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Methylation analysis of the neurofibromatosis type 1 (*NF1*) promoter in peripheral nerve sheath tumours

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Abstract

Peripheral nerve sheath tumours are hallmarks of neurofibromatosis type 1 (NF1). Development of plexiform neurofibromas to malignant peripheral nerve sheath tumours (MPNST) is common. The *NF1* gene promoter harbours a hypomethylated CpG island. Thus, methylation changes may be involved in the development of different types of neurofibromas and malignant transformation. We investigated NF1-associated dermal ($n = 9$) and plexiform neurofibromas ($n = 7$), MPNST ($n = 5$) and non-NF1 leucocyte samples ($n = 20$) for their methylation pattern by bisulphite genomic sequencing. We could not find global hypermethylation in the *NF1* promoter in our series. Nevertheless, site-specific methylation, involving transcription factor binding sites for SP1, CRE (−10), and AP-2, was observed. One region of the 5′-UTR (untranslated region) overlapping with a putative AP-2 binding site was methylated at 30–100% in 4/20 control samples. In conclusion, we did not find hypermethylation in NF1-associated tumours. Instead, low level methylation could parallel a global genomic hypomethylation in malignancy.

Keywords: Neurofibromatosis type 1; Neurofibromatosis; Promoter methylation; Nerve sheath tumour

1. Introduction

Multiple peripheral nerve sheath tumours are among prominent features of neurofibromatosis type 1 (NF1) [1], a common autosomal dominant disorder caused by defects in the *NF1* gene on chromosome 17q11.2. The benign peripheral nerve sheath tumours are classified as dermal, nodular, diffuse and plexiform neurofibromas [2,3]. Only plexiform neurofibromas grow across a nerve or along a plexus. They can infiltrate tissue diffusely [4] and possess the potential to progress to malignant peripheral nerve sheath tumours (MPNST). The reasons for the differential growth pattern of plexiform and other neurofibromas are unknown. Neurofibromas are assumed to arise from Schwann cells experiencing a second hit in the *NF1* gene, in addition to the germ-line mutation [5–7]. Further genetic events, such as *TP53* mutations or *CDKN2A* silencing [8], may contribute to their malignant progression. Although a classical tumour suppressor mechanism may be assumed for all NF1-associated neurofibromas, a second hit has been demonstrated in only 3–36% of neurofibromas [9]. This rather low frequency may be due to incomplete detection. However, somatic alterations in *NF1* currently do not suffice to explain both tumour development and the differential

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growth characteristics of plexiform and other neurofibromas. Nevertheless, strongly reduced levels of neurofibromin expression have been demonstrated for plexiform neurofibromas and MPNSTs [10,11] which may indicate a modulation of gene function by *NF1* promoter methylation.

The preferred site of methylation is the CpG dinucleotide, although recent studies also suggest a considerable extent of non-CpG methylation [12]. CpGs are underrepresented in the vertebrate genome and an expected frequency of CpGs is maintained only at CpG islands. Approximately half of all genes contain CpG islands in their promoters, being free of methylation and thus actively transcribed. In several human cancers *de novo* methylation of tumour suppressor gene promoters was shown to lead to gene silencing [13], as demonstrated for the retinoblastoma gene, RBI [14], Von Hippel Lindau gene [15], *BRCA1* [16], *CDKN2A* and *CDKN2B* [17,18].

The human *NF1* promoter contains a constitutively hypomethylated CpG island with conserved transcription start sites and transcription factor binding sites for SP1, CREB, AP-2 and SRE [19]. Only a small number of reports have analysed the methylation status of the *NF1* gene promoter, and the region and number of tumours examined were limited [20–22]. Neither transcription factor binding sites, nor the role of non-CpG methylation have been investigated extensively. Therefore, the objective of our study was to investigate the methylation pattern of all cytosine residues of the *NF1* CpG island in NF1-associated dermal and plexiform neurofibromas and in MPNST.

2. Materials and methods

2.1. Patients and sample preparation

21 surgical specimens from 9 NF1 patients (4 males, 5 females) were investigated: 7 patients (no. 23–29) contributed both dermal and plexiform neurofibroma, one patient (no. 21) contributed peripheral leucocytes and 3 MPNST, and one patient (no. 22) contributed peripheral leucocytes, a neurofibroma, a neurofibroma with increased proliferation and 2 MPNST (see Figs. 1–4). A diagnosis of NF1 was confirmed according to established criteria described in [1]. In addition, peripheral blood was taken from 20 healthy non-NF1 controls (10 males, 10 females). Samples were numbered from 1 to 20 for the non-NF1 controls and 21 to 29 for the NF1 patients.

Adjacent non-tumourous tissue was removed after sectioning of shock-frozen tumour tissue. DNA extraction was performed by standard ethanol–chloroform extraction.

