is a report suggesting that *p73* gene silencing is independent of promoter methylation in neuroblastomas [9] and reduced expression of *caspase 8* in primary tumours and neuroblastoma-derived cell lines has been correlated with methylation of the corresponding CpG island [10].

We, therefore, determined the methylation profile for 44 neuroblastic tumours, studying eleven genes that frequently show promoter region methylation in other neoplasms (lung, breast, gastric, colon cancer, brain tumours, etc). The genes were: the detoxifying gene glutathione S-transferase P1 (*GSTP1*), O^6-methylguanine-DNA methyltransferase (*MGMT*), death-associated protein kinase (*DAPK*), $p14^{ARF}$, thrombospondin-1 (*THBS1*), tissue inhibitor of metalloproteinase 3 (*TIMP-3*), *p73*, $p16^{INK4a}$, *RB1*, *TP53*, and *caspase 8*. These genes were selected for analysis on the basis of either, their localisation at genomic regions involved in chromosome deletions in neuroblastomas, or their key tumour development related function. As controls, two normal brain tissue samples and two adrenal medulla samples were also studied using polymerase chain reaction (PCR)-based techniques involving sodium bisulphite modification of DNA (MSP). We also determined the *MYCN* amplification and allelic status at 1p in the same series of tumours.

2. Materials and methods

2.1. Tissue samples and DNA purification

Unfixed frozen tumours and corresponding normal tissues (peripheral blood lymphocytes) from 44 children with neuroblastic tumours were studied. Histological diagnosis was performed according to the World Health Organization (WHO) guidelines [1] as: ganglioneuromas (G; five cases); ganglioneuroblastoma (GN; one sample) and neuroblastoma (N; 38 cases). Staging of the neuroblastomas was done according to the International Neuroblastoma Staging System (INSS) [2], and identified six stage 1 tumours; five stage 2 tumours; six stage 3 cases; 19 stage 4 tumours, and two stage 4S cases.

The tumour cell content was estimated by histological examination to be approximately 75–80%. In addition to tumours, two samples of non-neoplastic cerebral tissue and two samples of normal adrenal medulla were studied. DNA was prepared from frozen tissues and blood samples using standard methods as described in Ref. [11].

2.2. MYCN *amplification*

Normal and tumour DNAs were digested to completion with restriction enzymes, electrophoresed through 0.8–1% agarose gels, transferred to nylon membranes, and hybridised to a *MYCN* probe (pNb-1) radiolabelled with the random oligonucleotide-priming method as described in Ref. [11]. Probe pNb-1 consists of a *Bam*HI-*Eco*RI 1.0 Kb genomic *MYCN* DNA fragment cloned into a pBR322 plasmid (ATCC, Manassas, VA).

2.3. Loss of heterozygosity analysis

The allelic status at 1p was determined by analysis of CA repeat polymorphism analysis of seven 1p loci: D1S468 and D1S214 (located at 1p36.32), D1S199 (at 1p36.12), D1S200 (at 1p31.3), D1S438 (at 1p31.1), D1S207 (at 1p22.1) and D1S252 (at 1p12). Oligonucleotide primer pairs were obtained from GENSET, SA (Paris, France). PCR was performed in standard conditions using the BIOTOOLS DNA polymerase kit (Madrid, Spain), and the alleles were resolved on 6% polyacrylamide gels and then silver stained as previously described in Refs. [12,13].

2.4. Bisulphite treatment of DNA and methylation-specific polymerase chain reaction (MSP)

MSP was used to examine methylation at promoter regions of *MGMT* (located at 10q26), *GSTP1* (at 11q13), *DAPK* (at 9q34.1), $p14^{ARF}$ (at 9p21), *THBS1* (at 15q15), *TIMP-3* (at 22q12.3), *p73* (exon 1)(at 1p36.3), $p16^{INK4a}$ (at 9p21), *RB1* (at 13q14), *TP53* (at 17p13.1) and *caspase 8* (at 2q23). The primer sequences of these genes for the methylated and unmethylated reactions are shown in Table 1. The methods have been reported in detail in earlier reports [14,15]. Briefly, 2 μg of genomic DNA was denatured by NaOH and modified by sodium bisulphite treatment. DNA samples were purified using the DNA Clean Kit (Promega, Madison, WI), treated again with NaOH, precipitated with ethanol, and resuspended in water. Specific PCRs were performed for the

Table 1
Summary of primer sequences, annealing temperatures and PCR product sizes used for MSP

