Analysis of Mitochondrial DNA in Discordant Monozygotic Twins With Neurofibromatosis Type 1

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Neurofibromatosis type 1 (NF1) is the most frequent neurocutaneous disorder with autosomal dominant inheritance. Phenotype variability is high ranging from merely several café-au-lait spots to malignant peripheral nerve sheath tumors or severe disfigurement through plexiform neurofibromas. Identification of genetic factors that modify the NF1 phenotype would contribute to the understanding of NF1 pathophysiology and improve patient counselling. As even monozygotic (MZ) twins with NF1 may differ phenotypically, we wondered whether these variations might be inherited in a non-Mendelian fashion. Mitochondrial DNA (mtDNA) is inherited extrachromosomally through the cytoplasm of the oocyte and often harbours heteroplasmic sequence variations. At the time of blastomere separation, these variants may be skewedly distributed and effect phenotypic differences. Because of their co-localization with the tumor suppressor protein neurofibromin, which is mutated in NF1, mitochondria were particular attractive candidates for investigation. MtDNA was extracted from nucleated blood cells of four pairs of discordant MZ twins with NF1 and from cutaneous neurofibromas of one twin pair. We sequenced the entire mitochondrial genome and determined the state of heteroplasmy by investigating a microsatellite region of the mitochondrial D-loop (D310-tract). The clinical diagnosis was confirmed in all patients by detection of pathogenic mutations in the *NF1* gene. Monozygosity was verified by genotyping. However, we did not detect evidence for mtDNA sequence differences or for different degrees of heteroplasmy between individuals of the same twin pair. The phenotypic discordance of MZ twins with NF1 cannot be explained by skewed distribution of mtDNA mutations or polymorphisms.

Neurofibromatosis type 1 (NF1) is a frequent neurocutaneous disorder with autosomal dominant inheritance. It affects worldwide approximately 1 in 3500 individuals and is caused by mutations in the *NF1* gene. *NF1* encodes the tumor suppressor protein neurofibromin (Cawthon et al., 1990; Viskochil et al., 1990).

Clinically, NF1 is characterized by cutaneous neurofibromas and pigment anomalies such as café-au-lait spots and skinfold freckling. Further defining criteria include the presence of iris hamartomas (Lisch nodules), optic pathway gliomas, plexiform neurofibromas and bone dysplasias (NIH, 1988; Theos & Korf, 2006). The incidence of malignant tumors such as malignant peripheral nerve sheath tumors (MPNST) is increased. It is well known that NF1 disease severity may differ between patients to a large extent, even within a family. Some clinical reports document considerable phenotypic differences in monozygotic (MZ) twins. These comprise the age of disease onset and the severity of clinical signs and symptoms (Bauer et al., 1988; Easton et al., 1993).

The nuclear genome of MZ twins is thought to be identical — at least in terms of DNA markers — as the separation of the blastomeres occurs early between day 1 and 10 after conception (Machin, 1996). Therefore, the true nature of the phenotypic discordance in MZ twins remains poorly understood and speculations involve epigenetic mechanisms, genomic imprinting or the presence of chromosomal mosaics (Singh et al., 2002).

Recent studies have shown that polymorphisms in the mitochondrial DNA (mtDNA) may influence the phenotype of inbred animals with identical nuclear genetic background (Johnson et al., 2001; Nagao et al., 1998; Roubertoux et al., 2003). Additionally, is

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has been shown that neurofibromin, the protein affected by mutations in the *NF1*-gene, partially colocalizes with mitochondria thus suggesting a functional link (Roudebush et al., 1997). The mtDNA is therefore an obvious candidate for an extrachromosomal phenotype modifier.

The mtDNA is almost exclusively inherited through the maternal line. Every healthy women harbours a small percentage mutated mtDNA among the more than 100,000 copies that are present in the oocyte (Chen et al., 1995). These mutations might be skewedly distributed between the blastomeres through the 'mitochondrial bottleneck' and subsequently expand into different degrees of heteroplasmy in the fetuses (Kirches et al., 2001). Heteroplasmy is defined as the presence of two or more mtDNA populations in the same individual and different degrees of heteroplasmy have been shown to cause discordant symptoms in MZ twins with mitochondrial disorders (chronic progressive external ophthalmoplegia and Leber's hereditary optic neuropathy; Biousse et al., 1997; Blakely et al., 2004).

In search for the molecular basis of phenotypic differences in NF1 we screened the entire mitochondrial genome for unevenly distributed mtDNA sequence variants in four twin pairs with 'identical' nuclear genetic background.

