

# Monozygotic Twins With Neurofibromatosis Type 1 (NF1) Display Differences in Methylation of *NF1* Gene Promoter Elements, 5' Untranslated region, Exon and Intron 1

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Neurofibromatosis type 1 (NF1) is a common autosomal dominant disorder caused by heterozygotic inactivation of the *NF1* tumor suppressor gene at 17q11.2. The associated phenotypes are highly variable, and modifying genes have been proposed to explain at least in part the intriguing expressivity. Given that haploinsufficiency of the *NF1* gene product neurofibromin is responsible for some of the clinical manifestations, variations in expression of the wild-type *NF1* allele might modify the phenotype. We therefore investigated epigenetic molecular modifications that could result in variable expression of the normal *NF1* allele. To exclude confounding by DNA sequence variations, we analyzed monozygotic twin pairs with NF1 who presented with several discordant features. We fine-mapped the methylation pattern of a nearly 1 kb *NF1* promoter region in lymphocytes of 8 twin pairs. All twin pairs showed significant intra-pair differences in methylation, especially of specific promoter subregions such as 5'UTR, exon 1 and intron 1 (+7 to +622), transcription factor binding sites and promoter elements like *NF1HCS*. Furthermore, we detected significant intra-pair differences in cytosine methylation for the region from -249 to -234 with regard to discordance for optic glioma with a higher grade of methylation in glioma cases. In conclusion, our findings of epigenetic differences of the *NF1* promoter in leukocytes within monozygotic twin pairs may serve as a proof of principle for other tissues. The results point towards a role of methylation patterns of

the normal *NF1* allele for expression differences and for modification of the *NF1* phenotype.

**Keywords:** neurofibromatosis type 1, *NF1*, promoter, methylation, monozygotic twin pairs

Neurofibromatosis type 1 (NF1) is one of the most common autosomal dominant disorders in humans with an estimated birth incidence of 1:2500–1:3000. The disease is caused by mutations of the *NF1* gene. Major clinical manifestations are multiple café-au-lait spots, axillary and inguinal freckling, multiple dermal neurofibromas, and iris Lisch nodules. Affected individuals may have any of a wide range of additional features.

The *NF1* gene on chromosome 17q11.2 is known to act as a tumor suppressor since loss-of-function mutations are associated with the development of benign and malignant tumors in neural crest derived tissues and some myeloid malignancies (Shannon et al., 1994; Stiller et al., 1994). The great majority of known mutations are predicted to inactivate the mutant

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allele completely. The most common transcript codes for a cytosolic protein of 2818 amino acids termed neurofibromin (Gutmann et al., 2001; Li et al., 1995).

The NF1 phenotype shows a high degree of variability, even within families and between sibs and twins who carry the same *NF1* mutation, despite close to 100% penetrance. Numerous different mutations have already been detected (Messiaen L. 2008). But, for the majority of NF1 patients definite genotype-phenotype correlations have not yet emerged. There are two exceptions: patients with a 3 bp deletion in exon 17 exhibit a very mild phenotype, and those patients who carry large deletions of the *NF1* gene and flanking sequences (microdeletions) show a tendency towards a more severe phenotype (De Raedt et al., 2003; Leppig et al., 1997; Mensink et al., 2006; Spiegel et al., 2005). The latter finding was recently underlined by identification of four genes in the microdeletion region, *CENTA2*, *RAB11FIP4*, *C17orf79* and *UTP6*, which may potentially influence neurofibroma development (Bartelt-Kirbach et al., 2009). Given that haploinsufficiency of the *NF1* gene

product neurofibromin is responsible for some of the clinical manifestations of NF1 it seems plausible that variations in the expression of the wildtype *NF1* allele could modify the phenotype and clinical course. Such a variable expression by SNPs of the normal *NF1* allele was investigated recently, and no relation to NF1 variability was seen (Sabbagh et al., 2009). Alternatively, epigenetic modifications of the wildtype *NF1* allele could be responsible for NF1 phenotypic variability.

The DNA of most organisms is modified by the postsynthetic addition of a methyl group to carbon 5 of the cytosine ring. CpG dinucleotides as well as non-CpGs can be methylated in mammals (Haines et al., 2001; Laurent et al., 2010; Lister et al., 2009). CpG dinucleotides are underrepresented in the vertebrate genome: an expected frequency of CpGs is only maintained at CpG islands. About half of all genes contain CpG islands in their promoters that are free of methylation and actively transcribed. The human *NF1* promoter contains such a constitutively hypomethylated CpG island and is methylated far upstream of the transcriptional start site. Several regulatory elements,

**Table 1**  
Clinical Data of the Twin Pairs

Twin pair	Twin number	Age at investigation	Gender (F,M)	Lisch nodules	Freckling	Cafe-au-lait spots	Intra-cutaneous neuro-fibromas	Intra- and sub-cutaneous neuro-fibromas	Plexiform neuro-fibromas	MPNST	Optic glioma	Scoliosis
A	1	17	F	+	+	25	60	60	0	0	+	—
	2			+	+	15	40	40	2	0	—	+
B	3, 4	33	F	C	C	C	0.3	0.3	D	C	D	D
				—	+	5	10	10	0	0	—	+
				+	+	22	8	8	0	0	—	—
C	5, 6	18	M	D	C	D	0.2	0.2	C	C	C	D
				+	+	26	18	38	0	0	—	+
				+	+	18	26	26	1	0	—	—
D	7, 8	8	F	C	C	C	0.3	0.3	D	C	C	D
				—	+	12	7	7	0	0	+	—
				—	+	11	5	5	0	0	—	+
E	9, 10	15	M	C	C	C	0.3	0.3	C	C	D	D
				+	+	7	0	10	1	0	—	—
				—	+	8	0	14	1	0	+	—
F	13, 14	9	F	D	C	C	0	0.3	C	C	D	C
				—	+	7	0	0	0	0	—	—
				—	—	1	0	0	0	0	—	—
G	15, 16	6	F	C	D	D	0	0	C	C	C	C
				+	+	61	10	10	0	0	+	—
				+	+	42	0	0	0	0	—	—
H	17, 18	43	M	C	C	C	1	1	C	C	D	C
				/	+	9	30	30	2	0	—	—
				/	+	1	100	100	2	0	—	—
				/	C	D	0.7	0.7	C	C	C	C

Note: C stands for concordant features, D for discordant ones. To compare the difference in the number of cutaneous and subcutaneous neurofibromas a standard measure is given as described in material and methods.

conserved transcription start sites and transcription factor binding sites for SP1, CREB, AP-2 and SRE have been identified (Hajra et al., 1994; Zou et al., 2004). A few reports have investigated methylation of the *NF1* promoter in vitro or in NF1 associated tumors using different approaches (Andrews et al., 1996; Ebinger et al., 2005; Fishbein et al., 2005; Haines et al., 2001; Harder et al., 2004; Horan et al., 2000; Luijten et al., 2000; Mancini et al., 1999; Rodenhiser et al., 1993). It was also shown that methylation of the proximal *NF1* promoter inhibits expression of *NF1* constructs in luciferase assays (Zou et al., 2004).

