Chapter 12
NF1 Germline and Somatic Mosaicism

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12.1 Introduction

Neurofibromatosis type 1 (NF1, MIM 162200) is a common autosomal dominant genetic disorder affecting ~1/3,000 individuals worldwide (Huson et al. 1989). NF1 is a progressive disorder, with more symptoms typically developing with time. NF1 shows complete penetrance, although the disease presents with a high degree of phenotypic variability even within families and within monozygotic twins carrying the same mutation (Rieley et al. 2011). Patients present with multiple café-au-lait macules (CALM), skinfold freckles, iris Lisch nodules, and neurofibromas. Neurofibromas can be subdivided into dermal neurofibromas, benign tumors that increase in number and size with age but do not undergo malignant transformation, and congenital plexiform neurofibromas, tumors involving multiple fascicles which in ~5 % of cases may progress to malignant peripheral nerve sheath tumors (MPNSTs) (Korf 1999). Optic pathway gliomas and specific skeletal abnormalities of the sphenoid wing, long bones, and vertebrae are also typical clinical signs associated with NF1. In addition, macrocephaly, short stature, learning disabilities, and attention difficulties are frequently observed in NF1 patients. NF1 patients also have an increased risk of developing specific malignancies (reviewed by Brems et al. 2009).

NF1 is due to mutations in the 282-kb-long *NF1* gene located on 17q11.2, comprising 57 constitutive and at least three alternatively spliced exons. The 8,454 nucleotides of the open reading frame of the *NF1* transcript encode neurofibromin, which negatively regulates Ras-GTPases (Ballester et al. 1990; Xu et al. 1990). *NF1* functions as a tumor suppressor, and NF1-associated neoplasias, such as neurofibromas, gastrointestinal stromal tumors, glomus tumors,

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juvenile myelomonocytic leukemia, astrocytomas, and pheochromocytomas, have been shown to carry 2 mutant *NF1* copies (reviewed by Brems et al. 2009). *NF1* inactivation of both alleles has been documented in dermal as well as in plexiform neurofibromas, but additional genetic changes, including complex karyotypes, have only been identified in MPNSTs.

In addition, somatic "second hit" *NF1* mutations have also been identified in tissues from tibial pseudarthrosis (Stevenson et al. 2006) and CALMs (De Schepper et al. 2008; Maertens et al. 2007).

Biallelic *NF1* inactivation follows the Knudson "two-hit hypothesis". According to this model of tumorigenesis, one mutant allele (the "first hit" mutation) is inherited from an affected parent (for familial cases) or from a mutant germ cell of an otherwise unaffected parent. Alternatively, the first hit mutation results from a de novo mutation in the fertilized egg or occurs in very early stages of the developing embryo. By contrast, the "second hit" mutation affecting the other (wild-type) allele is then specifically present only in those somatic cells which represent the tumor or CALM initiating cells.

12.2 Mosaicism

NF1 affects ~1/3,000 individuals irrespective of gender or ethnic background and as many as 30–50 % of these patients present as "sporadic" or "founder" patients, that is, have no affected parent from whom the disorder was inherited (Friedman 1999; Evans et al. 2010). It follows that the *NF1* gene has a new mutation rate of $3.7–26 \times 10^{-5}$, that is, 10–100-fold higher to that which is observed in most other known human disease-associated genes (reviewed by Poyhonen et al. 2000 and references therein). The cause of this unusually high mutation rate is still unknown, the large size and complexity of the gene being possibly only partially contributory. Zlotogora proposed in 1993 that a proportion of the sporadic or "founder" NF1 patients may have the disease as the consequence of a somatic mutation after fertilization and the first molecularly proven somatic mosaic NF1 patient was reported in 1996: a multi-exon deletion of at least 100 kb encompassing exons 4–39 was identified in a 31-year-old patient with seven CALMs, bilateral axillary freckling, and multiple small cutaneous neurofibromas (Colman et al. 1996).

Genetic mosaicism refers to a condition where two or more genetically distinct cell populations, derived from the same fertilized egg, coexist in the same individual. Mosaicism in genetic disorders with significant skin manifestations, such as neurofibromatosis type 1, will be more readily visible than in disorders mainly affecting, for example, internal organs. With respect to NF1, mosaicism as discussed here refers to the fact that (only) a subpopulation of the body cells in a patient carries a "first hit" *NF1* mutation.

The following types of mosaicism for a "first hit" *NF1* mutation may be encountered. *Gonadal or germline mosaicism* occurs when a fraction of the germ cells (sperm or ova) carry a mutation, which may then lead to more than one affected offspring from clinically normal parents. Pure gonadal mosaicism is likely

