Ghent University, Faculty of Medicine and Health Sciences
Center for Medical Genetics

New insights in the molecular pathogenesis of neurofibromatosis type 1

this thesis is submitted as fulfillment of the requirements for the degree of
Ph.D. in Medical Sciences by Ophélia Maertens, 2006

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This thesis is dedicated to my parents.

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CHAPTER 1. INTRODUCTION AND RESEARCH OBJECTIVES

THE GENETIC BASIS OF CANCER

CANCER GENETICS

Cells have several safeguards to ensure that cell division, differentiation and death occur correctly and in a coordinated fashion, both during development and in the adult body. The strict regulation of these processes may be undermined through the accumulation of (epi)genetic defects, providing a single cell with a selective growth advantage. This unrestricted growth potential will lead to the clonal expansion of the abnormal cell that, in time, will form a tumor[1] (Figure 1).

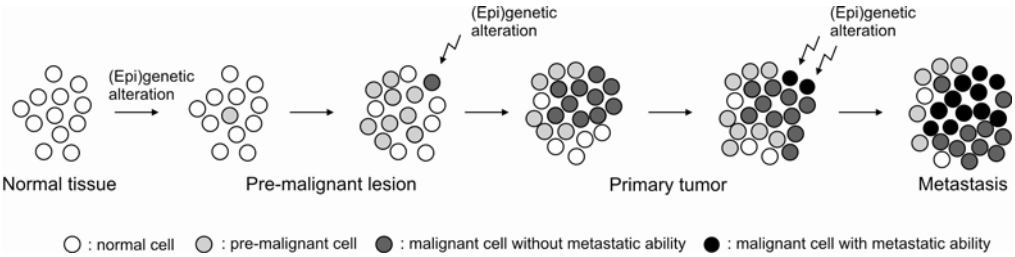

Figure 1. Model for stepwise progression of cancer in association with accumulation of (epi)genetic alterations in the cell (adapted from[2])**.**

During the course of tumor development most cancers acquire an essential set of functional capabilities, i.e. self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis[3]. These alterations in cell physiology are acquired, directly or indirectly, through successive genetic events that target two major classes of cancer-related genes: oncogenes and tumor suppressor genes.

Oncogenes

Oncogenes are mutated forms of proto-oncogenes. In normal cells, proto-oncogenes function as components of growth promoting signaling and anti-apoptotic pathways. Proto-oncogenes can act as cancer promoting genes when inappropriately (over-) expressed, which is caused by gain-of-function mutations[3]. Mutations leading to gain of function are dominant, since only one mutated allele is enough to push the cell towards the cancerous behavior. Examples of proto-oncogenes are growth factor receptors (e.g. *EGFR*), transcellular transducers (e.g. *K-RAS*), and DNA-binding nuclear proteins or transcription factors (e.g. *MYC*). Activation of oncogenes can occur through a wide variety of different mechanisms, such as gene amplifications, point mutations and chromosomal translocations.

Tumor suppressor genes

Tumor suppressor genes (TSGs) are targeted in the opposite way by genetic alterations: these genes are affected by loss-of-function mutations. According to Knudson's two-hit hypothesis[4], inactivation of both TSG alleles is required for tumor development (Figure 2). This two-hit paradigm was derived from mathematical modeling of cancer incidence in retinoblastoma, a rare embryonic pediatric tumor developing in the retina of the eye. Based on the remarkable age-of-onset difference between hereditary and sporadic forms of retinoblastoma, it was proposed that the presence of a germline mutation ('first hit') in heritable retinoblastoma followed by a rate-limiting somatic mutation ('second hit') on the other allele significantly accelerated the onset of the cancer phenotype. In sporadic tumors, both inactivating mutational events (first and second hit) have to occur in the same somatic cell and therefore it will take longer time for tumors to develop[4]. At the time this hypothesis was formulated, the nature of the genetic defect was not known. Subsequent molecular analyses on *RB1* and other TSGs confirmed the two-hit paradigm and illustrated that the general mechanisms to inactivate the remaining wild-type allele are gross chromosomal alterations, leading to loss of heterozygosity (LOH) over large regions of the chromosome[5]. More subtle inactivating mechanisms include intragenic mutations[6] and epigenetic silencing[7] due to hypermethylation of the CpG island spanning the promoter region and deacetylation of lysine residues on key histones in the chromatin.

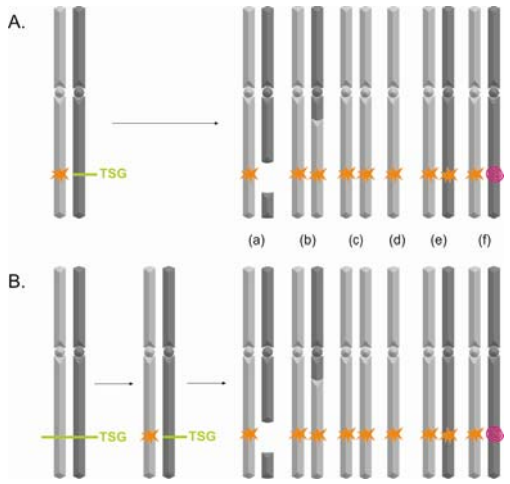

Figure 2. Knudson's two-hit model for the inactivation of a tumor suppressor gene (TSG).
In familial tumor syndromes (panel A), the initial inactivation of one allele is present in germ cells. The rate-limiting step for tumor formation is an additional inactivation event that affects somatically the second allele. Somatic inactivation events include subchromosomal deletion (a), mitotic recombination (b), nondisjunctional chromosome loss with (c)/ without (d) reduplication of the chromosome carrying the mutated TSG, intragenic mutation (e) or an epigenetic event (f). In sporadic tumors (panel B), the initial and second inactivating mutational events occur in the same somatic cell of an individual[4,5] (adapted from[8]).

Tumor suppressor genes have been classified into 'gatekeepers' and 'caretakers'[9]. 'Gatekeeper' genes directly inhibit cell growth by suppressing proliferation, inducing apoptosis or promoting differentiation. Their loss of function is rate-limiting for a particular step in multi-stage tumorigenesis and restoring the 'gatekeeper' function suppresses neoplasia. In patients with familial adenomatous polyposis (FAP), for example, an inherited defect in the *APC* gene leads to the development of hundreds of adenomatous polyps. Because of their great numbers, some polyps are virtually guaranteed to progress to cancer[9] (cfr. Infra). In contrast, 'caretaker' genes act indirectly to suppress growth by repairing mistakes made during normal DNA replication or induced by exposure to mutagens. These genes also control processes that are responsible for mitotic recombination and chromosomal segregation. The task of 'caretaker' genes is to keep genetic alteration to a minimum. Consequently, when these genes become inactivated it allows mutations in other genes (e.g. oncogenes and 'gatekeeper' genes) to accumulate. Defects in the mismatch repair genes *MSH2*, *MLH1* and *MSH6*, for example, are the genetic basis of hereditary non-polyposis colorectal cancer (HNPCC). While patients with HNPCC develop adenomatous polyps at about the same rate as the general population, these polyps progress to cancer much more often because of defective mismatch repair[9].

Some TSGs have been hypothesized to exert a selective advantage on a cell when only one allele is inactivated[10]. Potential mechanisms are a dominant negative mutation blocking the function of the remaining wild-type gene product, and haploinsufficiency, meaning that the protein product level of a single active allele is insufficient to suppress tumor development. One of the first examples of evidence for haploinsufficiency for tumor suppression was provided for the *p27* gene. *P27* acts to arrest the cell cycle and has been implicated as a critical player in the progression of a wide variety of neoplasms. Murine data show a strong selective advantage to tumor development with loss of a single *p27* allele. More specifically, $p27^{+/-}$ mice exhibit a tumor latency and aggressive tumor behavior between the phenotypes observed in $p27^{-/-}$ and $p27^{+/+}$ mice. In the tumors of the $p27^{+/-}$ mice, the *p27* wild-type allele is retained and continued to be expressed[11]. Although there is growing evidence supporting the concept of TSG haploinsufficiency in mouse models of cancer (reviewed in[10]), it is not clear yet whether loss of one wild-type allele confers a phenotype which is relevant to human carcinogenesis.

Recent evidence indicates that small non-protein-coding RNA molecules, called microRNAs (miRNAs), might also function as tumor suppressors and oncogenes. These short RNA sequences of 21 to 25 nucleotides play essential roles in many basic physiologic processes including proliferation, differentiation, and apoptosis. MicroRNAs negatively regulate protein-coding genes by binding to complementary or partially complementary target mRNAs and thereby targeting the mRNA for degradation or translational inhibition[12]. More than half of miRNAs are located at sites in the human genome that are frequently amplified, deleted, or rearranged in cancer, suggesting that miRNA abnormalities play a broad role in cancer pathogenesis[13]. Also consistent with this notion is the

observed dysregulation of miRNA expression in diverse cancer subtypes including lung cancer[14], breast cancer[15] and glioblastoma[16].

The multistep colorectal cancer model

Colorectal cancer is one of the most extensively investigated tumor types, and its model of progression due to the stepwise accumulation of genetic abnormalities serves as a paradigm for many other neoplasms. Germline mutations in the tumor suppressor gene *APC* result in familial adenomatous polyposis (FAP), one of the principal hereditary predispositions to colorectal cancer. Patients with FAP typically develop hundreds to thousands of colorectal adenomas and are prone to a variety of specific extracolonic features, such as gastrointestinal polyps, retinal lesions, desmoid tumors and brain tumors. Although the colorectal polyps are benign, some adenomas ultimately progress to malignant adenocarcinomas by 30-40 years of age[17]. Somatic mutations in *APC* are also found in the majority (85%) of sporadic colorectal cancers[18].

Inactivation of both *APC* alleles can be detected in the very earliest stages of the adenoma-carcinoma sequence, illustrating that this event is the initiating trigger for clonal evolution. Remarkably, the position and type of the second hit in FAP and sporadic polyps is determined by the localization of the first hit[19]. Apparently, the *APC* mutation spectrum reflects the fact that there exists an optimal *APC* genotype for providing the nascent tumor cell with a selective advantage[20,21]. Additional oncogenic mutations, often in *K-RAS*, are required for adenoma growth and progression. Subsequent clonal expansion and malignant transformation is driven by additional mutations and allelic losses in *TP53*, *SMAD4* and other 18q TSGs[9]. Taken together, not simply the accumulation, but rather also the type, position and temporal order of mutations determine the propensity for neoplasia.

The disease severity varies among FAP patients with respect to the number of adenomas which is related to the risk of developing cancer[22]. Disease severity is influenced by the site of the germline *APC* mutation[22,23] and there is evidence that additional inherited factors, determined by so-called modifier genes, influence the FAP phenotype[24]. The murine counterpart of human FAP, the $Apc^{Min/+}$ mouse model, shows great phenotypic variability depending on the genetic background[25]. Two modifier loci, *Mom1* and *Mom2* (Modifier of Min), have been identified that significantly affect polyp multiplicity[26,27]. The secretory phospholipase A2 (*Pla2g2a*) gene, which is part of the prostaglandin synthesis pathway involved in inflammation, has been associated with the *Mom1* locus[28]. There is little doubt that similar modifier genes exist in humans[29], however it seems that polymorphisms in the human orthologue of *Pla2g2a* do not account for significant variation in susceptibility to colorectal cancer[30].

The cancer stem cell hypothesis

Normal stem cells and cancer cells share several important properties. These include the capacity for self-renewal, the ability to differentiate, active telomerase expression, activation of anti-apoptotic pathways, increased membrane transporter activity and ability to migrate[31]. At present, an increasing body of evidence indicates that tumor initiation might be orchestrated by cancer stem cell-like cells. The existence of cancer stem cells was first documented in hematological malignancies where only a subset of cancer cells, representing the stem cell subset with the potential to self-renew and differentiate, were capable of forming new tumors[32]. More recently, cancer stem-cell like cells have also been identified in breast[33,34] and central nervous system tumors[35-38]. The cancer stem cell concept suggests that cancers originate in tissue stem or progenitor cells through dysregulation of self-renewal pathways. This subsequently leads to expansion of the cell population that then may undergo further genetic and epigenetic changes to become fully transformed. It is still not clear if the cancer stem cells are derived from true tissue-derived stem cells, bone marrow stem cells or mature cells that have undergone a de-differentiation process. Regardless, the observation that cancer stem cells give rise to phenotypically diverse cancers has fundamental implications for understanding the biology of carcinogenesis as well as important clinical implications for early detection, prevention, and therapy of human malignancies.

TUMOR MICROENVIRONMENT

Apart from genetically abnormal cancer cells, a tumor is composed of several other cell types including fibroblasts, endothelial cells and immune cells such as macrophages and mast cells. It is clear that tumor progression is the product of an evolving crosstalk between different cell types within the tumor and its surrounding supporting tissue, or tumor stroma[39]. Cancer cells generate a supportive microenvironment by producing stroma-modulating growth factors and proteases acting in autocrine and paracrine manners. The activated stromal cell types in turn, secrete additional fluxes of growth factors and proteases, resulting in an activated stroma that promotes tumor progression and metastasis. The extensive crosstalk between the microenvironment and the cancer cells induces a cascade of stromal reactions such as angiogenesis[40], inflammation[41] and remodeling of the extracellular matrix[42]. There is increasing evidence that the tumor stroma can also have a more direct role in tumorigenesis, by acting as a mutagen. As a consequence of mutations in stromal cells, the adjacent epithelial cells may be at an increased risk of neoplastic transformation[43]. The concept of neoplastic transformation as a result of an abnormal microenvironment, can be thought of as a ‘landscaper’ defect[44].

THERAPEUTIC TARGETING

Elucidating the molecular mechanisms leading to cancer development offers a unique potential for therapeutic intervention. During the last years, dramatic advances have come from agents specifically targeting critical genetic lesions. The tyrosine kinase inhibitor imatinib (Gleevec, Novartis), for example, has tremendously improved the treatment of advanced sporadic gastrointestinal stromal tumors, showing a clinical benefit in up to 80% of patients with *KIT* or *PDGFRA* mutations[45,46]. Another relatively new and particularly promising approach is the clinical targeting of the tumor microenvironment. It is clear that anti-angiogenic[47] and anti-inflammatory drugs[48], together with components restoring the extracellular matrix[49], can act as chemopreventive agents. Nevertheless, there is still an overwhelming need to develop more effective and safer agents to minimize cancer morbidity and mortality in the long term.

NEUROFIBROMATOSIS TYPE 1

Neurofibromatosis type 1 (NF1; OMIM 162200, http://www.ncbi.nlm.nih.gov/Omim) is one of the most common autosomal dominant genetic disorders, affecting approximately 1 in 3500 individuals worldwide[50,51]. The condition is caused by defects in the *NF1* tumor suppressor gene. The primary clinical features are café-au-lait spots, skin-fold freckling and neurofibromas. Characteristic for NF1 is its extreme clinical variability.

CLINICAL ASPECTS OF NF1

The most common manifestations in NF1 are pigmentary abnormalities, such as café-au-lait spots, skin-fold freckling and Lisch nodules (iris hamartomas), together with the development of benign peripheral nerve sheath tumors or neurofibromas. However, other complications, such as skeletal dysplasias, learning disabilities, mental retardation, seizures and optic gliomas fall within the wide clinical spectrum of the disease[50]. Table 1 displays the diagnostic criteria of NF1 following the National Institutes of Health (NIH) guidelines[52]. Patients are diagnosed with NF1 if they demonstrate two or more of these criteria.

Table 1. Diagnostic criteria for NF1 according to NIH Consensus Conference in 1987.

● Six or more café-au-lait spots of over 5 mm in prepubertal individuals and over 15 mm in postpubertal individuals
● Two or more neurofibromas of any type or one plexiform neurofibroma
● Freckling in the axillary or inguinal regions
● Optic glioma
● Two or more Lisch nodules (iris hamartomas)
● A distinctive osseous lesion such as sphenoid dysplasia or thinning of long bone cortex with or without pseudoarthrosis
● A first-degree relative (parent, sibling or offspring) with NF1 by the above criteria

THE NF1 GENE AND PROTEIN

NF1 gene

The *NF1* gene was identified by positional cloning in 1990[53-55]. It is located on chromosome 17q11.2 and spans approximately 300 kb of genomic DNA[56]. The *NF1* promotor is embedded in a ~10 kb CpG-rich region and contains several conserved transcription factor binding motifs[57]. From the start to the stop codon the *NF1* gene measures 278861 bp[58]. The *NF1* gene is composed of 60 exons with at

least 4 alternatively spliced exons which are expressed in a developmental- and tissue- specific pattern[59-62]. The processed full-length *NF1* mRNA transcript can be detected in nearly all adult tissues and is about 11 to 13 kb in length, with a 3.5 kb 3'-untranslated region[63-65].

Numerous *NF1*-related sequences are distributed throughout the human genome. These *NF1* pseudogenes are suggested to have arisen by duplication and transposition of the *NF1* locus. They are non-processed and non-functional[66,67]. Three genes, *OMGP*, *EVI2A* and *EVI2B*, are embedded in *NF1* intron 27b and are transcribed in the opposite orientation[68,69]. There is no apparent correlation between these genes and the NF1 phenotype.

The mutation rate in the *NF1* gene is one of the highest among human genes and about half of the NF1 patients are sporadic, i.e. they do not have a familial history for NF1[70,71]. Although mutation analysis has been proven to be difficult and labour intensive due to the large size of the gene, the presence of several pseudogenes, the lack of mutation hotspots and the very diverse spectrum of *NF1* lesions, the *NF1* mutation can be identified in the vast majority of NF1 patients fulfilling the NIH criteria if a comprehensive approach for mutation detection is applied[72].