Patients

Four pairs of MZ twins of European descent (A-D) with neurofibromatosis type 1 were enrolled into the study. All individuals provided written informed consent for all aspects of this study and the publication of the results according to the Declaration of Helsinki. All patients fulfilled the NIH diagnostic criteria (NIH, 1988). Monozygosity was verified by multi-locus DNA fingerprinting using synthetic simple repetitive oligonucleotides as probes (data not shown). In all individuals we detected *NF1* mutations (3 frameshift mutations and 1 nonsense mutation). All patients received a detailed physical examination of the entire skin, and a cranial MRI. The twins of each pair differed in their phenotype, mainly in height and weight, head circumference, number and localization of neurofibromas and café-au-lait spots, as well as in additional disease specific features such as optic glioma and plexiform neurofibromas and further clinical signs as scoliosis and hydrocephalus (Table 1).

Methods

Sample Collection and DNA Extraction

Blood samples of all individuals were collected by venipuncture. Additionally, we received specimens of neurofibromas from twins A1 and A2 that had been removed for cosmetic reasons by laser surgery, as well as blood from their mother. MtDNA was extracted from nucleated blood cells and from the cutaneous neurofibromas by standard procedures.

DNA Amplification and Sequencing

We screened the patients' DNA for sequence alterations in all exons of the *NF1* gene by temperature gradient gel electrophoresis of polymerase chain reaction (PCR) amplified genomic DNA fragments using intron-based primers as previously described (Klose et al., 1998). Silver staining was used for visualization of

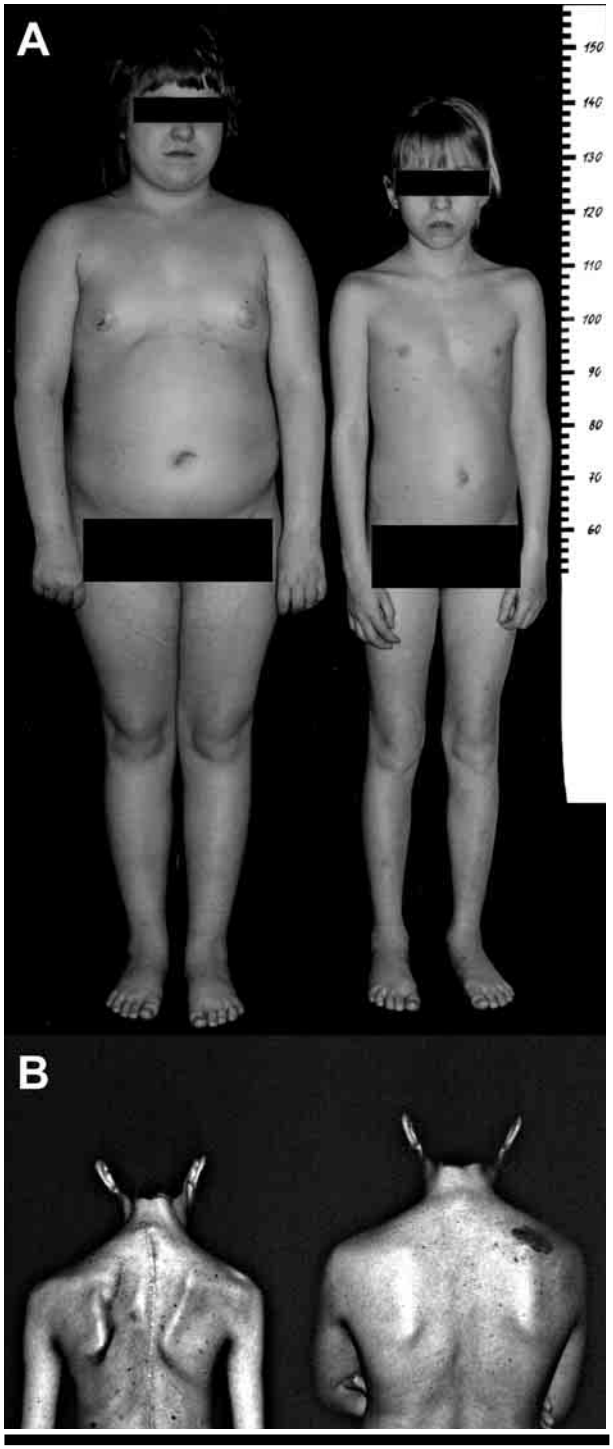

Figure 1

(A) Twin pair A at the age of 12 years, (B) twin pair B at the age of 17 years. The twin on the left has been operated on for severe scoliosis.