to be extremely rare in NF1, as only two such families have been reported to date (Lázaro et al. 1994; Bottillo et al. 2010). Mutations occurring early during embryonic development, before gastrulation and the formation and separation of the primordial germ cells, may lead to *gonosomal mosaicism* where somatic as well as germ cells can be mosaic. If mutations arise after the primordial germ cells were formed and set apart, patients may present solely with *somatic mosaicism*, implying that they would not be at risk of transmitting the disorder to the next generation. Ruggieri and Huson (2001) proposed the terms *mosaic-generalized* NF1 and *mosaic-localized* NF1 (or *segmental* NF1), reflecting the anticipated timing of the mutational event. Patients with *mosaic-generalized* NF1 present with typical NF1-related symptoms such as pigmentary changes and neurofibromas, not restricted to a few body segments. It may be particularly daunting to predict, solely from clinical observation, whether a sporadic patient has *constitutional* NF1 (i.e., with an *NF1* mutation in all body cells) versus *mosaic-generalized* NF1 and if in the latter patients, whether or not the gonads are involved. Patients with "*mosaic-localized*" or "*segmental*" NF1 present with NF-related symptoms confined to a limited body area. The affected area may present with pigmentary changes only, with neurofibromas only, or with both pigmentary changes and neurofibromas. Further, isolated plexiform neurofibromas also occur and recently the first patient to be described was proven to carry biallelic *NF1* inactivation in this isolated lesion (Beert et al. 2012).

In segmental NF1, the causative mutational event is assumed to have occurred at a later developmental time, offering an explanation for the clinically observed phenotype restricted to a specific body segment. It is most likely to assume that the germ cells of patients with segmental NF1 do not harbour the (first hit) *NF1* mutation. However, apart from the timing of the mutational event, the types of progenitor cells affected as well as the nature of the *NF1* mutation (hypomorphic versus, e.g., a total *NF1* gene deletion) and the age of the patient at clinical evaluation will all equally contribute to the observed widely variable phenotypic outcome in the patient at a given time point. Segmental NF1 is rare, with a frequency estimated to be at least 15 times less prevalent than NF1, between 0.0014 % and 0.002 % (Huson and Ruggieri 2000; Wolkenstein et al. 1995; Ingordo et al. 1995; Listernick et al. 2003). The first proof at the molecular level that *segmental NF1* is indeed due to postzygotic *NF1* mutations was provided through identification of an *NF1* microdeletion in ~15–24 % of the fibroblasts cultured from the CALMs of the affected, but not in the fibroblasts from the unaffected region, from an 18-year-old male with pigmentary lesions scattered over the left upper quadrant of the body (Tinschert et al. 2000). Importantly, however, thereafter some patients have been reported who phenotypically presented as having "*mosaic-localized*" or "*segmental*" NF1 disease, yet were confirmed as being gonosomal mosaic by molecular analysis and as ascertained after birth of their affected offspring (Consoli et al. 2005; Callum et al. 2012).

In the clinical literature, a number of segmental NF1 patients have been described having offspring with segmental NF1 (Rubenstein et al. 1983; Huson and Ruggieri 2000; Oguzkan et al. 2004); such vertical transmission is very difficult

to explain as a result of mosaicism for a mutation shared between parent and child. None of these reported cases had molecular analyses performed and hence the underlying cause remains obscure. In addition, apart from a number of clinical "*mosaic-localized*" or "*segmental*" NF1 cases which are very likely to carry *NF1* mutations in the affected cells, though it was not molecularly investigated and hence the precise underlying cause remains unknown (e.g., Hager et al. 1997; Ruggieri and Huson 2001; Listernick et al. 2003; Morais et al. 2010), some patients have been reported in the literature as having "segmental" NF1 even though they presented with, in addition to pigmentary abnormalities, some features clearly not associated with NF1 (e.g., Castori et al. 2008; Pascual-Castroviejo et al. 2008). As these patients were not analyzed at the molecular level, and hence not proven to carry an *NF1* mutation, it must be realized that their phenotype may well be due to a concurrent defect in more than one gene, including the *NF1* gene, or even might be due altogether to a defect in one or more other genes, not even involving *NF1*. As the molecular tools to investigate the genetics associated with NF1 disease have become more widely available, clinical reports of "segmental NF1" cases should be accompanied by appropriate supportive data using state-of-the-art molecular genetic analyses to prove *NF1* involvement, especially when patients present with features not associated with NF1.

Finally, *revertant mosaicism* refers to the situation where a mutant cellular phenotype is reversed back to normal in some somatic cells by, for example, true reverse point mutation, gene conversion, nondisjunction, or mitotic recombination. Revertant mosaicism has been demonstrated in many genetic conditions involving the skin, including epidermolysis bullosa, Bloom syndrome, and Fanconi anemia amongst others (reviewed by Lai-Cheong et al. 2011), yet has never been demonstrated so far in NF1. To date, only three cases have been specifically investigated for the presence/absence of revertant mosaicism: a woman with NF1 signs throughout the entire body with a few sharply delineated segments of the skin *un*affected, suggestive of revertant mosaicism (Vandenbroucke et al. 2004), and two pairs of monozygotic twins discordant for neurofibromatosis type 1 (Kaplan et al. 2010; Vogt et al. 2011).