2.2. NF1 promoter

The analysed region represents the major part of the *NF1* CpG island comprising the promoter, 5′-UTR, exon 1 and part of intron 1. The whole base sequence from position −286 to +650 was evaluated for methylation of all cytosine residues and sequence aberrations. All 324 cytosine residues including 119 CpNpGs (71 CpGs, 30 CCpGs, 10 CCCpGs, 5 CCCCpGs, 2 CCCCCpG and 1 CCCCCCpG) as well as 253 non-CpGs (147 CpCs, 41 CpAs and 65 CpTs) were analysed for methylation by comparison to the original sequence [19].

2.3. Sodium bisulphite reaction and polymerase chain reaction controls

Bisulphite genomic sequencing was used because it has been demonstrated to be the most sensitive technique for detecting CpG and non-CpG methylation [23,24]. DNAs were coded to disguise whether they originated from NF1 patients or controls. All samples were randomly assigned to batches processed in different experimental rounds. Bisulphite modification was performed in agarose beads as described by Olek and colleagues [25], with slight modifications: DNA was denaturated for 5 min in boiling water, immediately cooled on ice and fixed in agarose using 1 N NaOH. The bisulphite reaction was carried out for 5 h at 50 °C and stopped by 1× Tris/HCl/ethylene diamine tetra acetic acid (EDTA) (pH 7.0) and agarose beads were collected in one tube and purified three times with 1× Tris/HCl/EDTA. The supernatant was decanted and 0.2 M NaOH added (1 ml/bead) followed by the addition of 1 N HCl (200 μl/bead) after 15 min. After purification, the beads were separately frozen without any fluid at −20 °C.

In order to evaluate the quality of the chemical modification, two polymerase chain reactions (PCRs) were carried out with 4–5 beads of every DNA sample. A first control PCR was performed with primers that were able to bind only to the non-modified DNA: *TP53*-5bf: 5′-GTGGGTTGATTCCACACCCC-3′; *TP53*-5br: 5′-AACCAGCCCTGTCGTCTCTC-3′ (annealing at 59 °C, 30 cycles). When it was impossible to amplify a genomic *TP53* fragment of 162 bp, beads were used for further experiments. An unmodified DNA served as a positive control. A second control PCR was performed for a 150 bp *TP53* fragment with primers that were able to bind to bisulphite-converted DNA only: *TP53*-mod-ex6f: 5′-TTTTATTGATTGTTTTTAGGTTTGG-3′;*TP53*-mod-ex6r: 5′-CTCCTCCCAAAAACCCCAATTACA-3′ (annealing at 58 °C, 50 cycles). Beads were used for further experiments if a strong band was observed representing PCR product and indicating a successful bisulphite conversion.