NF1 protein

The *NF1* gene encodes neurofibromin, a 2818 amino acids (AA) containing protein that harbors a functional GAP (GTPase activating protein) related domain (GRD, AA 1205-1536) in its central region[73-75]. Neurofibromin is ubiquitously expressed and most abundant in the nervous system[58]. The protein is highly conserved among vertebrates[76] and shows 60% identity with the *Drosophila* homolog[77].

Neurofibromin functions as a negative regulator of Ras-mediated signaling (Figure 3). Ras proteins are small molecular weight GTPases that link cell surface receptors to intracellular effector pathways that regulate proliferation, differentiation and apoptosis. Downstream effectors of Ras include the Raf/Mek/Erk, PI3Kinase/AKT and Rho GTPase pathways. Ras also activates a family of GDP-GTP exchange factors for the Ral small GTPases (e.g. Ral-Gds). Ras switches between active, GTP-bound, and inactive, GDP-bound, conformations. This GTPase cycle is catalyzed by interactions with guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs)[78]. Through its GRD, neurofibromin accelerates the conversion of active GTP-bound Ras to the inactive GDP-bound form[73-75]. Increased activity of Ras and its downstream effectors has been observed in NF1-associated tumors and *NF1*-deficient cells[79-88]. Thus, *NF1* acts as a tumor suppressor gene since loss of neurofibromin function results in dysregulated Ras activity and aberrant growth. Importantly, the consequences of activated Ras are pleiotropic and the mechanisms and signaling pathways by which Ras effects its biological consequences might exhibit significant species-specific and tissue-specific differences[89]. Mutations of many genes in the Ras pathway have been detected in a variety of

human cancers[90]. Moreover, reduced expression of a conserved microRNA (let-7) that targets the 3'-untranslated region of Ras mRNA has been associated with elevated Ras activity in lung tumors[14,91].

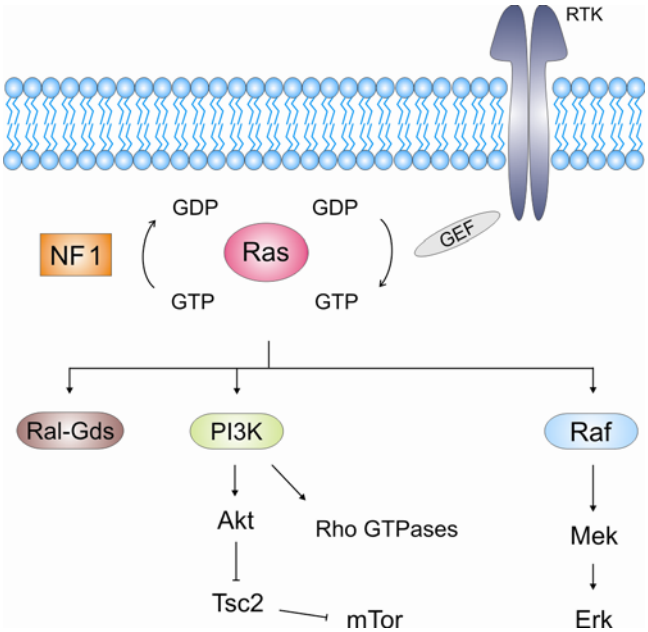

Figure 3. Neurofibromin (NF1) and Ras regulation.
Neurofibromin modulates intracellular signaling that originates from receptor tyrosine kinases (RTKs) by accelerating inactivation of active GTP-bound Ras. Loss of neurofibromin leads to increased activity of Ras and its downstream effectors resulting in dysregulated cell growth (adapted from[92]).

The Ras-GAP domain of neurofibromin comprises only 13% of the entire polypeptide, what has raised the question whether other regions of the molecule are important for modulating cell growth or other processes involved in NF1-related disease. Initial studies in *Drosophila* have shown that neurofibromin acts not only as a Ras-GAP, but also as a regulator of the cAMP pathway by regulating adenylyl cyclase (AC) activity (Figure 4)[77,93]. Activation of the Gα subunit of G-protein coupled receptors (GPCRs) upon ligand binding, stimulates the enzyme AC that synthesizes cyclic AMP (cAMP) from ATP. Cyclic AMP activates the cAMP-dependent protein kinase A (PKA), which in turn phosphorylates target proteins involved in a wide range of biological responses[94]. The NF1-regulated AC/cAMP pathway is important for controlling body size[77], neuropeptide responses[93] and learning[95] in flies, as well as neuropeptide-stimulated AC activity in both flies and mammals[96,97]. Only recently, it was shown that, in *Drosophila* brains, AC can be activated by at least three distinct pathways: an NF1/Ras-dependent, an NF1/Gα-dependent and a classical NF1-independent pathway[98]. Analysis of human *NF1* mutations and deletions, expressed in flies without NF1, showed that separate domains of neurofibromin control the different AC pathways. RasGAP activity is necessary for NF1/Ras-dependent but not NF1/Gα-dependent AC signaling, whereas part of the C-

terminal region (AA 1748-2818) is sufficient for NF1/Gα-dependent AC signaling and regulation of body size[98]. The functionality of human neurofibromin in the fly system, and the high degree of identity between human and fly homologs, suggests that distinct protein domains influencing different pathways for AC activation may also be operative in mammals.

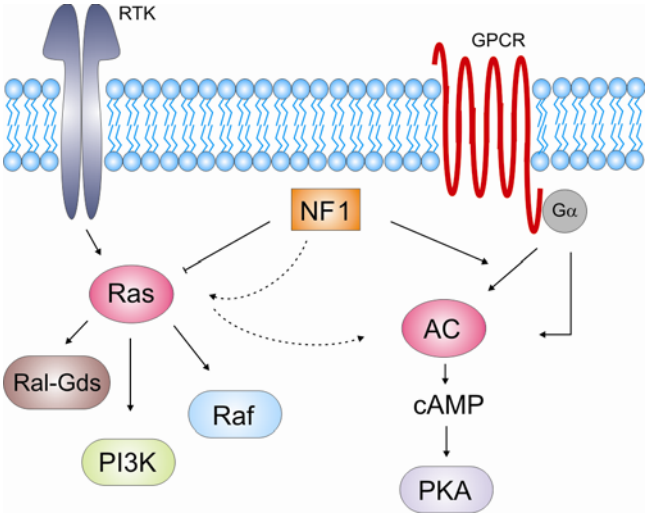

Figure 4: Neurofibromin (NF1) and adenylyl cyclase (AC) regulation.
In *Drosophila* brains, neurofibromin modulates intracellular signaling that originates from G-protein coupled receptors (GPCRs) by regulating AC activity. AC can be activated by at least three distinct pathways: an NF1/Ras-dependent, an NF1/Gα-dependent and a classical NF1-independent pathway (adapted from[98]).

Homology searches have revealed several other putatively functional domains in the NF1 protein. Neurofibromin shares high homology with the lipid-binding domain of *Saccharomyces cerevisiae* Sec14p[99,100], which mediates the exchange of phospholipids between membrane bilayers[101]. The inhibition of neurofibromin RasGAP activity by specific lipids[102,103] suggests that Ras GTPase function may be co-regulated by this putative lipid-binding domain (AA 1562-1714). The Sec14p domain overlaps with a protein sequence containing four caveolin-binding domains (AA 1601-2110)[104]. Caveolin proteins have been shown to bind to signaling molecules, such as Ras, within cholesterol-rich lipid raft microdomains[105,106]. Both putative lipid-binding domains may target neurofibromin to the plasma membrane, where the Ras protein is located.
Yeast two-hybrid screening revealed that two widely separated regions in neurofibromin (AA 1356-1562 and AA 2616-2812) can bind to syndecan[107]. The syndecan family of transmembrane proteins is believed to play roles in cell adhesion and intercellular signaling, often acting as binding sites for growth/differentiation factors and as co-receptors for conventional receptors[108,109]. The binding of neurofibromin to syndecan provides again a potential mechanism for localizing neurofibromin in specialized molecular microdomains with specific cell surface receptors and other cell signaling molecules.

A functionally active nuclear localization signal has been characterized in exon 43 of *NF1*[110]. Translocation to the nuclear compartment could be a mechanism to regulate the RasGAP function of neurofibromin by sequestration from the cytoplasm. Alternatively, neurofibromin may have a specific but yet unknown function in the nucleus.
Upstream of the GRD, neurofibromin contains a cysteine- and serine-rich domain (CSRD, AA 543-909), harboring three potential PKA recognition sites which are constitutively phosphorylated[64,111]. Also the C-terminal region of the protein (AA 2260-2818) is constitutively phosphorylated by PKA[112]. Therefore, phosphorylation-dependent NF1-associated proteins may have regulatory effects on the function of neurofibromin.
Neurofibromin has been shown to associate with the three major cytoskeletal systems: the microtubule (tubulin)[113-115], microfilament (actin)[116-118], and filamentous (keratin)[119,120] cytoskeleton. Closely related to the cytoskeleton is the association of neurofibromin with the motor protein kinesin-1[121]. Moreover, an interaction between the amyloid precursor protein (APP) and the GRD of neurofibromin was identified[122]. APP has been proposed to function as a vesicle cargo receptor for kinesin-1 in neurons[123] and melanocytes[124]. Together, these observations suggest a role for neurofibromin in cytoskeletal-mediated intracellular signal transduction pathways and vesicular cargo transport.

GENOTYPE-PHENOTYPE CORRELATIONS IN NF1

A hallmark of NF1 is the extreme clinical heterogeneity, even among related individuals carrying the same *NF1* mutation. Although the major diagnostic features are present in the large majority of affected individuals, the number and location of lesions varies enormously. In addition, NF1 patients are prone to other phenotypic features. As a result, the clinical phenotype varies from mild manifestations to severe morbidity. Epidemiologic studies suggest that the molecular basis underlying this phenotypic variability is determined to a large extent by the genotype at modifying loci and that these modifying genes are trait specific[125-128]. Unlike the highly penetrant mutations in TSGs, polymorphic modifier genes exert a more subtle effect by changing the efficiency of different steps in tumorigenesis. These steps could occur within the tumor cell, such as the efficiency of cell cycle checkpoints or DNA repair, or systematically in the organism, such as the efficiency of antitumor immune responses[129].

Five percent of NF1 patients have a constitutional microdeletion that encompasses *NF1* and its neighbouring genes. A total of seventeen genes are located in the typical ~1.5 Mb *NF1* microdeletion region[130]. In comparison with the general NF1 population, individuals with an *NF1* microdeletion frequently show a phenotype with more dermal neurofibromas at an earlier age, a lower average IQ[131] and facial dysmorphy[132-135]. In addition, an increased risk for the development of malignant peripheral nerve sheath tumors (MPNSTs) has been reported[136]. As yet, the biological basis for this association remains unclear.

NF1-ASSOCIATED TUMORS

The most common NF1-associated tumor is the benign peripheral nerve sheath tumor or neurofibroma. Neurofibromas have been subdivided into localized neurofibromas, which are discrete lesions associated with a single peripheral nerve, diffuse neurofibromas, which are more extended beyond the confines of the nerve, and plexiform neurofibromas, which involve multiple nerve fascicles. In a small percentage of NF1 patients, plexiform neurofibromas progress to malignant peripheral nerve sheath tumors (MPNSTs). NF1 patients are also predisposed to astrocytic brain tumors, spinal tumors, pheochromocytoma, myeloid leukemia and gastrointestinal stromal tumors[50,137].

Neurofibroma

Cutaneous neurofibromas grow as discrete lesions in the dermis or subcutis and are the most common type of neurofibroma encountered in NF1. They typically arise during the second decade of life, are often associated with the onset of puberty, and continue to increase in number and size throughout adulthood. In females, there is often a clear increase in number and size during pregnancy. While these tumors are benign, patients can develop thousands of them which, depending on location, can be painful and disfiguring. Plexiform neurofibromas are more diffuse and can develop internally, sometimes involving an entire limb or body region. They represent congenital tumors that seem to enlarge more rapidly during the first decade of life. They can be debilitating and can progress to malignancy[50]. Plexiform neurofibromas are virtually pathognomonic for NF1. Diffuse neurofibromas are an uncommon but distinctive form of neurofibroma occuring principally in the head and neck region of children and young adults. About 10% of patients with this lesion also have NF1.

Histopathology

Normal peripheral nerve

Peripheral nerves are complex well-organized structures in which single peripheral nerve fibers are wrapped by one Schwann cell that forms a protective sheath. The peripheral nerve sheath provides structural and metabolic support to nerves and, in the case of myelinated fibres, potentiates the conduction of action potentials. Several nerve fibers and associated Schwann cells are clustered into a nerve bundle (or fascicle) and each fascicle is surrounded by concentric layers of perineurial cells. Multiple nerve fascicles, bound by loose connective tissue, constitute an individual nerve (Figure 5a). Different cell types are present in a normal nerve fascicle: neural cells, Schwann cells, perineurial cells, fibroblasts, endothelial cells and occasionally mast cells. During development and regeneration following injury, the migration, proliferation, survival, growth arrest, and differentiation of each of these cell types is dependent on cues from surrounding cells[138].

Peripheral nerve sheath tumor

During neurofibroma formation, cell-cell interactions are disrupted, leading to the loss of the normal nerve structure. Schwann cells are found dissociated from nerves and the perineurium is disrupted. In addition, neurofibromas contain increased numbers of all cell types present in the normal peripheral nerve. The most abundant cell type in this complex are Schwann cells, which comprise 60-80% of the cell population (Figure 5b)[139]. The mixed cell composition in a myxoid background is important for diagnosis of neurofibromas and can be confirmed by immunohistochemistry, which shows a subset of S100 protein-positive cells (Schwann cells). The lesions are often transversed by nerve axons (positive neurofilament immunostaining) and may contain varying amounts of collagen fibers and mast cells[140].

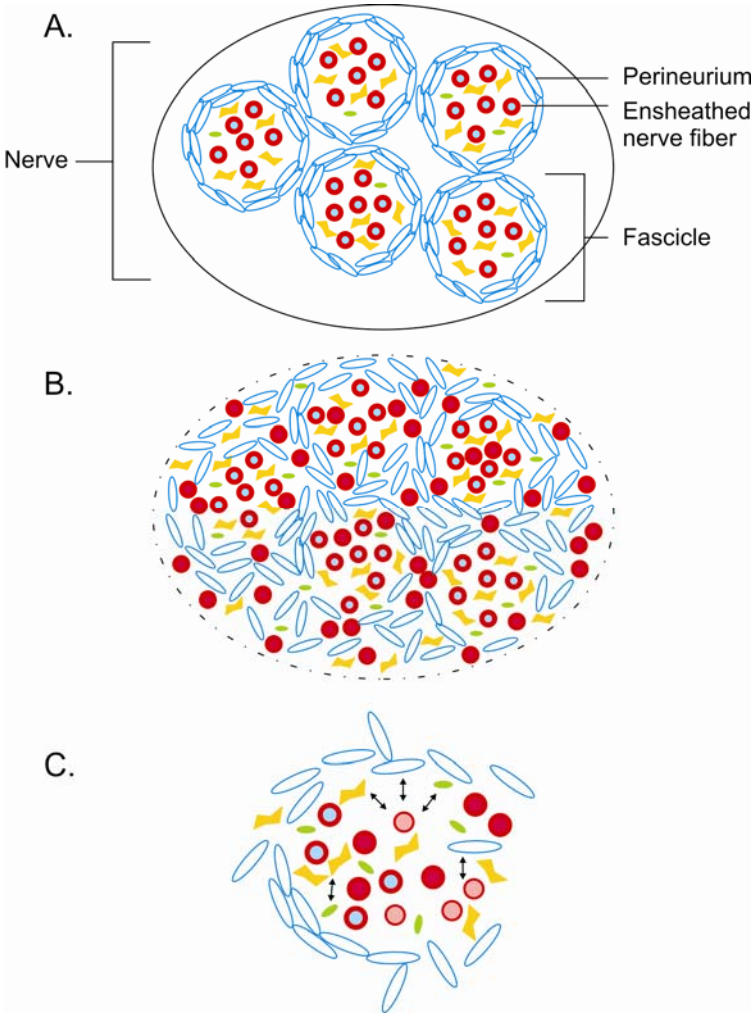

Figure 5: Normal peripheral nerve and model of neurofibroma development.

(A) Peripheral nerves are comprised of multiple cell types: neurons (pale blue) with surrounding Schwann cells (red), perineurial cells (blue) and fibroblasts (yellow). Occasional mast cells are also observed (green). (B) Neurofibromas exhibit an increased number of all cell types. In addition, Schwann cells are often dissociated from neurons and the perineurium is disrupted. (C)

Neurofibroma development is initiated by mutation or loss of the inherited wild-type *NF1* allele in Schwann cells (pink cells are $NF1^{-/-}$). This may trigger tumor development by initiating a cascade of changes in other cell types as a result of their interdependency and/or haploinsufficiency (adapted from[141]).