All cases *with* molecular data supporting mosaicism for a "first hit" *NF1* mutation, reported as of May 2012, are summarized in Table 12.1. Highlights from these studies and case reports include the following:

- The frequency of mosaicism in sporadically affected NF1 patients is currently still largely unknown. A first estimate was obtained for patients carrying specifically a total gene deletion (TGD) interphase FISH analysis in 146 patients carrying a TGD showed mosaicism in blood in 9.6 % (14/146) (Messiaen et al. 2011). At least 10/14 carried a 1.2-Mb NF1 type 2 *or* atypical TGD deletion (see Chap. 14).
- The typical 1.4-Mb type 1 TGD, previously uniquely found as a result of nonallelic homologous recombination during maternal meiosis between the paralogous recombination sites 1 and 2 (PRS 1 and 2), may also rarely result

Table 12.1 Overview of the mutational and clinical data in all published patients in whom molecular genetic studies were performed

		Phenotype			Molecular analysis				Percentage of cells carrying the mutation in		
Type	Patient ID	Age Years	Gender	Clinical features	NF1 first hit mutation	Methods	Parental origin[b]	Offspring affected	Peripheral blood/ cultured lymphocytes	Others	References
GO	XAT27: I1	–	M	No signs, but has two affected children	Del ivs31-ivs39 and Ins 30 bp	Haplotype analysis, BP cloning, sequencing, cDNA analysis, q-PCR, southern blot	–	Mutation detected	N/D	~10 % in sperm cells	Lázaro et al. (1994, 1995)
GEN	UF161[a]	31	F	7 CALMs, bilateral axillary freckling, multiple small cutaneous NFs on face, trunk and extremities, first NFs developed at age 26 after 2nd pregnancy, macrocephaly, normal development; no segmental distribution of features	Del ex4-ivs39 (~100 kb)	LOH analysis, cloning	Mat (UF162)	2 sons: UF394:1CALM; UF395:no signs)	~98.5 %	Mosaic in NFs	Colman et al. (1996) and Rasmussen et al. (1998)
GEN	867	21	F	Multiple CALMs, axillary freckling, multiple small NFs, and bilateral Lisch nodules; cranial CT and MRI scans normal	Del ~ivs27-ivs41	Haplotype analysis	Mat	–	Mosaic	–	Ainsworth et al. (1997)
GEN	5b	40.4	F	>100 cutaneous/ subcutaneous NFs, normal IQ	Microdeletion	FISH	–	Daughter 5a: severely affected	83 %	N/D in fibroblasts from normal skin	Tonsgard et al. (1997)

(continued)

Table 12.1 (continued)

		Phenotype			Molecular analysis				Percentage of cells carrying the mutation in		
Type	Patient ID	Age Years	Gender	Clinical features	NF1 first hit mutation	Methods	Parental origin[b]	Offspring affected	Peripheral blood/ cultured lymphocytes	Others	References
GEN	95-870-P[a]	0.6	F	Large congenital cervical PNF and multiple CALMs in a generalized distribution, facial dysmorphism with facial nerve palsy	Microdeletion (~700 kb)	FISH	–		77–84 %	N/D in fibroblasts	Wu et al. (1997) and Riva et al. (2000)
GEN	UF113	10	?	Multiple CALMs, 2 cutaneous NFs, axillary freckling, no macrocephaly, normal development, mild hemihypertrophy, bilateral sensorineural hearing loss	Intragenic Del	LOH analysis, Southern blot	Mat	–	Small fraction	–	Rasmussen et al. (1998)
GEN	–	6	M	Severe NF1: multiple CALMs of up to 8 × 5 cm in a generalized distribution, axillary freckling, pigmentation of the genitalia, PNF, growth retardation, unable to crawl, walk, talk. Subdermal NFs below the left nipple and at the right neck. Facial anomalies, severe and early-onset psychomotor retardation, seizures, spasticity, microcephaly	Microdeletion (>1.7 Mb)	FISH	–	–	33 %	58 % in fibroblasts	Streubel et al. (1999)

GEN	N.R.	47	M	CALMs, freckling, Lisch nodules, NFs, macrocephaly, no PNF	Microdeletion	FISH (locus-specific probes)	–	–	Mosaic	–	Riva et al. (2000)
SEG	–	18	M	Freckling and CALMs on the left upper quadrant: side of the neck, upper trunk, axilla and left arm. No NFs and Lisch nodules	Microdeletion	FISH	–	–	~0–2 %	~15–24 % in fibroblasts from CALMs, N/D in normal skin, N/D in hair follicles	Tinschert et al. (2000)
SEG	–	50	M	Multiple soft, skin-colored tumors on the left front, glabella, upper eyelid, nose, nostril. No other symptoms of NF1	N/D (fibroblasts cultured from NFs)	PTT, enzymatic mutation detection, FISH	–	–	N/D	N/D in fibroblasts from NFs and normal skin	Schultz et al. (2002)
GEN	IL39[a]	60	F	4 CALMs on her thighs and forearms; minor freckling in the left axilla; no dermal NFs or Lisch nodules	Microdeletion (type 2)	FISH, BP PCR, haplotype analysis	–	2 severely affected sons	70 %	15 % in fibroblast	Petek et al. (2003) Kehrer-Sawatzki et al. (2004) and Steinmann et al. (2007, 2008)
GEN	WB[a]	65	F	>20 NFs, no facial dysmorphism, no mental retardation	Microdeletion (type 2)	FISH, BP cloning, polymorphic markers analysis	–	Daughter SB: severely affected	94 %	–	Kehrer-Sawatzki et al. (2004) and Steinmann et al. (2007, 2008)
GEN	659	47	F	>1,000 NFs, no mental retardation	Microdeletion (atypical HKS, personal communi- cation)	FISH, BP cloning, polymorphic markers analysis	–	–	96 %	52 % in buccal smear	Kehrer-Sawatzki et al. (2004)
GEN	928[a]	35	F	>6 CALMs, freckling, Lisch nodules, 9 subcutaneous and 20 cutaneous NFs, 1 PNF. No facial dysmorphism, no mental retardation	Microdeletion (type 2)	FISH, BP cloning, polymorphic markers analysis	–	–	97.2–99.8 %	80 % in NFs, 55 % in buccal smear, 61.3 % in urine	Kehrer-Sawatzki et al. (2004, 2012), Steinmann et al. (2007, 2008), and Roehl et al. (2012)
GEN	697[a]	11	F	No NFs, no facial dysmorphism or mental	Microdeletion (type 2)	FISH, BP cloning,	Mat	–	95.4–98.7 %	59 % in buccal smear, 27.6 % in urine	Kehrer-Sawatzki et al. (2004), Steinmann et al.