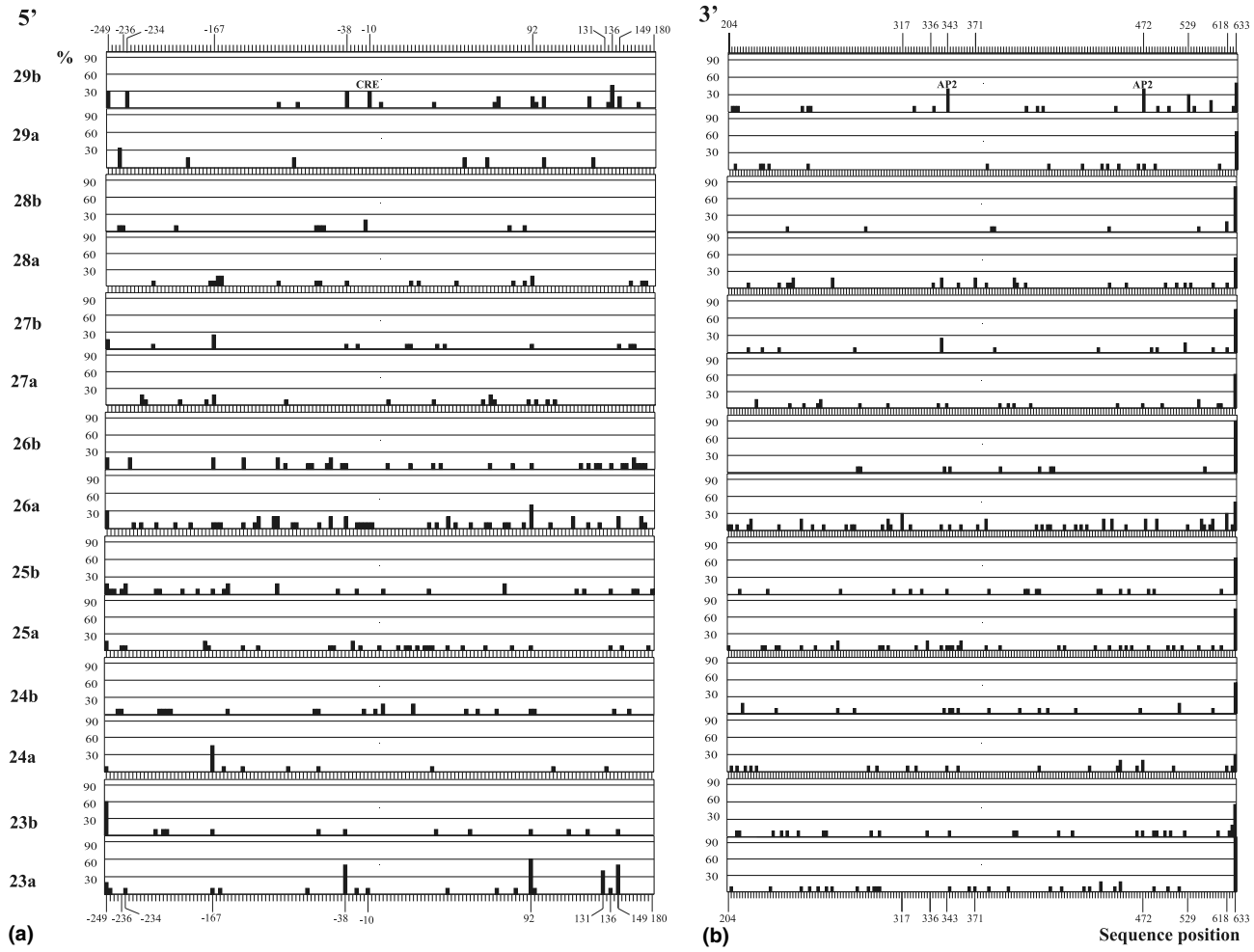

Fig. 1. Methylation pattern of the NF1 promoter in dermal (a) and plexiform (b) neurofibromas from NF1 patients 23–29. Promoter analysis was separated into a 5′-fragment from position −286 to +203, and into a 3′-fragment from +182 to +650 according to Hajra and colleagues described in [19]. Black bars indicate percentage of methylated clones per cytosine residue. Tick marks represent all cytosine residues of the whole promoter sequence (first residue at −249, last residue at +633). Tick marks with numbers indicate positions found to be site-specifically methylated (in $\geqslant$ 30% of clones) in the samples investigated. Transcription factor binding sites affected by site-specific methylation are shown.

2.4. Polymerase chain reaction of bisulphite-treated DNA

The *NF1* promoter was investigated by using two primer pairs from −286 to +203 (P5/P8, 5′-fragment, 489 bp, annealing 60.6 °C) and +182 to +650 (P7/P6, 3′-fragment, 468 bp, annealing 59.6 °C). Positions were numbered according to Hajra and colleagues [19], Genbank accession number: U09106.

P5: 5′-AGTTTAAGTTGAGAGTATAGTTTTTT-TAGG-3′; P8: 5′-TCTCCCCACAACCATCACAAT-CC-3′; P7: 5′-GATTGTGATGGTTGTGGGGAG-3′; P6: 5′-CAAAACCTAAAACAACC(AG)CAAAAA-AAAC-3′. For reactions with primers P 5,6,7 and 8, the Taq PCR Core Kit (Qiagen) was used. PCR included a pre-denaturation step of 98 °C for 5 min, a hot start and 40–45 cycles. Reverse primers for the 3′-fragment-PCR from position +182 to +650 are located in a part of intron 1 which can be aligned to positions 16574–16669 of sequence AC079915 from BAC RP-14206 in Genbank:

5′-GCGGGCGGGAGGTGGGAGCGGAGTGG-GGGTGGGGACAGAATAGGTGAAGGGGAGG-TAGGAGCGGCCGCCTCCCCGCGGCTGCCTC-AGGCTCTGGA-3′ (+558 to +652).

2.5. Cloning and sequencing

Sodium bisulphite-modified PCR products were eluted and cloned using the TOPO TA Cloning® kit (Invitrogen) with vector pCR® 4-TOPO® and One Shot® TOP10 competent *E. coli* cells. White colonies were cultured overnight and plasmids were extracted using standard procedures. Inserts were checked by *EcoRI* digestion. A minimum of 10 positive plasmids/clones per sample were subjected to semi-automated sequencing (Applied Biosystems, model 377) using the