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Table 1

Clinical Features and *NF1* Mutations of the Examined Patient Twin Pairs

Twin pair	A		B		C		D	
Age [years], Sex	17, Female		18, Male		33, Female		8, Female	
Country of origin	Germany		Croatia		Germany		Germany	
mtDNA haplotype	J1		J1		T1		K	
Twin number	#1	#2	#1	#2	#1	#2	#1	#2
Mutation in *NF1*	c.1541delAG		c.499delTGTT		c.4839T>G		c.3737delTGTT	
Height [cm]	158	164	173	183.5	156	152	122	122
Weight [kg]	80	51	47	78	56	70	27	24
Head circumference [cm]	56	54	57.5	60.5	58	64.5	53	51
Cutaneous neurofibromas [*n*]	60	40	18	26	10	8	7	5
Subcutaneous neurofibromas [*n*]	—	—	20	—	—	—	—	—
Plexiform neurofibromas [*n*]	—	2	—	1	—	—	—	—
Café-au-lait spots > 1.5 cm [*n*]	25	15	26	18	5	22	12	11
Skinfold freckling	+	+	+	+	+	+	+	+
Lisch nodules	+	+	+	+	—	+	—	—
Optic glioma	+	—	—	—	—	—	+	—
Scoliosis	—	+	+	—	+	—	—	+
Hydrocephalus	+	—	—	—	—	+	—	—
Other discordant features	(1) (2)	(3)			(4)	(5) (6)		

Note: (1) pulmonal valvular stenosis, (2) atrial septal defect, (3) ventricular septal defect, (4) hyopthyreoidism, (5) vitiligo, (6) strabism.

deviating bands. Prior to bidirectional automatic sequencing, PCR products were isolated and extracted from agarose gels. In order to preclude the analysis of mtDNA pseudogenes (Woischnik & Moraes, 2002), we first amplified the major portion of the 16.5 kbp mtDNA molecule by long range PCR using the LA PCR Kit v2.1 (TaKaRa), with mtDNA specific oligonucleotid primers (f) 5'-TGA GGC CAA ATA TCA TTC TGA GGG GC-3' and (r) 5'-TTT CAT CAT GCG GAG ATG TTG GAT GG-3'. The long-range PCR product was then used as template for 17 nested PCR reactions with fragment lengths varying between 800 and 1200 bp. The complete list of primers used for the nested PCR reaction and for automatic cycle sequencing is available as supplementray material. A fragment of 250 bp within the cytochrome b (*MT-CYB*) gene that bridged the gap between the long-range PCR oligonucleotid primers was amplified by conventional PCR from native patient mtDNA samples. Therefore, coamplification of interfering nuclear pseudogenes in this region cannot be entirely excluded. For cycle sequencing on the ABI Prism 3700 DNA Sequencer (Applied Biosystems) we used the BigDye Terminator® v2.0 protocol (Applied Biosystems) and nested primers (see supplementary material). Overlapping sequences were aligned to the revised Cambridge Reference Sequence (rCRS) and analysed with the software 'Sequence Navigator v1.0.1' (Applied Biosystems). Analysis of polymorphic sites and patient assignment into mtDNA haplogroups were performed with data from the Human Mitochondrial Genome Database (mtDB; Ingman & Gyllensten, 2005).

Verification of the MZ state of the twins was done through multi-locus DNA fingerprinting. We used the synthetic oligonucleotide probe $(GTG)_5$ for the simultaneous detection of about 20 minisatellite loci (Neitzel et al., 1991). Prior to agarose electrophoresis, genomic DNA was digested with the restriction endonucleases *Mbo* I and *Hinf* I. Identical patterns of up to 50 DNA fingerprint bands were observed in cases of MZ twins as apposed to a drastically reduced band sharing rate of only 50 to 60% in cases of dizygotic twins (data not shown).

