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ARTICLE

Microsatellite instability and promoter methylation as possible causes of *NF1* gene inactivation in neurofibromas

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Neurofibromatosis type 1 (NF1) is a frequent hereditary disorder. One of the characteristic features of this disease is the development of neurofibromas. Since the *NF1* gene is supposed to be a tumour suppressor gene, these neurofibromas should develop upon inactivation of both *NF1* alleles. So far, mutation and deletion have been found to be involved in *NF1* gene inactivation. However, these inactivating mechanisms explain the development of only a limited fraction of analysed neurofibromas. In this study, we investigated microsatellite instability (MSI) and promoter methylation as potential contributors to *NF1* gene inactivation. As site-specific methylation in the *NF1* promoter inhibits binding of transcription factors Sp1 and CREB, we studied the methylation status of their binding sites in particular. We analysed 20 neurofibromas and three neurofibrosarcomas, but did not find evidence for microsatellite instability or *NF1* promoter methylation in any of the tumours. Thus, our data suggest that both microsatellite instability and promoter methylation are unlikely to be the major causes of *NF1* gene inactivation in these tumours.
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Keywords: neurofibroma; *NF1* gene; microsatellite instability; promoter methylation

Introduction

Neurofibromatosis type 1 (NF1) or von Recklinghausen disease is one of the most common hereditary diseases of man. Characteristic features of this autosomal dominant disorder are the development of café-au-lait spots, Lisch nodules on the iris, and of benign dermal or plexiform neurofibromas.[1] The *NF1* gene on chromosome arm 17q is supposed to be a tumour suppressor gene, of which, according to Knudson's two-hit model for tumorigenesis,[2] both alleles have to be inactivated for tumour formation to occur. Indeed, inactivation of both *NF1* alleles has been demonstrated in four dermal neurofibromas,[3,4] in three plexiform neurofibromas,[4–6] and in a neurofibrosarcoma,[7] a malignant peripheral nerve sheath tumour (MPNST) for which NF1 patients are at increased risk. However, mutation and/or deletion of the *NF1* gene, the latter often detectable as loss of heterozygosity (LOH), have only been reported for a limited fraction of the analysed neurofibromas. LOH rates range from 0 to 25–35% for dermal neurofibromas[5,6,8,9] and from 20 to 60% for plexiform neurofibromas,[4,5] whereas mutations in the *NF1* gene have only been found in a few tumours.[3,4,6]

Cases for which no LOH or mutation as inactivating mechanism could be demonstrated may be explained by the presence of subtle deletions, escaping detection by LOH, or by inadequate mutation detecting systems. We wondered whether other means of *NF1* gene inactivation, especially microsatellite instability (MSI) and promoter methylation, might contribute to inactivation of the *NF1* gene in neurofibromas and neurofibrosarcomas. MSI reflects genomic instability, which is normally caused by loss of mismatch repair function. Genomic instability could, among other targets, disrupt the *NF1* gene.[10,11] MSI has been investigated in neurofibromas, but with conflicting results. Ottini *et al*[12] found MSI in eight out of 16 neurofibromas (50%), whereas Serra *et al*[9] were not able to detect MSI in any of 60

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neurofibromas investigated. Gene inactivation through hypermethylation of CpG dinucleotides in the promoter region has been demonstrated for several tumour suppressor genes, like the retinoblastoma (*Rb1*) gene,[13] the von Hippel Lindau (*VHL*) gene,[14] and the *BRCA1* gene.[15]

Mancini *et al*[16] have shown that the region in the vicinity of the *NF1* gene transcription start site is unmethylated in normal DNA. This region contains several transcription factor binding motifs, including a Sp1 binding site and a cAMP responsive element (CRE).[17] By *in vitro* studies, it was furthermore demonstrated that binding of Sp1 protein and CRE binding protein (CREB) is inhibited by methylation of specific cytosine residues in their respective binding site motifs. They suggest that inhibition of Sp1 binding would additionally result in hypermethylation of the normally unmethylated *NF1* CpG island. Taken together, these data suggest a model in which methylation of the Sp1 and CRE motifs in NF1-related tumours would cause transcriptional silencing and, thus, inactivation of the *NF1* gene.