Model of neurofibroma development

Neoplastic Schwann cell

Molecular analyses of neurofibromas have shown that only a subpopulation of the Schwann cells ($SC^{NF1-/-}$) in these tumors exhibit biallelic inactivation of the *NF1* gene[142-144]. First, genotyping the *NF1* locus in an NF1-associated neurofibroma and the derived Schwann cells and fibroblasts, revealed loss of heterozygosity (LOH) in both the original tumor and the Schwann cells, but not in the tumor-derived fibroblasts[142]. Second, it was demonstrated that fibroblasts isolated from neurofibromas carried at least one normal *NF1* allele and expressed both *NF1* mRNA and protein, whereas the S100 positive cells typically lacked the *NF1* transcript[143]. Third, using a cell culture system that allows isolation and selective expansion of human Schwann cells from neurofibromas[145], pure populations of Schwann cells and fibroblasts were grown from 10 neurofibromas with characterized constitutional and somatic *NF1* mutations. It was found that Schwann cells, but not fibroblasts, harbored the somatic *NF1* mutation in all studied tumors. Moreover, by culturing neurofibroma-derived Schwann cells under different *in vitro* conditions, the authors were able to obtain two genetically distinct ($NF1^{+/-}$ and $NF1^{-/-}$) Schwann cell subpopulations[144]. The other cellular elements are genetically heterozygous for an inactivating *NF1* mutation ($NF1^{+/-}$). Accordingly, based on Knudson's two-hit hypothesis[4], Schwann cells can be considered as the initiating cell type in neurofibroma formation (Figure 5c). Neurofibromin-deficient Schwann cells demonstrate increased Ras activity and have a substantial growth advantage compared with the other neurofibroma-derived cellular components[80,146]. Moreover, they have angiogenic and invasive properties[147]. It is unclear whether interdependence between both hits, as described for FAP and sporadic polyps, exists.

NF1 mouse models confirm that *NF1* loss in Schwann cells is the genetic bottleneck for neurofibroma formation. Because $Nf1^{+/-}$ mice don't develop neurofibromas[148] and $Nf1^{-/-}$ mice die during embryogenesis[148,149], the question of second-hit mutation in murine neurofibroma development was addressed experimentally through the creation of chimeric mice partially composed of $Nf1^{-/-}$ cells[150,151]. Nearly all of these mice develop numerous neurofibromas that histologically resemble human plexiform neurofibromas. To identify the initiating cell type, Zhu and coworkers generated a conditional NF1 mouse model in which a floxed *Nf1* allele is deleted by a Cre transgene under control of the Schwann cell-specific promoter, Krox-20. All progeny with the $Nf1^{flox/-}$;Krox20-Cre genotype develop plexiform neurofibromas, confirming that *Nf1* loss in the Schwann cell lineage is sufficient to generate tumors[152].

Tumor microenvironment

To address whether the heterozygous state of *NF1* in somatic cells other than the tumor-initiating Schwann cell plays an active role in neurofibroma formation, Zhu and coworkers constructed $Nf1^{flox/flox}$;Krox20-Cre mice that have intact *Nf1* function in all cells ($Nf1^{+/+}$), except in the Schwann cells ($SC^{Nf1-/-}$). In contrast to the wide-spread plexiform neurofibromas of the $Nf1^{flox/-}$;Krox20-Cre mice, the $Nf1^{flox/flox}$;Krox20-Cre mice only developed small, infrequent hyperplastic lesions[152]. This finding has led to the emerging view that *NF1* heterozygosity in the tumor environment actively contributes to neurofibroma formation. Implicit is the requisite that heterozygous inactivation of *NF1* has functional consequences (haploinsufficiency).

Mast cells accumulate in the stroma of certain tumors and selectively secrete molecules involved in growth promotion, neovascularization and remodeling of the ECM[153]. Mast cells show marked infiltration in neurofibromas and have been considered as a major player in neurofibroma formation[154,155]. It has been demonstrated that $Nf1^{+/-}$ mast cells have increased survival and proliferation, and are hypermigratory compared to wild-type cells in response to Kit Ligand (KitL) secreted by $Nf1^{-/-}$ Schwann cells. Reintroduction of the GRD into $Nf1^{+/-}$ mast cell reduces their migration to wild-type levels in response to KitL, providing direct evidence that an $Nf1^{+/-}$ motile phenotype is secondary to increased Ras activity[84,156,157]. Together, these findings illustrate that $Nf1^{-/-}$ neoplastic cells alter the tumor microenvironment by secreting growth factors and that the haploinsufficient state of non-neoplastic cells might be permissive for the progression of tumorigenesis.

It is likely that other cells within the tumor microenvironment contribute to neurofibroma progression by similar paracrine interactions. Previous studies, for example, have shown that $Nf1^{-/-}$ Schwann cells secrete increased concentrations of various angiogenic growth factors, such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and platelet-derived growth factor (PDGF)[82,158-160]. Further, $Nf1^{+/-}$ mast cells secrete increased concentrations of VEGF and bFGF[161]. There is recent genetic and biochemical evidence that, in response to bFGF and VEGF, $Nf1^{+/-}$ endothelial cells have increased migration and proliferation properties[162-164] and that $Nf1^{+/-}$ fibroblasts are hypersensitive to low concentrations of PDGF[165,166], both via hyperactivation of the Ras-Erk pathway. In addition, $Nf1^{+/-}$ Schwann cells are able to induce angiogenesis and are more invasive than their wild-type counterparts[167] though the biochemical mechanisms for these observations are not determined.

Taken together, a growing body of experimental evidence supports the idea that *Nf1* haploinsufficiency in the tumor environment promotes neurofibroma formation in mice. The *Nf1* gene may hence play the role of 'gatekeeper' and 'landscaper' depending on the tissue type. The tumors that have been observed in the murine model to date have been plexiform neurofibromas derived from dorsal root ganglia and cranial nerves[152]. Whether this is a consequence of the expression pattern of the Cre transgene used in those experiments or alternatively reflects a slightly

different pathogenesis from the dermal neurofibromas remains an important question. Moreover, the recurrent discrepancies in the tumor phenotypes between humans and mice, especially in the context of Ras-related neoplasms[168], emphasize the value of dissecting tumorigenesis in human cells.

MPNST

Approximately 10% of individuals with NF1 will develop an MPNST, a clinically aggressive, often fatal cancer, which is poorly responsive to chemotherapy or radiation. Some MPNSTs arise in pre-existing plexiform neurofibromas implying a model of multi-step tumor progression, as has been demonstrated for the adenoma-carcinoma sequence. Besides *NF1* inactivation, additional genetic changes have been identified in MPNSTs but not in benign neurofibromas. Alterations required for malignant progression include homozygous deletion of *CDKN2A* which encodes $p16^{INK4A}$ and $p14^{ARF}$ [169,170], *TP53* loss[171,172], and inactivation of *CDKN1B* encoding $p27^{KIP}$ [173]. Aberrant expression of the epidermal growth factor receptor (EGFR) has also been implicated in the development of MPNST in humans and in mouse models[174-176].

Mice carrying *Nf1* and *Trp53* mutations on the same chromosome (cis*Nf1/Trp53* mice) develop tissue sarcomas that resemble MPNSTs[150,151]. Tumor susceptibility in this mouse model appears to be highly dependent on the genetic background[177]. Recent evidence suggests that genetic interaction between an imprinted locus, linked to *Nf1* and *Trp53,* and other polymorphic susceptibility loci strongly modifies the incidence of MPNSTs in mice[129]. The relevance of these results to NF1 patients will need to be tested by human association studies.

Other NF1-associated tumors

While defects in glial cells in the peripheral nervous system (Schwann cells) underlie neurofibroma development, NF1 patients are also predisposed to developing gliomas or astrocytomas, tumors derived from glial cells in the central nervous system (astrocytes). Between 15% and 50% of NF1 patients develop some type of glioma, although they are often indolent in nature[58]. Similar to neurofibroma formation, *Nf1* loss in astrocytes alone is insufficient for murine glioma formation[178]. Factors produced by the $Nf1^{+/-}$ brain environment are possibly required for the formation of low-grade tumors[179]. High-grade glioma has been observed in cis*Nf1/Trp53* mice[177]. However, high-grade gliomas in individuals with NF1 do not develop from pre-existing low-grade tumors.

Juvenile myelomonocytic leukaemia (JMML) is a relentless myeloid malignancy of young children characterized by overproduction of myeloid lineage cells that infiltrate hematopoietic and non-hematopoietic tissues[180,181]. The incidence of JMML is increased more than 200-fold in children with NF1[182]. Studies on human NF1 leukemia-derived tumor cells have shown that neurofibromin loss in bone marrow cells[183,184], which is associated with hyperactive Ras[87], results in hypersensitivity to

granulocyte-macrophage colony stimulating factor (GM-SCF), which promotes the survival, proliferation and differentiation of myeloid lineage cells[185].

Spinal nerve sheath tumors are described as symptomatic findings in ~2% of NF1 patients, but can be visualized by magnetic resonance imaging (MRI) in ~40% of NF1 patients[186]. The wide, symmetrical distribution of spinal neurofibromas occurring in all adult affected members of the same family and segregating in an autosomal dominant fashion, is however extremely rare. Besides café-au-lait spots, other typical cutaneous manifestations of NF1 are usually absent or very mild in these patients. Familial spinal neurofibromatosis (FSNF, OMIM 162210) has therefore been considered as an 'alternate' form of NF1. It is noteworthy that the *NF1* mutations found in the FSNF families are mild mutations, i.e. truncating mutations at the very 3' end of the gene, splicing and missense mutations[187,188]. It has been suggested that modifier genes might cooperate with the *NF1* mutation to result in FSNF[187].

Pheochromocytomas usually arise within the adrenal medulla and are catecholamine-secreting tumors that can present with hypertension. Inherited cancer syndromes with pheochromocytoma as a component feature include von Hippel-Lindau syndrome (OMIM 193300), multiple endocrine neoplasia type 2 (OMIM 171400) and, to a much lesser extent, NF1[189]. Somatic *NF1* inactivation is the underlying mechanism of NF1-associated pheochromocytoma development[190].

Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal tumors of the gastrointestinal tract[191]. *KIT* and *PDGFRA* activating mutations are the oncogenic mechanisms in most sporadic GISTs[192,193]. In addition to sporadic occurrences, GISTs are increasingly being recognized in association with NF1[137], yet the underlying pathogenic mechanism remains elusive.

THERAPEUTIC TARGETING

Therapeutic interventions designed to inhibit Ras function have been proposed as treatments for NF1[194]. One approach is the administration of molecules that inhibit the enzyme farnesyl transferase. Post-translational farnesylation of Ras proteins is absolutely necessary for Ras function since this modification is required for anchoring of Ras to the plasma cell membrane where it exerts its function[195]. Preclinical studies have shown that farnesyl transferase inhibitors (FTIs) decrease hyperproliferation of neurofibromin-deficient Schwann cells[167,196]. FTIs have found their way into clinical trials for NF1-associated plexiform neurofibromas[197,198]. Statins, commonly used agents to prevent cardiovascular disease, function in the mevalonate pathway as small-molecule inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase. Besides reducing cholesterol levels, statins also inhibit the formation of lipid isoprenoids such as farnesyl and geranylgeranyl pyrophosphate with beneficial effects on cancer prevention[199]. Recently, it was reported that lovastatin decreased the enhanced brain Ras-MAPK activity in $Nf1^{+/-}$ mice and reversed their

learning and attention deficits[200]. These observations demonstrate that statins may prove useful in the treatment of various NF1-related symptoms. In addition, it might be important to consider targeting more specific downstream effectors of Ras. Aberrant activation of mTOR in *Nf1*-deficient cells, for example, depends on Ras and PI3K, which inactivate the *TSC2* gene product tuberin via AKT (Figure 3)[83]. Importantly, tumor cell lines derived from NF1 patients are highly sensitive to the mTOR inhibitor rapamycin[83], suggesting the potential utility of rapamycin and its derivatives in treating NF1 tumors. Another biological-based therapeutic approach may include pharmacological inhibition of KIT activity by Gleevec in order to reduce mast cell infiltration into neurofibromas[156].

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RESEARCH OBJECTIVES

One major effort in NF1 research has been to dissect the genetic events underlying different aspects of disease development. While determining when and in what cell type the crucial molecular defect actually occurs, is critical for understanding the basic pathology of any phenotype, this exercise has proven to be surprisingly difficult for many NF1-related symptoms. Impeding factors include the large size of the *NF1* gene, the presence of several *NF1* pseudogenes, the complex interactions between cell types within affected tissues, and the *NF1* heterozygous state of all cells in the body. In order to gain insight in the developmental concepts underlying various NF1-related symptoms, this thesis aimed at exploiting improved somatic mutation detection strategies on a diverse spectrum of NF1-associated cellular entities.

(1) Characterization of the somatic mutation spectrum in neurofibromas

Since characterization of somatic mutational spectra may provide important clues about different aspects of tumorigenesis, the first research objective of this thesis was to investigate thoroughly the status of the somatically affected *NF1* allele in neurofibromas. To this purpose, we developed an improved somatic mutation detection strategy on selectively cultured Schwann cell populations and screened a large panel of neurofibromas. In a next step, we compared the germline versus the somatic mutation spectrum, studied the interdependence between first and second hits and searched evidence for the existence of modifier genes as triggers for particular mechanisms of somatic inactivation preceding tumorigenesis. Our initial analyses were suggestive for a different mechanism of somatic inactivation between *NF1* microdeletion patients and the general NF1 population. In order to study the putative association between germline mutation and type of second hit in further detail, we compared a larger number of neurofibromas from both NF1 patient groups. The results of these studies are reported in Papers 1 and 2.

(2) Molecular dissection of isolated disease features in mosaic NF1 patients

As opposed to classic NF1 where all cells of the body bear at least one mutated *NF1* allele, NF1 segmental phenotypes provide the opportunity to study cell populations differing only with regard to the mutation(s) giving rise to the localized disease manifestations. As a second research objective, this thesis aimed at molecularly dissecting different clinical subtypes emerging within mosaic NF1 in order to gain insight into developmental concepts underlying particular NF1-related disease features. To this purpose, we developed a real-time quantitative PCR assay capable of detecting low-percentage mosaic point mutations against backgrounds of wild-type and pseudogene alleles and investigated mosaic NF1 patients with different clinical manifestations (neurofibromas only, pigmentary changes only, and association of both symptoms) at the molecular level. The results of these studies are reported in Papers 3 and 4.

(3) Identification of the molecular pathogenesis underlying the development of NF1-related gastrointestinal stromal tumors

While *KIT* and *PDGFRA* activating mutations are the oncogenic mechanisms in most sporadic gastrointestinal stromal tumors (GISTs), the underlying pathogenic mechanism for NF1-related GIST development remains elusive. A third goal of this thesis was to gain insight into the mechanisms underlying GIST formation in NF1 patients. For this purpose, we studied several NF1-associated GISTs with a combination of different techniques: mutation analysis (*KIT*, *PDGFRA* and *NF1*), Western blotting, array CGH and *ex vivo* imatinib response experiments. The results of these studies are reported in Paper 5.

CHAPTER 2

Results

CHAPTER 2. RESULTS

HUMAN MUTATION 27(10), 1030–1040, 2006

RESEARCH ARTICLE

Comprehensive *NF1* Screening on Cultured Schwann Cells from Neurofibromas

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Neurofibromatosis type 1 (NF1) is mainly characterized by the occurrence of benign peripheral nerve sheath tumors or neurofibromas. Thorough investigation of the somatic mutation spectrum has thus far been hampered by the large size of the *NF1* gene and the considerable proportion of *NF1* heterozygous cells within the tumors. We developed an improved somatic mutation detection strategy on cultured Schwann cells derived from neurofibromas and investigated 38 tumors from nine NF1 patients. Twenty-nine somatic *NF1* lesions were detected which represents the highest *NF1* somatic mutation detection rate described so far (76%). Furthermore, our data strongly suggest that the acquired second hit underlies reduced *NF1* expression in Schwann cell cultures. Together, these data clearly illustrate that two inactivating *NF1* mutations, in a subpopulation of the Schwann cells, are required for neurofibroma formation in NF1 tumorigenesis. The observed somatic mutation spectrum shows that intragenic *NF1* mutations (26/29) are most prevalent, particularly frameshift mutations (12/29, 41%). We hypothesize that this mutation signature might reflect slightly reduced DNA repair efficiency as a trigger for *NF1* somatic inactivation preceding tumorigenesis. Joint analysis of the current and previously published *NF1* mutation data revealed a significant difference in the somatic mutation spectrum in patients with a *NF1* microdeletion vs. non-microdeletion patients with respect to the prevalence of loss of heterozygosity events (0/15 vs. 41/81). Differences in somatic inactivation mechanism might therefore exist between *NF1* microdeletion patients and the general NF1 population. Hum Mutat 27(10), 1030–1040, 2006.

KEY WORDS: neurofibromatosis type 1; *NF1*; somatic mutation analysis

INTRODUCTION

Neurofibromatosis type 1 (NF1; MIM# 162200) is a genetic disorder affecting one in 3,500 individuals worldwide. The primary clinical features are café-au-lait spots, skin-fold freckling, and benign peripheral nerve sheath tumors or neurofibromas [Riccardi, 1992]. Characteristic for NF1 is its extreme clinical variability. Epidemiologic studies suggest that the molecular basis underlying this phenotypic variability is determined to a large extent by the genotype at modifying loci and that these modifying genes are trait specific [Easton et al., 1993; Szudek et al., 2000, 2002, 2003].

The *NF1* gene spans 350 kb of genomic DNA, contains 60 exons, and is transcribed in an 11–13 kb transcript coding for the protein neurofibromin with an open reading frame of 2,818 amino acids [Cawthon et al., 1990; Viskochil et al., 1990; Wallace et al., 1990; Marchuk et al., 1991; Li et al., 1995]. The only known functional domain of neurofibromin is the GAP related domain (GRD) which is involved in Ras downregulation [Ballester et al., 1990; Martin et al., 1990; Xu et al., 1990; Bollag et al., 1996]. In accordance with Knudson's two hit hypothesis [Knudson, 1971], both copies of the *NF1* gene have been found to be inactivated in NF1 related tumors [Xu et al., 1992; Legius et al., 1993; Shannon et al., 1994; Colman et al., 1995]. *NF1* promoter methylation appears to be an uncommon event in NF1 tumorigenesis [Horan et al., 2000; Luijten et al., 2000; Harder et al., 2004; Upadhyaya et al., 2004; Fishbein et al., 2005].