Genotyping of the D310 Tract of the mtDNA

The D-loop of the mtDNA contains two hypervariable regions (HRVs) between nt16024-16383 (HVR1) and nt57-372 (HVR2). The mtDNA 5' to the thymine at nt310 contains a poly-cytosine tract which is called 'D310 tract'. The D310 tract generally occurs in heteroplasmic form, with several length variants, and can be used for genotyping (e.g., for forensic purposes). In our patients we used the genotyping method described earlier (Kirches et al., 2001). Briefly, PCR was performed with a FAM-labelled forward-primer (FAM-GCC ACT TTC CAC ACA GAC ATC ATA-3', (r) 5'-TTA AAA GTG CAT ACC GCC AAA AG-3'). The ensuing products were digested with *Hae* III and the fluorescent fragment sizes were determined with the ABI Prism® 3100 Genetic Analyzer (Applied Biosystems). With the 'Genescan Analysis Software v3.1' (Applied Biosystems), signals were transformed into curve diagrams, and the integral below the curve was taken as the

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Table 2

Sequence Variants that Deviated from the Revised Cambridge Reference Sequence (rCRS) in the Four Twin Pairs

			Twinpair	A	B	C	D
			mtDNA haplotype	J1	J1	T1	K
			Tissue of investigation	NBC and skin	NBC	NBC	NBC
			GenBank accession	DQ358973	DQ358974	DQ358975	DQ358976
Locus	Polymorphism	% of normal population	Exchange of AA				
MT-DLOOP	73G	84.16	—	x	x	x	x
	146C	9.86	—				x
	152C	21.45	—			x	
	185A	3.21	—	x	x		
	188G	1.17	—	x			
	195C	12.58	—			x	x
	228A	3.45	—	x	x		
	263G	99.63	—	x	x	x	x
	295T	4.37	—	x	x		
	462T	3.68	—	x	x		
	489C	36.98	—	x	x		
12sRNA	709A	17.23	—			x	
	789C	**0.04**	—	**x**			
	1189C	3.17	—				x
	1438G	96.83	—	x	x	x	x
16sRNA	1811G	7.07	—				x
	1888A	5.61	—			x	
	2706G	80.66	—	x	x	x	x
	3010A	24.76	—	x	x		
MT-ND1	3480G	4.10	L = L				x
	4216C	9.51	Y > H	x	x	x	
MT-ND2	4769G	98.90	M = M	x	x	x	x
	4917G	4.92	D > N			x	
	5004C	1.06	L = L			x	
MT-CO1	5913A	**0.41**	D > N				**x**
	7028T	81.43	A = A	x	x	x	x
	7211A	**0.04**	M = M				**x**
MT-ATP6	8697A	4.88	M = M			x	
	8860G	99.76	T > A	x	x	x	x
	9055A	4.23	A > T				x
	9123A	2.52	L = L				
MT-CO3	9698C	4.39	L = L				x
	9899C	1.14	H = H			x	
MT-ND3	10143A	**0.41**	G > S			**x**	
MT-ND3	10398G	53.80	T > A	x	x		x
MT-TR	10463C	4.92	—			x	
MT-ND4L	10550G	3.66	M = M				x
	10646A	0.53	V = V				x
MT-ND4	11251G	9.10	L = L	x	x	x	
	11299C	4.35	T = T				x
	11467G	**0.53**	L = L				**x**
	11719A	77.69	G = G	x	x	x	x

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Table 2 (CONTINUED)

Sequence Variants that Deviated from the Revised Cambridge Reference Sequence (rCRS) in the Four Twin Pairs

			Twinpair	A	B	C	D
			mtDNA haplotype	J1	J1	T1	K
			Tissue of investigation	NBC and skin	NBC	NBC	NBC
			GenBank accession	DQ358973	DQ358974	DQ358975	DQ358976
Locus	Polymorphism	% of normal population	Exchange of AA				
MT-TL2	12308G	11.05	—				x
MT-ND5	12372A	12.80	L = L				x
	12612G	4.43	V = V	x	x		
	12633A	1.46	S = S			x	
	12738G	**0.16**	A = A				**x**
	13194A	**0.45**	L = L				**x**
	13368A	5.08	G = G			x	
	13708A	6.87	A > T	x	x		
	13934T	**0.98**	T > M		**x**		
	14002G	**0.37**	T > A		**x**		
MT-ND6	14167T	4.19	Q = Q				x
	14281T	**0.45**	G = G			**x**	
MT-CYB	14766T	77.41	I > T	x	x	x	x
	14798C	7.52	F > L	x	x		x
	14905A	7.52	M = M			x	
	15326G	99.31	T > A	x	x	x	x
	15394C	**0.12**	N = N		**x**		
	15452A	9.14	L > I	x	x	x	
	15607G	5.61	K = K			x	
MT-TT	15928A	5.04	—			x	
	15947G	**0.00**	—	**x**			
MT-DLOOP	16069T	4.25	—	x	x		
	16126C	9.48	—	x	x	x	
	16163G	1.17	—			x	
	16186T	1.42	—			x	
	16189C	27.22	—			x	
	16224C	4.13	—				x
	16291T	2.71	—		x		
	16294T	6.03	—			x	
	16311C	14.73	—				x
	16519C	57.64	—	x		x	x