To test this model and to determine the potential contribution of MSI to *NF1* gene inactivation, we characterised in this study a series of neurofibromas and three neurofibrosarcomas for the presence of LOH, MSI and specific methylation of Sp1 and CRE motifs.

Materials and methods

Patients and tumour samples

Tumour and corresponding blood samples were obtained from 23 unrelated patients. Genomic DNAs were isolated using standard procedures. Type and location of the tumour and NF1 status of the patient are given in Table 1. Individuals without NF1 were not reported to be affected by other diseases.

LOH

The LOH status of the *NF1* gene region was determined by analysing the following markers: *D17S1871* – (2 cM) – *D17S959* – (3 cM) – *IVS27AAAT2.1* – *IVS27AC28.4* – *IVS38GT53.0* – (10 cM) – *D17S250*. Markers *IVS27AAAT2.1* and *IVS27AC28.4* are located in intron 27 of the *NF1* gene, and marker *IVS38GT53.0* is in intron 38 of the *NF1* gene. For each tumour, the LOH status was derived from at least two informative markers. Primer sequences and PCR conditions for these markers were taken from the Genome Database (http://www.gdb.org). PCR was performed as described previously.[18] LOH was determined by visual inspection of autoradiographs. LOH in neurofibromas is never complete due to variable contribution of cells without LOH. Therefore, in tumours with less significant visual loss of one allele, LOH was additionally assessed by laser scanning densitometry (Phosphorimager, Molecular Dynamics, Sunnyvale, CA, USA).

Microsatellite instability (MSI)

Tumour and corresponding blood samples were subjected to PCR using microsatellite markers *BAT25*, *BAT26*, *BAT40*, *D2S123*, *D5S346*, and *D17S250* from the reference panel for

Table 1 Type, location, and LOH status of the tumours and NF1 status of the patients

Case no.	*Tumour type*	*Location*	*NF1*[a]	*LOH*[b]
569	Neurofibroma, dermal	sacrum	+	–
789	Neurofibroma, dermal	sacrum	+	–
806	Neurofibroma, dermal	back	+	+
1121	Neurofibroma, dermal	unknown	–	–
2752	Neurofibroma, dermal	back (Th4–Th6)	–	–
2753	Neurofibroma, dermal	mamma	+	+
2755	Neurofibroma, dermal	sacrum	+	–
427	Neurofibroma, plexiform	plexus brachialis	+	–
452	Neurofibroma, plexiform	N, saphenus, groin	+	–
2701	Neurofibroma, plexiform	plexus brachialis	+	+
2703	Neurofibroma, plexiform	N. medianus	–	–
2754	Neurofibroma, dorsal root	C2	+	–
363	Neurofibroma	orbita	+	+
443	Neurofibroma	neck (C6–C7)	–	–
444	Neurofibroma	orbita	+	+
746	Neurofibroma	N. ischiadicus	–	–
1087	Neurofibroma	orbita	–	–
1122	Neurofibroma	unknown	+	–
2700	Neurofibroma	N. vagus	+	–
2702	Neurofibroma	plexus brachialis	+	–
475	MPNST[c] (neurofibrosarcoma)	plexus cervicalis	+	+
784	MPNST (neurofibrosarcoma)	N. ischiadicus	+	+
2764	MPNST (neurofibrosarcoma)	sacrum	+	+

[a]+: NF1 patient; –: no NF1 patient, [b]+: loss of heterozygosity; –: retained heterozygosity, [c]MPNST: malignant peripheral nerve sheath tumour.