Nevertheless, data on the status of methylation of *NF1* alleles in normal tissues of patients with NF1 are lacking. Differences in methylation of the *NF1* gene between relatives, sibs and twins with NF1 have not been investigated in detail so far. Since it has been shown that methylation of the *NF1* promoter influences gene expression (Zou et al., 2004), we propose that the intriguing clinical variability of NF1 could be related to differences in methylation patterns of the *NF1* promoter region. To exclude confounding by DNA sequence variations we analyzed eight genetically identical monozygotic twin pairs with NF1, who presented with discordant features, for methylation in their promoter regions of the *NF1* gene. We fine-mapped the methylation pattern by genomic sequencing of clones of bisulphite treated DNA. Thus our approach involves research into epigenetic molecular factors that could result in variation in expression of the normal *NF1* allele.

## Material and Methods

### Patients and Material

Eight pairs of monozygotic twins with NF1 were analyzed (A-H). All twin pairs but one were descending from Germany, twin pair C originated from Croatia. Clinical features of pairs A, B, C, and D have already been published (Detjen et al., 2007). Monozygosity was verified in all cases. All twins except one (twin 14 of pair F) fulfilled diagnostic criteria of NF1 according to NIH (1988). A mutation analysis confirmed a pathogenic *NF1* mutation in 6/8 twin pairs. A *SPRED1* mutation was ruled out in twin pair F where we diagnosed only one twin according to diagnostic criteria to exhibit NF1 and did not find a *NF1* mutation by comprehensive analysis. One twin pair did not subject DNA for mutation analysis, but both twin had full-blown NF1. All patients provided written informed consent of this study and the publication of the results.

Physical examination of the affected sibs was carried out by clinical geneticists or pediatricians including cranial MRI in twin pairs A-E and G. The clinical features are summarized in Table 1. All individual twin pairs differed at least slightly in their phenotype. We assigned NF1 features to be of discordant (D) or concordant (C) occurrence (Table 1). Furthermore, we defined a standard (normalized) measure for the difference in number of cutaneous and

subcutaneous neurofibromas ( $\Delta Nf$ ) within each twin pair by the formula:  $(Nf_1 - Nf_2) / Nf_1$  ( $Nf_1$ , higher value of neurofibroma number;  $Nf_2$ , lower value of neurofibroma number; in case of absence of neurofibromas ( $Nf_1 = 0$ ) we replaced 0 by a value slightly larger than 0 (to be able to apply the formula)).

We analyzed DNA of peripheral leukocytes for methylation, which was extracted by standard procedures. 3  $\mu$ g of DNA was necessary for the bisulphite modification by our procedure.

### Analyzed *NF1* Region

The analyzed *NF1* region (NCBI NG\_009018.1, GenBank U17084) spanned -286 to +650 of the promoter, 5'UTR, exon 1 and part of intron 1. Base numbering was according to Hajra et al. (Hajra et al., 1994) and was used throughout this publication to facilitate comparisons with other studies. We mapped methylation of every cytosine residue in accordance to recent studies which showed that besides CpG methylation also non-CpG methylation is important (Lister et al., 2009).

Several subregions were chosen for detailed analysis with regard to their previously published relevance: The binding region (-337 to -234) of the human TATA-binding protein-like factor (TLF) which was shown to increase transcription from the *NF1* promoter and to regulate *NF1* expression (Chong et al., 2005) overlapped in part with our analyzed region (5'-CGGCAGAGATCCGCGC-3', -249 to -234). The *cis* regulatory element between -236 and -3 able to repress *NF1* gene expression via binding of its *trans* regulator Tax of HTLV-1 (human T-lymphotropic virus type 1) or Tax-regulated genes (Feigenbaum et al., 1996), was included in our analyzed sequence. Additionally, a region with a 100% homology between different species (NF1HCS, *NF1* 5' Highly Conserved Sequence) being identical in mouse, human and rat and being speculated to represent a core promoter element or a strong transcriptional activator (Lee and Friedman 2005) was investigated in between +151 to +174 (5'-ACTTCCGGTGGGGTGTTCATGGCGG-3'). The -141 SP1 as well as the -16 CRE binding site were analyzed since they were shown to have an impact on *NF1* gene transcription (Zou et al., 2004). Furthermore, binding of the CREB protein to its recognition element (5'-ATGACGTCA-3') as well as SP1 binding (5'-GGGCGG-3') was found to be methylation sensitive in vitro (Mancini et al., 1999).

### Sodium Bisulphite Reaction, PCR, Cloning and Sequencing

Bisulphite modification was carried out as described in detail previously (Harder et al., 2004) using the agarose bead method since it assures full denaturation during the chemical conversion at 50°C. The *NF1* promoter was analyzed within two overlapping fragments using primer pairs for sequences from -286 to +203 (P5/P8, 489 bp) and from +182 to +650 (P7/P6, 469 bp). Sequences and PCR conditions had already been published (Harder et al., 2004). Sodium bisulphite-

modified PCR products were eluted and cloned using the TOPO TA Cloning kit (Invitrogen) with vector pCR 2.1-TOPO and One Shot TOP10F' competent *E. coli* cells. White colonies were cultured overnight. Plasmids were extracted, checked for insert by EcoRI digestion, and finally sequenced by capillary sequencing using M13 forward and reverse primers.

#### Determining Methylation Patterns

The method of sequencing individual plasmids/clones from a transfected PCR product of a bisulphite converted DNA fragment allows to obtain exact quantitative figures for the percentage of methylation for every cytosine residue over an analyzed region. We sequenced 8–46 clones per sample (mean 25 per sample). Percentages of methylation were calculated for each cytosine residue (e.g. if 3 out of 30 cytosines at a specific residue were methylated, the percentage of methylation was 10%). The mean percentage for a given fragment was calculated by summing up all values of percentages of methylation of the fragment and dividing this sum by the number of cytosine residues (or CpGs) of the fragment, as proposed by Siegmund and Laird (Siegmund and Laird 2002).