Neurofibromas are comprised of multiple cell types, including Schwann cells, mast cells, perineurial cells, fibroblasts, and endothelial cells [Riccardi, 1992]. Schwann cells are the most

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prevalent cell type in this complex and have been proposed to be the tumorigenic cell population [Kluwe et al., 1999; Rutkowski et al., 2000; Serra et al., 2000]. Notably, only a subpopulation of the Schwann cells harbors the somatic *NF1* alteration [Serra et al., 2000].

Thorough investigation of the somatic mutation spectrum has thus far been hampered by the large size of the *NF1* gene and the considerable proportion of *NF1* heterozygous cells within the tumors. Here we show that these problems can be resolved by performing comprehensive analysis of the *NF1* gene on selectively cultured Schwann cell populations of neurofibromas.

Comparison of the germline vs. the somatic mutation spectrum and the study of the interdependence between first and second hits might reveal genotypes with a higher risk for tumorigenesis. Furthermore, comparison of somatic mutation spectra in different types of tumors and patients might reflect distinct mutational mechanisms and might provide evidence for the existence of modifier genes as triggers for particular mechanisms of somatic inactivation preceding tumorigenesis.

MATERIAL AND METHODS

Tumor Specimens

Thirty-eight dermal neurofibromas were obtained from nine unrelated NF1 patients who signed informed consent. All patients fulfilled the NF1 clinical criteria established by the NIH [Stumpf et al., 1988]. Since patients with a high tumor burden are more likely to undergo neurofibroma excision for reasons of discomfort, the individuals included in this study may have been biased towards those NF1 patients with a more severe tumor phenotype. Indeed, compared to the age-matched NF1 population [Huson et al., 1988], most individuals suffered from a severe to excessive neurofibroma burden.

Schwann Cell Culture

Culture conditions for neurofibroma derived Schwann cells were as described by Rosenbaum et al. [2000] and Serra et al. [2000]. The presence of forskolin in the Schwann cell medium promotes proliferation of cells bearing only the germline mutation (SC F+). Replacement of proliferation medium by serum-free N_2 medium [Bottenstein and Sato, 1979] followed by proliferation medium without forskolin, promotes proliferation of cells containing both hits (SC F-). To estimate the purity of Schwann cell cultures, immunofluorescence was performed. Briefly, fixated cells were incubated for 1 hr at room temperature with rabbit S100 primary antibody (1/1000) (Dako; www.dako.com). Alexa Fluor 488 goat anti-rabbit antibody (1/1000) (Molecular Probes; http://probes.invitrogen.com) was added for 45 min at room temperature. Cells were mounted with Vectashield (Vector; www.vectorlabs.com) plus 4′,6-diamidino-2-phenylindole (DAPI, Sigma Aldrich; www.sigmaaldrich.com) and analyzed on a Zeiss (www.zeiss.com) fluorescence microscope. Cells were passaged when cultures were confluent and harvested not earlier than at passage four.

NF1 Mutation Analysis

NF1 mutation analysis (GenBank reference sequence NM_000267.1, www.ncbi.nlm.nih.gov/GenBank) was performed essentially as described by Messiaen et al. [2000]. Briefly, genomic DNA (gDNA) was extracted from Schwann cell cultures with the QiaAmp procedure (Qiagen; www.qiagen.com) following the manufacturer's instructions. Schwann cell cultures were treated with puromycin (200μg/ml, 4–6 hr) before RNA extraction (RNeasy kit, Qiagen). The entire *NF1* complementary DNA (cDNA) was sequenced using the ABI3100 genetic analyzer (Applied Biosystems; www.appliedbiosystems.com). All mutations found at the cDNA level were confirmed on gDNA by cycle sequencing. Detection of loss of heterozygosity (LOH) in the *NF1* gene was performed by genotyping two microsatellite markers distal from (3′NF1-3, 3′NF1-1) [Lopez Correa et al., 1999] and four within the *NF1* gene (Alu, IVS27AC33.1, IVS38GT53.0, IVS27TG24.8) [Xu et al., 1991; Lazaro et al., 1993a,b, 1994]. Genomic DNA from paired Schwann cell and lymphocyte cultures was investigated by three sets of multiplex PCR for the microsatellite markers and subsequently analyzed on the ABI3100 genetic analyzer (Applied Biosystems) with the Genescan software version 3.7 (Applied Biosystems). In case no somatic hit was detected with the previous techniques, multiplex ligation-dependent probe amplification (MLPA) analysis using the P081/P082 kit (MRC Holland; www.mrc-holland.com) was performed following the manufacturer's instructions.

Semiquantitative Mutation Screening

In order to determine putative traces of $SC^{NF1-/-}$ in SC F+ cultures and $NF1^{+/-}$ cells in SC F− cultures, a semiquantitative assay was performed on the tumors with detected somatic hits (SC F−) from patients NF96-1 (tumors A, B, C, and E), NF253-UHG (tumors D and E), and L-004 (tumors B and D). SC F+, SC F− and control gDNA samples were subjected to PCR amplification using primers surrounding the respective somatic hit, except for tumors E and B of patients NF253-UHG and L-004, respectively. PCR products were sequenced following the BigDye Terminator Sequencing protocol (Applied Biosystems). A liz-tagged size standard was added and the samples were analyzed on a ABI3130XL platform (Applied Biosystems) using a standard fragment analysis protocol. Using the Genemapper analysis software version 3.7 (Applied Biosystems), peak height ratios of the somatic hit were calculated relative to an internal control peak of the same nucleotide type at least five nucleotides apart and these ratios were compared to the control gDNA sample (wild-type sequence). Each gDNA sample was PCR amplified and sequenced in triplicate. SC F+, SC F−, and control cDNA samples of tumor B from patient L-004 were amplified in a multiplex PCR using primers in *NF1* exon 17 (internal control) and primers surrounding *NF1* exon 23.1. Relative peak heights of amplified fragments (with/without exon 23.1 and exon 17) were analyzed by the Genemapper analysis software version 3.7 (Applied Biosystems). All analyses were performed in triplicate. For tumor E of patient NF253-UHG, LOH analysis was performed using six microsatellite markers as described under the *NF1* mutation analysis paragraph; two markers were informative.

In order to evaluate allele-specific *NF1* expression in tumors D and E from patient NF253-UHG, SC F+ and SC F− cDNA samples were amplified in a multiplex PCR using primers located in *NF1* exon 17 (exclusive amplification of the allele not bearing the germline *NF1* mutation, 100 bp) and *TBP* (a stable reference gene in Schwann cells as determined by geNorm (http://medgen.ugent.be/genorm/) [Vandesompele et al., 2002b], 90 bp). Relative peak heights of amplified fragments (*TBP* vs. *NF1*) were analyzed by the Genemapper analysis software version 3.7 (Applied Biosystems). All analyses were performed in triplicate.

Real-Time Quantitative PCR

In order to evaluate the correlation between *NF1* messenger RNA(mRNA) expression, somatic mutation detection, and cell

admixture, a real-time quantitative PCR assay was performed on the tumors from patients NF96-1, NF253-UHG, and L-004. Relative *NF1* expression levels were determined using an optimized two-step SYBR Green I RT-PCR assay [Vandesompele et al., 2002a] in Schwann cell cultures (SC F+ and parallel SC F−). PCR reagents were obtained from Eurogentec (www.eurogentec.com) as SYBR Green I mastermixes and used according to the manufacturer's instructions. Primers in *NF1* exons 45 and 46 were previously described [Vandenbroucke et al., 2002]. Reactions were run on an ABI5700 instrument (Applied Biosystems). *NF1* mRNA expression levels were normalized using the geometric mean of the three most stably expressed reference genes in Schwann cells (i.e., *TBP*, *HPRT1*, and *HMBS*) as determined by the geNorm software [Vandesompele et al., 2002b]. Automated analysis of real-time quantitative PCR data was performed using our in-house developed qBase software which employs a delta-Ct relative quantification model with PCR efficiency correction and multiple reference gene normalization (http://medgen.ugent.be/qbase/). Each sample was tested in duplicate and all PCR runs were performed twice, starting from newly synthesized cDNA.

Mismatch Repair Gene Analysis

As a first attempt to determine the putative contribution of DNA repair variation in NF1 tumor predisposition, we screened the mismatch repair (MMR) genes *MLH1* (GenBank NM_000249.2, www.ncbi.nlm.nih.gov/GenBank), *MSH2* (GenBank NM_000251.1, www.ncbi.nlm.nih.gov/GenBank), and *MSH6* (GenBank NM_000179.1, www.ncbi.nlm.nih.gov/GenBank) in peripheral blood lymphocytes of all nine NF1 patients (eight Belgian and one Dutch). The entire coding regions and the splice junctions were amplified by PCR using 23 primer pairs for each gene. All amplicons were subjected to denaturing gradient gel electrophoresis (DGGE) except for exons 12 (*MLH1*), exons 1 and 5 (*MSH2*), and exons 1 and 10 (*MSH6*), which were analyzed by direct cycle sequencing. Amplification and DGGE analysis was performed as described [Wu et al., 1998]. The observed MMR gene variants p.I219V (*MLH1*), p.G322D (*MSH2*), and p.G39E (*MSH6*) were screened in a control population (non-NF1 individuals, geographically matched to the NF1 patients under study) to determine the respective allele frequencies. The p.G322D (*MSH2*) variant was tested in 15 additional geographically matched NF1 patients selected for an excessive neurofibroma burden compared to the age-matched NF1 population [Huson et al., 1988].

Microsatellite Instability Analysis

In order to evaluate microsatellite instability in the neurofibromas from patients L-002 and NF482-UHG, SC F− cultures and corresponding blood samples were subjected to PCR using microsatellite markers for BAT25, BAT26, D2S123, D5S346, and D17S250 from the reference panel for evaluation of microsatellite instability in colorectal tumors [Boland et al., 1998], together with D18S55, D18S58, and D18S61. PCR products were analyzed on the ABI3100 genetic analyzer (Applied Biosystems) with the Genescan software version 3.7 (Applied Biosystems).

Statistical Analysis

A comprehensive overview of the current findings and previously reported data is shown in Table 4. Only those studies applying a systematic somatic mutation detection approach (combination of LOH analysis and search for *NF1* intragenic mutations) were taken into account. These comprise the studies of Sawada et al. [1996], Eisenbarth et al. [2000], Serra et al. [2001], Wiest et al. [2003], and Upadhyaya et al. [2004]. Moreover, only those neurofibromas for which both germline and somatic mutations were characterized were included in the survey. In total, 96 neurofibromas derived from 34 NF1 patients were withheld: 29 neurofibromas from this report and 67 from the literature. For both groups of mutations (germline and somatic), distinct categories were made according to the type of mutation (splice, missense, nonsense, *NF1* microdeletion, deletion, insertion, LOH) and their respective region of occurrence within the *NF1* gene (GenBank reference sequence NM_000267.1: 5′ (proximal to c.3613), GRD (c.3613_c.4608), 3′ (distal to c.4608)). Fisher's Exact and Binomial tests were performed using SPSS 12.0 (www.spss.com) for Windows.

RESULTS

Schwann Cell Culture

By using selective culture media with and without forskolin, we cultured from each tumor Schwann cells expected to carry only the first hit ($SC^{NF1+/-}$) in parallel with cells expected to carry both hits ($SC^{NF1-/-}$). In general, SC F+ cultures grew at a slower pace than SC F− cultures and fibroblast contamination, determined by immunofluorescence staining and morphological evaluation of cell cultures, appeared to be the most persistent problem. The purity of 22 out of 38 SC F- cultures, estimated by S100 immunofluorescence staining, is presented in Table 1.

NF1 Mutation Analysis and Semiquantitative Somatic Mutation Screening

Germline mutations were identified in all patients and confirmed on blood lymphocytes. *NF1* somatic mutation analysis was performed on Schwann cell cultures of 38 dermal neurofibromas derived from nine NF1 patients. Somatic mutations were found in 29 tumors. Of these, 26 represented subtle mutations of different types whereas three samples showed LOH of the *NF1* region. In summary, 12 small deletions ranging from 1 to 19 bp, one single exon deletion, seven nonsense mutations, six splicing mutations (four leading to out-of-frame exonic dropouts), and three LOH events were detected (Table 1). Semiquantitative estimation of the percentage of somatic hit in SC F+ and SC F− cultures derived from tumors of patients NF96-1, NF253-UHG, and L-004 are presented in Table 2.

NF1 mRNA Expression in Schwann Cell Cultures

Data from two independent real-time quantitative PCR experiments were combined (average of normalized logarithmic *NF1* expression levels) and are presented in Table 2 and Figure 1. The average *NF1* expression ratio (SC F+/SC F−) of the eight neurofibromas without detected second hit (tumor D from patient NF-96-1; tumors A, B, C, and F from patient NF253-UHG; and tumors A, C, and E from patient L-004) equals 1.08 (95% confidence interval [CI]: 0.79–1.48). The average *NF1* expression ratio (SC F+/SC F-) of six of the eight neurofibromas with detected second hit (tumors A, B, and C from patient NF-96-1; tumors D and E from patient NF253-UHG; and tumor B from patient L-004) falls outside this 95% CI. Notably, only in the SC F− cultures derived from the six latter tumors (average fold reduction in *NF1* expression being 3.33 [95% CI: 2.20–5.05]), was a substantially high percentage of somatic hit (31–100%, Table 2) detected compared to the parallel SC F+ cultures. Moreover, semiquantitative evaluation of allele-specific *NF1* expression in tumors D and E from patient NF253-UHG revealed that

TABLE 1. Germline and Somatic *NF1* Mutations in 38 Dermal Neurofibromas Derived from 9 NF1 Patients Together With Estimated Purity of SC F- Cultures*

		NF1		
Patient	Tumor	Germline	Somatic	SC F–
L-001	Tumor A	g.AC127024.4_AC129917.6del (= *NF1* microdeletion)	c.5026_5032del	95%
	Tumor B		c.3189T>A (p.C1063X)	95%
	Tumor C		c.4086_4092del	95%
	Tumor D		c.396_403del	95%
	Tumor E		c.3774G>A (p.W1258X)	95%
L-002	Tumor A	c.1246C>T (p.R416X)	c.655-1G>T	95%
	Tumor B		c.3757_3764del	95%
	Tumor C		LOH	95%
	Tumor D		c.1105C>T (p.Q369X)	95%
	Tumor E		c.1153del	95%
	Tumor F		c.246_247del	95%
NF96-1	Tumor A	g.AC127024.4_AC129917.6del(= *NF1* microdeletion)	c.[7395–1G>A; 7395G>A]	NA
	Tumor B		c.3330del	NA
	Tumor C		c.7438del	NA
	Tumor D		ND	NA
	Tumor E		c.2050C>T (p.P684X)	NA
L-004	Tumor A	c.6791dup	ND	NA
	Tumor B		c.3871-?_3974+?del	NA
	Tumor C		ND	NA
	Tumor D		c.4729del	NA
	Tumor E		ND	NA
L-005	Tumor A	c.3113+1G>A	LOH	NA
NF253-UHG	Tumor A	c.2851–2A>G	ND	75%
	Tumor B		ND	70%
	Tumor C		ND	10%
	Tumor D		c.1663_1666del	95%
	Tumor E		LOH	95%
	Tumor F		ND	95%
NF339-UHG	Tumor A	g.AC127024.4_AC129917.6del(= *NF1* microdeletion)	c.4697T>A (p.L1566X)	95%
	Tumor B		c.2851–2A>G	80%
	Tumor C		c.[1007G>A; 1008G>A] (p.W336X)	95%
	Tumor D		c.2409+1G>A	95%
	Tumor E		ND	70%
NF482-UHG	Tumor A	c.3525_3526del	c.2252-30_2252-6delinsT	NA
	Tumor B		c.603_621del	NA
	Tumor C		c.1185+1G>A	NA
	Tumor D		c.359_375del	NA
NF116-UHG	Tumor A	c.5122dup	c.4537C>T (p.R1513X)	NA

*GenBank *NF1* reference sequence NM_000267.1 with mutation numbering beginning with +1 as A of the ATG codon.
LOH, loss of heterozygosity; ND, not detected; SC F-, estimated Schwann cell percentage of SC F- cultures determined by S100 immunofluorescence staining; NA, no data available.

decreased expression is due to the allele not bearing the germline *NF1* mutation (data not shown). Since the constitutional mutation in patient NF96-1 is a *NF1* microdeletion, the same holds true for the tumors of this individual. Taken together, these data strongly suggest that the acquired second hit results in reduced *NF1* expression (P = 0.0023; Fisher's Exact test).

Mismatch Repair Gene Analysis

In all patients, several alterations in *MLH1*, *MSH2,* and *MSH6* were detected (data not shown). All alterations were known polymorphisms (InSight database, www.insight-group.org). Those variants leading to amino acid alterations were selected for further evaluation, i.e., p.I219V (*MLH1*), p.G322D (*MSH2*), and p.G39E (*MSH6*). Respective allele frequencies of these variants were 74/184 (~40%), 5/364 (~1%), and 37/186 (~20%) in a control non-NF1 population, geographically matched to the NF1 patients under investigation, and 6/18 (~33%), 2/18 (~11%), and 4/18 (~22%) in the NF1 patients (Table 3). Only the frequency of the p.G322D (*MSH2*) polymorphism is statistically different (P = 0.038; Fisher's Exact test) in the NF1 patients compared to the control population, but this significance at the 5% level disappeared after correcting for multiple testing. Analysis of 15 additional geographically matched NF1 patients with a similar tumor burden did not reveal the *MSH2* polymorphism in any individual. No microsatellite instability could be detected in any of the analyzed tumors.