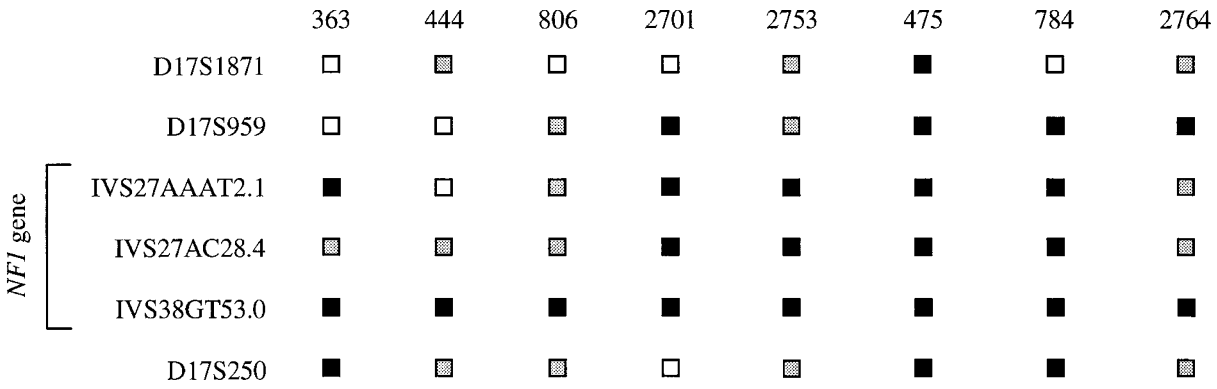

Figure 1 LOH profiles of neurofibro(sarco)mas with allelic losses in the *NF1* gene region. Tumours 475, 784, and 2764 are neurofibrosarcomas, the other tumours are neurofibromas. Black squares denote LOH, white squares no LOH, and grey squares uninformative loci.

evaluation of MSI in colorectal tumours.[19] Primers amplifying these microsatellites and PCR conditions were taken from Parsons *et al*.[20] PCR products were analysed on 6% polyacrylamide sequencing gels.

Sequencing of the antisense strand of the Sp1 site

The region harbouring the Sp1 site was PCR-amplified from bisulfite modified tumour DNA. To amplify the antisense strand, the following primers were used: 5'-CTA-AAA-AAA-AAA-CTA-ACC-CCA-AAA-3' and 5'-GGT-TGG-GAA-AGG-GGA-3', corresponding to, respectively, nucleotides –232 to –209 and 92 to 78 in the *NF1* promoter sequence.[17] The resulting 300 bp products were excised from polyacrylamide gels and extracted by incubation at 65°C for 15 min in 300 µl of 10 mM Tris-HCl, pH 7.6, 1 mM EDTA. After purification using Spin-X columns (Costar, Acton, MA, USA), DNAs were ethanol precipitated and dissolved in 25 µl H_2O. A second PCR was performed on 50 ng of purified DNA, using the same primers. The resulting PCR products were sequenced on an Applied Biosystems Model 377 automated sequencer using the Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer, Foster City, CA, USA). Sequencing was performed in triplicate using the reverse primer.

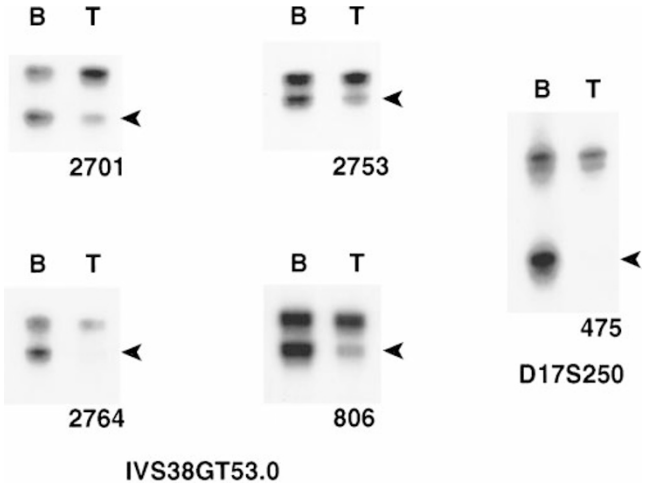

Figure 2 LOH for markers in the *NF1* gene region in neurofibro(sarco)mas. Left: examples of LOH for marker *IVS38GT53.0* in neurofibromas 806, 2701, and 2753 and in neurofibrosarcoma 2764; right: example of LOH for marker *D17S250* in neurofibrosarcoma 475. Arrowheads indicate lost alleles.