To compare the differences in methylation between twin pairs, we subtracted the lower mean value (mean percentage of cytosine or CpG methylation) from the higher mean value within each twin pair. This allows to exclude the different levels of methylation between

pairs and to obtain one value as a measure for difference for each pair.

#### Statistics

We used a two-sided one-sample *t*-test to test the hypothesis that the mean within-pair difference in methylation for a given fragment of the *NF1* promoter was significantly different from 0. To account for multiple testing, we additionally adjusted the type 1 error by widening the confidence interval of the variable to 99%. We tested all elements that had been previously investigated functionally such as the TLF binding region (Chong et al., 2005), the tax element (Feigenbaum et al., 1996), -141 SP1 and -16 CRE binding sites (Zou et al., 2004), all putative binding sites proposed by Hajra et al. (2004) such as AP2 binding sites (-166 to -157, -139 to -130, +264 to +273, +335 to +344, +463 to +472), SP1 binding sites (-165 to -160, -141 to -136, +416 to +421, +460 to +465), the MT1 element (-100 to -95), the SRE binding site (-14 to -7) as well as the NF1HCS element (Lee and Friedman 2005), repressor elements from +144 to +474 (Viskochil 1998) and from +231 to +539 (Zou et al., 2004), a redefined promoter region from -228 to +373, core promoter elements from -21 to +131 and from +248 to +399 (Lee and Friedman 2005) and a minimal proximal promoter from -249 to +230 (Zou et al., 2004).

**Table 2**

CpG Methylation of the Respective NF1 Regions

Region of interest	Ref.	Mean % of methylation	Difference of methylation (%)	P
<b>promoter, 5'UTR, exon 1, intron 1</b> (-249 to +622)	[1]	3.47 ± 0.57	1.23 ± 0.42	.022
<b>5'UTR, exon 1, intron 1</b> (+7 to +622)	[1]	3.39 ± 0.57	1.46 ± 0.35	<b>.004*</b>
<b>promoter</b> (-249 to -13)	[1]	3.69 ± 0.69	1.75 ± 0.52	.012
<b>5'UTR</b> (+7 to +474)	[1]	3.17 ± 0.49	1.21 ± 0.51	.049
<b>exon 1</b> (+489 to +537)	[1]	5.53 ± 1.52	4.44 ± 1.54	.024
<b>intron 1</b> (+549 to +622)	[1]	2.52 ± 0.67	2.06 ± 0.61	.012
<b>minimal proximal promoter</b> (-249 to +222)	[2]	3.71 ± 0.64	1.83 ± 0.66	.027
<b>redefined promoter</b> (-208 to +345)	[3]	3.19 ± 0.57	1.61 ± 0.64	.039
<b>core promoter</b> (-13 to +131)	[3]	3.44 ± 0.79	2.99 ± 1.14	.035
<b>core promoter</b> (+291 to +345)	[3]	1.73 ± 0.38	1.20 ± 0.22	<b>.001*</b>
<b>TLF</b> (-249 bis -234)	[6]	6.58 ± 1.50	5.99 ± 2.07	.023
<b>tax cis element</b> (-236 to -13)	[5]	3.23 ± 0.64	1.49 ± 0.57	.036
<b>CRE (SRE)</b> (-13)	[1], [7]	4.37 ± 1.42	4.66 ± 1.91	.045
<b>SP1 (AP2)</b> (-138)	[1], [7]	0.95 ± 0.55	1.24 ± 0.91	.216
<b>AP2</b> (-163/-166)	[1]	3.91 ± 0.77	4.02 ± 0.75	<b>.001*</b>
<b>repressor</b> (+156 to +474)	[4]	3.03 ± 0.49	1.17 ± 0.31	<b>.007*</b>
<b>NF1HCS</b> (+156 to +172)	[3]	3.38 ± 0.77	3.59 ± 1.03	.010
<b>repressor</b> (+247 to +537)	[2]	3.33 ± 0.79	2.08 ± 0.83	.040

Note: Mean values of methylation with standard error of the mean (SEM) are given in percentage for all twins (*n* = 16) of the 8 pairs. Mean values of differences (%) in CpG methylation of all twin pairs (*n* = 8) are given with SEM. Differences within twin pairs were calculated by subtracting the lower mean value (% of methylation) from the higher mean value within each twin pair. Base numbering is according to Hajra et al. (Hajra et al., 1994). P values for statistical analysis with a 95% confidence interval (CI) are given. An asterisk (\*) marks those values that remained statistically significant after adjusting for multiple testing (99% CI). References: (Hajra et al., 1994) — [1]; (Zou et al., 2004) — [2]; (Lee and Friedman 2005) — [3]; (Viskochil, 1998) — [4]; (Feigenbaum et al., 1996) — [5]; (Chong et al., 2005) — [6]; (Mancini et al., 1999) — [7].

**Table 3**  
Cytosine Methylation of the Respective NF1 Regions

Region of interest	Ref.	Mean % of methylation	Difference of methylation (%)	P
<b>promoter, 5'UTR, exon 1, intron 1</b> (-249 to +622)	[1]	2.76 ± 0.50	0.99 ± 0.37	.030
<b>5'UTR, exon 1, intron 1</b> (+5 to +622)	[1]	2.71 ± 0.47	1.01 ± 0.26	<b>.006*</b>
<b>promoter</b> (-249 to -4)	[1]	2.91 ± 0.70	1.77 ± 0.07	.004
<b>5'UTR</b> (+5 to +483)	[1]	2.50 ± 0.41	0.81 ± 0.31	.034
<b>exon 1</b> (+488 to +541)	[1]	5.55 ± 1.51	4.14 ± 1.44	.024
<b>intron 1</b> (+548 to +622)	[1]	2.36 ± 0.54	1.55 ± 0.73	.071
<b>minimal proximal promoter</b> (-249 to +226)	[2]	3.12 ± 0.68	1.83 ± 0.81	.058
<b>redefined promoter</b> (-222 to +373)	[3]	2.57 ± 0.49	1.19 ± 0.47	<b>.004*</b>
<b>core promoter</b> (-13 to +131)	[3]	3.33 ± 0.83	3.02 ± 1.10	.028
<b>core promoter</b> (+252 to +398)	[3]	1.89 ± 0.37	0.97 ± 0.29	.012
<b>TLF</b> (-249 bis -234)	[6]	5.17 ± 1.29	5.57 ± 1.75	.015
<b>tax cis element</b> (-236 to -4)	[5]	2.79 ± 0.69	1.63 ± 0.74	.064
<b>CRE</b> (-13/-10)	[1], [7]	4.43 ± 1.28	4.23 ± 1.82	.053
<b>SP1</b> (AP2) (-138)	[1], [7]	0.95 ± 0.55	1.24 ± 0.91	.216
<b>AP2</b> (-163/-166)	[1]	3.91 ± 0.77	4.02 ± 0.75	<b>.001*</b>
<b>repressor</b> (+148 to +474)	[4]	2.25 ± 0.39	1.12 ± 0.24	<b>.003*</b>
<b>NF1HCS</b> (+152 to +172)	[3]	3.41 ± 0.71	4.12 ± 0.69	<b>.001*</b>
<b>repressor</b> (+233 to +537)	[2]	2.43 ± 0.50	1.39 ± 0.47	.022
<b>AP2</b> (+463 to +472)	[1]	2.11 ± 0.39	1.14 ± 0.28	<b>.004*</b>