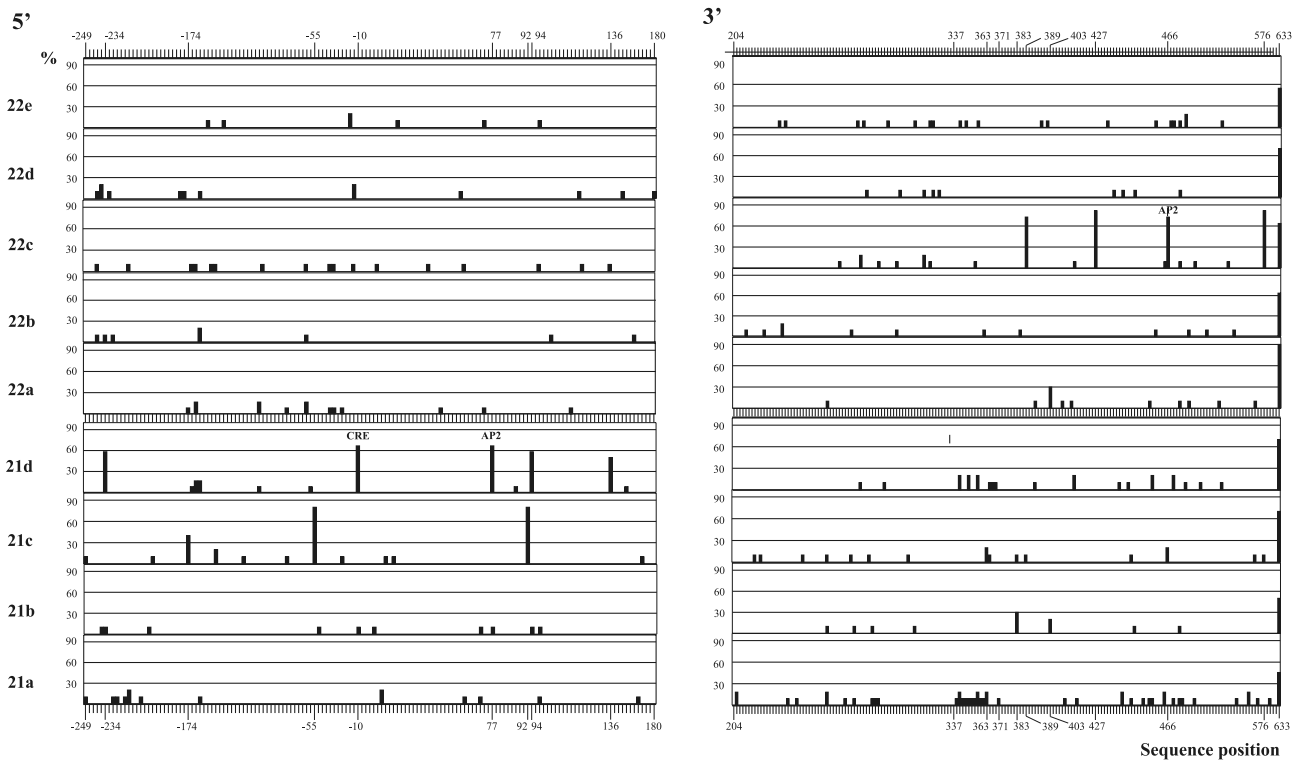

Fig. 2. Methylation in blood and tumours from different surgical interventions and divergent histology from NF1 patients 21 and 22: 21a – blood; 21b,c,d – MPNST; 22a – blood, 22b – neurofibroma, 22c – neurofibroma with increased proliferation, 22d,e – MPNST. Tick marks with numbers indicate positions found to be site-specifically methylated (in $\geqslant$ 30% of clones) in the samples investigated. Transcription factor binding sites affected by site-specific methylation are shown.

BigDye Terminator Cycle Sequencing kit (Applied Biosystems) and the M13 reverse primer.

2.6. Determining site-specific methylation

Site-specific methylation was defined if a cytosine position was methylated in $\geqslant$ 30% of clones. This level was chosen as it represents a conservative approach in comparison with previously published studies that scored methylation in individual positions of less than 10% as insignificant [21].

2.7. Loss of heterozygosity screening in NF1-associated tumours

Tumours were searched for loss of heterozygosity (LOH) using established protocols. Markers *Alu I/II* (intron 27b) and D17S250 downstream of *NF1* were investigated by non-radioactive microsatellite analysis. D17S33 upstream of *NF1* was investigated by *Rsa I* digestion of the 306 bp PCR product.

2.8. Statistical analysis

The mean frequency of methylation in a sample was calculated by counting every methylated residue in the whole sequence in every clone out of all of the positions that could be methylated in all of the clones examined over the whole sequence. Data are presented as medians and ranges. As the data were not normally distributed, the Mann–Whitney *U*-test was used to compare the groups. For comparison of the pairs of cutaneous and plexiform neurofibromas and for comparison of CpG *versus* non-CpG methylation, the Wilcoxon test was used (Statistical Package for the Social Sciences (SPSS) for windows 10.0; SPSS Inc., Munich, Germany). A *P*-value of <0.05 was considered significant.