Methylation-specific PCR (MSP)

The methylation status of the strand of the CRE site in the *NF1* promoter was determined by MSP following sodium bisulfite treatment of 2 µg of DNA.[21] Primers used for unmethylated reactions of the CRE motif were: 5'-TGT-TTG-TTA-GAT-GGT-TTA-GAG-GAG-TTA-GAT-GAT-3' (sense) and 5'-AAA-AAC-AAA-AAA-AAA-AAC-AAC-CTA-CCA-CA-3' (antisense), and for methylated reactions: 5'-CGT-TAG-ACG-GTT-TAG-AGG-AGT-TAG-ATG-AC-3' (sense) and 5'-AAA-AAA-AAA-ACG-ACC-TAC-CGC-G-3' (antisense). PCR reactions were hot-started at 95°C for 5 min before the addition of 1.25 units of Taq polymerase. Amplification of 50 ng of bisulfite-treated DNA was performed by 35 cycles (30 s at 95°C, 30 s at 62°C, and 30 s at 72°C), followed by a final 5-min extension at 72°C. All PCRs included no DNA control as well as positive controls for both unmethylated and methylated alleles. For the latter, human placental DNA was treated with excess *Sss*I methyltransferase (New England Biolabs, Beverly, MA, USA), according to the manufacturer's protocol. PCR products were directly loaded on to nondenaturing 8% polyacrylamide gels, and visualised under UV illumination after ethidium bromide staining.

Results

We analysed 20 neurofibromas and three neurofibrosarcomas from 23 patients for the presence of LOH in the *NF1* gene region and for MSI. Furthermore, we determined the methylation status of the Sp1 binding site and the CRE site near the

transcription start site of the *NF1* gene. As shown in Table 1, 17 tumours were from NF1 patients.

LOH for the *NF1* gene region was detected in five of 20 neurofibromas (25%) and in the three neurofibrosarcomas (Table 1 and Figure 1). Due to variable contribution of cell types without LOH in the neurofibromas, allele loss was never found to be complete (Figure 2). The tumours showed LOH for all intragenic markers, except for tumour 444 in which the *NF1* gene seems to be only partially deleted.

The presence of MSI in the tumours was determined by analysis of mononucleotide repeat markers *BAT25, BAT26,* and *BAT40,* and dinucleotide repeat microsatellite markers *D2S123, D5S346,* and *D17S250*. Corresponding leukocyte DNAs were used as negative control, whereas DNA from a MSI-positive duodenum carcinoma from a patient with Muir-Torre syndrome was used as positive control. We did not detect MSI in any of the tumours. Representative examples, showing presence of MSI for marker *BAT26* in the duodenum carcinoma but absence of MSI in the neurofibromas, are given in Figure 3.

By *in vitro* studies, it has been demonstrated that methylation of the first two cytosines in the antisense strand of the Sp1 recognition sequence nearby the transcription start site impedes binding of the Sp1 protein (see Figure 4).[16] To evaluate the methylation status of these cytosine residues, we treated each tumour DNA with sodium bisulfite and sequenced the antisense strand of the Sp1 motif in every tumour. In Figure 5, the sequencing electrophoretogram in 5'–3' orientation for the Sp1 site in neurofibroma 443 is shown. The two cytosines have been converted into thymidines (marked by arrows), suggesting that in this tumour these cytosines were unmethylated. Figure 5 is representative