Note: Mean values of methylation with SEM are given in percentage for all twins ( $n = 16$ ) of the 8 pairs. Mean values of differences (%) in CpG methylation of all twin pairs ( $n = 8$ ) are given with SEM. Differences within twin pairs were calculated by subtracting the lower mean value (% of methylation) from the higher mean value within each twin pair. Mean percentage of cytosine methylation over a region was calculated by summing up all individual values of percentages of the fragment and dividing the sum by the number of cytosine residues (including CpGs, CpNpGs, non-CpGs). Base numbering is according to Hajra et al. (Hajra et al., 1994). P values for statistical analysis with a 95% confidence interval (CI) are given. An asterisk (\*) marks those values that remained statistically significant after adjusting for multiple testing (99% CI). References are equal to Table 2.

To analyze whether the appearance of optic glioma in discordant twin pairs could be explained by differences in methylation of specific subregions we used the following approach: We hypothesized that the difference in methylation within discordant pairs (affected co-twin minus unaffected co-twin) would be significantly greater than within unaffected twin pairs (unaffected co-twin minus unaffected co-twin). Both groups (discordant twin pairs vs. concordant twin pairs) were compared using Mann-Whitney U-test.

Spearman's rank correlation test was applied to correlate the standard measure of neurofibroma number with the differences in methylation of given *NF1* regions.

## Results

### Mean Percentage of *NF1* Methylation

We analyzed 72 CpGs, 20 CCGs, 5 CCCGs, 3 CCCCCGs, 1 CCCCCG, and 1 CCCCCCG as well as 255 non-CpGs including 43 CpAs, 64 CpTs and 148 CpCs. Data are shown in Figure 1 illustrating different patterns when comparing methylation of all cytosine residues (including CpGs, CpNpGs and non-CpGs) with CpG methylation.

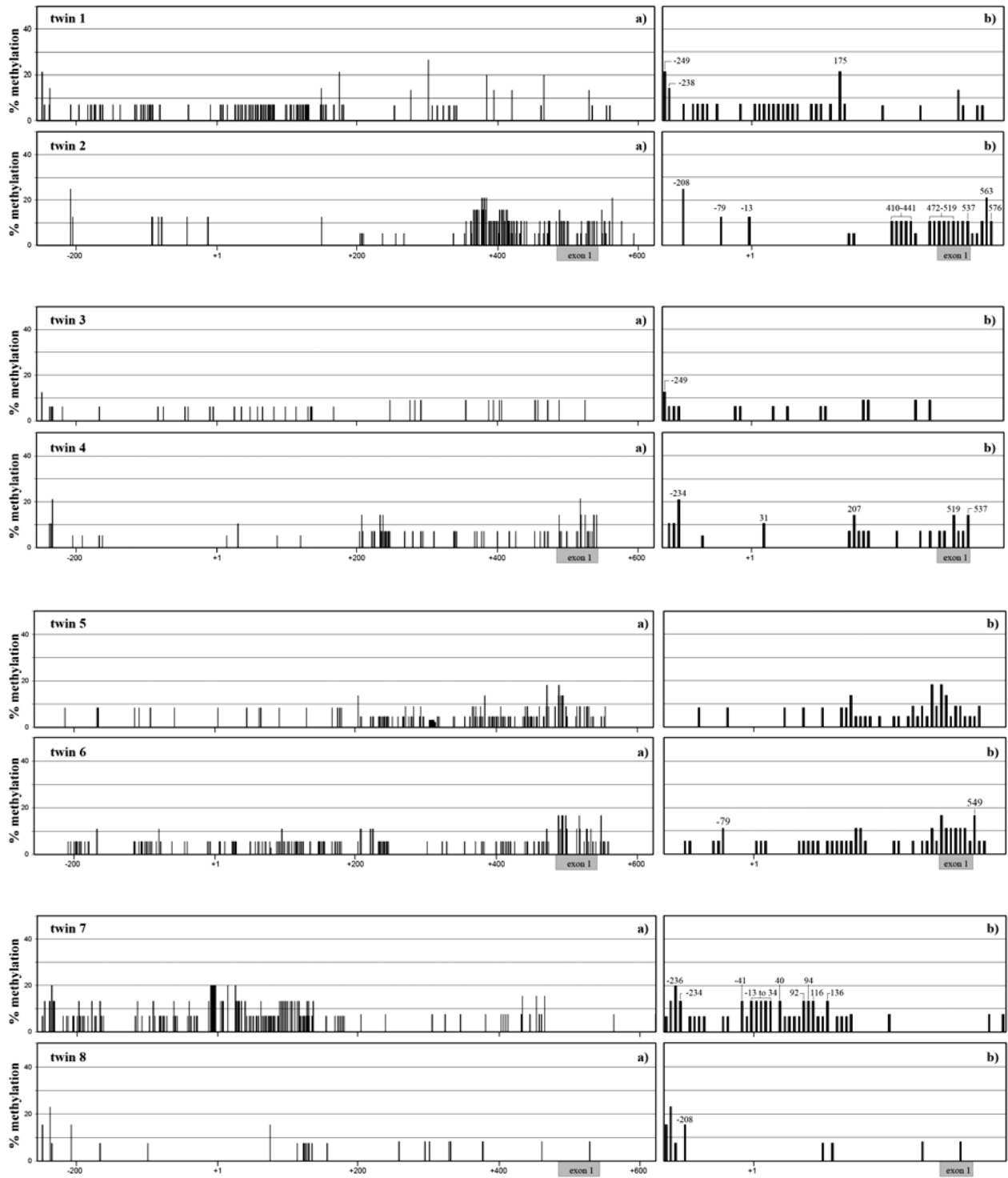
The individual levels of methylation in the *NF1* region which covered the CpG island ranged between 0% up to 40.5% per residue. We first calculated the

mean percentage of methylation. In all twins ( $n = 16$ ) mean percentage of methylation for the whole fragment was  $2.76\% \pm 1.98\%$  for cytosine methylation and  $3.47\% \pm 2.26\%$  for CpG methylation (Tables 2 and 3). For all calculations primer sequences were excluded, therefore we present data for a region from -249 to +622. We also calculated mean percentages of methylation for subregions (promoter, 5'UTR, exon 1, intron 1) and several promoter elements (TLF binding site, tax element, SP1 and CRE binding sites, NF1HCS) according to their functional properties since methylation showed clustering in some regions. Mean percentages are shown in Tables 2 and 3. Twin pairs A, B and G showed marked differences of the mean percentage of methylation in exon 1, and pair D for the promoter 'only' region. Twin pair D represented the highest differences (0 versus 16.67%) of the mean percentage of methylation of the CRE site (-10/-13).