Germline vs. Somatic Mutation Spectrum

In order to compare the germline and somatic mutation spectrum and to explore a putative interdependence between both, a descriptive and statistical analysis on the germline and somatic *NF1* mutation data set of 96 neurofibromas from 34 NF1 patients was performed. Twenty-nine neurofibromas are described in this report and the remaining 67 were compiled from the literature (Table 4). In summary, the 34 germline mutations include seven splice and nine nonsense mutations, nine small deletions (1–4 bp), four insertions, and five total *NF1* deletions. Nineteen of these reside in the 5′ region of the *NF1* gene, while two and eight mutations, respectively, are present in the GRD and the 3′ region. The 96 somatic hits include 15 splice, 15 nonsense

TABLE 2. Association Between Somatic Mutation Detection, Cell Admixture and *NF1* Expression for the Tumors of Patients NF96-1, NF253-UHG, and L-004*

Patient	Germline	Tumor	Somatic	C	SQ	qPCR
NF96-1	*NF1* microdeletion	A	c.[7395-1G>A; 7395G>A]	F+	0%	3.70 ± 0.34
				F–	72%	1.00 ± 0.09
		B	c.3330del	F+	0%	3.74 ± 0.75
				F–	31%	1.00 ± 0.09
		C	c.7438del	F+	0%	6.10 ± 1.52
				F–	100%	1.00 ± 0.12
		D	ND	F+	NA	0.91 ± 0.14
				F–	NA	1.00 ± 0.11
		E	c.2050C>T (p.P684X)	F+	0%	1.18 ± 0.22
				F–	9%	1.00 ± 0.18
NF253-UHG	c.2851–2A>G	A	ND	F+	NA	0.74 ± 0.08
				F–	NA	1.00 ± 0.12
		B	ND	F+	NA	1.40 ± 0.17
				F–	NA	1.00 ± 0.27
		C	ND	F+	NA	1.01 ± 0.27
				F–	NA	1.00 ± 0.07
		D	c.1663_1666del	F+	0%	3.01 ± 0.56
				F–	50%	1.00 ± 0.21
		E	LOH	F+	0%	2.97 ± 0.22
				F–	94%	1.00 ± 0.07
		F	ND	F+	NA	1.20 ± 0.28
				F–	NA	1.00 ± 0.17
L-004	c.6791dup	A	ND	F+	NA	1.38 ± 0.24
				F–	NA	1.00 ± 0.26
		B	c.3871-?_3974+?del	F+	0%	1.81 ± 0.45
				F–	100%	1.00 ± 0.13
		C	ND	F+	NA	1.94 ± 0.17
				F–	NA	1.00 ± 0.06
		D	c.4729delA	F+	80%	1.46 ± 0.53
				F–	100%	1.00 ± 0.19
		E	ND	F+	NA	0.61 ± 0.45
				F–	NA	1.00 ± 0.13

*GenBank *NF1* reference sequence NM_000267.1.
C, culture condition; F+, with forskolin; F–, without forskolin; SQ, semiquantitative estimation of percentage $NF1^{-/-}$ cells; ND, not detected; NA, not applicable; qPCR, relative *NF1* expression in SC F+ vs. the parallel SC F– cultures (rescaled to 1 ± Standard error of the mean [SEM]) determined by real-time quantitative PCR.

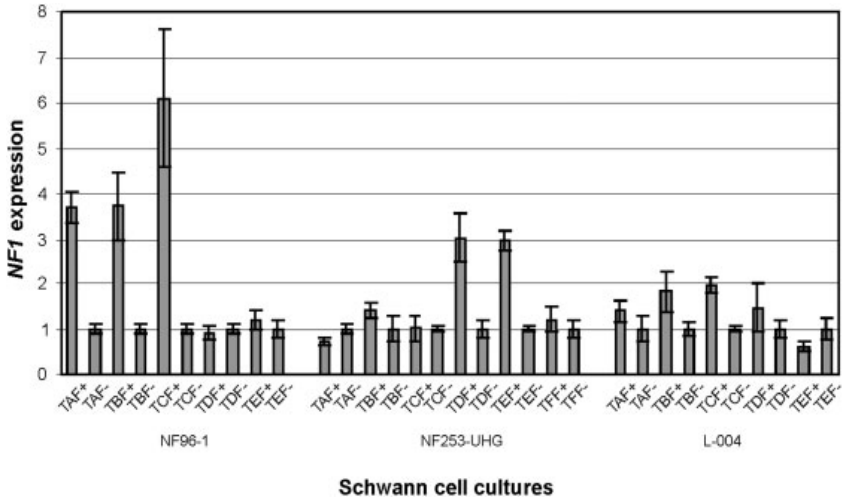

FIGURE 1. *NF1* mRNA expression levels in Schwann cell cultures (SC F+ left from SC F–, whereby SC F– levels are rescaled to one for each tumor) derived from different tumors from patients NF96-1, NF253-UHG, and L-004 (mean of two independent experiments ± standard error of the mean [SEM]).

and one missense mutation, 22 small deletions (1–31 bp), one single exon deletion, one insertion, and 41 LOH events. Thirty-one of the intragenic somatic mutations reside in the 5′ region, 10 in the GRD, and 14 in the 3′ region of the *NF1* gene. Cross tabulation did not reveal a statistically significant association between the location of the germline mutation and the location or type of somatic mutation. The somatic mutation spectrum in patients with a *NF1* microdeletion vs. patients with a minor lesion as germline mutation was significantly different with respect to the prevalence of LOH events (0/15 vs. 41/81: P = 0.0001; Fisher's Exact test) (Table 5). Thirteen of the 15 neurofibromas with a germline *NF1* microdeletion are described in this report. To avoid

TABLE 3. Mismatch Repair Gene Variants Leading to Amino Acid Changes Detected in Nine NF1 Patients*

Patient	*MLH1*	*MSH2*	*MSH6*	Tumor phenotype (age/♯NFB)
Severe tumor burden and NF1 microdeletion				
L-001	c.655A>G (p.I219V)	—	—	49 years/100–500
NF96-1	—	—	—	33 years/>100
Severe tumor burden without NF1 microdeletion				
L-002	c.655A>G (p.I219V) (hom)	c.965G>A (p.G322D)	c.116G>A (p.G39E) (hom)	34 years/TNTC
L-004	c.655A>G (p.I219V)	—	—	58 years/100–500
L-005	c.655A>G (p.I219V)	—	c.116G>A (p.G39E)	55 years/TNTC
NF482-UHG	—	c.965G>A (p.G322D)	c.116G>A (p.G39E)	48 years/TNTC
NF116-UHG	—	—	—	49 years/>100
Moderate tumor burden				
NF253-UHG	c.655A>G (p.I219V)	—	—	33 years/<50
Mild tumor burden				
NF339-UHG	—	—	—	41 years/segmental

*GenBank *MLH1* reference sequence NM_000249.2, *MSH2* reference sequence NM_000251.1, *MSH6* reference sequence NM_000179.1.
hom, homozygous alteration; NFB, neurofibroma; TNTC, too numerous to count.

TABLE 4. Overview of Germline and Somatic *NF1* Mutations in Neurofibromas Revealed by a Combination of LOH Analysis and *NF1* Mutation Detection Techniques*

NF1 germline			NF1 Somatic			
Mutation	Type	Region	Mutation	Type	Region	Reference
g.AC127024.4_AC129917.6del	Microdeletion	–	c.5026_5032del	Deletion	3′	Current report
			c.3189T>A (p.C1063X)	Nonsense	5′	
			c.4086_4092del	Deletion	GRD	
			c.396_403del	Deletion	5′	
			c.3774G>A (p.W1258X)	Nonsense	GRD	
c.1246C>T (p.R416X)	Nonsense	5′	c.655–1G>T	Splice	5′	
			c.3757_3764del	Deletion	GRD	
			LOH	LOH	–	
			c.1105C>T (p.Q369X)	Nonsense	5′	
			c.1153del	Deletion	5′	
			c.246_247del	Deletion	5′	
g.AC127024.4_AC129917.6del	Microdeletion	–	c.[7395–1G>A; 7395G>A]	Splice	3′	
			c.3330del	Deletion	5′	
			c.7438del	Deletion	3′	
			c.2050C>T (p.P684X)	Nonsense	5′	
c.6791dup	Insertion	3′	c.3871-?_3974+?del	Deletion	GRD	
			c.4729del	Deletion	3′	
c.3113+1G>A	Splice	5′	LOH	LOH	–	
c.2851-2A>G	Splice	5′	c.1663_1666del	Deletion	5′	
			LOH	LOH	–	
g.AC127024.4_AC129917.6del	Microdeletion	–	c.4697T>A (p.L1566X)	Nonsense	3′	
			c.2851–2A>G	Splice	5′	
			c.[1007G>A; 1008G>A] (p.W336X)	Nonsense	5′	
			c.2409+1G>A	Splice	5′	
c.3525_3526del	Deletion	5′	c.2252-30_2252-6delinsT	Splice	5′	
			c.603_621del	Deletion	5′	
			c.1185+1G>A	Splice	5′	
			c.359_375del	Deletion	5′	
c.5122dup	Insertion	3′	c.4537C>T (p.R1513X)	Nonsense	GRD	
g.AC027793_AC004526del	Microdeletion	–	c.543_546del	Deletion	5′	Sawada et al. [1996]
c.1260+1G>A	Splice	5′	c.4021C>T (p.Q1341X)	Nonsense	GRD	Eisenbarth et al. [2000]
			c.4084C>T (p.R1362X)	Nonsense	GRD	
c.1246C>T (p.R416X)	Nonsense	5′	LOH	LOH	–	
c.2041C>T (p.R681X)	Nonsense	5′	c.2246C>G (p.S749X)	Nonsense	5′	Serra et al. [2001]
			LOH	LOH	–	
			LOH	LOH	–	
c.3525_3526del	Deletion	5′	c.2928_2940del	Deletion	5′	
			c.2266C>T (p.Q756X)	Nonsense	5′	
			c.1260+1604A>G	Splice	5′	
			c.1260+1G>A	Splice	5′	
			c.4514+1G>C	Splice	GRD	
			c.587-8_587-3del	Splice	5′	
			c.7676–2A>T	Splice	3′	
			c.2815del	Deletion	5′	
			c.5774del	Deletion	3′	
			c.6292_6322del	Deletion	3′	

TABLE 4. Continued

NF1 germline			NF1 Somatic			
Mutation	Type	Region	Mutation	Type	Region	Reference
			LOH (12)	LOH (12)	–	
c.979delinsTT	Deletion	5′	LOH	LOH	–	
			LOH	LOH	–	
c.3419C>G (p.S1140X)	Nonsense	5′	LOH	LOH	–	
			LOH	LOH	–	
c.5749+332A>G	Splice	3′	LOH	LOH	–	
c.910C>T (p.R304X)	Nonsense	5′	LOH	LOH	–	
c.2251+2T>C	Splice	5′	LOH	LOH	–	
			LOH	LOH	–	
c.3870+1G>T	Splice	GRD	LOH	LOH	–	
c.910C>T (p.R304X)	Nonsense	5′	LOH	LOH	–	
c.1754_1757del	Deletion	5′	LOH	LOH	–	
c.3525_3526del	Deletion	5′	LOH	LOH	–	
c.801G>A (p.W267X)	Nonsense	5′	c.1641+1G>A	Splice	5′	Wiest et al. [2003]
			c.1528-14_1546del	Splice	5′	
			c.3049C>T (p.Q1017X)	Nonsense	5′	
			c.3916C>T (p.R1274X)	Nonsense	GRD	
			c.5205+1G>A	Splice	3′	
			c.5767_5770del	Deletion	3′	
			LOH	LOH	–	
			LOH	LOH	–	
c.801G>A (p.W267X)	Nonsense	5′	c.1381C>T (p.R461X)	Nonsense	5′	
			c.7237_7253del	Deletion	3′	
			c.3303_3314+7del	Splice	5′	
			c.4750_4751ins	Insertion	3′	
			LOH	LOH	–	
c.2731del	Deletion	5′	c.7285C>T (p.R2429X)	Nonsense	3′	Upadhyaya et al. [2004]
c.6788_6791del	Deletion	3′	c.1888del	Deletion	5′	
c.6791dup	Insertion	3′	c.2033del	Deletion	5′	
			c.4374_4375del	Deletion	GRD	
			LOH	LOH	–	
			LOH	LOH	–	
			LOH	LOH	–	
g.AC127024.4_AC129917.6del	Microdeletion	–	c.6387A>C (p.R2129S)	Missense	3′	
c.2851-2A>G	Splice	5′	LOH	LOH	–	
c.4537C>T (p.R1513X)	Nonsense	GRD	LOH	LOH	–	
c.6788_6791del	Deletion	3′	LOH	LOH	–	
c.7267dup	Insertion	3′	LOH	LOH	–	
c.7268_7269del	Deletion	3′	LOH	LOH	–	

*GenBank *NF1* reference sequence NM_000267.1.
5′, proximal to c.3613; GRD, GAP-related domain (c.3613-4608); 3′, distal to c.4608.

TABLE 5. Comparison *NF1* Somatic Mutation Spectrum of *NF1* Microdeletion (MD) and Non-microdeletion Patients

Somatic *NF1* mutation		Germline *NF1* mutation		
Type	Number	MD	Non-MD	Fisher's Exact test ($\alpha = 0.008$)
Splice	15	3	12	P = 0.7
Nonsense	15	5	10	P = 0.06
Missense	1	1	0	P = 0.2
Deletion	23	6	17	P = 0.2
Insertion	1	0	1	P = 1
LOH	41	0	41	P = 0.0001
Total	96	15	81	

an ascertainment bias against LOH, only those neurofibromas derived from NF1 patients with an intragenic germline mutation were taken into account for further comparison. The frequency of LOH in our cohort (3/16, 19%) is similar to literature data (42/205, 20%) [Colman et al., 1995; Daschner et al., 1997; Serra et al., 1997; Eisenbarth et al., 2000; Rasmussen et al., 2000; Serra et al., 2001; Wiest et al., 2003; Upadhyaya et al., 2004] but is significantly different from the group of tumors with both characterized hits derived from the systematic somatic mutation detection studies (3/16 vs. 38/65: P = 0.0053; Fisher's Exact test).

DISCUSSION

Unbiased *NF1* somatic mutation screening has been challenging due to the large size of the *NF1* gene, the diversity of lesions found within the *NF1* gene, and the cellular heterogeneity of NF1 related tumors. Here, we present an improved strategy for *NF1* somatic mutation detection. Extensive screening of the *NF1* gene in selectively grown Schwann cells derived from neurofibromas resulted in the highest *NF1* somatic mutation detection rate described up to now (76%). Moreover, it was demonstrated that the acquired somatic hit reduces *NF1* mRNA expression. As a reduction in *NF1* expression was not observed for SC F- cultures with undetectable somatic *NF1* mutations (Table 2) and Schwann cell purity could not be assessed for several of these (Table 1), we postulate that a high proportion of $NF1^{+/-}$ cells was still present in at least some of these cultures. Fibroblast admixture in Schwann cell cultures is a persistent problem in this context and the purity of Schwann cell subpopulations during successive passages may

depend on the composition of the original tumor. Regardless, the high somatic mutation detection rate and the observed *NF1* mRNA reduction in SC F− cells illustrate that two inactivating events, in a subpopulation of the Schwann cells, are required for neurofibroma formation and emphasize the importance of genetic inactivation mechanisms, instead of epigenetic factors, in NF1 tumorigenesis.