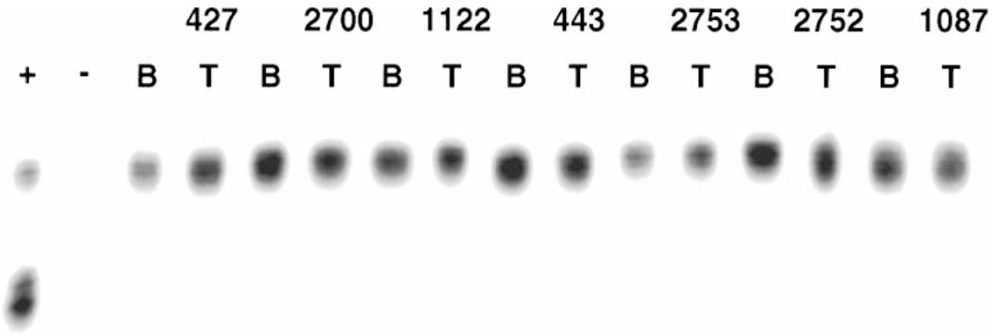

Figure 3 Determination of MSI in neurofibromas using microsatellite marker *BAT26*. Tumour (T) DNA and corresponding blood (B) DNA was subjected to PCR using *BAT26*-specific primers. PCR products were analysed on 6% polyacrylamide gels; +: duodenum carcinoma with known MSI from a patient suffering from Muir-Torre syndrome; –: no DNA. Case numbers of tumours are indicated.

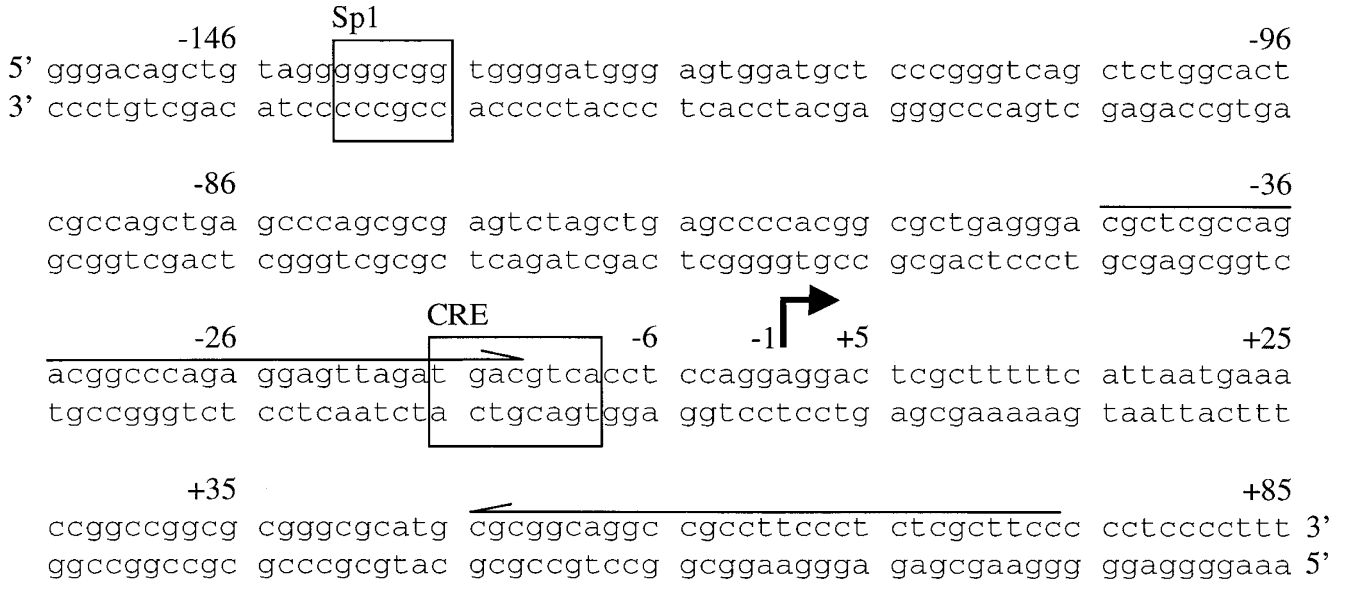

Figure 4 DNA sequence of part of the human *NF1* promoter region. The major transcription initiation site is indicated by an arrow. The sequence is numbered relative to this site and harbours, among other transcription binding site motifs, a cAMP response element (CRE) and an Sp1 site. Both sequences are boxed. Positions of primers for unmethylated reactions in MSP for examination of the sense strand of the CRE site are indicated.