### Methylation Within Twin Pairs Differs Specifically in Subregions and CpG Sites

As displayed in Figure 1 methylation patterns were different within the twin pairs. Although overall methylation levels were low, it must be taken into consideration that mean percentages of methylation do not reflect the regional clustering with much higher values within the fragments analyzed. To detect spatial clustering of methylation as well as methylation of a

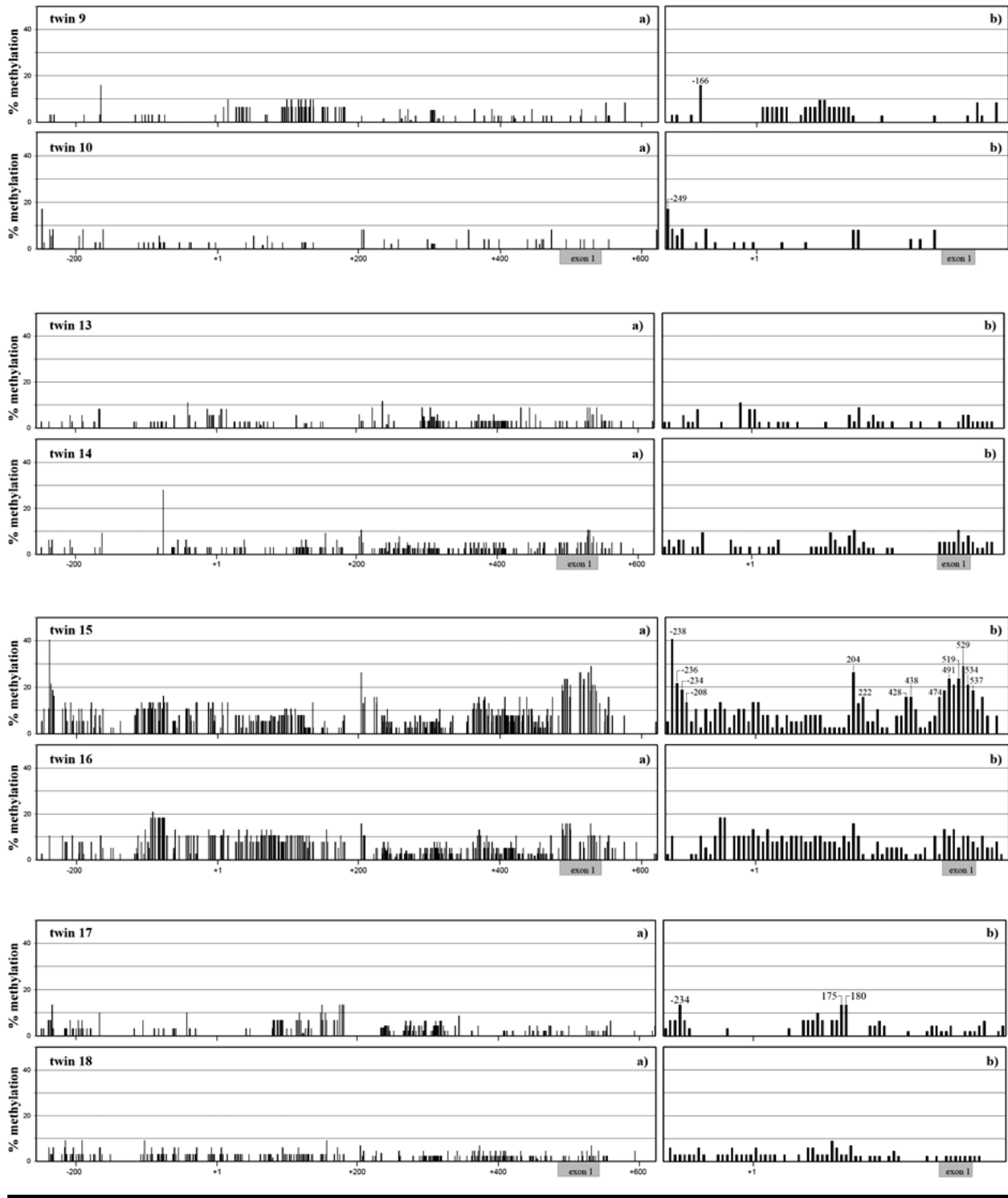




*continued over*

**Figure 1**

Percentages of methylation of all cytosine residues (sequence from -249 to +622) for all twin pairs (A–H) are depicted. On the left side (a) percentage of methylation of all cytosine residues is shown (including CpGs, CpNpGs and non-CpGs). On the right side (b) percentages of methylation of only CpG dinucleotides of the same region is presented. Those CpGs which showed a different percentage of methylation (at least 10 %) between the twins of the each pair are indicated. Position +112 was omitted from evaluation according to Hajra et al. 1994.



**Figure 1 (continued)**

Percentages of methylation of all cytosine residues (sequence from -249 to +622) for all twin pairs (A–H) are depicted. On the left side (a) percentage of methylation of all cytosine residues is shown (including CpGs, CpNpGs and non-CpGs). On the right side (b) percentages of methylation of only CpG dinucleotides of the same region is presented. Those CpGs which showed a different percentage of methylation (at least 10 %) between the twins of the each pair are indicated. Position +112 was omitted from evaluation according to Hajra et al. 1994.

large amount of single residues we especially applied a method for fine mapping of the region. When comparing twins of each pair, we regarded methylation to be different when the percentage of methylation was at least 10% different between the same residues. Those CpG sites are marked in Figure 1.