The high prevalence of somatic minor lesion mutations (26/29) compared to LOH events in our cohort (3/29) is striking. This is in contrast with previous somatic mutation reports where LOH has been detected more often than small somatic lesions. This might be explained by the fact that: 1) LOH is technically easier to detect than intragenic mutations, 2) somatic mutation detection approaches were used that were not sensitive enough to detect all types of mutations, 3) the coding *NF1* region was not entirely analyzed, and 4) some patient groups might exist with a tendency to acquire a particular type of somatic alteration. The high prevalence of frameshift mutations (12/29, 41%) as second hit is remarkable. The occurrence of deletions comprising ≥ 4 nucleotides (7/29, 24%) is especially high in comparison with the germline mutation spectrum (71/804, 9%; own unpublished data) ($P = 0.012$; Binomial test). Recently, *NF2* frameshift mutations were reported to be more prevalent in sporadic tumors, particularly with increasing age at diagnosis, compared to classic and mosaic neurofibromatosis type 2 [Evans et al., 2005]. The high occurrence of somatic frameshift mutations might therefore be explained by an age related shift in mutation mechanism, possibly due to an age related decline in DNA repair efficiency [Evans et al., 2005]. Compared to the general age matched NF1 population [Huson et al., 1988], most individuals included in the current report suffer from a severe to excessive neurofibroma burden. While it is well known that *NF1* microdeletion patients develop more neurofibromas at an earlier age [Kayes et al., 1994; Cnossen et al., 1997; Leppig et al., 1997; Tonsgard et al., 1997], for other NF1 patients this remarkable tumor phenotype remains unexplained. It is tempting to speculate that reduced DNA repair efficiency influences the *NF1* somatic mutation rate and hence tumor development and tumor burden. Several lines of evidence strengthen this hypothesis. First, there is increasing evidence that mild reductions in DNA repair capacity, assumed to be the consequence of common genetic variation, affect cancer predisposition [Mohrenweiser et al., 2003]. Second, the *NF1* gene has been shown to be a mutational target in cells deficient for the MMR process [Wang et al., 2003]. Third, epidemiologic studies suggest that the molecular basis underlying the phenotypic variability in NF1 is determined to a large extent by the genotype at modifying loci [Easton et al., 1993; Szudek et al., 2000, 2002, 2003]. Genes involved in eukaryotic MMR are responsible for the repair of base:base and single to larger base insertion/deletion mispairs [Kolodner and Marsischky, 1999]. The somatic mutation signature (high occurrence of larger frameshift mutations) might reflect impairment of the latter DNA repair mechanism. In a first attempt to determine the putative contribution of DNA repair variation in NF1 tumor predisposition, we screened the MMR genes *MLH1, MSH2,* and *MSH6* in peripheral blood lymphocytes of all patients in this report. In accordance with the extensive genetic variation found in repair genes in the general population, distinct polymorphisms were detected for all genes in all NF1 patients. Only those variants leading to amino acid alterations were selected for further evaluation, i.e., p.I219V (*MLH1*), p.G322D (*MSH2*), and p.G39E (*MSH6*), because of their potential impact on protein structure and activity. The *Polyphen* algorithm (http://tux.embl-heidelberg.de/ramensky; [Xi et al., 2004]) indicates that, of the three selected polymorphisms, only p.G322D in *MSH2* could have a potential impact on the respective protein activity. This finding is supported by functional assays in yeast since quantitative in vivo DNA MMR assays in *Saccharomyces cervisiae* indicate that the homologous yeast p.G317D allele variant exhibits a slightly reduced efficiency of MMR compared with the wild-type yeast *MSH2* [Drotschmann et al., 1999; Ellison et al., 2001]. The triggers for development of multiple tumors might be different in *NF1* microdeletion patients (17 genes are present in the typical *NF1* microdeletion region) compared to individuals with an intragenic *NF1* mutation. Therefore, we subdivided the patient group with a severe tumor burden into two different entities: 1) severe tumor burden and *NF1* microdeletion (L-001, NF96-1) and 2) severe tumor burden without *NF1* microdeletion (L-002, L-004, L-005, NF482-UHG, and NF116-UHG). While the frequency of the *MLH1* and *MSH6* variants in the NF1 patients and the control population is roughly similar, the occurrence of the *MSH2* variant in NF1 patients with multiple neurofibromas and an intragenic *NF1* germline mutation (five patients) is higher than expected (2/5 vs. 5/182: $P = 0.011$; Fisher's Exact test). Microsatellite instability analysis of the tumors of the two NF1 patients was negative. Analysis of 15 additional NF1 patients selected with a similar tumor burden did not reveal the *MSH2* polymorphism in any individual. One might expect that genetic predisposition to tumor development is the result of an "at-risk" genotype composed of small contributions of functionally significant variants in multiple DNA repair genes. Therefore, epidemiological studies in large NF1 patient groups will be essential for evaluating the association between variant DNA repair genotypes and tumor predisposition.

To study the interdependence between germline and somatic hits. we made an overview of the current findings together with previously reported cases (Table 4). In total, combined germline and somatic mutation data of 96 neurofibromas derived from 34 NF1 patients were subjected to descriptive and statistical analysis. These results have to be interpreted with caution. Inadequate detection techniques and the mixed cellular pools that were used for mutation analysis in previous studies together with the selection of only those neurofibromas for which both germline and somatic mutations were characterized, might have led to biased results. More specifically, somatic *NF1* minor lesion mutations might be underestimated compared to LOH events, and certain types of small somatic lesions might be under/overrepresented due to the specific technologies applied in the former studies.

Both intragenic germline and somatic mutations tend to reside in the 5′ region of the *NF1* gene (19/29, 66% and 31/55, 56%, respectively). In contrast with observations in familial adenomatous polyposis (MIM♯ 175100) [Lamlum et al., 1999; Crabtree et al., 2003], no association was found between the location of the germline and the location or type of the somatic *NF1* mutation. When focusing on the minor somatic lesions, the frequency of somatic missense mutations (1/55) is remarkably low in comparison with the *NF1* intragenic germline mutation spectrum (114/851, 13%; own unpublished data) ($P = 0.003$; Binomial test). It is tempting to speculate that the described somatic missense mutation might have only a mild impact on protein activity and will only contribute to tumorigenesis when occurring in combination with a "dramatic" constitutive hit such as the *NF1* microdeletion (leading to loss of 17 genes) in the particular tumor. More tumors will need to be studied, including tumors from patients with a missense alteration as the constitutive hit, to clarify this further. The somatic mutation spectrum of *NF1* microdeletion

and non-microdeletion patients was revealed to be significantly different with respect to the prevalence of LOH events (0/15 vs. 41/81: $P = 0.0001$; Fisher's exact test) (Table 5). This finding points to a putative difference in somatic inactivation mechanism between both NF1 patient groups. This phenomenon was also observed in patient UHG-339, who is mosaic for a *NF1* microdeletion. We investigated this hypothesis in more detail on an extended series of tumors [De Raedt et al., 2006]. A similar discrepancy in the frequency of LOH between patients with a germline total gene deletion vs. a germline intragenic mutation has been observed in tumors of patients with the von Hippel-Lindau syndrome and in patients with retinoblastoma [Hagstrom and Dryja, 1999; Vortmeyer et al., 2002; Wait et al., 2004].

In conclusion, we developed an improved strategy for somatic screening of the *NF1* gene on selectively cultured Schwann cells and analyzed the somatic mutation spectrum in neurofibromas. The high somatic mutation detection rate (76%) together with the observed *NF1* mRNA reduction in SC F− cultures illustrate that two inactivating mutations, in a subpopulation of the Schwann cells, are required for neurofibroma formation. Given the high occurrence of somatic frameshift mutations, a putative role of reduced DNA repair efficiency as a trigger for *NF1* somatic inactivation and hence tumorigenesis was suggested. The absence of LOH as second hit in *NF1* microdeletion patients compared to the general NF1 population, led to the hypothesis that differences in somatic inactivation mechanism might exist between both patient groups. Studies on a larger series of tumors are needed to confirm these observations and will be important to further elucidate the molecular basis underlying tumorigenesis in NF1.

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Somatic Loss of Wild Type *NF1* Allele in Neurofibromas: Comparison of *NF1* Microdeletion and Non-microdeletion Patients

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Neurofibromatosis type I (NF1) is an autosomal dominant familial tumor syndrome characterized by the presence of multiple benign neurofibromas. In 95% of NF1 individuals, a mutation is found in the *NF1* gene, and in 5% of the patients, the germline mutation consists of a microdeletion that includes the *NF1* gene and several flanking genes. We studied the frequency of loss of heterozygosity (LOH) in the *NF1* region as a mechanism of somatic *NF1* inactivation in neurofibromas from NF1 patients with and without a microdeletion. There was a statistically significant difference between these two patient groups in the proportion of neurofibromas with LOH. None of the 40 neurofibromas from six different *NF1* microdeletion patients showed LOH, whereas LOH was observed in 6/28 neurofibromas from five patients with an intragenic *NF1* mutation ($P = 0.0034$, Fisher's exact). LOH of the *NF1* microdeletion region in *NF1* microdeletion patients would *de facto* lead to a nullizygous state of the genes located in the deletion region and might be lethal. The mechanisms leading to LOH were further analyzed in six neurofibromas. In two out of six neurofibromas, a chromosomal microdeletion was found; in three, a mitotic recombination was responsible for the observed LOH; and in one, a chromosome loss with reduplication was present. These data show an important difference in the mechanisms of second hit formation in the 2 NF1 patient groups. We conclude that NF1 is a familial tumor syndrome in which the type of germline mutation influences the type of second hit in the tumors.

INTRODUCTION

Neurofibromatosis type I (NF1) is an autosomal dominant disorder with a prevalence of 1/4,000 (Huson, 1989). It is caused by mutations in the *NF1* tumor suppressor gene located at chromosome band 17q11.2 (Legius et al., 1993). Neurofibromin, the *NF1* gene product, is a negative regulator of the Ras-Map kinase pathway. The main features of the NF1 phenotype are multiple café-au-lait spots, axillary freckling, Lisch nodules, benign neurofibromas, and learning disabilities. Most individuals with NF1 show a mutation in the *NF1* gene (point mutation, small deletion, insertion, or duplication) (Messiaen et al., 2000). Five percent of NF1 individuals have a microdeletion (Clementi et al., 1996; Cnossen et al., 1997; Rasmussen et al., 1998; Kluwe et al., 2004) that encompasses *NF1* and its neighboring genes. Individuals with an *NF1* microdeletion exhibit on average a larger neurofibroma burden, have a lower average IQ (Descheemaeker et al., 2004; Venturin et al., 2004) compared with non-microdeletion patients, and often show distinct facial characteristics (Venturin et al., 2004). In addition, an increased risk for the development of malignant peripheral nerve sheath tumors has been reported (De Raedt et al., 2003). Two recurrent types of *NF1* microdeletions have been described.

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The type I microdeletion is the most prevalent, is meiotic in origin (Lopez-Correa et al., 2000a), and has a length of 1.4 Mb. The breakpoints are located in the low copy repeats (NF1REP A and C) flanking the *NF1* microdeletion region (Lopez-Correa et al., 2001; Kehrer-Sawatzki et al., 2004), and 17 genes are localized in this region (De Raedt et al., 2004). The type II *NF1* microdeletion is smaller (about 1.2 Mb) and mitotic in origin. The breakpoints are located in the *JJAZ1* (*SUZ12*) gene/pseudogene region, and a total of 16 genes are deleted. These patients are often mosaic for the *NF1* microdeletion, which may be the reason why their phenotype is less severe (Petek et al., 2003; Kehrer-Sawatzki et al., 2004).

A common mechanism for the somatic inactivation of a tumor suppressor gene is loss of heterozygozity (LOH), i.e., loss of the wild type allele at a heterozygous locus. This chromosomal event may arise by several mechanisms such as deletion of part of the chromosome, mitotic recombination between the centromere and the locus of the tumor suppressor gene, or chromosome loss with or without reduplication of the homologous chromosome. Mitotic recombination has been suggested as a common mechanism for LOH at the *NF1* locus in neurofibromas of patients with NF1 (Serra et al., 2001b) and has been reported in malignant myeloid cells from children with NF1 (Cooper et al., 2000). LOH in the *NF1* region in neurofibromas from individuals with a known *NF1* germline mutation has been reported in 42 of 208 neurofibromas (21%) (Colman et al., 1995; Lothe et al., 1995; Sawada et al., 1996; Daschner et al., 1997; Serra et al., 1997; Eisenbarth et al., 2000; Rasmussen et al., 2000; Serra et al., 2001a; Wiest et al., 2003; Upadhyaya et al., 2004). Thus far, we found only three publications describing LOH data each in one neurofibroma from an *NF1* microdeletion patient. (Sawada et al., 1996; Serra et al., 2001a; Upadhyaya et al., 2004). This is the first study that systematically compares the frequency of somatic loss of the *NF1* wild type allele in a large number of neurofibromas from both *NF1* microdeletion and non-microdeletion patients.

MATERIALS AND METHODS

Samples

Forty neurofibromas from six patients with a constitutional type I *NF1* microdeletion and 28 neurofibromas from six NF1 patients without a microdeletion were investigated. Twenty neurofibromas were formalin-fixed and paraffin-embedded (FFPE), and 42 had been fresh frozen. In addition, neurofibroma-specific Schwann cell cultures were available from six other neurofibromas (Table 2). All neurofibromas investigated were located cutaneously or s.c., and none were plexiform or spinal neurofibromas. Peripheral blood leukocyte DNA from each patient was used as matched control DNA. All patients fulfilled the NIH diagnostic criteria for NF1 (Stumpf et al., 1988). None of the 6 *NF1* microdeletion patients are mosaic, and all deletions are *de novo* and of type I. They have their proximal breakpoint located in the low copy repeats flanking *NF1* (Lopez-Correa et al., 2001) between chromosome 17 reference positions (build 35) 25996120 and 26022120. Their distal breakpoint is located between positions 27412117 and 27438208. The other six patients have intragenic *NF1* mutations (GenBank reference sequence NM_000267.1): c.2851-2A>G (NF253-UHG), c.4515-2A>T (NF44-UHG), c.1261-19G>A (NF93-UHG), c.5546G>A (p.R1849Q) (NF37-UHG), c.1246C>T (p.R416X) (L-002) and c.988_989insA (NF24-UHG).

DNA Extraction from Frozen Tissue and Cultured Cells

Frozen tumor tissue fragments were pulverized with a mortar. Culture conditions for neurofibroma-derived Schwann cells were applied as described in (Rosenbaum et al., 2000; Serra et al., 2000). In both cases, gDNA was extracted using the QiaAmp procedure (Qiagen, The Netherlands) following the manufacturer's instructions.

DNA Extraction from FFPE Tissue

Ten-micrometers thick unstained slides were dewaxed through successive xylene and ethanol washes. DNA was extracted from archival FFPE neurofibroma tumor samples following manual microdissection of the neoplastic regions, visualized by comparison with one parallel H&E stained slide. DNA was extracted from these microdissected tissue fragments using the QiaAmp procedure (Qiagen, The Netherlands) following the manufacturer's instructions and was subsequently purified and concentrated on Microcon filters (Millipore, Belgium).

LOH Analysis

Genomic regions at both sides of the *NF1* microdeletion region were sequenced, and heterozygous SNPs were detected in all patients (Table 1). Tumor and control DNA samples were subjected to PCR amplification (Hotgoldstar mix, Eurogentec, Belgium) using primers for the informative SNPs

TABLE 1. Overview of SNPs Used in the LOH Analysis and Clones Used in Array CGH Experiments in Positional Reference to *NF1*

SNP number	Chr17 reference position	Polymorphism
rs8082669	10335232	A/G
rs4791544	13125881	A/T
rs1634421	18792051	C/T
Centromere		
RP11-138P22	23.14 Mb	
rs1018190	24571352	C/G
rs6505129	24777744	A/G
RP11-104I20	25.11 Mb	
rs29001484	25562640	G/A
rs4583306	25562840	T/C
Proximal BP	25996120-26022120	
NF1	26.44 Mb	
Distal BP	27412117-27438208	
rs9891455	27534262	C/T
RP11-474K4	27.58 Mb	
rs8074061	27625458	C/T
rs753750	27638817	A/T
rs2055091	27961859	A/T
rs28909978	28053334	T/C
rs11869264	28135095	C/T
RP11-47L3	30.68 Mb	

BP, breakpoint.

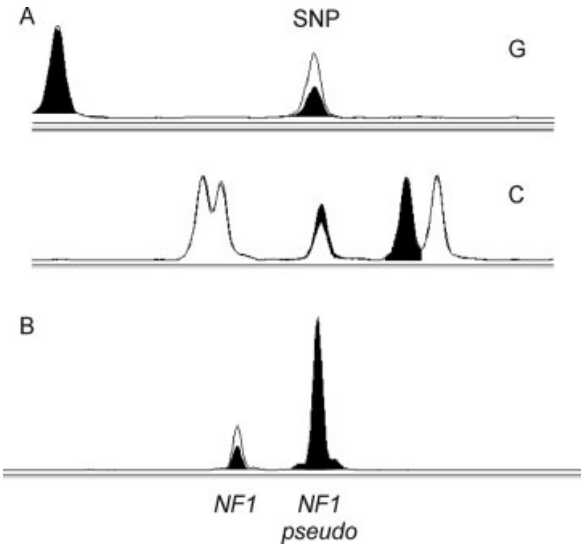

Figure 1. Example of LOH assay. A: Overlay of the traces of SNP rs1018190 (G/C polymorphism) from blood and tumor 32 of individual NF253-UHG, only the G- (top) and the C-trace (bottom) are shown. The SNP peak and the control peak of the tumor DNA are represented in black. It is clear that in tumor 32 the G-allele of rs1018190 is lost compared with that in the blood. B: Overlay of the traces of the semi-quantitative PCR used to determine if the LOH of *NF1* is caused by a copy number loss. Blood DNA of patient L-002 was compared with DNA of tumor 3 (represented in black). The first peak (103 bp) represents *NF1*, and the second peak (107 bp) represents the *NF1* pseudogene located on chromosome 15. Tumor 3 has copy number loss of *NF1*.

(primers available on request). For each SNP, a short amplicon could be designed (<130 bp). In the individuals NF44-UHG (c.4515-2A>T), L-002 (c.1246C>T), NF93-UHG (c.1264-19G>A), and NF37-UHG (c.5546G>A), the intragenic constitutional *NF1* mutation was used as an additional intragenic SNP. PCR products were sequenced following the BigDye Terminator Sequencing protocol (ABI, Applied Biosystems, Belgium). A liz-tagged size standard was added, and the samples were analyzed on an ABI3100 machine using a standard fragment analysis protocol. Using the Genescan analysis software (Applied Biosystems, Belgium), peak height ratios of the two alleles of the SNP were calculated relative to an internal control peak of the same nucleotide at least five nucleotides apart, and these ratios were compared between tumor and corresponding control DNA. Every DNA sample was PCR amplified (35 cycles) and sequenced for each SNP in triplicate. LOH for an SNP was scored when the average ratio (SNP nucleotide/control nucleotide) of the two alleles in tumor tissue fell outside the 95% confidence interval of the ratios observed in control DNA of the same patient and when the average ratios in tumor versus control tissue were at least 20% different. If one SNP showed LOH, a second SNP was tested to confirm the presence of LOH in the tumor. Given the cutoff of 20% used in this study, LOH will not be detected if less than 20% of the tumor cells are pathogenic. Figure 1A shows the typical output when LOH is observed for a SNP (tumor 33 of patient NF253-UHG, SNP rs1018190).