3. Results

3.1. Methylation in peripheral nerve sheath tumours

Investigation of *NF1* CpG island methylation in NF1-derived tumour tissues revealed low level and random methylation of several cytosine residues. Data are shown in Figs. 1 and 2. The median percentage of methylation was 1.35% for the dermal neurofibromas (range 0.86–3.46; $n = 7$), 1.35% for the plexiform neurofibromas (range 0.53–1.94; $n = 7$), and 0.82% for the MPNST (range 0.65–1.9; $n = 5$). Comparison of all

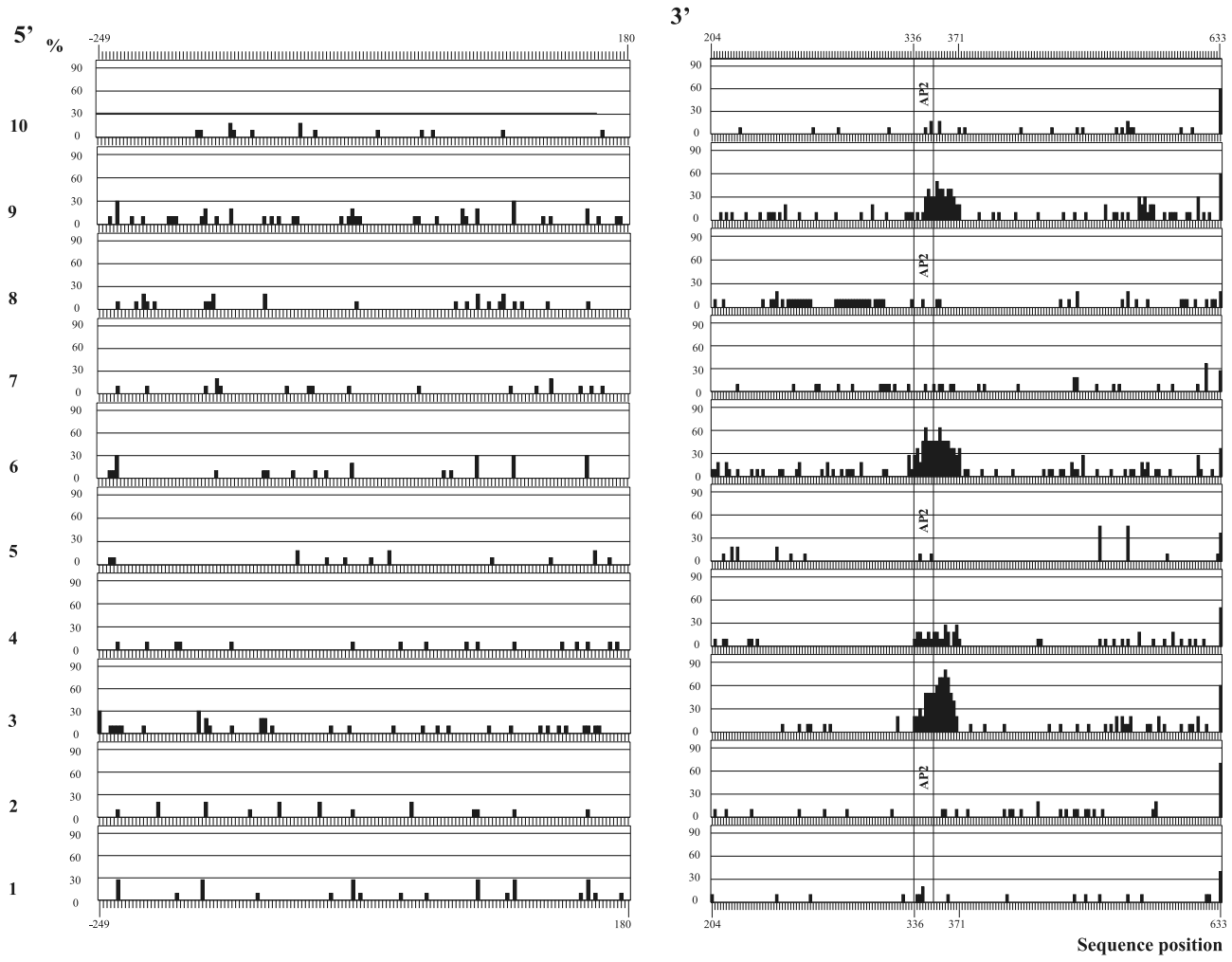

Fig. 3. Methylation in leucocytes of female control probands 1–10. Transcription factor binding site for AP-2 affected by site-specific methylation is indicated.

methylated positions in the 7 patients who each contributed a dermal and plexiform neurofibroma revealed no significant differences in methylation patterns. Furthermore, comparing methylation results between neurofibromas of any type with MPNST did not lead to statistically significant differences.

In all of the tumours ($n = 21$), cytosine methylation frequently involved CpG sites, although non-CpGs were also involved: median percentage was 1.6% for CpG sites (range 0.54–5.21), 0.98% for CpT sites (range 0.13–3.08), 1.05% for CpC sites (range 0.26–2.79), and 1.23% for CpA sites (range 0.0–3.41).

3.2. Methylation in leucocytes from non-NF1 controls

Peripheral leucocyte DNA from 20 non-NF1 controls exhibited low level methylation of cytosine residues. The median percentage of methylation was 1.4% for all of the control samples (range 0.65–4.77; $n = 20$), 1.7% for the female samples (range 0.99–4.77; $n = 10$), 1.25% for the male samples (range 0.65–4.32; $n = 10$). Data are shown in Figs. 3 and 4.