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Table 12.1 (continued)

		Phenotype			Molecular analysis				Percentage of cells carrying the mutation in		
Type	Patient ID	Age Years	Gender	Clinical features	NF1 first hit mutation	Methods	Parental origin[b]	Offspring affected	Peripheral blood/ cultured lymphocytes	Others	References
				retardation. Delayed in writing/reading		polymorphic makers analysis					(2007, 2008), and Roehl et al. (2012)
GEN	488[a]	33	F	>6 CALMs, freckling, Lisch nodules, 80 subcutaneous and 140 cutaneous NFs, no facial dysmorphism, no mental retardation	Microdeletion (type 2)	FISH, BP cloning, [olymorphic makers analysis	–	–	96.8–100 %	56 % in buccal smear, 46.5 % in urine	Kehrer-Sawatzki et al. (2004, 2012), Steinmann et al. (2007, 2008), and Roehl et al. (2012)
GEN	938[a]	31	F	>6 CALMs, freckling, Lisch nodules, 1 subcutaneous and 20 cutaneous NFs. 1 internal tumor and MPNST, no facial dysmorphism, no mental retardation	Microdeletion (type 2)	FISH, BP cloning, polymorphic makers analysis	–	–	93.5–99.6 %	80 % in buccal smear, 23.9 % in urine	Kehrer-Sawatzki et al. (2004, 2012), Steinmann et al. (2007, 2008), and Roehl et al. (2012)
GEN	KCD[a]	34	F	>100 NFs, no facial dysmorphism, no mental retardation	Microdeletion (type 2)	FISH, BP cloning, polymorphic makers analysis	–	–	92 %	51 % in fibroblasts	Kehrer-Sawatzki et al. (2004) and Steinmann et al. (2007, 2008)
GEN	736[a]	68	F	>6 CALMs, freckling, Lisch nodules, ~2,000 cutaneous NFs, no facial dysmorphism, no mental retardation	Microdeletion (type 2)	FISH, BP cloning, polymorphic makers analysis	–	–	93.8–99.6 %	59 % in buccal smear, 26.7 % in urine	Kehrer-Sawatzki et al. (2004, 2012), Steinmann et al. (2007, 2008), and Roehl et al. (2012)
GEN	NF296-UHG[a]	38	F	NF1 disease manifestations throughout the whole body, but leaving a few sharply delineated segments of the skin	Del ex13-ex28	Long-range RT-PCR, FISH, microsatellite analysis, q-PCR, MLPA	– (father unknown—mother deceased)	–	20.5 %	2.4 % in fibroblasts from CALMs, 0.9 % in fibroblasts from normal skin	Vandenbroucke et al. (2004) and Wimmer et al. (2006)

				unaffected (segmental *absence* of symptoms. Multiple CALMs, NFs, axillary and inguinal freckling, bilateral Lisch nodules, congenital pseudarthrosis of the right tibia						
SEG/GOSO	–	–	F	An area of freckling and CALMs confined to the left upper quadrant, with ipsilateral Lisch nodules in one eye	p.Arg1947*	dHPLC, DNA sequencing, cloning, allele-specific PCR	–	1 child with classic NF1	N/D	~17.6 % in fibroblasts and ~40 % in keratinocytes from CALMs, N/D in normal skin	Consoli et al. (2005)
SEG	–	40	M	Regional presentation of NF restricted to the right chest wall	N/D	Comprehensive NF1 testing	–	–	N/D		Fortino et al. (2005)
N/A	NF362-UAB	–	–	–	Del ex10b-ex49	MLPA, RT-PCR, microsatellite analysis	–	–			Wimmer et al. (2006)
N/A	NF467-UHG	–	–	–	Del ex28-ex40	MLPA, RT-PCR, microsatellite analysis	–	–			Wimmer et al. (2006)
Atypical (mosaic Ring chr17)	–	9.5	F	Mild scoliosis, gait wide-based. Multiple CALMs on the skin, mild axillary and inguinal freckling. Mental retardation, autistic features, cognitive and language delay	Mosaic non-supernumerary ring chr17 (0.6–2.5 Mb on 17p, and up to about 10 Mb on 17q)	FISH, MLPA	–	–	12 %		Havlovicova et al. (2007)
SEG	SNF1-1	46	F	*NFs only*: several small (1–4 mm) cutaneous NFs clustered within a limited body region on her trunk	c.2041C>T (p. Arg681*) identified in 3 different NFs	Comprehensive NF1 testing, q-PCR, FISH	–	(2 unaffected sons)	3.7 %	8–19.4 % in fibroblasts and 41–47.4 % in Schwann cells	Maertens et al. (2007)