Newly identified SNPs were submitted to the NCBI SNP database (http://www.ncbi.nlm.nih.gov/snp).

NF1 Somatic Mutation Analysis

Selective Schwann cell cultures ($SC^{NF1-/-}$) derived from the neurofibromas of patient L-001 were treated with puromycin before RNA extraction (RNeasy kit, Qiagen, Belgium). The entire *NF1* cDNA was sequenced using the ABI3100 genetic analyzer (Applied Biosystems) (Messiaen et al., 2000). All mutations found at the cDNA level were confirmed on gDNA by cycle sequencing. Comparison of the mutations found in the $SC^{NF1-/-}$, the parallel $SC^{NF1+/-}$ cultures, and blood lymphocytes allowed to conclude which mutation represented the somatic alteration. Tumors from patients C174, C176, and C186 were screened for a deletion of the *NF1* gene, using FISH (clones P1-9 and P1-12; Leppig et al., 1996), dHPLC, and direct sequencing as described by Upadhyaya et al. (2004), combined with MLPA and deletion PCR.

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Identification of the Mechanism of LOH

Semiquantitative PCR

Several pseudogenes of *NF1* are present in the human genome, some of which contain deletion/insertions when compared with the functional copy of *NF1*. PCR primers were designed in an area present in both the *NF1* gene and an *NF1* pseudogene in such a way that both loci would be amplified with the same primers in the same PCR reaction. The PCR amplification would result in two PCR products with a size difference of only a few basepairs (bp). A semiquantitative PCR can be used to test whether one or two copies of *NF1* are present in the tumor in relation to the pseudogene. Using a pseudogene as control fragment has an advantage over a classical semiquantitative PCR because only one primer set is needed and variations caused by PCR efficiency of test and control fragment is minimized, resulting in increased accuracy of the assay. Primer pairs were designed to amplify a small fragment of *NF1* exon 22 located at 17q11.2 (103 bp) together with the corresponding fragment of its pseudogene located on chromosome 15 (107 bp) and characterized by a 4-bp insertion. Tumor and normal DNA samples were subjected to 35 cycles of PCR (Hotgoldstar mix, Eurogentec, Belgium). Relative peak heights of amplified fragments were analyzed by the Genescan software (Applied Biosystems, Belgium), and the ratios of gene versus pseudogene fragments were compared between tumor and normal DNA for each patient. Every analysis was performed in triplicate. Similar to SNP LOH analysis, copy number loss was defined when the average ratio (*NF1*/*NF1* pseudogene) fell outside of the 95% confidence interval of the corresponding ratio in normal DNA with a minimum difference of at least 20%. Figure 1B shows the output of this semiquantitative PCR for *NF1*. DNA from a tumor with copy number loss of *NF1* was compared to the blood DNA of the same individual (L-002 tumor 3).

LOH analysis of markers on the p arm of chromosome 17

To distinguish LOH caused by mitotic recombination from LOH caused by deletion and reduplication of the homologous chromosome 17, SNPs rs8082669, rs1634421, and rs4791544 located on the p arm of chromosome 17 were analyzed. If LOH of *NF1* was caused by a mitotic recombination, markers on chromosome 17p would not show LOH. Only tumors showing LOH not caused by a somatic deletion were tested (i.e., tumor 32 (NF253-UHG), tumor 5 and 12 (L-002), and tumor 41 (NF44-UHG)).

Array CGH

The array CGH experiments were performed according to the protocol described by Vermeesch et al. (2005). The arrays were constructed using a 1 Mb Clone Set and contain 3527 BAC and PAC clones (Fiegler et al., 2003) spotted in duplicate. Tumor DNA was directly compared to DNA extracted from blood leukocytes of the same individual, and both were labeled by a random prime labeling system (Bioprime DNA Labeling System, Invitrogen, Belgium) using Cy3- and Cy5-labeled dCTPs (Amersham Biosciences, Belgium). Following incubation of about 36 hr, the slides were washed and scanned at 532 nm (Cy3) and 635 nm (Cy5) on the Agilent G2565BA MicroArrayScanner System (Agilent; Palo Alto, CA). Image analysis was performed using ArrayVision software (Imaging Research; St Catharines, Ontario, Canada). Further analysis was performed with Excel (Microsoft; Diegem, Belgium). For each clone, a normalized $\log_2$ ratio was calculated. Subsequently, a 2D Lowess normalization was performed (Yang, 2003). Datapoints for which the variation between the intensity ratios of the duplicated spots was larger than 10% were excluded from the analysis. The quality of an array experiment was considered good when the SD was lower than 0.096 and the hybridization efficiency was higher than 90%. The fold change of a single clone is considered significantly different if it falls outside of the $\pm|(\log_2(1.5) - 2 \times \mathrm{SD})|$ interval. The fold change of two or more consecutive clones is considered significantly different if it falls outside of the $\pm 4 \times$ SD interval (Vermeesch et al., 2005).

Real-time quantitative PCR

Real time quantitative PCR (primers available on request) was performed on *C17orf41* with *HPRT1* as housekeeping gene as described by Jun et al. (2001), with the exception that an ABI PRISM 7000 instrument (Applied Biosystems, Belgium) was used.

RESULTS

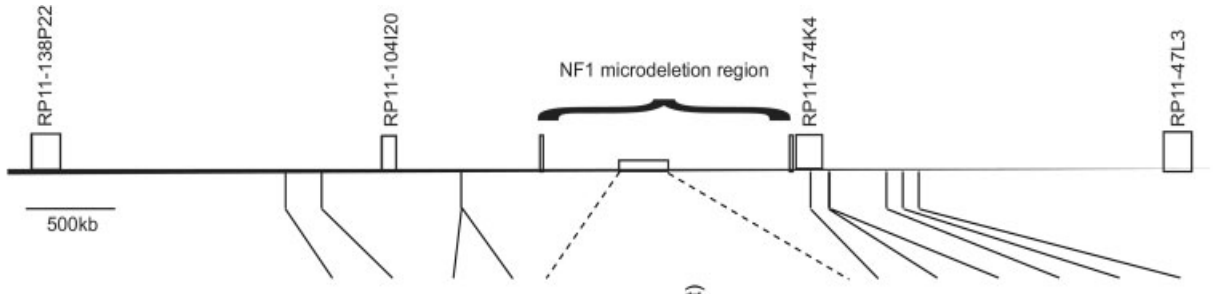
Detection of LOH

As the somatic point mutation in *NF1* had already been identified in 10 neurofibromas of microdeletion patients, these samples were excluded from LOH analysis. These somatic mutations are shown in Table 2.

LOH in the Neurofibromas

LOH of *NF1* was detected in six of the 28 neurofibromas (21%) from *NF1* non-microdeletion patients, compared with none of the 40 neurofibromas from the *NF1* microdeletion patients. This is a significant difference in LOH frequency ($P = 0.0034$, Fisher's exact test). The percentage LOH observed varied between 30% and 75%. Table 2

TABLE 2. Overview of Results of the LOH Analysis



Germ line	Patient	Tumor	17p	Origin	rs1018190	rs6505129	rs29001484	rs4583306	*NF1* (germ line)	*NF1* SQ	*NF1* (somatic mut)	rs9891455	rs8074061	rs753750	rs2055091	rs28909978	rs11869264
DELETION	NF96-1	1	ND	F	NI	H	NI	NI	NI	1	ND[k]	H	NI	NI	NI	NI	NI
		2	ND	F	NI	H	NI	NI	NI	1	ND	H	NI	NI	NI	NI	NI
		4	ND	F	NI	H	NI	NI	NI	1	ND	H	NI	NI	NI	NI	NI
		5	ND	F	NI	H	NI	NI	NI	1	ND	H	NI	NI	NI	NI	NI
		6	ND	F	NI	H	NI	NI	NI	1	ND	H	NI	NI	NI	NI	NI
		7	ND	F	NI	H	NI	NI	NI	1	ND	H	NI	NI	NI	NI	NI
		8	ND	F	NI	H	NI	NI	NI	1	ND	H	NI	NI	NI	NI	NI
		9	ND	F	NI	H	NI	NI	NI	1	ND	H	NI	NI	NI	NI	NI
		10	ND	F	NI	H	NI	NI	NI	1	ND	H	NI	NI	NI	NI	NI
		12	ND	F	NI	H	NI	NI	NI	1	ND	H	NI	NI	NI	NI	NI
		13	ND	F	NI	H	NI	NI	NI	1	ND	H	NI	NI	NI	NI	NI
		14	ND	F	NI	H	NI	NI	NI	1	ND	H	NI	NI	NI	NI	NI
		15	ND	F	NI	H	NI	NI	NI	1	ND	H	NI	NI	NI	NI	NI
		16	ND	F	NI	H	NI	NI	NI	1	ND	H	NI	NI	NI	NI	NI
		17	ND	F	NI	H	NI	NI	NI	1	ND	H	NI	NI	NI	NI	NI
		18	ND	F	NI	H	NI	NI	NI	1	ND	H	NI	NI	NI	NI	NI
		19	ND	F	NI	H	NI	NI	NI	1	ND	H	NI	NI	NI	NI	NI
	L-001	A	ND	SC	ND	ND	ND	ND	NI	ND	c.5026_5032del	ND	ND	ND	ND	ND	ND
		B	ND	SC	ND	ND	ND	ND	NI	ND	p.C1063X	ND	ND	ND	ND	ND	ND
		C	ND	SC	ND	ND	ND	ND	NI	ND	c.4086_4092del	ND	ND	ND	ND	ND	ND
		D	ND	SC	ND	ND	ND	ND	NI	ND	c.396_403del	ND	ND	ND	ND	ND	ND

(Continued)

TABLE 2. Overview of Results of the LOH Analysis (Continued)

		E	ND	SC	ND	ND	ND	ND	NI	ND	p.W1258X	ND	ND	ND	ND	ND	ND
	NF99-1	15	ND	P	H	NI	NI	NI	NI	1	ND	NI	NI	NI	H	NI	H
		16	ND	P	H	NI	NI	NI	NI	1	ND	NI	NI	NI	H	NI	H
		17	ND	P	H	NI	NI	NI	NI	1	ND	NI	NI	NI	H	NI	H
		19	ND	P	H	NI	NI	NI	NI	1	ND	NI	NI	NI	H	NI	H
		20	ND	P	H	NI	NI	NI	NI	1	ND	NI	NI	NI	H	NI	H
		21	ND	P	H	NI	NI	NI	NI	1	ND	NI	NI	NI	H	NI	H
		22	ND	P	H	NI	NI	NI	NI	1	ND	NI	NI	NI	H	NI	H
		23	ND	P	H	NI	NI	NI	NI	1	ND	NI	NI	NI	H	NI	H
		24	ND	P	H	NI	NI	NI	NI	1	ND	NI	NI	NI	H	NI	H
		25	ND	P	H	NI	NI	NI	NI	1	ND	NI	NI	NI	H	NI	H
		26	ND	P	H	NI	NI	NI	NI	1	ND	NI	NI	NI	H	NI	H
		27	ND	P	H	NI	NI	NI	NI	1	ND	NI	NI	NI	H	NI	H
		28	ND	P	H	NI	NI	NI	NI	1	ND	NI	NI	NI	H	NI	H
	C176	1	ND	F	ND	ND	ND	ND	NI	ND	p.Y1604X	ND	ND	ND	ND	ND	ND
		2	ND	F	ND	ND	ND	ND	NI	ND	p.W1976X	ND	ND	ND	ND	ND	ND
		3	ND	F	ND	ND	ND	ND	NI	ND	c.4110+1G>A	ND	ND	ND	ND	ND	ND
	C186	1	ND	F	ND	ND	ND	ND	NI	ND	c.2990+1G>A	ND	ND	ND	ND	ND	ND
	C174	1	ND	F	ND	ND	ND	ND	NI	ND	c.2326-?_2409+?del	ND	ND	ND	ND	ND	ND
NONDELE	NF253-UHG	31	ND	P	H	ND	NI	NI	NI	ND	ND	H	H	NI	NI	NI	ND
		32	H[a]	P	L	ND	NI	NI	NI	2	ND	L	L	NI	NI	NI	ND
		33	ND	P	L	L	NI	NI	NI	2	ND	H	H	NI	NI	NI	ND
	NF44-UHG	1	ND	F	H	NI	NI	NI	L	1	ND	L	NI	NI	NI	NI	NI
		41	L[b]	P	L	NI	NI	NI	U	2[b]	ND	L	NI	NI	NI	NI	NI
	NF93-UHG	3	ND	F	H	NI	NI	NI	H	ND	ND	NI	H	NI	NI	NI	NI
	NF37-UHG	2	ND	P	NI	NI	H	NI	H	ND	ND	NI	NI	H	NI	NI	NI
		15	ND	SC	NI	NI	H	NI	H	ND	ND	NI	NI	H	NI	NI	NI
	L-002	1	ND	F	ND	ND	H	H	H	ND	ND	NI	NI	NI	H	NI	H
		2	ND	F	ND	ND	H	H	H	ND	ND	NI	NI	NI	H	NI	H
		3	ND	F	ND	ND	L	L	L	1	ND	NI	NI	NI	L	NI	L
		4	ND	F	ND	ND	H	H	H	ND	ND	NI	NI	NI	H	NI	H
		5	H[a]	F	ND	ND	L	L	L	2	ND	NI	NI	NI	L	NI	L

(Continued)

TABLE 2. Overview of Results of the LOH Analysis (Continued)

T		6	ND	F	ND	ND	H	H	H	ND	ND	NI	NI	NI	H	NI	H
I		7	ND	F	ND	ND	H	H	H	ND	ND	NI	NI	NI	H	NI	H
		8	ND	F	ND	ND	H	H	H	ND	ND	NI	NI	NI	H	NI	H
O		9	ND	F	ND	ND	H	H	H	ND	ND	NI	NI	NI	H	NI	H
		10	ND	F	ND	ND	H	H	H	ND	ND	NI	NI	NI	H	NI	H
N		11	ND	F	ND	ND	H	H	H	ND	ND	NI	NI	NI	H	NI	H
		12	H[a]	F	ND	ND	L	L	L	2	ND	NI	NI	NI	L	NI	L
		13	ND	F	ND	ND	H	H	H	ND	ND	NI	NI	NI	H	NI	H
		15	ND	F	ND	ND	H	H	H	ND	ND	NI	NI	NI	H	NI	H
		17	ND	F	ND	ND	H	H	H	ND	ND	NI	NI	NI	H	NI	H
		18	ND	F	ND	ND	H	H	H	ND	ND	NI	NI	NI	H	NI	H
		19	ND	F	ND	ND	H	H	H	ND	ND	NI	NI	NI	H	NI	H
		20	ND	F	ND	ND	H	H	H	ND	ND	NI	NI	NI	H	NI	H
	NF24-	35	ND	P	NI	NI	NI	H	NI	ND	ND	NI	NI	NI	NI	H	NI
	UHG	38	ND	P	NI	NI	NI	H	NI	ND	ND	NI	NI	NI	NI	H	NI

The column *NF1* (germ line) shows if LOH was observed using the germline *NF1* mutation of the individual; the column *NF1* (somatic mut) shows the somatic mutation found in the respective tumor.

NF1 SQ, semiquantitative PCR (number of *NF1* copies indicated) relative to *NF1* pseudogene; F, frozen; H, marker heterozygous; SC, Schwann cell culture; P, paraffin; L, marker not heterozygous (LOH); U, amplification failed; NI, not informative; ND, not determined; 17p, 17p marker analysis.

[a]Tumors have LOH of *NF1* and not of markers on 17p; LOH is thus caused by a mitotic recombination.

[b]Tumors have LOH of *NF1* and for markers on 17p; no copy number loss of NF1 is observed; LOH is thus caused by chromosome loss and reduplication. The dark gray areas represent the minimal region of LOH due to a somatic deletion. The light gray areas represent the minimal region of LOH due to a mitotic recombination.

gives an overview of the results obtained in the different tumors. In addition to these six neurofibromas with LOH tumor 33 from patient NF253-UHG (a nondeletion patient) has LOH of two markers (rs6505129 and rs1018190) located centromeric of *NF1*. The semiquantitative PCR (*NF1* (pseudo)exon 22) did not show any evidence of copy number loss of *NF1* in this tumor. Because of the poor quality of the DNA, we were unable to prove the involvement of *NF1* in the area with LOH. Therefore, we did not include this tumor in our calculations. In the individuals NF44-UHG (c.4515-2A>T), L-002 (c.1246C>T), NF93-UHG (c.1264-19G>A), and NF37-UHG (c.5546G>A), the intragenic constitutional *NF1* mutation was used as an additional intragenic SNP for testing LOH. Each time LOH was observed in *NF1* (Table 2), the wild type allele was lost in the tumor.