Gene	Forward primer (5′->3′)	Reverse primer (5′->3′)	Annealing temperature (°C)	Product size (bp)
MGMT	M: TTTCGACGTTCGTAGGTTTTCGC	M: GCACTCTTCCGAAAACGAAACG	66	81
	U: TTTGTGTTTTGATGTTTGTAGGTTTTTGT	U: AACTCCACACTCTTCCAAAAACAAAACA	66	93
DAPK	M: GGATAGTCGGATCGAGTTAACGTC	M: CCCTCCCAAACGCCGA	64	98
	U: GGAGGATAGTTGGATTGAGTTAATGTT	U: CAAATCCCTCCCAAACACCAA	64	106
p14^{ARF}	M: GTCGAGTTCGGTTTTGGAGG	M: AAAACCACAACGACGAACG	61	160
	U: TGAGTTTGGTTTTGGAGGTGG	U: AACCACAACAACAAACACCCCT	65	165
THBS1	M: TGCGAGCGTTTTTTTAAATGC	M: TAAACTCGCAAACCAACTCG	62	74
	U: GTTTGGTTGTTGTTTATTGGTTG	U: CCTAAACTCACAAACCAACTCA	62	115
TIMP-3	M: CGTTTCGTTATTTTTTGTTTTCGGTTTC	M: CCGAAAACCCCGCCTCG	59	116
	U: TTTTGTTTTGTTATTTTTTGTTTTTGGTTTT	U: CCCCCAAAAACCCCACCTCA	59	122
p73	M: GGACGTAGCGAAATCGGGGTTC	M: ACCCCGAACATCGACGTCCG	67	60
	U: AGGGGATGTAGTGAAATTGGGGTTT	U: ATCACAACCCCAAACATCAACATCCA	65	69
p16^{INK4a}	M: TTATTAGAGGGTGGGGCGGATCGC	M: GACCCCGAACCGCGACCGTAA	65	150
	U: TTATTAGAGGGTGGGGTGGATTGT	U: CAACCCCAAACCACAACCATAA	60	151
RB1	M: GGGAGTTTCGCGGACGTGAC	M: ACGTCGAAACACGCCCCG	66	172
	U: GGGAGTTTTGTGGATGTGAT	U: ACATCAAAACACACCCCA	58	172
Caspase-8	M: TAGGGGATTCGGAGATTGCGA	M: CGTATATCTACATTCGAAACGA	50	320
	U: TAGGGGATTTGGAGATTGTGA	U: CCATATATCTACATTCAAAACAA	53	321
TP53	M: TTCGGTAGGCGGATTATTTG	M: AAATATCCCCGAAACCCAAC	60	193
	U: TTGGTAGGTGGATTATTTGTTT	U: CCAATCCAAAAAAACATATCAC	58	247
GSTP1	M: TTCGGGGTGTAGCGGTCGTC	M: GCCCCAATACTAAATCACGACG	55	91
	U: GATGTTTGGGGTGTAGTGGTTGTT	U: CCACCCCAATACTAAATCACAACA	55	97

PCR, polymerase chain reaction; MSP, methylation-specific polymerase chain reaction; bp, base pairs; M, methylated; U, unmethylated.

methylated and unmethylated alleles in standard conditions with different (55 °C to 66 °C) annealing temperatures. Ten microlitres of each PCR reaction were loaded directly onto non-denaturing 6% polyacrylamide gels or 2–3% agarose gels, stained with ethidium bromide, and visualised under ultraviolet (UV) illumination. Samples giving signals approximately equivalent to the positive control were designated as methylated. As a positive control for methylated alleles, we treated lymphocyte DNA from healthy volunteers with Sss1 methyl-transferase (New England Biolabs) (Beverly, MA), then subjected it to bisulphite treatment. The identity of PCR products was verified by purification and sequencing (after PCR re-amplification with the same primer set) using the ABI PRISM Byg-Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer Applied Biosystems) (Foster City, CA) on the model 310 or 377 Applied Biosystem DNA sequencers. Each amplicon was sequenced bidirectionally.

2.5. Statistics

We used the methylation index (MI) to determine the overall methylation rate in individual samples. MI is defined as a fraction representing the number of genes methylated/the number of genes tested. Statistical analysis was performed using X^2 and Fisher's exact test for differences between groups, and Student's t and Mann-Whitney tests for differences between means.

3. Results

As shown in Table 2, *MYCN* amplification (10-50-fold amplification) was detected in 11 samples. All of them corresponded to the stage 4 neuroblastomas. Allelic loss at 1p was found in 16 tumours: 13 N4, two N3, and one G. Both anomalies were present in nine N4 tumours. Examples of *MYCN* amplification and 1p allelic loss are shown in Fig. 1.

The results of the MSP for the 11 loci are shown in Tables 2 and 3. A total of 42 of the 44 samples (95%) showed methylation in at least one of these loci. The methylation frequency for each locus in this tumour series varied from 0 to 64%. Representative examples of the MSP analysis are shown in Fig. 2. A high frequency of methylation (64%) was detected in *THBS1*, whereas an intermediate frequency was found in *TIMP-3* (30%), *MGMT* (27%), *p73* (25%) and *RB1* (18%). The remaining loci (*DAPK*, *p14*^{ARF}, *p16*^{INK4a} and *caspase 8*) showed methylation frequencies of 14%, while *TP53* and *GSTP1* showed no methylation at all.