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Table 12.1 (continued)

		Phenotype			Molecular analysis				Percentage of cells carrying the mutation in		
Type	Patient ID	Age Years	Gender	Clinical features	NF1 first hit mutation	Methods	Parental origin[b]	Offspring affected	Peripheral blood/ cultured lymphocytes	Others	References
				and neck. Intestinal ganglioneuroma. A glomus tumor at the distal falanx of the right middle finger						from NFs, 1.8 % in hair follicles	
SEG	SNF1-2	23	M	*Pigmentary defects only*: several CALMs within a background of hyperpigmented skin involving right leg, hip, and lower back. Inguinal freckling in the affected segment. no NFs, no Lisch nodules, no LD	Microdeletion (atypical) in CALM and hyperpigmented skin	Comprehensive NF1 testing, q-PCR, FISH	–	–	2 % (400 interphase cells analyzed)	Mosaic in melanocytes from CALMs, N/D in normal skin	Maertens et al. (2007)
GEN	SNF1-3	15	F	>6 CALMs over her body, several small cutaneous and subcutaneous NFs on her right hand within an overlying CALM, a putative NF on her left shoulder identified by total body MRI, no LD, no skinfold freckling	Microdeletion (1.84–2.8 Mb) or c.2325+1G>A in multiple NFs and CALMs	Comprehensive NF1 testing, q-PCR, FISH	–	–	~4 % N/D	Mosaic in melanocytes from CALMs and Schwann cells from NFs 54.9–97.1 % in melanocytes from CALMs, 12.3 % in fibroblasts and 67.2–88.8 % in Schwann cells from NFs	Maertens et al. (2007)
GEN	1630[a]	15	F	>6 CALMs, freckling, Lisch nodules, no NFs, no large hands and feet,	Microdeletion (type 2)	Polymorphic markers analysis, BP	–	–	94.1–98.2 %	29.3 % in urine	Steinmann et al. (2007, 2008), Kehrer-Sawatzki

				none of the other features frequently seen in patients with large deletions		analysis, MLPA, aCGH					et al. (2012), and Roehl et al. (2012)
GEN	1104[a]	36	F	Hyperpigmented brownish areas with chessboard pattern on upper back, left shoulder, left arm, left breast, right shoulder, lumbar region, right side of the abdomen; No axillary and inguinal freckling, no Lisch nodules, 4–6 NFs on abdomen, thighs, forearms	Microdeletion (type 2)	Polymorphic markers analysis, BP analysis, MLPA, aCGH	–	–	84–94 %	8 % in buccal smear, 15 % in urine	Steinmann et al. (2007, 2008)
GEN	1502[a]	26	F	Multiple CALMs, axillary and inguinal freckling, Lisch nodules, <10 subcutaneous NFs, IQ90, cerebral and whole-body MRI did not reveal any tumors or other anomalies	Microdeletion (type 2)	Polymorphic markers analysis, BP analysis, MLPA, aCGH	–	–	95.9–99.3 %	70 % in buccal smear, 54.6 % in urine	Steinmann et al. (2007, 2008), Kehrer-Sawatzki et al. (2012), and Roehl et al. (2012)
GEN	811-M[a]	–	F	–	Microdeletion (type 2)	Polymorphic markers analysis, BP analysis, MLPA, aCGH	–	1 affected daughter	93 %	–	Steinmann et al. (2007, 2008)
GEN	HC[a]	–	M	–	Microdeletion (type 2)	Polymorphic markers analysis, BP analysis, MLPA, aCGH	Mat (PH)	–	100 %	Mosaic in buccal smear	Steinmann et al. (2007, 2008)
GEN	1860-M[a]	28, 54	F	LD, developmental delay. Axillary and inguinal freckling, Lisch	Microdeletion (Atypical)	MLPA, haplotype analysis, FISH	Mat (1860-M)	Affected son	94.4–95.9 %	50.9 % in urine	Steinmann et al. 2008 and Roehl et al. 2012

(continued)

Table 12.1 (continued)