Note: This table depicts all sequence variants that deviated from the revised Cambridge Reference Sequence (rCRS) in the four twin pairs. Length polymorphisms of long C-stretches in HVS1 and HVS2 and the dinucleotide repeats at nt522-523 and the 3107delC were disregarded. The third column lists the percentage of occurrence of specific sequence variants in relation to the normal population as derived from the mitochondrial genomes of 1622 to 2461 individuals listed in the mtDB database. Amino acid exchanges in the coding regions were depicted if present. Polymorphisms that occur in less than 1% of the world population are highlighted. NBC, nucleated blood cells; AA, amino acid.

relative amount of the respective length fragment. For generation of a length standard for genotyping, we cloned a mtDNA fragment containing the D310 tract of a healthy individual into the pGEM-T® vector (Promega), and determined the length of the poly cytosine tract by automatic sequencing. The D310 tract length was analysed in mtDNA preparations from blood cells of all individuals and from the cutaneous neurofibromas of twin pair A.

Results

Sequence Analysis of the mtDNA

In the four twin pairs we found a total of 73 mtDNA sequence variants that deviated from the revised Cambridge Reference Sequence (rCRS). With the exception of 15947G in *MT-TT* (twin pair A), all variants were listed in the Human Mitochondrial Genome Database (mtDB). The variants placed the patients

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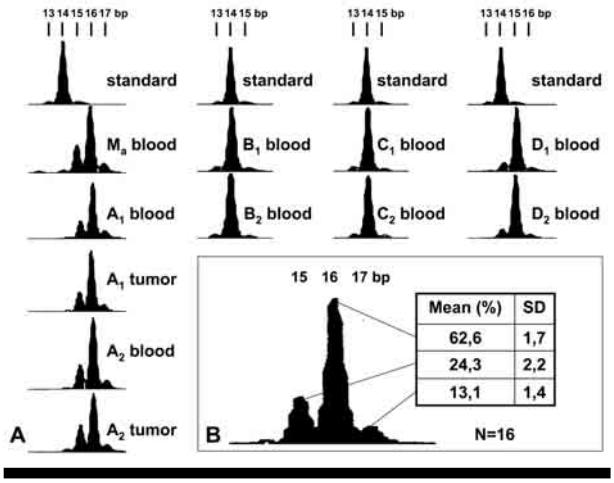

Figure 2

(A) D310 Tract length heteroplasmy in nucleated blood cells (B) and in cutaneous neurofibromas (T) from twin pair A and their mother (M_a) as well as in nucleated blood cells from twin pairs B-D. (B) From twin A1 and A2 we additionally examined D310 tract length heteroplasmy in cutaneous neurofibromas. The distribution of D310 length variants between 16 different cutaneous neurofibromas of patient A1 is nearly the same.

into the mtDNA haplogroups J1, T1 and K. The 15947G variant was only listed in the MITOMAP database. Half of the sequence variants (54%) were found in the 12 protein-coding genes (40/73), 28% (21/73) of the variants were in the D-loop, 11% (8/73) in rRNA and 5% (4/73) in tRNA genes (*MT-TR, MT-TL2, MT-TT*). We did not detect any sequence variants in *MTATP8* or in the remaining 19 tRNA genes. Twelve sequence variants were rare and occur worldwide in less than 1% of the normal population (Table 2). All variants were homoplasmic and we did not detect any difference in the presence of these variants between the individuals of the same twin pair. Additionally, we did not find any sequence differences between mtDNA preparations from blood and cutaneous neurofibromas in twin pair A.

Genotyping of the D310 Tract of the mtDNA

As the D-loop is difficult to sequence due to sequence heteroplasmy, we analysed the D310 tract by fragment length analysis. In the rCRS, the tract between nt303-316 has a length of 14 bp and length variations only occur in the poly-cyosine tract 5' to D310 (Parella et al., 2003). Therefore, length analysis of the fragment spanning the D310 region accurately reflects the polymorphisms in the 5' polycytosine tract. All patients were heteroplasmic with 3 length variants of the D310 tract (Figure 2). The twin pairs B and C had a predominant length variant of 14 bp. In pair D there was heteroplasmy with 14-16 bp length variants. Twins A1 and A2 as well as their mother had three length variants of 15-17 bp in their mtDNA from blood cells. The distribution of the length variants was nearly the same in the children and their mother, with a tract length of 16 bp dominating with about 64% (Figure 2). We did not detect any significant differences in the degree of heteroplasmy between mtDNA preparations from blood and 16 cutaneous neurofibromas in twin pair A (Figure 2).