Twin pair A presented the highest amount of intra-pair differences of methylation. The 17-year-old females were discordant for the occurrence of plexiform neurofibromas, optic glioma and scoliosis. Differences in methylation occurred at single sites as well as in clusters at 69 cytosine residues including CpGs at -249, -238, -208, -79, -13, +175, +410, +417, +428, +438, +441, +472, +474, +489, +491, +500, +519, +537, +563, and +576. Twin 2 who had plexiform neurofibromas showed remarkable clustering of cytosine and CpG methylation of 5'UTR, exon 1 and intron 1 between +355 to +575 with very dense clustering between +355 to +441. In this region putative SP1 and AP2 binding sites (+336 to +345, +416 to +421; +460 to 465) are located. Both twins also differed by site-specific CpG methylation. Twin 1 who presented with an optic glioma showed higher methylation levels of CpG -249 (21.43% vs. 0%) and CpG -238 (14.29% vs. 0%) both located in the TLF binding region (see Fig. 2) as well as of CpG +175. Twin 2 had a markedly higher methylation at CpG -208 (25.0% vs. 7.14%), CpG +563 (21.05% vs. 0%) and at the CpG covering the CRE site at -13 (12.5% vs. 0%).

Twin pair B, women at age 33, differed mainly in presentation of pigmentary lesions and scoliosis. Differences in methylation occurred at 10 cytosine residues including CpGs at -249, -234, +31, +207, +519, and +537. A remarkable difference of methylation was seen in exon 1. Both twins had no optic glioma and presented with a comparable amount of methylation concerning the TLF binding site (-249 to -234), although twin 4 had higher values.

Twin pair C, 18-year-old males, had relatively similar methylation patterns with slight clustering at low values. Only four sites displayed differences of methylation greater than 10% involving CpG -79 and CpG +549. Exon 1 region showed increased methylation levels in both. The twins differed by the presence of subcutaneous neurofibromas, a plexiform neurofibroma and scoliosis.

Twin pair D presented with discordance for an optic glioma, scoliosis and seven versus five cutaneous neurofibromas at the age of 8 years. Nevertheless, the girls displayed marked differences of methylation at single sites as well as in certain clusters. Differences in methylation occurred at 46 single sites as well as in clusters including CpGs at -236, -234, -208, -41, -13, +7, +27, +31, +34, +40, +92, +94, +116, and +136. Twin 7 who had an optic glioma had a higher methylation of the promoter and upstream 5'UTR up to CpG +136 including 14 different CpG sites and dense clustering of cytosine methylation between -13 to +34 and +88 to +110 and furthermore covering the region for TLF

binding (-249 to -234). In the region between -13 to +34 a functionally relevant CRE is located, whereas in between +88 to +110 an AP2 binding site is predicted.

Twin pair E differed by the presence of Lisch nodules and an optic glioma at the age of 15. The boys both had no cutaneous, but nearly the same amount of subcutaneous neurofibromas. Their methylation was relatively low and only at two CpG sites (-249, -166) different. CpG at -249 is located in the TLF binding region and had higher methylation in twin 10 (17.4%) who suffered from an optic glioma compared to twin 9 (0%) without an astrocytoma.

Twin pair F had the lowest amount of intra-pair differences of methylation. These female twins had the mildest manifestation of NF1 features compared to all other twin pairs. They differed only in the number of café-au-lait spots and freckling and had no tumors at the age of 9 years. Only at cytosine residue -76 there was a marked difference (28.13% vs. 0%).

Twin pair G was the youngest twin pair in our cohort with an age of six. The girls had many pigmentary lesions and differed by neurofibroma manifestation and presence of an optic glioma. They showed the highest methylation levels and regional clustering although the spatial pattern was quite similar (Fig. 1). Differences in methylation occurred at 33 cytosine residues. Twin 15 who had more café-au-lait spots, an optic glioma and neurofibromas presented with higher methylation levels of 14 CpGs (-238, -236, -234, -208, +204, +222, +428, +438, +474, +491, +519, +529, +534, +537) and higher values of overall cytosine methylation clustering in between +488 to +537 representing *NF1* exon 1 as well as in between -246 to -215, the TLF binding region. In the latter region the differences were quite remarkable such as for CpG -238 (40.54% vs. 10.53%) and CpG -236 (21.62% vs. 0%) which is shown in Figure 2.

Twin pair H was the oldest twin pair with an age of 43 at the time of investigation. The men differed strongly in neurofibroma burden as well as in pigmentary lesions. Instead, their methylation levels were low and not much different except for a small cluster between +175 to +180. Differences in methylation occurred at 5 cytosines including CpGs at -234, +175, and +180, twin 17 displayed higher methylation values.

#### **Methylation of 5'UTR, Exon 1 and Intron 1 and Specific Promoter Elements Shows Significant Differences Within Monozygotic NF1 Twin Pairs**

To obtain a measure for the "epigenetic distance" which quantitatively reflects the difference in methylation between co-twins being concordant or discordant for different clinical features, we calculated differences of mean percentages of methylation for every twin pair. Then mean differences in methylation of all twin pairs were compared for different regions by one sample t-test against 0, thereby testing the null hypothesis that there is no intra-pair difference in methylation. Data for the most important regions are



shown in Tables 2 and 3. Regarding CpG and overall cytosine methylation, nearly all *P*-values indicated that the mean values of differences in methylation within twin pairs were significantly different from 0 both for the whole region investigated as well as for single elements. Remarkably, the SP1 binding site at -141 (CpG at -138) did not show significant differences at all. Intron 1, the minimal proximal promoter, the tax element and the CRE site at -13/-10 differed only significantly in CpG methylation. After adjusting for multiple testing, the region containing 5'UTR, exon 1 and intron 1 (+1 to +622), the repressor between +144 to +474, and an element for putative SP1/AP2 binding (-163, -166) were consistently different in cytosine as well as in CpG methylation. Interestingly, a putative AP2 binding site (+463 to +472), and NF1HCS (+156 to +172) were only significantly different from 0 in cytosine methylation whereas the core promoter element (+291 to +345) was only significantly different from 0 in CpG methylation.