		Phenotype			Molecular analysis				Percentage of cells carrying the mutation in		
Type	Patient ID	Age Years	Gender	Clinical features	NF1 first hit mutation	Methods	Parental origin[b]	Offspring affected	Peripheral blood/ cultured lymphocytes	Others	References
				nodules. ~1,000 cutaneous and subcutaneous NFs, internal tumors in the brachial plexus and lumbar region							
GO	NF-307: I1	–	M	NO signs of NF1	Del ivs27b-ex30 (c.4773-3622-?_5749+?)	q-PCR, microsatellite analysis, MLPA, RNA-based analysis	–	2 affected daughters and 1 granddaughter	N/D	10–17 % in sperm cells	Bottillo et al. (2010)
GOSO (affected twin)/? (discordant MZ twin)	–	57	F	Affected twin: CALMs, cutaneous NF, PNF, Lisch nodules, freckling. Implication of gonosomal mosaicism	p.Arg1968*	Sequencing, STR markers, aCGH	Mat	2 affected children, 1 unaffected child	Present	Present in fibroblasts and buccal smear	Kaplan et al. (2010)
				Unaffected twin: No signs of NF1; has 2 unaffected children				No	Present	Present in buccal smear, N/D in fibroblasts	
GEN	#3	36	M	9 CALMs and macrosomia, bilateral axillary freckling; Normal cognition without LD, NO segmental distribution. Possibly 1 NF	c.2866dupA	SNaPshot, pyrosequencing	–	1 affected son, 2 unaffected children	24 %	–	Muram-Zborovski et al. (2010)
GEN	–	34	F	20 CALMs on trunk and upper extremities, axillary and submammary freckling,	c.3198-314G>A (leaky splice mutation)	DNA-based sequencing, SNaPshot	–	–	~100 %	~100 % in urine, ~20–35 % in buccal smear,	Fernández-Rodríguez et al. (2011)

				mild scoliosis, <50 minuscule NFs which developed since age 18 years, no Lisch nodules, no dysmorphism, no LD						hair follicles and skin	
GEN	UAB-r3302	15	M	>6 CALMs, axillary freckling, cutaneous NFs on left neck, arm, trunk and PNF on left arm. A few freckles and CALMs on right arm. Bilateral Lisch nodules. No spinal NFs or optic gliomas	Microdeletion (type 1)	MLPA, microsatellite, FISH, BP spanning PCR, SNP analysis	–	–	~80 %	–	Messiaen et al. (2011)
GEN	UAB-r7332	10	M	>6 CALMs, bilateral axillary and inguinal freckles. Bilateral Lisch nodules. 2–6 cutaneous and 2–6 subcutaneous NFs, symptomatic optic glioma on left optic nerve. Scoliosis	Microdeletion (likely type 1 according to MLPA)	MLPA, microsatellite, FISH, BP spanning PCR, SNP analysis	–	–	~97 %	–	Messiaen et al. (2011)
GEN	UAB-r3222	27	F	2 CALMs, no freckles. >100 cutaneous NFs, no PNF. Bilateral Lisch nodules. No optic gliomas or skeletal abnormalities. Normal development	Microdeletion (type 1)	MLPA, microsatellite, FISH, BP spanning PCR, SNP analysis	–	–	50 %	–	Messiaen et al. (2011)
SEG	P067	45	F	~20 small dermal NFs, freckling on upper right back and under right breast. Inguinal freckling. No CALMs or Lisch nodules. No facial dysmorphy, LD	Microdeletion (type 1)	MLPA, microsatellite, FISH, BP spanning PCR, SNP analysis	–	–	N/D	Present in Schwann cells from NFs	Messiaen et al. (2011)
SEG	UAB-M1	14	M	3 CALMs, freckling hyperpigmentation on left upper thigh, left	Microdeletion (likely type 1	MLPA, microsatellite, FISH, BP	–	–	N/D	Present in melanocytes from CALMs,	Messiaen et al. (2011)

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Table 12.1 (continued)

		Phenotype			Molecular analysis				Percentage of cells carrying the mutation in		
Type	Patient ID	Age Years	Gender	Clinical features	NF1 first hit mutation	Methods	Parental origin[b]	Offspring affected	Peripheral blood/ cultured lymphocytes	Others	References
				inguinal region, no NFs or PNFs, no Lisch nodules	according to MLPA)	spanning PCR, SNP analysis				N/D in fibroblasts	
GEN (discordant MZ twins)	–	3	M	Affected twin: >6 CALMs, axillary and inguinal freckling	c.4108C>T (p. Gln1370*)	SNP/allelic marker analysis, SNaPshot, Cloning	Mat	–	~30–40 %	~8 % (4 % allele) in buccal smear, N/D in urine	Vogt et al. (2011)
				Unaffected twin: 2 CALMs	N/D			–	N/D	N/D in buccal smear, urine	
SEG	–	13	M	An isolated plexiform neurofibroma on the lumbosacral area. No CALMs, no Lisch nodules, and no other NF1 features	1st hit: ins(17;1) (q11.2;p35p36) 2nd hit: 8.28 Mb del on 17q11.2q12	Karyotyping, FISH, aCGH	–	–		61 % in Schwann cells (21 % by FISH) 13 % in Schwann cells by FISH, 15–20 % by aCGH	Beert et al. (2012)
GOSO	–	–	M	Sperm donor: only 4 hyperpigmented spots, midline back. No developmental disability, no Lisch nodules, no freckling, no NFs	Del ex11-23.1	RT-PCR, breakpoint cloning MLPA, aCGH	–	–	Present (detected by reverse-transcriptase PCR)	~20 % in sperm cells	Callum et al. (2012)
GEN	1956[a]	9	M	>6 CALMs, axillary freckling, Lisch nodules, scoliosis, normal development	Microdeletion (type 2)	FISH	–	–	96.7–99.1 %	60 % in urine	Kehrer-Sawatzki et al. (2012) and Roehl et al. (2012)
GEN	2442[a]	40	F	>6 CALMs, axillary freckling, Lisch nodules, ~250 cutaneous NFs,	Microdeletion (type 2)	FISH	–	–	98.6–100 %	60 % in urine	Kehrer-Sawatzki et al. (2012) and Roehl et al. (2012)