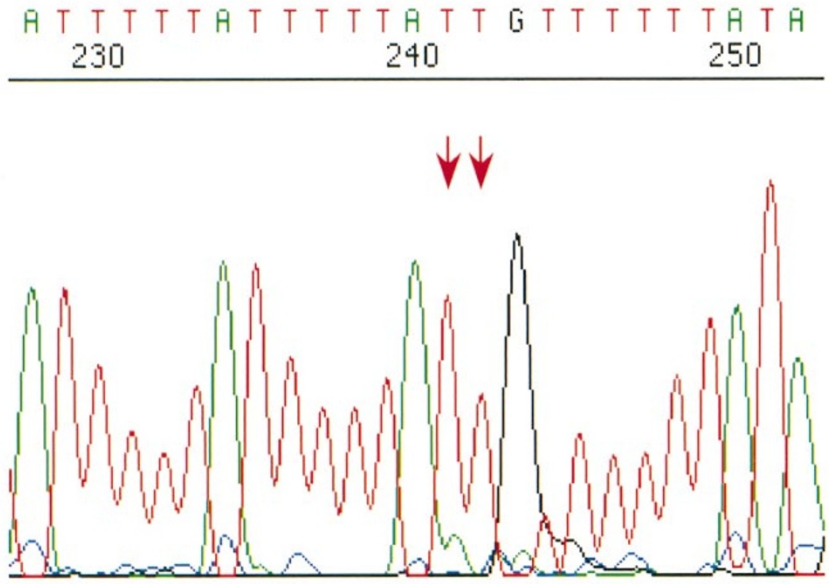

Figure 5 Sequence of the antisense strand of the Sp1 site in neurofibroma 443 after bisulfite treatment. Arrows indicate thymidines corresponding to the first two cytosines in the antisense strand sequence of the Sp1 site.

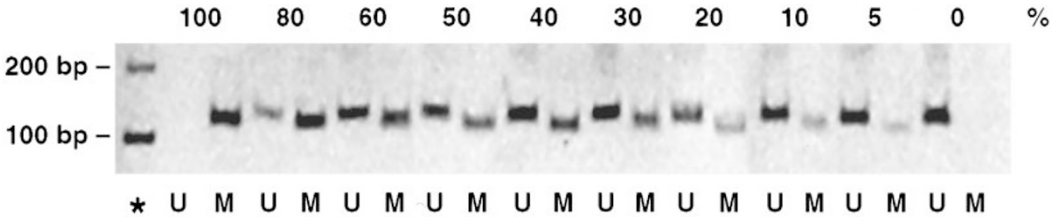

Figure 6 Sensitivity of MSP to detect methylation of the CRE site in the *NF1* promoter. *In vitro* methylated DNA was gradually diluted with unmethylated DNA. % denotes percentage of methylated DNA. Each PCR reaction contained in total approximately 50 ng of DNA. U: PCR products using primers specific for unmethylated DNA; M: PCR products using primers specific for methylated DNA; *: 100 bp and 200 bp fragments of a 100 bp ladder.

for all tumours: we did not detect any methylation of the two cytosine residues in our tumours.

Mancini *et al*[16] have shown that *in vitro* binding of CREB is inhibited by cytosine methylation of the CpG dinucleotide in the CRE motif. To assess its methylation status in the tumours, we performed methylation-specific PCR (MSP), using a forward primer that ends in the CpG dinucleotide (see Figure 4). Considering the variable contribution of non-tumour cells to the tumour tissues, we first checked whether MSP under the conditions applied was sensitive enough to detect methylation. This was performed by gradually diluting *in vitro* methylated DNA with unmethylated DNA. As shown in Figure 6, methylation of the CRE site is easily detectable under conditions in which the contribution of methylated DNA is only 5%. Subsequently, we studied the methylation status of the CRE site in all tumours. However, for none of them methylation was found (Figure 7).