The frequency of methylation of multiple loci in a tumour was determined using the MI (Table 2), and ranged from 0 to 0.45, with an overall mean of 0.2. When the MI's for the tumour groups are considered (Table 3), one can observe that the N1, N2 and N4 groups displayed MI values equal to/or greater than 0.2. In contrast, the N3 and G tumour groups were characterised by slightly lower MI values (0.15 and 0.16,

Table 2
Summary of methylation of all 11 genes, MYCN amplification and LOH 1p studies in neuroblastic tumours

				Methylation status											Methylation index
	Gender/ Age*	*MYCN* amplification	LOH 1p	*MGMT*	*DAPK*	*p14^{ARF}*	*THBS1*	*TIMP-3*	*p73*	*p16^{INK4a}*	*RB-1*	*Caspase-8*	*TP53*	*GSTP-1*	
G-1	M/3y	–	+	–	–	–	+	+	–	–	–	–	–	–	0.18
G-15	M/7y	–	–	+	–	–	+	+	–	+	–	–	–	–	0.36
G-37	F/2y	–	–	–	–	–	–	–	–	–	–	–	–	–	0
G-38	M/12y	–	–	+	–	–	–	–	–	–	–	–	–	–	0.09
G-41	F/5y	–	–	+	–	–	+	–	–	–	–	–	–	–	0.18
GN-5	F/4y	–	–	–	–	–	+	+	–	–	–	–	–	–	0.18
N1-7	M/7m	–	–	–	–	–	–	–	–	–	–	–	–	–	0
N1-34	F/6m	–	–	–	+	–	+	–	–	–	+	–	–	–	0.27
N1-35	M/1m	–	–	–	+	–	+	–	+	+	+	–	–	–	0.45
N1-36	F/16m	–	–	–	+	+	+	–	+	–	–	–	–	–	0.36
N1-39	F/12m	–	–	+	+	–	+	–	–	–	+	–	–	–	0.36
N1-40	M/5m	–	–	–	–	–	–	+	–	–	+	–	–	–	0.18
N2-22	F/4y	–	–	–	–	–	+	–	+	–	–	–	–	–	0.18
N2-26	F/18m	–	–	+	+	+	–	–	–	–	–	–	–	–	0.27
N2-30	F/2y	–	–	+	–	–	+	+	–	+	+	–	–	–	0.45
N2-31	F/1m	–	–	–	–	–	+	–	+	–	+	–	–	–	0.27
N2-43	F/9m	–	–	–	–	–	–	+	–	–	–	–	–	–	0.09
N3-2	M/5y	–	+	–	–	–	–	–	+	–	+	–	–	–	0.18
N3-6	F/3y	–	–	–	–	–	+	–	–	–	–	–	–	–	0.09
N3-13	M/3y	–	–	+	–	–	–	+	–	–	–	–	–	–	0.36
N3-14	M/10y	–	+	–	–	–	+	–	–	–	–	–	–	–	0.09
N3-25	F/3m	–	–	–	–	–	–	–	–	+	–	–	–	–	0.09
N3-27	F/18d	–	–	–	–	+	–	–	–	–	–	–	–	–	0.09
N4-3	M/5y	–	–	–	+	–	+	–	–	–	–	–	–	–	0.18
N4-4	F/2y	+	+	–	–	+	+	–	–	–	–	–	–	–	0.18
N4-8	F/18m	+	–	+	–	+	–	+	–	+	–	+	–	–	0.45
N4-9	M/3y	–	+	–	–	–	+	–	–	–	–	–	–	–	0.09
N4-10	F/2m	+	+	–	–	–	+	–	+	–	–	+	–	–	0.27
N4-11	M/11m	–	+	–	–	–	+	–	–	+	+	+	–	–	0.36
N4-12	F/2.5y	+	+	–	–	–	+	–	–	–	–	+	–	–	0.18
N4-16	F/6y	+	+	+	–	–	–	–	–	–	–	–	–	–	0.09
N4-17	M/6m	+	+	–	–	–	–	+	–	–	–	–	–	–	0.09
N4-18	M/11m	–	+	–	–	–	–	+	–	–	–	–	–	–	0.09
N4-19	F/6y	+	+	+	–	–	–	–	+	–	–	–	–	–	0.18
N4-20	F/4y	+	+	–	–	–	+	–	+	–	–	+	–	–	0.27
N4-21	M/3.5y	–	–	–	–	–	+	+	+	–	–	–	–	–	0.27
N4-23	M/4y	+	–	–	–	–	+	+	–	–	–	+	–	–	0.27
N4-24	M/9y	–	–	–	–	–	+	–	+	–	–	–	–	–	0.18
N4-28	F/1m	–	–	–	–	–	+	–	–	–	–	–	–	–	0.09
N4-29	F/4y	+	+	–	–	–	+	–	–	–	–	–	–	–	0.09
N4-32	F/7m	–	+	+	–	–	+	+	–	–	–	–	–	–	0.27
N4-42	F/2y	+	+	+	–	+	–	–	+	–	–	–	–	–	0.27
N4S-33	M/3m	–	–	–	–	–	+	–	–	–	–	–	–	–	0.09
N4S-44	F/3m	–	–	–	–	–	+	–	–	–	–	–	–	–	0.09