				foraminal tumors, normal brain, optic pathway and orbital MRI, scoliosis, short stature (<5th PC)							
GEN	UC172	–	–	–	Microdeletion (type 2)	FISH, Microsatellite analysis, breakpoint cloning	–	–	91.9–97.9 %	64.5 % in urine	Roehl et al. (2012)
GEN	585	–	–	–	Microdeletion (type 2)	FISH, microsatellite analysis, breakpoint cloning	–	–	94.3–96.7 %	81.9 % in urine	Roehl et al. (2012)
GEN	3304	47	M	Multiple CALMs, >1,000 cutaneous NFs, muscular hypotonia, large soft hands, funnel chest, difficulties with reading/writing, developmental delay	Microdeletion (atypical or type 1)	FISH, microsatellite analysis, breakpoint cloning	–	Yes affected daughter	16.9–19.3 %	25 % in urine	Roehl et al. (2012)

GO gonadal mosaicism, *GOSO* gonosomal mosaicism, *GEN* generalized mosaicism, *SEG* segmental mosaicism, *NF* neurofibroma, *PNF* plexiform neurofibroma, *CALM* café-au-lait macule, *Del* deletion, *Ins* insertion, *N/D* not detected, *M* male, *F* female, *Mat* maternally derived, *Pat* paternally derived, *LD* learning disability, *BP* breakpoint

[a]Described in multiple studies, as indicated.

[b]Parental origin of the allele carrying the mutation in the mosaic proband.

from recombination during mitotic post-zygotic cell divisions, as now proven in three patients carrying a mosaic TGD type 1 in blood (Messiaen et al. 2011).

- Mosaicism in sporadic patients carrying a TGD type 2 is very high, estimated to occur at least in 70 % of founders (Messiaen et al. 2011). There seems to be a clear preponderance of females amongst mosaic founder patients with a TGD type 2 (Kehrer-Sawatzki et al. 2004; Steinmann et al. 2007).
- Several studies found the percentage of hematopoietic cells carrying an *NF1* mutation, especially when carrying a TGD type 2, to be higher as compared to urine and buccal epithelial cells or fibroblasts from affected as well as unaffected regions in patients with generalized-mosaic NF1 (Kehrer-Sawatzki et al. 2004, 2012; Vandenbroucke et al. 2004; Steinmann et al. 2007; Kehrer-Sawatzki and Cooper 2008; Roehl et al. 2012). Two mosaic patients were, however, found by FISH analysis in blood to carry the deletion in all cells, whereas a lower percentage was found in the buccal cells (Steinmann et al. 2007; Roehl et al. 2012). These tissue-specific differences suggest a selective growth advantage of hematopoietic stem cells carrying an *NF1* deletion (Roehl et al. 2012).
- Patients with mosaic type 2 TGD have milder disease manifestations, with a significantly lower external and internal plexiform neurofibroma burden, no facial dysmorphisms, and no delayed cognitive development compared to patients with a type 1 TGD (which typically is constitutional) (Kehrer-Sawatzki et al. 2012). This can probably be attributed to the presence of normal cells in the mosaic patients. However, since the risk of malignant transformation remains in any plexiform lesions present, special clinical management to allow early detection is still required.
- The line separating generalized mosaic NF1 from segmental NF1 may not always be clear. In general, a mutation in blood is not detected in patients with clear segmental presentation. Gonosomal mosaicism has, however, been proven in an adult male with four pigmentary lesions on the midline of his back as the sole NF1-related manifestation (Callum et al. 2012). This intragenic multi-exon deletion, present in <20 % of the blood cells and in ~20 % of the sperm, was detected because of the preferential amplification of the shorter transcript by reverse-transcriptase PCR as part of a comprehensive *NF1* testing (Callum et al. 2012). Low-level mosaic mutations typically escape detection by Sanger sequencing and array comparative genome hybridization (aCGH) or multiplex ligation-dependent probe amplification (MLPA).
- The aggregated data obtained from all patients with mosaic NF1 ("*generalized*" or "*segmental*") clearly indicate that the percentage of cells carrying the *NF1* "first hit" mutation often is too low to be reliably detected in blood (or even is absent) and hence may be missed (Tinschert et al. 2000; Consoli et al. 2005; Maertens et al. 2007; Callum et al. 2012).
- A subpopulation of the Schwann cells within the neurofibromas carries the second *NF1* hit, in accordance with the two-hit tumor-suppressor hypothesis (Serra et al. 2000; Maertens et al. 2006). In the CALMs of NF1 patients, *melanocytes*, but *not* keratinocytes or fibroblasts, carry a first *and* second hit in the *NF1* gene (De Schepper et al. 2008). Maertens et al. (2007) demonstrated

that accurate diagnosis of mosaic or segmental NF1 necessitates comprehensive mutation analysis specifically of those neural-crest derived cells that are relevant, that is, the Schwann cells in the neurofibromas and the melanocytes in the CALMs. In these specific cells, but not (or only at too low a level) in blood, fibroblasts, or keratinocytes, a common first *NF1* hit can be found in the different lesions (neurofibromas or CALMs) of segmental NF1 patients. This may also explain why no mutations were found in the fibroblasts of the patient described by Schultz et al. (2002).