Methylated positions were distributed randomly, with one exception. From position +336 to position +371, methylation of 30–100% of individual clones was detected in 4/20 control samples (1 male, 3 female; no. 3, 6, 9, 20) and to a lower degree in 1 female control (no. 4) and 1 male NF1 patient (no. 21a). This position locates to the 5′-UTR (+1 to +483) region and overlaps with a putative conserved AP-2 binding site from +335 to +344 (recognition sequence: TCCCCACCCC). Median percentages of methylation were evaluated analysing only the region of 336–371. Comparing the median percentage of 2.6% in all of the leucocyte samples (range 0.52–43.75; $n = 20$) with the median percentage of 1.3% in all of the tumours (range 0.0–5.0; $n = 21$) revealed a significantly higher methylation level in the leucocytes ($P = 0.019$). Comparing the median percentage of 3.1% in the female leucocyte samples (range 1.88–43.75; $n = 10$) with the median percentage of 0.9% in

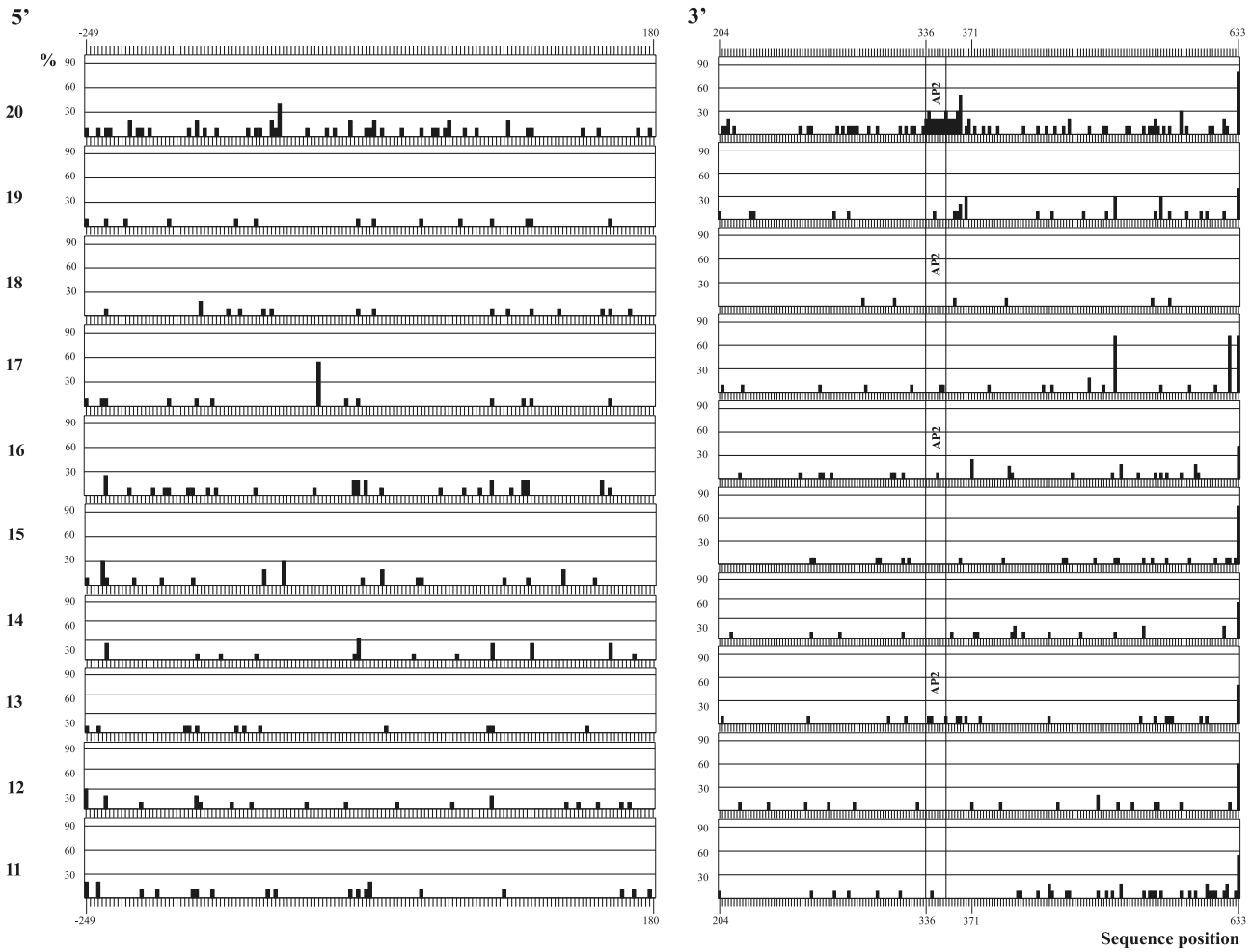

Fig. 4. Methylation in leucocytes of male controls 11–20. Transcription factor binding site for AP-2 affected by site-specific methylation is indicated.

the males (range 0.52–21.88; $n = 10$) revealed a significantly higher methylation in the females ($P = 0.029$).