* Age: y: years; m: months; d: days; M, male; F, female; LOH, loss of heterozygosity.

respectively). No statistical differences were found when the MI values were compared between the tumour groups. The data for the GN and N4S groups are not informative as these groups are composed of one and two samples, respectively. The overall MI value for the neuroblastomas with concurrent *MYCN* amplification and 1p loss (proposed as an aggressive neuroblastoma subgroup) did not reach statistical significance when compared with the corresponding MI value in the rest of the neuroblastomas (0.18 versus 0.21). The overall MI value for the six tumours with *caspase 8* methylation was 0.3; this parameter was 0.15 for the N4 tumours with normal *caspase 8*. DNA from the four normal tissue samples (brain and adrenal medulla) displayed no positive signals for methylation in the 11 loci analysed.

The sequencing of the representative PCR products of all of the tested loci showed that cytosines outside the CpG sites were converted to thymine, thus excluding the

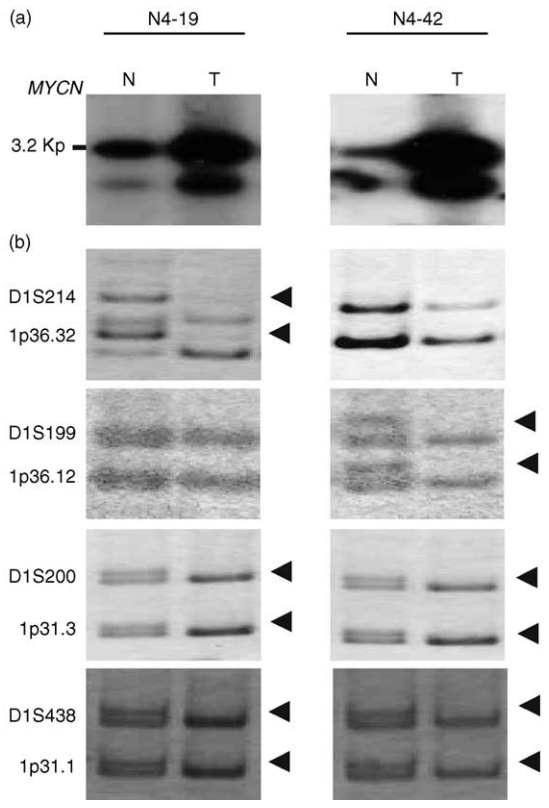

Fig. 1. *MYCN* amplification (a) and LOH at 1p (b) in neuroblastomas N4-19 and N4-42. The 1p markers analysed and their location is shown to the left. Arrows: allele lost. N: constitutional DNA; T: tumour DNA.

possibility that PCR amplification could be the result of incomplete bisulphite conversion. Sequence analysis demonstrated that cytosine methylation occurred only at the CpG sites located inside the genomic fragments amplified with the corresponding primer set.

No significant association could be determined between the methylation status of each locus and data on the age/gender of the patients. The overall MI values for patients with an age lower/over 1.5 years were 0.195 and 0.23, respectively. The MI data according to the gender distribution were 0.19 and 0.2 for male and female patients, respectively.

4. Discussion

This study of a series of 44 neuroblastic tumours analysed the methylation status of a panel of loci, which have been identified as frequently methylated in other cancers or cell lines. Moreover, the chosen loci are located at genomic regions involved in chromosomal deletions in neuroblastomas (*p73* at 1p; *caspase 8* at 2q; $p14^{ARF}$ and $p16^{INK4a}$ at 9p; *GSTP1* at 11q) or they play key tumour-related functions (*RB1*, *TP53*, *MGMT*, etc.). Our findings also evaluated the analysis of *MYCN* amplification and the allelic status of chromosome arm