- In total, 49 unrelated patients, mosaic for a "first hit" post-zygotic *NF1* mutation in the gonads and/or somatic cells, were reported and only 6/49 carried a minor lesion mutation affecting 1 nucleotide, all resulting in a premature stop codon. This probably reflects the technical difficulties associated with the detection of mosaic point mutations.
- In 43/49 of these cases, the mutation was either an intragenic multi-exon deletion or a large deletion. At least 17 of these deletions were type 2 TGD spanning 1.2-Mb.
- Finally, one pair of discordant twins was reported by Kaplan et al. (2010), with a woman carrying an *NF1* nonsense mutation in both the Epstein–Barr virus-transformed lymphoblastoid cells propagated from her B-lymphocytes and her buccal epithelial cells, but not in her skin fibroblasts, yet being asymptomatic as of the age of 57 years. In another pair of discordant twins (Vogt et al. 2011), the affected 3-year-old twin was shown to be mosaic in blood and buccal cells for a nonsense mutation p.Gln1370*, which was not found in the unaffected twin. This can be explained by a post-zygotic mutation occurring after the twinning event took place, that is, after day 3–4 of embryonic life.

12.3 Molecular Diagnosis of NF1

Molecular diagnosis of NF1 is challenging owing to the large size of the gene, the existence of multiple highly homologous non-processed pseudogenes, the lack of mutational hotspots, and the complex mutational spectrum including a significant fraction of unusual splice mutations such as deep-intronic splice mutations or mutations affecting exonic splice enhancers, mimicking nonsense, missense, or even silent mutations at the genomic level (Messiaen and Wimmer 2008). The highest sensitivity and specificity are obtained using a multistep approach including an RNA-based center assay (Messiaen et al. 2000; Wimmer et al. 2006; Valero et al. 2011).

Clinical applications of mutational analysis have increased in relevance since some clear genotype–phenotype correlations have unfolded, with large deletions resulting in a more severe clinical phenotype (Upadhyaya et al. 1998; Riva et al. 2000; Mautner et al. 2010) and with the 3-bp in-frame deletion of 1 amino acid in exon 17 resulting in a milder phenotype without cutaneous or superficial plexiform neurofibromas (Upadhyaya et al. 2007). Further, limited genetic heterogeneity has

been found by identification of a novel disorder, Legius syndrome, caused by mutations in the *SPRED1* gene (Brems et al. 2007). Patients with Legius syndrome have multiple CALMs with or without skinfold freckling and macrocephaly, but do not develop the typical NF1-associated tumors (Brems et al. 2012). In patients presenting solely with CALMs with/without freckling, a correct diagnosis of constitutional NF1 versus mosaic NF1 (for sporadic patients) versus Legius syndrome cannot be made exclusively based on the clinical manifestation.

In ~95% of patients presenting with "classic NF1", including CALMs, skinfold freckling and neurofibromas, the *NF1* mutation is identified if a comprehensive approach including RNA-based sequencing is applied (Messiaen et al. 2009). However, the detection rate in the blood lymphocytes drops in sporadic patients, even if they present with CALMs, skinfold freckling and neurofibromas probably because some of them present with mosaicism for an *NF1* mutation undetectable/absent in the blood.

The majority of generalized mosaic patients reported to date present with a type 2 or atypical TGD, apparently detectable in a higher percentage of blood lymphocytes compared to urine-derived or buccal epithelial cells (Kehrer-Sawatzki et al. 2004, 2012; Vandenbroucke et al. 2004; Steinmann et al. 2007; Kehrer-Sawatzki and Cooper 2008; Roehl et al. 2012). Hence, blood represents a good sample from which to start analyses. In order to ascertain mosaicism, it seems to be sensible to pursue quantitative testing such as FISH in urine-derived epithelial cells in sporadic patients carrying a type 2 or atypical TGD identified in blood. Establishing a diagnosis of *mosaic* versus *constitutional* NF1 has important repercussions for counseling the patients and family with regard to recurrence risk.

Identification of the cell of origin in the CALMs and neurofibromas, that is, the melanocyte (De Schepper et al. 2008) and Schwann cell (Serra et al. 2000; Maertens et al. 2006), can now be applied to the precise identification of the common "first hit" *NF1* mutation in the melanocytes or Schwann cells cultured from the affected regions of segmental patients (Maertens et al. 2007) or suspected mosaic patients in whom no mutation could be identified in the blood after comprehensive testing. Identification of such a common "first hit" establishes the unambiguous diagnosis of segmental or mosaic NF1. Moreover, it provides a marker that can be used for family planning, if desired, as the mutation may be at risk of being transmitted to the next generation and, if so, would result in constitutional NF1 (with all body cells carrying the inherited mutation). Refinement of the risk for transmission can be pursued in mosaic/segmental male patients, through analysis of the sperm, but cannot be provided in female patients, given the inaccessibility of the eggs for screening.

Future progress in diagnosis of founder patients is likely to come from novel technologies such as deep sequencing and may provide a means to detect low-level mosaicism in a reliable and sensitive way.

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