Discussion

In neurofibromatosis type 1 (NF1) the concordance of phenotypic features, such as the number of neurofibromas and café-au-lait spots, is high between MZ twins, and declines with lower degrees of relationship (Easton et al., 1993). According to our hypothesis, polymorphisms or mutations of the mtDNA, which are inherited extrachromosomally through the maternal line, could make a difference between the genetic make-up of MZ twins and thus modify their phenotype. In order to exclude confounding factors of the nuclear genetic background, we investigated the mtDNA of discordant MZ twins. However, we did not detect any mtDNA differences between patients of the same twin pair, which renders mtDNA polymorphisms an unlikely cause for predisposition of phenotype variation, at least in our cohort of NF1 patients. Kösel et al. chose a similar approach and examined the mtDNA-encoded genes of complex I and the tRNAs in five pairs of identical twins with Parkinson's disease (Kösel et al., 2000). Four of their twins were discordant for the disease at the time of analysis but no mtDNA sequence differences were found. Wilichowski et al. analysed the mtDNA of a pair of MZ twins one of whom was affected with X-linked adrenoleukodystrophy and did not detect any differences either (Wilichowski et al., 1998). Most polymorphisms that we found were already noted in the mtDB database as sequence variants. The accumulation of sequence variants within the D-loop and the *MT-CYB* gene is not unexpected since these regions exhibit the highest genetic variability in population studies (Vega et al., 2004). In contrast to most pathogenic mtDNA mutations that occur in heteroplasmic form (Kirches et al., 2001), all sequence variants detected by us were homoplasmic and therefore probably without influence on the NF1 phenotype.

To search for late embryonic, somatic mtDNA mutations that might only be present in tissues of ectodermal origin, we also sequenced the entire mtDNA from cutaneous neurofibromas of twin pair A — the only one of whom the material was available — but did not find any differences either. As mutations with a low degree of heteroplasmy (< 10–15%) might have gone unnoticed by the BigDye® sequencing technology, we additionally used the genotyping technique of the D310-tract to detect quantitative differences between mtDNA extracted from the cutaneous neurofibromas and from nucleated blood cells. A quantitative shift could have served as additional, albeit indirect evidence, whether somatic segregation

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of heteroplasmic mtDNA mutations might have taken place, but we detected none.

In the absence of mtDNA alterations the discordance in our patients must be generated through other mechanisms. One might be the modification of genes or their expression patterns through epigenetic influences. It is known, that methylation may inactivate mismatch repair genes (Fraga et al., 2005; Jones & Laird, 1999) and the interaction of transcription factors with their binding sites can be altered by methylation (Mancini et al., 1999) which may lead to decreased *NF1* gene expression. Other reasons might be the accumulation of random somatic (second hit) mutations in the *NF1* gene (Serra et al., 2000).

In conclusion, mitochondrial DNA polymorphisms do not seem to contribute to the phenotypic variability of neurofibromatosis type 1. Other factors such as epigenetic influences or second hit somatic mutations in the *NF1* gene might be reasons for this phenomenon. Future studies of MZ twins should thus focus on regulatory and noncoding DNA sequences.