Table 3
Methylation of multiple genes in neuroblastic tumours

Tumour type	*MGMT*	*DAPK*	$p14^{ARF}$	*THBS1*	*TIMP-3*	*p73*	$p16^{INK4a}$	*RB1*	*Caspase 8*	*TP53*	*GSTP1*	MI
G	60% (3/5)	0% (0/5)	0% (0/5)	60% (3/5)	40% (2/5)	0% (0/5)	20% (1/5)	0% (0/5)	0% (0/5)	0% (0/5)	0% (0/5)	0.16
GN	0% (0/1)	0% (0/1)	0% (0/1)	100% (1/1)	100% (1/1)	0% (0/1)	0% (0/1)	0% (0/1)	0% (0/1)	0% (0/1)	0% (0/1)	–
N1	17% (1/6)	67% (4/6)	17% (1/6)	67% (4/6)	17% (1/6)	33% (2/6)	17% (1/6)	67% (4/6)	0% (0/6)	0% (0/6)	0% (0/6)	0.27
N2	40% (2/5)	20% (1/5)	20% (1/5)	60% (3/5)	40% (2/5)	40% (2/5)	20% (1/5)	40% (2/5)	0% (0/5)	0% (0/5)	0% (0/5)	0.25
N3	17% (1/6)	0% (0/6)	17% (1/6)	33% (2/6)	17% (1/6)	17% (1/6)	17% (1/6)	17% (1/6)	0% (0/6)	0% (0/6)	0% (0/6)	0.15
N4	26% (5/19)	5% (1/19)	16% (3/19)	68% (13/19)	32% (6/19)	32% (6/19)	11% (2/19)	5% (1/19)	32% (6/19)	0% (0/19)	0% (0/19)	0.20
N4S	0% (0/2)	0% (0/2)	0% (0/2)	100% (2/2)	0% (0/2)	0% (0/2)	0% (0/2)	0% (0/2)	0% (0/2)	0% (0/2)	0% (0/2)	0.09
Totals	27% (12/44)	14% (6/44)	14% (6/44)	64% (28/44)	30% (13/44)	25% (11/44)	14% (6/44)	18% (8/44)	14% (6/44)	0% (0/44)	0% (0/44)	

No methylated templates of the 11 genes were detected by MSP in the four non-tumour samples studied. MI: Methylation Index. G, ganglioneuroma; GN, ganglioneuroblastoma; N1, neuroblastoma stage 1; N2, neuroblastoma stage 2; N3, neuroblastoma stage 3; N4, neuroblastoma stage 4; N4S, neuroblastoma stage 4S.

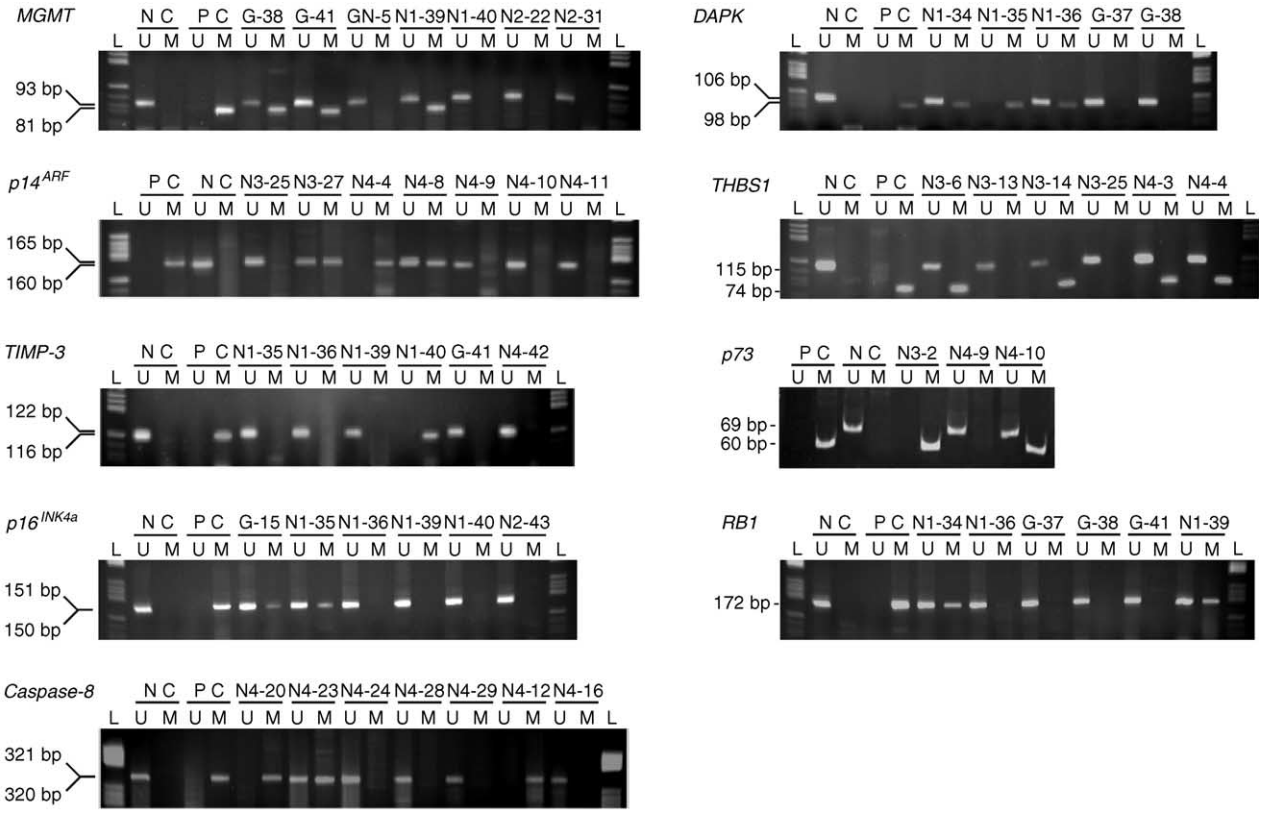

Fig. 2. Methylation analysis in neuroblastic tumours by MSP. The gene studied is given at the left of each panel. *Lane U*, amplified product with primers recognising unmethylated sequence; *Lane M*, amplified product with primers recognising methylated sequence. PC: positive control for methylation. NC: non-malignant tissue control. The PCR product sizes of all of the genes are shown to the left. L: ladder.