Discussion

We detected LOH of the *NF1* gene region in five of 20 neurofibromas and in the three neurofibrosarcomas (Table 1 and Figures 1 and 2). All tumours showing LOH were from patients with NF1, who are assumed to carry one constitutively inactivated *NF1* allele. Assuming that the wild type *NF1* was lost by LOH, the development of these tumours may be explained by Knudson's two-hit model for tumorigenesis.[2] At least one hit, a germ line mutation, is supposed to be present in the tumours of the remaining NF1 patients. In the other tumours, no hit was detected. In these cases, besides subtle deletions escaping detection by LOH or point

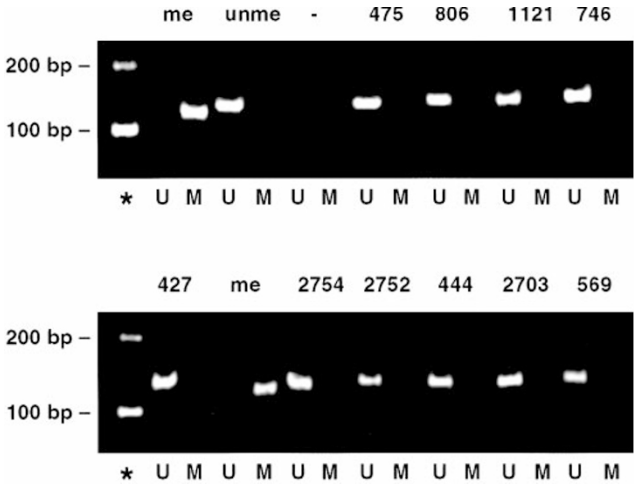

Figure 7 Analysis by MSP of the methylation status of the CRE site in the *NF1* promoter region in neurofibro(sarco)mas. Case numbers of analysed tumours are indicated. Tumour 475 is a neurofibrosarcoma, all other tumours are neurofibromas. me: *in vitro* methylated DNA; unme: unmethylated DNA. Each PCR reaction contained in total approximately 50 ng of DNA. U: PCR products using primers specific for unmethylated DNA; M: PCR products using primers specific for methylated DNA; * 100 bp and 200 bp fragments of a 100 bp ladder.

mutations, the *NF1* gene might have been inactivated by alternative mechanisms. Here, we have investigated microsatellite instability (MSI) and *NF1* promoter methylation as potential contributors to *NF1* gene inactivation. We analysed six microsatellite markers with proven efficiency for MSI detection in colorectal tumours, and we could not find MSI in any tumour (Figure 3). Our findings support the results presented by Serra *et al*,[9] who could not detect MSI in 60 neurofibromas, but are in contrast to those of Ottini *et al*,[12] who found MSI in eight of 16 cases.

Hypermethylation of the promoter region may inactivate the *NF1* gene by abolishing its expression, as has been shown for several other tumour suppressor genes.[13–15] In addition, the data of Mancini *et al*[16] suggest that *in vitro* binding of the transcription factors Sp1 and CREB is inhibited by methylation of specific cytosine residues in their respective recognition sequences in the *NF1* promoter region. In the tumours we examined, there was no indication for methylation of the two cytosines in the antisense strand of the Sp1 site or for cytosine methylation in the CpG dinucleotide of the CRE site (Figures 5 and 7). Moreover, the methylation-specific PCR (MSP) that was used for examination of the CpG in the CRE site additionally excluded hypermethylation of the analysed region.

In conclusion, our data suggest that MSI and promoter methylation are unlikely to be involved in *NF1* gene inactivation in neurofibromas.

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