#### Comparison of Intra-Pair Differences of Methylation With NF1 Features

We created a standard measure ranging from 0 to 1 for the difference of cutaneous and subcutaneous neurofibromas between the twins of each pair (see material and methods). Applying this measure, 0 indicated no difference whereas 1 indicated maximal discordance. For example, twin pair G that was maximally discordant in neurofibroma burden (1.0) showed also a high amount of intra-pair differences in CpG methylation (14 CpGs). We wanted to test if increasing differences of methylation (higher intra-pair differences of methylation) correlated to an increasing number of neurofibromas. To test this by a statistical approach, we applied Spearman's rank correlation and

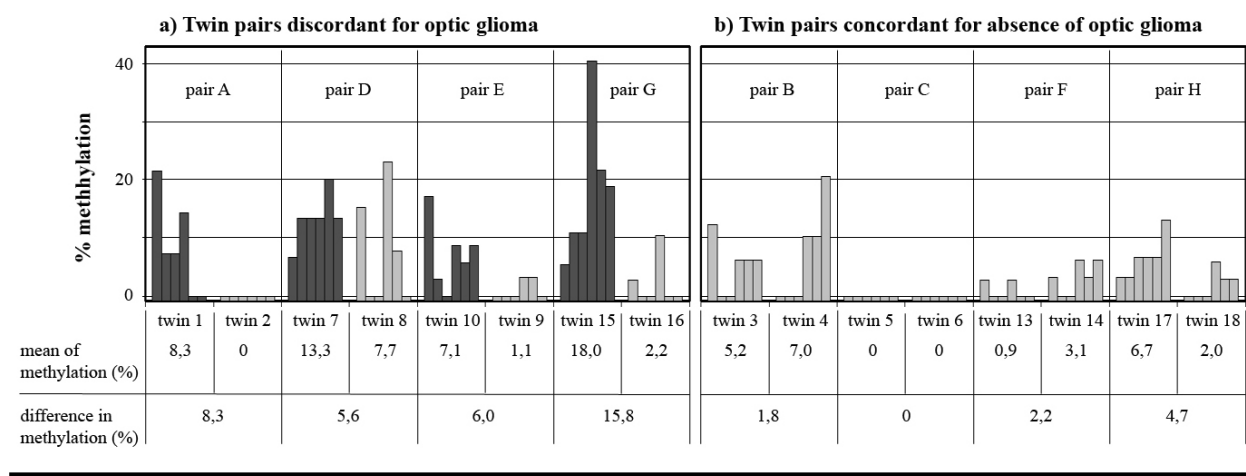
compared the standard measure of each pair with differences of methylation of subregions of the analyzed *NF1* region. Significant differences comparing the degree of intra-pair differences in neurofibroma number and intra-pair differences of methylation were not seen. Thus, the increasing difference of the number of neurofibromas did not correlate significantly with an increasing degree of *NF1* methylation.

We furthermore did not see a relation between intra-pair differences and other *NF1* features such as plexiform neurofibromas and pigmentary lesions in the twin pairs.

#### In Twin Pairs Discordant for Optic Glioma the Affected Co-twin Shows Significantly Higher Percentages of Methylation From -249 to -234

Finally, we wanted to compare *NF1* promoter methylation of twin pairs being concordant with those being discordant for certain *NF1* features. As displayed in Table 1 we were able to create two groups (concordant and discordant for the feature optic glioma and scoliosis) which contained the same number of pairs ( $n = 4$ ). The limited number of twin pairs did not allow us to perform statistical analysis for other features such as Lisch nodules, freckling and café-au-lait spots since the group sizes would have been far too asymmetric.

We compared differences in methylation between four twin pairs being discordant for optic glioma with four pairs concordant for the absence of this feature. Comparing a total of 26 regions we detected significant differences for the region from -249 to -234 overlapping with the TLF binding site (Chong et al., 2005): The difference in cytosine methylation was significantly different between the two groups ( $p = .021$ , U-test). In Figure 2 percentages of methylation as well as intra-pair differences are shown. Those twins who were concor-



**Figure 2**

*NF1* region covering -249 to -234 is overlapping with the TLF binding site (-337 to -234). Percentages of methylation are given separately for the twins of pairs being discordant (left side, a) for the presence of an optic glioma ( $n = 4$ ) and concordant (right side, b) for the absence of an optic glioma ( $n = 4$ ). Vertical bars display percentage of methylation of the six cytosine residues following each other from left to right: -249, -246, -239, -238, -236, and -234. This order is repeated for every twin. Black bars were used for patients with an optic glioma, grey bars for those without an optic glioma. Beneath, for every twin the mean percentage of methylation for this site (mean of 6 cytosine residues) and the intra-pair difference of cytosine methylation is given. Differences of cytosine methylation were significantly different between the two groups ( $p = .021$ , Mann-Whitney-U-test).

dant to have no optic glioma showed not only lower intra-pair differences of mean methylation levels but also lower general methylation levels at this site. Twins being discordant for this feature presented higher differences of methylation for this site that was due to higher methylation levels in those twins who presented with optic glioma. Since we were not able to detect a *NF1* mutation in twin pair F and only one twin was diagnosed to fulfil NF1 criteria within this pair, we excluded this twin pair from analysis to rule out a bias due to a twin pair with a probable mosaic mutation in *NF1*. However, also in this restricted data set methylation still showed a significant group difference ( $p = .034$ , U test).

All other regions tested did not show significant group differences (data not shown). We also compared differences in methylation between four twin pairs being discordant for severe scoliosis with four pairs being concordant by Mann-Whitney U-test, but did not find significant group differences (data available upon request from the authors).

## Discussion

The variability of the NF1 phenotype is discussed to be determined by modifying factors since except patients with microdeletions or with a 3 bp deletion in exon 17, no genotype-phenotype correlations exist. Easton et al. (Easton et al., 1993) were the first to provide evidence for modifying genes in NF1. In their study 175 individuals with NF1 including six monozygotic twin pairs and 76 pairs of sibs were analyzed, and significant intrafamilial correlations for the number of café-au-lait spots, number of cutaneous neurofibromas and head circumference were found. Concordance for dermal neurofibromas between monozygotic twins was much higher than between sibs, suggesting a genetic factor. Szudek et al. (Szudek et al., 2002) examined familial aggregation of NF1 features in 373 families. They found that Lisch nodules and café-au-lait spots had significantly higher correlations among first-degree relatives than among second-degree relatives, which could be due to modifying genes at unlinked loci or environmental factors. Intertriginous freckling, subcutaneous neurofibromas, plexiform neurofibromas and café-au-lait spots had higher correlations between sibs than between parents and children which could be due to functional polymorphisms of the normal *NF1* allele and environmental factors. Therefore, patterns of familial correlations suggest that genetic factors involved in determining the occurrence of various clinical features of NF1 vary, depending on the feature. A recent large scale analysis of 750 NF1 patients including six monozygotic twin pairs showed significant familial aggregation of nearly all NF1 features and indicated a strong genetic modifier unlinked to *NF1* (Sabbagh et al., 2009).