1p, two characteristic features of neuroblastomas. These molecular alterations primarily presented in stage 4 tumours (*MYCN* amplification in 11 cases and 1p loss in 13 cases), and were concurrent in nine samples, thus corroborating previous findings demonstrating that approximately 90% of cases with *MYCN* amplification also display 1p loss [6]. Allelic loss at 1p was also found in one ganglioneuroma (case G-1). The biological relevance of this deletion in this tumour group remains controversial, as this genomic anomaly is associated with a poor prognosis in neuroblastoma.

Transcriptional silencing by hypermethylation of CpG islands in the promoter regions is accepted as a primary mechanism involved in the inactivation of tumour-related genes [7]. Previous studies have demonstrated that the genes analysed here are methylated in a variety of human cancers. Furthermore, when the CpG islands we analysed are methylated, the expression of the corresponding gene is generally silenced, while in the matching normal tissues the same genes remain unmethylated [8]. In our series, 95% of the neuroblastic tumours were methylated in one or more loci, and the number of methylated loci averaged 2.25 per 11 tested loci, with a range of 0 to 5. In contrast, normal tissue samples did not show methylation at any of the tested loci. These findings indicate that aberrant CpG island methylation is a relatively frequent and tumour-related change in neuroblastic tumours.

The highest frequency of methylation was detected in *THBS1*, an angiogenesis inhibitor whose altered expression may contribute to neo-vascularisation and metastasis in human cancer [16]. Intermediate methylation frequencies were observed in four genes (*MGMT*, *TIMP-3*, *p73* and *RB1*) with key functions related to carcinogenesis. *MGMT* is a DNA repair gene, and it has been proposed that silencing the gene with promoter hypermethylation confers on the cancer cells an additional mutational capacity [17]. *TIMP-3* is necessary to suppress tissue remodelling, angiogenesis, invasion and metastasis [18]. Finally, *RB1* and *p73* play key roles in cell cycle-control or apoptosis [19,20]. Thus, a possible transcription silencing of these genes may be involved in tumour formation, and CpG island methylation would help enhance neuroblastoma development. In fact, the *RB1* promoter gene is frequently hypermethylated in malignant gliomas [14,21]. This epigenetic anomaly has been proposed as invariably associated with gene silencing and so might occur in neuroblastic tumours. On the other hand, it has been proposed that *p73* silencing in these neoplasms (primarily neuroblastomas) is independent of promoter and/or exon 1 methylation [9], a mechanism that appears to be critical

in haematological malignancies with regard to *p73* activity [22].

Methylation frequencies of 14% were obtained in four genes in our study ($p16^{INK4a}$, $p14^{ARF}$, *DAPK* and *caspase 8*). The INK4/ARF locus encodes two cell cycle regulatory proteins (p16 and p14) which share an exon, but have different reading frames [19]. They are located at 9p, a region frequently deleted in neuroblastomas, common regions of loss occur at 9p21 and 9p22-24 [23,24]. Deletion of 9p21, where the INK4/ARF locus is mapped, has been reported to correlate with a poor prognosis in neuroblastomas [4]. Furthermore, the $p16^{INK4a}$ gene is not expressed as a result of aberrant promoter methylation in approximately two-thirds of neuroblastoma cell lines [4]. Our findings in primary tumours thus suggest that methylation might silence $p16^{INK4a}$ in a subgroup of neuroblastomas, thus confirming this gene as a candidate tumour suppressor in neuroblastomas. Inactivation of $p14^{ARF}$ by this epigenetic mechanism may interfere with the p53 network in a subset of neuroblastomas since $p14^{ARF}$ interacts *in vivo* with the MDM2 protein to neutralise MDM2-mediated degradation of p53 [25]. Only one tumour in our series displayed concurrent aberrant methyaltion of both $p16^{INK4a}$ and $p14^{ARF}$ (case N4-8), thus suggesting once again that methylation of the two genes is an independent event in each gene, as occurs in colon carcinomas or non-small cell lung cancers [26].

DAPK is a positive mediator of apoptosis [27]. We found this gene to be aberrantly hypermethylated in stage 1 neuroblastomas (4 of 6 cases in our series), although only two additional tumours displayed this anomaly (one stage 2 and one stage 4 neuroblastoma). The significance of this epigenetic aberration in this tumour type should be further investigated, since it has been proposed as a potential metastasis inhibitor gene in nasopharyngeal carcinomas and lung cancers [28,29]. Furthermore, *DAPK* is frequently hypermethylated in brain metastasis from solid tumours [30]. Concurrent *DAPK* and *RB1* aberrant methylation was found in three stage 1 neuroblastomas. This is an irregular finding as methylation of these genes has been correlated with a higher tumour aggressiveness and metastasis in other neoplasms [14,21,28–30]. If confirmed in a larger series of tumours, this finding might be indicative of a low-grade neuroblastoma subgroup with a potentially distinct (aggressive?) biological behaviour; the clinical follow-up of these patients should clarify this aspect.