Since tumours appeared to be less methylated than leucocytes, comparison of all methylated residues in leucocytes ($n = 20$) with those in peripheral nerve sheath tumours ($n = 21$) over the whole region was performed, but the results were statistically non-significant. There was a tendency for more variable and higher methylation levels in females compared with males.

In all non-NF1 leucocytes samples ($n = 20$), cytosine methylation frequently involved CpG sites, although a proportion of low level non-CpG methylation was also found in the leucocyte samples: median percentage was 2.8% for CpG sites (range 0.85–6.34), 1.1% for CpT sites (range 0.28–5.14), 1.0% for CpC sites (range 0.39–4.34), and 1.7% for CpA sites (range 0.44–6.99).

3.3. CpG versus non-CpG methylation

The median percentages for CpG methylation were 1.7% in the neurofibromas (range 0.59–5.21; $n = 16$), 1.4% in the MPNST (range 0.54–3.3; $n = 5$), and 2.8% in the leucocyte samples (range 0.85–6.34; $n = 20$). The median percentages for non-CpG (CpT + CpC + CpA) methylation were 1.2% in the neurofibromas (range 0.13–3.41; $n = 16$), 0.8% in the MPNST (range 0.0–3.3; $n = 5$), and 1.2% in the leucocyte samples (range 0.28–6.99; $n = 20$). Statistical analysis revealed that the CpG methylation level was significantly higher than non-CpG methylation in all 21 tumours ($P = 0.005$), in all 16 neurofibromas ($P = 0.001$), in 10 female and 10 male leucocytes ($P = 0.047$ and $P = 0.017$), but not in the MPNST samples ($P = 0.225$).

3.4. Site-specific methylation in tumours

Overall, 24 positions were methylated site-specifically in at least 30% the of clones examined. This specific pattern affected 11 CpGs (−249, −234, −55, +92, +94, +131, +136, +472, +529, +576, +618), 7 CpCs (−174, −167, +149, +343, +383, +427, +466), 3 CpAs (−38, −10, +403) and 3 CpTs (+77, +317, +389). Position +633 was excluded from the evaluation because of its localisation in a degenerate primer. Some positions were

methylated in tumours from different patients: −249 and +92 were methylated site-specifically in 3 tumours and positions −234, −38, −10, +136 in 2 different tumours.

Comparison of tumours from 7 NF1 patients with dermal and plexiform neurofibromas revealed site-specific methylation in 6/14 tumours (no. 23a, 23b, 24a, 26a, 29a, 29b), and comparison of multiple tumours and peripheral leucocytes in two NF1 patients (Fig. 2) revealed site-specific methylation in a neurofibroma (22c) and in 3 MPNSTs (21b,c,d).

3.5. Methylation of transcription factor binding sites

In tumours, five putative transcription factor binding sites for SRE and CRE (−10) and AP-2 (+77, +343, +466, +472) showed methylation of single cytosines. Position −10 at the −16 CRE motif was methylated site-specifically in a plexiform neurofibroma and a MPNST (see Figs. 1 and 2). In leucocytes, the region +336 to +371 overlapping with a putative AP-2 binding site was frequently methylated (see 3.2).

3.6. LOH screening in NF1-associated tumours

All tumours were investigated for LOH. Two markers flanking *NF1* (D17S250, D17S33) and one intragenic marker (ALU repeat, exon 27b) were used. LOH was observed in only one patient who contributed 2 neurofibromas and 2 MPNST samples. While the MPNST showed LOH in all 3 markers, the benign neurofibroma showed LOH in ALU and maintained heterozygosity in D17S250 and D17S33. The neurofibroma with increased proliferation exhibited LOH in ALU and a partial loss of the signals for D17S250 and D17S33. All other 17 samples investigated revealed no evidence for a second hit. Nine tumours (3 patients) were informative for 3, 6 tumours (3 patients) were informative for 2 markers and 4 tumours (2 patients) were informative for one marker.

3.7. Deviations from the published sequence of the NF1 promoter

While sequencing the plasmids, three nucleotides were found to differ from the published sequence probably reflecting minor typing errors, since all clones in the present study displayed the same deviation: C → G (−53), C → G (−68), G → T (−76).

4. Discussion

Reduced expression of *NF1* RNA and neurofibromin in peripheral nerve sheath tumours has already been demonstrated [12,13,26] indicating transcriptional silencing of the *NF1* gene in these tumours. These findings and low *NF1* somatic mutation rates in nerve sheath tumours may be explained by the adapted model of Knudson's two-hit hypothesis suggesting promoter hypermethylation as an equivalent event to gene mutation or LOH [27]. Interestingly, we could not find global hypermethylation in the *NF1* promoter in our series of 21 NF1-associated tumours. This contradicts the concept of global *NF1* promoter methylation and our initial hypothesis. Low methylation of the *NF1* CpG island probably parallels the global genomic hypomethylation frequently seen in neoplasia [28], and other second and third hit mechanisms need to be elucidated in NFI-associated tumours.