Despite extensive research, only some reports found evidence for modifiers so far: Mismatch repair is supposed to modify the NF1 tumor burden, and site-specific methylation of *MSH2* was found to be

linked to a higher neurofibroma burden in NF1 patients (Titze et al., 2010; Wang et al., 2003; Wiest et al., 2003). Recently, the genes *CENTA2*, *RAB11FIP4*, *C17orf79* and *UTP6* located within the microdeletion region of *NF1* were identified to be potential modifiers for neurofibroma development (Bartelt-Kirbach et al., 2009). In a mouse model, two loci, *NSTR1* and *NSTR2*, protected the mice to develop peripheral nerve sheath tumors and astrocytomas (Walrath et al., 2009). Finally, the neurotrophin GDNF was identified to be a modifier for a NF1 phenotype associated with intestinal neuronal dysplasia type B (Bahau et al., 2001).

In this study, we analyzed whether differences of *NF1* gene methylation between twins of monozygotic pairs are related to their phenotypic variability since epigenetic patterns of the *NF1* promoter might be responsible for differences of the expression of the normal *NF1* allele and in turn influences the variable expressivity of NF1. For a long time, twin studies have been a tool for studying genetic disorders. But still, incomplete concordance between monozygotic twins is not fully understood. Many pairs of monozygotic twins with NF1 have been described in the literature being concordant as well as discordant for NF1 features, but there is no twin study up to now analyzing epigenetic modifications of *NF1* that may contribute to the discordance in those twins.

We fine-mapped the methylation pattern of more than 900 bp of the *NF1* promoter region in eight monozygotic twin pairs with NF1, and we were able to show that the genetically identical monozygotic twins with NF1 presented significant intra-pair differences in methylation concerning the spatial distribution as well as levels of region- and site-specific methylation of the *NF1* gene promoter. When testing if intra-pair differences of methylation are significant for the whole fragment as well as for *NF1* subregions, all twin pairs showed significant results for the majority of regions and elements analyzed. Moreover, some regions presented highly consistent differences of methylation when widening the confidence interval to 99% to overcome the problem of multiple testing. These regions, which are known to be highly important for *NF1* gene transcription and in part were also shown to be methylation sensitive, included the region containing 5'UTR, exon 1 and intron 1 (+1 to +622), the 'redefined promoter' region, a core promoter element, a repressor between +156 to +474, and an element for putative SP1/AP2 binding (-163, -166) as well as one putative AP2 binding site between +463 to +472, and furthermore NF1HCS.

Our findings are in accordance with recent reports showing that monozygotic twins (non-NF1) display remarkable differences of their content and genomic distribution of methylation at more than 20.000 genomic sites as well as of their histone acetylation (Fraga et al., 2005; Kaminsky et al., 2009). Therefore, differences of methylation not only genome wide but of single genes represent a plausible explanation for

different phenotypes in twins who share the same genotype. Studies of single genes such as *DRD2*, *COMT*, *DLX1* and at *KCNQ1OT1* support this idea since they also detected epigenetic differences between monozygotic twins discordant for certain features (Kaminsky et al., 2008; Mill et al., 2006; Petronis et al., 2003; Weksberg et al., 2002). Thus, we would propose that methylation of specific *NF1* gene promoter regions, as cited above, can be regarded as a modifier for *NF1* variability since significant intra-pair differences of methylation are associated with discordant *NF1* features in the twin pairs investigated here.

Nevertheless, there are limitations of our study. First, we analyzed blood cells for methylation, although methylation patterns might be tissue specific. Second, due to the fact that we had only DNA material for our analysis, we were not able to show that methylation of certain sites, which seem to have a high relevance for *NF1* gene transcription, actually lead to changes of *NF1* gene transcription. The first problem can be solved by investigating other tissues of these monozygotic twins in further studies. Interestingly, there were already studies to show that some genes did not show differences in methylation between different tissues such as for *COMT* between blood and brain in a twin study (Murphy et al., 2005). *NF1* methylation of lymphocytes can nevertheless be regarded as a proof of principle that there exist epigenetic differences in monozygotic twins. The second limitation can probably be overcome by detailed in-vitro luciferase reporter gene analyses of constructs that contain spatially defined methylated regions, but those experiments are still technically limited. Furthermore, to link the exact amount of *NF1* mRNA to methylation, it would be necessary to obtain RNA and DNA samples from exactly the same time point of investigation, since methylation pattern might change over time.

Beside the main result that *NF1* twin pairs show intra-pair differences in methylation, we detected significant intra-pair differences in cytosine methylation for the region from -249 to -234 between twins concordant and discordant for an optic glioma. This region overlaps with a TLF binding site (Chong et al., 2005). As shown in Figure 2, twins who had no optic glioma displayed lower methylation levels compared to twins with a symptomatic optic glioma. Differences of methylation were higher in twin pairs with discordance for this tumor compared to those who were concordant to have no glioma. A previous study investigating a 119 bp fragment of *NF1* covering the transcriptional start site by MSP did not detect methylation in astrocytomas including optic gliomas. However, the region -249 to -234 was not included in their analysis (Ebinger et al., 2005). The basal transcription factor TLF is expressed in various tissues including brain, and its binding to *NF1* stimulates transcription (Chong et al., 2005). Since there is not enough knowledge on the tissue specific role of TLF and TLF binding to *NF1*, conclusions should be made

with caution. But if TLF binding to *NF1* would be methylation sensitive, than altered TLF binding might affect *NF1* gene transcription. Hence, a scenario in which epigenetically driven decrease of *NF1* gene expression would promote growth of low grade optic gliomas can be imagined.

In contrast, we detected that the important regulatory SP1 site at -141, which is known to be methylation sensitive (Mancini et al., 1999) was not significantly different in methylation between the twins of the pairs in all calculations. This may be addressed to the fundamental necessity of this binding site to be methylation-free to assure a stable *NF1* gene transcription.

Interestingly, in our small cohort we did not see that methylation or intra-pair differences increased with age. The oldest twin pair (H) displayed marked differences of methylation at three CpGs whereas the youngest pair (G) was different in methylation at 14 CpGs. The highest amount of differences in CpG methylation was seen in young patients: in twin pair A (17 years of age) and pair D (8 years of age). There are contrary findings in the literature on methylation and age. Some studies presented data of increasing methylation with age, others of a decrease of methylation with age (Fraga et al., 2005; Fuke et al., 2004). Due to the small number of twins in this study we cannot reliably state on this, but our findings may be consistent with a decrease of methylation in leukocytes as described by Fuke and coworkers.

To conclude, we presented the first report that focussed on epigenetic differences of the *NF1* gene promoter, on *NF1* DNA-methylation in monozygotic twins with *NF1* displaying discordant *NF1* features. We observed significant intra-pair differences of methylation in subregions of the *NF1* promoter and therefore argue that epigenetic distances of *NF1* methylation might be a modifier for *NF1* phenotypic variability.

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