We also detected aberrant methylation of *caspase 8* (another apoptosis-related gene) in six tumours, all of them diagnosed as stage 4 neuroblastomas. This gene plays a key role in mediating tumour necrosis factor (TNF-) and Fas- induced apoptosis [31], and previous reports have shown it is silenced through methylation in neuroblastomas [10,32,33]. *Caspase 8* inactivation (primarily due to aberrant methylation) is frequently associated with *MYCN* amplification, as occurs in five of the six cases in our series; thus, it has been proposed that *caspase 8* might act as a tumour suppressor gene in a subset of aggressive neuroblastomas [10,32,33]. Interestingly, the six tumours in our series with *caspase 8* methylation displayed a higher overall MI value than the neuroblastomas with the unmethylated gene; nevertheless the biological significance of this association also needs to be explored in further studies. Cases with *caspase 8* methylation also correlated with 1p loss: 4 of 6 tumours in our series displayed this association. However, methylation of the other genes we studied failed to show any association with either *MYCN* amplification or 1p loss. Accordingly, a higher aggressiveness (represented at the molecular level by concurrent *MYCN* amplification and 1p loss) is not paralleled by an accumulation of CpG island methylation in neuroblastomas. No sample in our series displayed methylation of either *TP53* or *GSTP1*, suggesting that this epigenetic mechanism does not contribute to their inactivation in neuroblastic tumours.

In conclusion, we have examined CpG island methylation in neuroblastic tumours by analysing the methylation status of 11 genes using MSP. We found that CpG island methylation is a relatively frequent event in these neoplasms and, in agreement with previous reports, *caspase 8* was hypermethylated in a subset of neuroblastomas that, in this study, were all stage 4. The latter finding, *caspase 8* hypermethylation, has been associated with an increased biological aggressiveness [33].

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References

1. Schwab M, Shimada H, Joshi V, Brodeur GM. Neuroblastic tumours of adrenal gland and sympathetic nervous system. In Kleihues P, Cavenee WK, eds. *Pathology and Genetics of Tumours of the Nervous System. World health Organizatin Classification of Tumours* . Lyon, IARC Press, 2000, 153–161.
2. Brodeur GM, Pritchard J, Berthold F, *et al*. Revision of the international criteria for neuroblastoma diagnosis, staging and response to treatment. *J Clin Oncol* 1993, **11**, 1466–1477.