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Appendix A

Supplementary Table 1

Oligonucleotide Primer Pairs Used for Nested PCR Reactions

Forward-Primer	Position	Reverse-Primer	Position	Length
5′-CCCATCCTACCCAGCACACACA-3′	500–521	5′-CGCCTATACTTTATTTGGGTAA-3′	1750–1729	1251bp
5′-ACTTGGACGAACCAGAGTG-3′	1590–1608	5′-ATGGGTACAATGAGGAGTAGGAGGT-3′	3344–3320	1755bp
5′-AGGACAAGAGAAATAAGGCC-3′	3130–3149	5′-AAGATGGTAGAGTAGATGACGG-3′	4510–4489	1381bp
5′-CCAGCATTCCCCCTCAAACCTA-3′	4280–4301	5′-GGGGTTTTGCAGTCCTTAGC-3′	5604–5585	1325bp
5′-CGCTACTCCTACCTATCTCC-3′	5470–5489	5′-AGAATAGTCAACGGTCGGCG-3′	5928–5909	459bp
5′-CTCAGCCATTTTACCTCACCC-3′	5877–5897	5′-TTGAAAAAGTCATGGAGGCCAT-3′	7516–7495	1640bp
5′-ATGCCCCCCACCCTACCACAC-3′	7394–7414	5′-GGCTCTAGAGGGGGTAGAGGGG-3′	8294–8273	901bp
5′-TCGTCCTAGAATTAATTCCC-3′	8211–8230	5′-TCTGAGGCTTGTAGGAGGGTA-3′	9745–9725	1535bp
5′-TCCAAGCCTACGTTTTCACAC-3′	9152–9172	5′-AAGGCTAGGAGGGTGTTGATT-3′	10,102–10082	951bp
5′-GAAGCCGCCGCCTGATACTG-3′	9912–9931	5′-GAGGATATGAGGTGTGAGCG-3′	10,555–10536	644bp
5′-GAGTGACTACAAAAAGGATT-3′	10,371–10390	5′-GTGCGATGAGTAGGGGAAGG-3′	11,258–11238	888bp
5′-CCACTATCACGAAAAAAACTC-3′	11,020–11040	5′-AGGGTTAGGGTGGTTATAGT-3′	12,371–12352	1352bp
5′-CAACATGGCTTTCTCAACTTT-3′	12,250 –12270	5′-AAGCGGATGAGTAAGAAGAT-3′	13,124–13105	875bp
5′-CAACACAGCACGGATTCAAG-3′	12,828–12847	5′-CTATTGAGGAGTATCCTGAGGC-3′	14,453–14432	1626bp
5′-TCTTCTTCCCACTCATCCTAA-3′	14,108–14128	5′-TTTCATCATGCGGCGCTGTGGATGG-3′	14,816–14790	709bp
5′-CACCGACCAATGATATGAAAAACC-3′	14,695–14717	5′-GGAAAAAGGTTTTCATCTCCG-3′	15,946–15926	1252bp
5′-GCATCCGTACTATACTTCAC-3′	15,806 –15825	5′-GGGTGAACTCACTGGAACGG-3′	726–707	1489bp