However, against a background of low methylation levels, individual positions in 10/21 tumours showed marked methylation peaks. Such site-specific methylation is in accordance with a previous study of 11 NF1-associated tumours describing a tumour-specific methylation of 6 of 70 CpGs, but no hypermethylation [21]. Of the 23 positions found to be methylated site-specifically in our study, 5 involved binding sites for SRE, CRE (−10) and AP-2 (+77, +343, +466, +472) [19,20]. In leucocytes, the region from position +336 to +370/71, containing a putative AP-2 binding site [19], was methylated in 4/20 control cases and to a lower degree in 1 NF1 patient. This region was significantly more methylated in leucocytes compared with tumours, and in female control leucocytes compared with male controls. AP-2 binding sites are known to be methylation-sensitive and are involved in gene regulation [29,30]. Interestingly, a *NF1* reporter gene construct consisting of the 5'UTR, exon 1 and intron 1 (+144 to +474) and including this AP-2 site possessed independent promoter activity and was reported to repress an *NF1* activator [31]. Therefore, this specific AP-2 binding site may influence *NF1* promoter activity by triggering an enhancer function. Recently, AP-2 was shown to regulate the timing of Schwann cell development [32]. Our observation of AP-2 site methylation in the *NF1* promoter, predominantly in female leucocytes, may indicate a regulatory function.

In our study, binding sites for SRE, CRE (−16) were methylated site-specifically in a malignant peripheral nerve sheath tumour at −10. Previously, two specific *NF1* sites for SP1(−141) and CRE (−16) binding proteins have been shown to be sensitive for methylation *in vitro* [20]. Another study analysing these two binding sites by methylation-specific PCR [22] did not find evidence for methylation. By contrast, our data demonstrate, for the first time, *in vivo* methylation at the −16 CRE motif at position −10 in a plexiform neurofibroma and a MPNST. This finding may have been overlooked in the above study [22], since bisulphite genomic sequencing of plasmids, being assumed to be the most sensitive method for determining cytosine methylation, was not applied. Because altered cyclic adenosine

monophosphate (cAMP) levels affect *NF1* gene expression [33], methylation of this specific motif may affect *NF1* gene expression in nerve sheath tumours.

Interestingly, a high percentage of low level methylation detected was due to non-CpG methylation. Since CpG dinucleotides are known to be the primary targets for methylation in eukaryotic cells, the role for methylation at non-CpGs or CpNpGs is a topic of debate. A recent study reported 15–20% non-CpG methylation in murine embryonic stem cells, but not in somatic tissues [24]. Other studies favour a potent role in gene regulation for CpNpG methylation in mammalian cells [34]. For example, non-CpG methylation has been shown to abolish binding of the murine *trans*-acting factor Ctcf to *Tsix* [35]. In human tissues, non-CpG methylation was detected in the *IFNG* promoter in T cells from umbilical cord blood and, to a lesser extent, in adult T cells [36]. Non-CpG methylation in the *NF1* gene was demonstrated in a 2-cell embryo and, at markedly lower levels, in sperm and adult tissues [12]. Our findings may indicate that the role of non-CpG methylation is currently underestimated. We do not think that non-CpG methylation in our study is a random artifact, since a protocol strongly favouring complete bisulphite conversion [25], and stringent conditions for denaturation and conversion were applied. In addition, efficient conversion was verified by control PCRs, similar to established protocols for methylation-sensitive PCR [37]. Furthermore, CpG methylation was significantly higher than non-CpG methylation in benign tumours and leucocytes, but not in MPNST. This could reflect that tissues keep a specific pattern of CpG methylation which disappears due to a genomic hypomethylation process in malignant tumours.

In our study, we did not find *NF1* CpG island hypermethylation in NF1-associated peripheral nerve sheath tumours. However, our experimental design does not allow us to distinguish between the *NF1* wild-type promoter (expected second hit) and the potential presence of a promoter of a mutant germ-line *NF1* allele (first hit) that is not completely deleted by extensive allelic loss. For those cases, we cannot exclude that random selection of 10 clones includes potentially non-methylated sequences from the remaining promoter of the mutant germ-line *NF1* gene copy, and that the second allele escaped our analysis. Thus, the extent of methylation would be underestimated in these cases. This problem can only be solved by matching promoter sequences to the *NF1* gene copies. Given the size of the *NF1* gene and the number of cases included in our series, such analyses are beyond the scope of this study.

In conclusion, promoter hypermethylation in NF1-associated peripheral nerve sheath tumours was not observed. Since loss of neurofibromin unquestionably contributes to the formation of nerve sheath tumours, other mechanisms of *NF1* gene inactivation or incomplete mutation detection need to be considered.

Conflict of interest statement

The authors have disclosed any financial and personal relationships with other people or organisations that could inappropriately influence their work.

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