3. Kong XT, Choi SH, Inoue A, *et al*. Alterations of the tumour suppresor gen DCC in neuroblastoma. *Eur J Cancer* 1997, **33**, 1962–1965.
4. Takita J, Hayashi Y, Nakajima T, *et al*. The p16 (CDKN2A) gene is involved in the growth of neuroblastoma cells and its expression is associated with prognosis of neuroblastoma patients. *Oncogene* 1998, **17**, 3137–3143.
5. Versteeg R, Caron H, Cheng NC, *et al*. 1p36: every subband a suppressor? *Eur J Cancer* 1995, **31A**, 538–541.
6. Fong CT, Dracopoli NC, White PS, *et al*. Loss of heterozygosity for the short arm of chromosome 1 in human neuroblastomas: correlation with N-myc amplification. *Proc Natl Acad Sci USA* 1989, **86**, 3753–3757.
7. Baylin SB, Herman JG, Graff JR, Vertino PM, Issa JP. Alterations in DNA methylation: a fundamental aspect of neoplasia. *Adv Cancer Res* 1998, **72**, 141–196.
8. Esteller M, Corn PG, Baylin SB, Herman JG. A gene hypermethylation profile of human cancer. *Cancer Res* 2001, **61**, 3225–3229.
9. Banelli B, Casciano I, Romani M. Methylation-independent silencing of the *p73* gene in neuroblastoma. *Oncogene* 2000, **19**, 4553–4556.
10. Teitz T, Wei T, Valentine MB, *et al*. Caspase 8 is deleted or silenced preferentially in childhood neuroblastomas with amplification of *MYCN*. *Nature Medicine* 2000, **6**, 529–535.
11. Rey JA, Bello MJ, Jimenez-Lara A, *et al*. Loss of heterozygosity for distal markers on 22q in human gliomas. *Int J Cancer* 1992, **51**, 703–706.
12. Bello MJ, de Campos JM, Vaquero J, Ruiz-Barnes P, Kusak ME, Rey JA. *hRAD54* gene and 1p high-resolution deletion-mapping analyses in oligodendrogliomas. *Cancer Genet Cytogenet* 2000, **116**, 142–147.
13. Bello MJ, de Campos JM, Vaquero J, Kusak ME, Sarasa JL, Rey JA. High-resolution analysis of chromosome arm 1p alterations in meningiomas. *Cancer Genet Cytogenet* 2000, **120**, 30–36.
14. Gonzalez-Gomez P, Bello MJ, Alonso ME, *et al*. CpG island methylations status and mutation analysis of the RB1 gene essential promoter region and protein-binding pocket domain in nervous system tumours. *Br J Cancer* 2003, **88**, 109–114.
15. Gonzalez-Gomez P, Bello MJ, Alonso ME, *et al*. Frequent death-associated protein-kinase promoter hypermathylation in brain metastases of solid tumors. *Oncology Rep* 2003, **10**, 1031–1033.
16. Roberts DD. Regulation of tumor growth and metastasis by thrombospondin1. *FASEB J* 1996, **10**, 1183–1191.
17. Esteller M, Risques R-A, Minoru T, *et al*. Promoter hypermethylation of the DNA repair gene O6-Methylguanine-DNA methyltransferase is associated with the presence of G:C to A:T transition in p53 in human colorectal tumorigenesis. *Cancer Res* 2001, **61**, 4689–4692.
18. Bachman KE, Herman JG, Corn PG, *et al*. Methylation-associated silencing of the tissue inhibitor of *metalloproteinase-3* gene suggest a suppressor role in kidney, brain, and other human cancers. *Cancer Res* 1999, **59**, 798–802.
19. Sherr CJ. Cancer cell cycles. *Science* 1996, **274**, 1672–1677.
20. Melino G, De Laurenzi V, Vousden KH. p73: friend or foe in tumorigenesis. *Nature Revies Cancer* 2002, **2**, 1–11.
21. Nakamura M, Yonekawa Y, Kleihues P, Ohgaki H. Promoter hypermethylation of the RB1 gene in glioblastomas. *Laboratory Invest* 2001, **81**, 77–82.
22. Corn PG, Kuerbitz SJ, van Noesel MM, *et al*. Transcriptional silencing of the p73 gene in acute lymphoblastic leukemia and Burkitt's lymphoma is associated with 5′ CpG island methylation. *Cancer Res* 1999, **59**, 3352–3356.
23. Takita J, Hayashi Y, Kohno T, *et al*. Deletion map of chromosome 9 and p16(CDKN2A) gene alterations in neuroblastoma. *Cancer Res* 1997, **57**, 907–912.
24. Giordani L, Iolascon A, Servedio V, Mazzocco K, Longo L, Tonini GP. Two regions of deletion in 9p22-p24 in neuroblastoma are frequently observed in favorable tumors. *Cancer Genet Cytogenet* 2002, **135**, 42–47.
25. Zhang Y, Xiong Y, Yarbrough WG. ARF promotes MDM2 degradation and stabilizes p53: ARF-INK4a locus deletion impairs both the Rb and p53 tumor suppression pathways. *Cell* 1998, **92**, 725–734.
26. Esteller M, Tortola S, Toyota M, *et al*. Hypermethylation-associated inactivation of $p14^{ARF}$ is independent of $p16^{INK4a}$ methylation and p53 mutational status. *Cancer Res* 2000, **60**, 129–133.
27. Sanchez-Cespedes M, Esteller M, Wu L, *et al*. Gene promoter hyperemthylation in tumors and serum of head and neck cancer patients. *Cancer Res* 2000, **60**, 892–895.
28. Tang X, Khuri FR, Lee JJ, *et al*. Hypermethylation of the death-associated protein (DAP) kinase promoter and aggressiveness in stage I non-small-cell lung cancer. *J Natl Cancer Inst* 2000, **92**, 1511–1516.
29. Wong TS, Chang HW, Tang KC, *et al*. High frequency of promoter hypermethylation of the death-associated protein-kinase gene in nasopharyngeal carcinoma and its detection in the peripheral blood of patients. *Clin Cancer Res* 2002, **8**, 433–437.
30. Gonzalez-Gomez P, Bello MJ, Arjona D, *et al*. CpG island methylation of tumor-related genes in three primary central nervous system lymphomas in immunocompetent patients. *Cancer Genet Cytogenet* 2003, **142**, 21–24.
31. Boldin MP, Goncharov TM, Goltsev YV, Wallach D. Involvement of MACH, a novel MORT1/FADD-interacting protease, in FAS/APO-1- and TNF receptor-induced cell death. *Cell* 1996, **85**, 803–815.
32. Takita J, Yang HY, Chen YY, *et al*. Allelic imbalance on chromosome 2q and alterations of the caspase 8 gene in neuroblastoma. *Oncogene* 2001, **20**, 4424–4432.
33. Teitz Y, Lahti JM, Kidd VJ. Aggressive childhood neuroblastomas do not express caspase-8: an important component of programmed cell death. *J Mol Med* 2001, **79**, 428–436.