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Appendix B

Supplementary Table 2

Nested Oligonucleotid Primers for Sequencing of the mtDNA

Primer-Sequence	Position	Orientation
5′-CCCATCCTACCCAGCACACACA-3′	500–521	FORWARD
5′-CGGTCACACGATTAACCCAAG-3′	900–920	FORWARD
5′-TCCCAGTTTGGGTCTTAGCTATTG-3′	1075–1052	REVERSE
5′-ACTTGGACGAACCAGAGTG-3′	1590–1608	FORWARD
5′-CGCCTATACTTTATTTGGGTAA-3′	1750–1729	REVERSE
5′-TCATTATTACCCTCACTGTCAACCC-3′	2404–2428	FORWARD
5′-AACCTTTCCTTATGAGCATGCCT-3′	2456–2434	REVERSE
5′-AAACAGGCGGGGTAAGATTTG-3′	2499–2479	REVERSE
5′-ACCTCGGAGCAGAACCCAA-3′	2820–2838	FORWARD
5′-AGGACAAGAGAAATAAGGCC-3′	3130–3149	FORWARD
5′-TCCTATTTATTCTAGCCACCTC-3′	3611–3632	FORWARD
5′–AAGATGGTAGAGTAGATGACGG-3′	4510–4489	REVERSE
5′–CCAGCATTCCCCCTCAAACCTA-3′	4280–4301	FORWARD
5′–ACCGCATCCATAATCCTTCTA-3′	4661–4681	FORWARD
5′-GCCTTCTCCTCACTCTCTCAA-3′	4923–4943	FORWARD
5′-GGGTTTTGCAGTCCTTAGCTGTT-3′	5603–5581	REVERSE
5′-CGCTACTCCTACCTATCTCC-3′	5470–5489	FORWARD
5′-AGAATAGTCAACGGTCGGCG-3′	5928–5909	REVERSE
5′-TTATGTTGTTTATGCGGGGAA-3′	6204–6184	REVERSE
5′-CAACCTTCTAGGTAACGACCACATC-3′	6038–6062	FORWARD
5′-CGTCCTAATCACAGCAGTCCT-3′	6464–6484	FORWARD
5′-GTTCTTTTTTTCCGGAGTAGT-3′	6702–6682	REVERSE
5′-ATTTTGGCGTAGGTTTGGTCT-3′	7138–7118	REVERSE
5′-TTGAAAAAGTCATGGAGGCCAT-3′	7516–7495	REVERSE
5′-ATGCCCCCCACCCTACCACAC-3′	7394–7414	FORWARD
5′-GGCTCTAGAGGGGGTAGAGGGG-3′	8294–8273	REVERSE
5′-TGAGGGAGGTAGGTGGTAGTTTGT-3′	8479–8456	REVERSE
5′-TCGTCCTAGAATTAATTCCC-3′	8211–8230	FORWARD
5′-ACCCGCCGCAGTACTGATCAT-3′	8577–8597	FORWARD
5′ CACACCTACACCCCTTATCCC-3′	8922–8942	FORWARD
5′-TCCAAGCCTACGTTTTCACAC-3′	9152–9172	FORWARD
5′-TCTGAGGCTTGTAGGAGGGTA-3′	9745–9725	REVERSE
5′-AAGGCTAGGAGGGTGTTGATT-3′	10,102-10082	REVERSE
5′-GAAGCCGCCGCCTGATACTG-3′	9912–9931	FORWARD
5′-GAGGATATGAGGTGTGAGCG-3′	10,555-10536	REVERSE
5′-GAGTGACTACAAAAAGGATT-3′	10,371–10390	FORWARD
5′-GTTTTTTTCGTGATAGTGGT-3′	11,039–11020	REVERSE
5′-TCAATCTCCAACACATATGGCCT-3′	10,707–10729	FORWARD
5′-GTGCGATGAGTAGGGGAAGG-3′	11,258–11238	REVERSE
5′-CCACTATCACGAAAAAAACTC-3′	11,020–11040	FORWARD
5′-TACGCCTCACACTCATTCTCA-3′	11,490–11510	FORWARD
5′-GGACTTCAAACTCTACTCCC-3′	11,798–11817	FORWARD
5′-AGGGTTAGGGTGGTTATAGT-3′	12,371–12352	REVERSE
5′-GCTATCCATTGGTCTTAGGCCC-3′	12,283–12304	FORWARD
5′-GCTAAGGCGAGGATGAAACC-3′	12,896–12877	REVERSE
5′-AAGCGGATGAGTAAGAAGAT-3′	13,124–13105	REVERSE

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Appendix B

Supplementary Table 2 (CONTINUED)

Nested Oligonucleotid Primers for Sequencing of the mtDNA

5′-TACCGCTAACAACCTATTCCAACTG-3′	12,732–12756	FORWARD
5′-CAACACAGCAGCCATTCAAG-3′	12,828–12847	FORWARD
5′-TCTTCTTACTCATCCGCTTCC-3′	13,105–13125	FORWARD
5′-TAGAGGGGGATTGTTGTTTGGA-3′	13,790–13769	REVERSE
5′-TCCCCACCCTTACTAACATTAACG-3′	13,646–13669	FORWARD
5′-TCTTCTTCCCACTCATCCTAA-3′	14,108–14128	FORWARD
5′-TAAACCCATATAACCTCCCCCAA-3′	14,515–14537	FORWARD
5′-AGGTCGATGAATGAGTGGTT-3′	14,786–14767	REVERSE
5′-CACGACCAATGATATGAAAAACC-3′	14,695–14717	FORWARD
5′-CTGAGTAGCCTCCTCAGATTCAT-3′	15,254–15232	REVERSE
5′-GCAACTATAGCAACAGCCTTC-3′	15,110–15130	FORWARD
5′-GAATGGGAGGTGATTCCTAGG-3′	15,390–15370	REVERSE
5′-CCTCCCATTCCGATAAAATC-3′	15,381–15400	FORWARD
5′-GGAAAAAGGTTTTCATCTCCG-3′	15,946–15926	REVERSE
5′-TAAGGGTGGGTAGGTTTGTTGGT-3′	16,299–16277	REVERSE
5′-GCATCCGTACTATACTTCAC-3′	15,806–15825	FORWARD
5′-GGATGAGGCAGGAATCAAAGAC-3′	151– 130	REVERSE
5′-CAACCCTCAACTATCACACAT-3′	16,218–16238	FORWARD
5′-CACCCTATTAACCACTCACGGG-3′	15–36	FORWARD
5′-TTATGGGGTGATGTGAGCCC-3′	644–625	REVERSE
5′-GGGTGAACTCACTGGAACGG-3′	726–707	REVERSE

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