Mechanism Leading to LOH

In three tumors (L-002 tumor 5 and 12 and NF253-UHG tumor 32), the observed LOH resulted from a mitotic recombination event as LOH for markers on 17q was shown in the presence of two copies of the *NF1* gene without any LOH on 17p. Two neurofibromas had lost one copy of *NF1* because of a deletion on chromosome 17 (L-002 tumor 3, NF44-UHG tumor 1). These two samples were used for array CGH analysis. In both cases, array CGH confirmed the presence of a somatic deletion that affected the *NF1* region on chromosome 17 (Fig. 2A). The deletions were large and different in size. The somatic deletion of NF44-UHG tumor 1 was at least 2.5 Mb and encompassed clones RP11-104I20 to RP11-474K4 (25.11–27.58 Mb on the ENSEMBL contig of chromosome 17). L-002 tumor 3 had a somatic deletion of at least 7.5 Mb encompassing clones RP11-138P22 to RP11-47L3 (23.14–30.68 Mb on the ENSEMBL contig of chromosome 17). For reference, *NF1* is located at position 26.44 Mb. More than the entire *NF1* microdeletion region was somatically deleted in these two tumors. No copy number aberrations were observed for clones in other regions of chromosome 17 or on other chromosomes (Fig. 2B). Array CGH was also performed on a neurofibroma with LOH resulting from a mitotic recombination (L-002 tumor 12), and on a

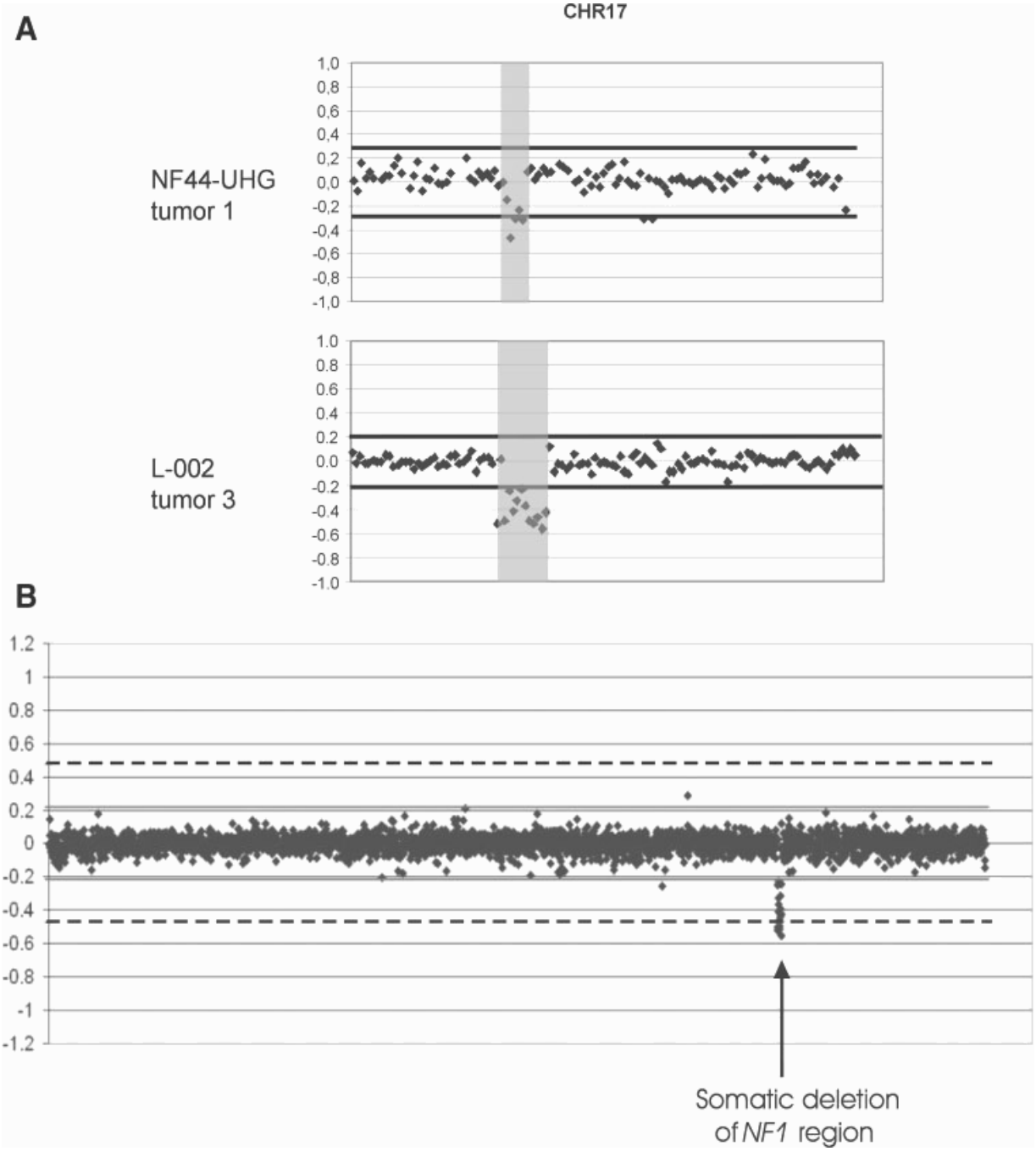


Figure 2. Array CGH output of neurofibromas. A: Array CGH output of NF44-UHG tumor 1 (top) and L-002 tumor 3 (bottom). The normalized $\log_2$ ratio for each clone from chromosome 17 is shown. The clones are arranged from chromosome 17pter to 17qter. The deleted region is indicated in gray. *NF1* is deleted in both neurofibromas. B: Array CGH output of L-002 tumor 3. The normalized $\log_2$ ratio of all clones is depicted. The clones are arranged from pter on chromosome 1 on the left to qter of the Y chromosome on the right. The fold change of a single clone is considered significant if it falls outside of the $\pm|(\log_2(1.5) - 2 \times SD)|$ interval (indicated by the dashed line on the figure). The fold change of two or more consecutive clones is considered significant if it falls outside of the $\pm 4 \times$ SD interval (indicated by the bold line on the figure). Similar to all other neurofibromas tested on array CGH, L-002 tumor 3 has a stable karyotype and a somatic deletion only in the *NF1* region (indicated by the arrow). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

neurofibroma without LOH (L-002 tumor 1). As expected, neither of these DNA samples showed any copy number changes across the genome. Tumor 41 of individual NF44-UHG did not show any copy number loss of *NF1*. Besides LOH in the *NF1* region, LOH was also present for markers located on 17p. This points to the mechanism of chromosome loss and reduplication.

Real Time PCR of C17orf41

C17orf41 is located in the *NF1* microdeletion region and is possibly essential for the survivals of

cells. The expression of *C17orf41* was tested with real time PCR on seven cell lines from Schwann cells of neurofibromas (four cell lines of *NF1* microdeletion patients and three of non-microdeletion patients). On average, the expression of *C17orf41* was five times lower than the housekeeping gene *HPRT1* ($\Delta Ct = 2.25$, South Dakota = 0.80). There was no difference in expression between both patient groups.

DISCUSSION

The tumor suppressor *NF1* can be inactivated in tumors by different mechanisms. In this report, we showed that the relative proportion of one of these mechanisms (LOH) differs significantly in *NF1* microdeletion patients when compared with that in NF1 patients with an intragenic *NF1* mutation. Thus, although LOH was responsible for the somatic inactivation of *NF1* in a quarter of the neurofibromas from *NF1* non-microdeletion patients (6/28 = 21%; 95% CI, 8–41%), LOH was never observed in 40 neurofibromas (0/40; 95% CI, 0–7%) from known *NF1* microdeletion patients ($P = 0.0034$, Fisher's exact test). The finding of LOH in neurofibromas from NF1 patients with an intragenic mutation are in concordance with published data from the literature: LOH being detected in DNA from 42/205 neurofibromas (21%; 95% CI, 15–27%) from patients in whom the germline mutation was not a microdeletion (Colman et al., 1995; Lothe et al., 1995; Sawada et al., 1996; Daschner et al., 1997; Serra et al., 1997; Eisenbarth et al., 2000; Rasmussen et al., 2000; Serra et al., 2001a; Wiest et al., 2003). In addition, LOH has not been described in three neurofibromas from *NF1* microdeletion patients reported in the literature (Sawada et al., 1996; Serra et al., 2001a; Upadhyaya et al., 2004). Combining the data on *NF1* microdeletion patients presented here and in the literature, none of the 43 neurofibromas from microdeletion patients showed LOH (0%; 95% CI, 0–6.7%) versus 48 of 233 neurofibromas from NF1 individuals without a microdeletion (21%; 95% CI, 16–26%) (X^2; $P = 0.001$).

A similar discrepancy in the frequency of LOH between patients with a germline gene deletion and a germline intragenic mutation has been observed in tumors of patients with the von Hippel-Lindau (VHL) syndrome and in patients with retinoblastoma. In VHL patients with a germline deletion of *VHL,* no LOH was observed in eight tumors analyzed (0/8 tumors, 95% CI, 0–31%) (Vortmeyer et al., 2002; Wait et al., 2004), while this is a frequent event in tumors without a germline deletion (81/132 tumors = 61%, 95% CI, 52–70%) (Crossey et al., 1994; Zeiger et al., 1995; Prowse et al., 1997; Bender et al., 2000; Glasker et al., 2001; Vortmeyer et al., 2002). Also, no LOH was observed for *RB1* in 12 retinoblastoma patients with a germline deletion of *RB1* (0/12 tumors, 95% CI, 0–22%), whereas 69% of the tumors from individuals without a germline deletion had LOH (101/146 tumors, 95% CI, 61–77%) (Hagstrom and Dryja, 1999). Germline/somatic mutation correlations have also been observed in familial adenomatous polyposis patients. In this disorder, the location and the type of somatic mutation in *APC* depends on the position of the germline mutation in *APC*. If the germline *APC* mutation is near codon 1300 (codon 1285–1398), then the inactivation of the wild type allele is associated with LOH and is usually due to a mitotic recombination. If a germline truncating point mutation is present before codon 1285, then LOH is very rare and all somatic mutations are located after codon 1285. However, when the germline mutation is located after codon 1399, then the majority of somatic mutations are located before codon 1285 (Crabtree et al., 2003).

Several hypotheses can be put forward to explain the observed difference in LOH in the two NF1 patient groups. The type I *NF1* microdeletion region is known to contain at least 17 genes, and thus LOH of this region, whether due to mitotic recombination or a microdeletion, would lead to a nullizygous state for all genes located within this region.

Several hypotheses can be put forward to explain the present findings:

1. Neurofibromas contain a mixture of cells. Only the Schwann cells show a complete inactivation of the *NF1* gene (Serra et al., 2000). It could be possible that for some unknown reason the percentage of cells from microdeletion neurofibromas showing LOH in the *NF1* region is lower than the 20% detection limit. If less than 20% (criteria used for classification of LOH) of the cells in the neurofibroma are affected, LOH would not be detected resulting in a bias against LOH. It was estimated by sequence analysis of the five frozen tumors from microdeletion patients C174, C176, and C186 that the second hit in the *NF1* gene was present in at least 30–60% of cells. Moreover, in an additional five neurofibromas of *NF1* microdeletion patients, a second hit in the *NF1* gene was found in cultured Schwann cells

and none of these tumors showed LOH (Table 2). Also, on the basis of marker analysis in the neurofibromas of non-microdeletion patients, the minimum percentage of cells showing LOH was 30%.

2. One or more of the genes in the microdeletion region may be essential for the survival of the cell. The complete loss of (some of) these genes following LOH would therefore be lethal. Gene *C17orf41* (OMIM No. 609534), also known as *FLJ12735* or *FRAG1*, is a good candidate to support this hypothesis. Real-time quantitative PCR demonstrated that *C17orf41* is expressed in Schwann cells. It is located in the *NF1* microdeletion region and *in vitro* experiments have shown that mouse cells with a reduced amount of C17orf41 protein enter the apoptosis pathway. More specifically, a reduced expression of *C17orf41* leads to the induction of apoptosis through the release of Rad9 (Ishii et al., 2005). Hence, one can imagine that, in humans, complete loss of *C17orf41* resulting from LOH in a cell with an *NF1* microdeletion might induce apoptosis. Not a lot is known on the effect of a nullizygous state of the genes present in the *NF1* microdeletion region. Besides the *NF1* knock-out mouse models, *OMGP* is the only gene in the *NF1* microdeletion region of which a knock-out mouse model exists (Huang et al., 2005). These nullizygous *OMGP* mice are perfectly viable. Other genes present in the *NF1* microdeletion region might also cause lethality; however, any direct evidence is lacking at this moment.
3. A more mechanistic hypothesis is that the presence of a *NF1* microdeletion on one chromosome 17 may suppress mitotic recombination within the region. For a mitotic recombination to occur, two chromatids of homologous chromosomes need to align. The presence of a microdeletion close to the centromere (17q11.2) might reduce the likelihood of a mitotic recombination occurring between the centromere and *NF1*. The end result would be a lower frequency of LOH. Mitotic recombination has however been demonstrated to be a common mechanism of LOH at the *NF1* locus in tumors of patients with NF1 (Serra et al., 2001b), an observation confirmed in the present study. However, we also demonstrate that the loss of copy number of *NF1* due to a somatic deletion is a frequent mechanism underlying LOH in neurofibromas (3/6 neurofibromas with LOH). This *NF1* copy number loss was thoroughly investigated using both semiquantitative PCR and array CGH analysis. If LOH due to a mitotic recombination is impossible in *NF1* microdeletion patients, then one would still expect LOH to occur because of a somatic deletion of the *NF1* region. However, we failed to observe any evidence of LOH in 40 neurofibromas from *NF1* microdeletion patients. This is in contrast to non-microdeletion patients, where three cases of a somatic deletion of *NF1* were found in 28 neurofibromas. This difference is only of borderline significance ($P = 0.065$, Fisher's exact test).
4. The observed data might also result from the frequent use of alternative second hit mechanisms in microdeletion patients, thus greatly reducing the proportion of LOH observed in neurofibromas of these patients. Assuming that the absolute number of LOH events in Schwann cells is similar in both *NF1* microdeletion and non-microdeletion patients, then the proportion of LOH in neurofibromas would be lower in *NF1* microdeletion patients if alternative mechanisms leading to the inactivation of the normal *NF1* allele were more frequent. The observed difference in LOH frequency could then point to the presence of a mechanism that made the wild type *NF1* allele in *NF1* microdeletion patients more vulnerable to other somatic mutations, excluding LOH, than in non-microdeletion patients. *NF1* microdeletion patients lack the homologous allele of *NF1* and 16 other genes. The repair of double-strand breaks (DSBs) cannot be performed by the error-free mechanism of homologous recombination at the moment during the cell cycle when sister chromatids are not present (G1 phase). This would entail that in these phases of the cell cycle, DSB in the *NF1* region on the normal chromosome 17 can only be repaired by the error-prone mechanisms of non-homologous end joining or single-strand annealing (Pfeiffer et al., 2004). This would cause an excess of small somatic mutations in the wild type alleles of the 17 genes in the *NF1* microdeletion region. The question remains why this potential mechanism would not be at play in other familial cancer syndromes where germline deletions are not associated with a more severe tumor phenotype (NF2 and VHL) (Lopez-Correa et al., 2000b; Wait et al., 2004).

We believe that the observed findings are best explained by the hypothesis that the presence of one copy of certain genes in the *NF1* microdeletion

region is essential for the survival of Schwann cells and/or by the hypothesis that the wild type genes in the *NF1* microdeletion region on the normal chromosome 17 are more vulnerable to mutation.

Nonmosaic *NF1* microdeletion patients have on average a higher tumor burden than do non-microdeletion patients. One explanation might be that the wild type *NF1* gene is more vulnerable to mutation. Another hypothesis is that haplo-insufficiency for one or more genes present in the *NF1* microdeletion results in an aspecific growth advantage of different cell types, including Schwann cells. *NF1* microdeletion patients are known to have a general tendency to overgrowth. Thus, children with an *NF1* microdeletion are often relatively tall, and have large hands and feet, and sometimes they even show a real overgrowth phenotype in infancy (van Asperen et al., 1998). Recently Spiegel et al. (2005) reported an advanced childhood height growth in *NF1* microdeletion patients. It can be hypothesized that because of an aspecific growth advantage of cells with an *NF1* microdeletion, the Schwann cells of subclinical neurofibromas could grow at a faster pace and hence give rise to more visible tumors at a given age. Therefore, the number of visible tumors might be higher in *NF1* microdeletion patients because of a faster growth rate of the tumors. Aside from the overgrowth phenotype observed in *NF1* microdeletion patients, we do not have additional arguments for this hypothesis.

In conclusion, we have demonstrated that a significant difference exists in the somatic inactivation mechanisms of *NF1* in neurofibromas of *NF1* microdeletion versus non-microdeletion patients. Hence, it is clear that both patient groups differ not only at the phenotypic (different average tumor burden) and the constitutional level (presence/absence of a microdeletion), but also at the somatic level (LOH as rare/frequent mechanism of *NF1* inactivation). This new insight might open new avenues for a better understanding of the genetic basis underlying the high tumor burden of *NF1* microdeletion patients.

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Notes & Tips

Real-time quantitative allele discrimination assay using 3′ locked nucleic acid primers for detection of low-percentage mosaic mutations

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Although fluorescent in situ hybridization (FISH)[1] and loss of heterozygosity (LOH) analysis can pick up mosaicism for gross chromosomal rearrangements relatively easily, detection of small mosaic lesion mutations remains technically challenging. Moreover, the presence of highly homologous pseudogenes throughout the human genome may complicate minor lesion mutation analysis at the genomic DNA (gDNA) level. In an attempt to detect low-percentage mosaicism for point mutations against a background of normal and pseudogene alleles, we have developed a nested real-time quantitative PCR (qPCR) assay taking advantage of 3′ locked nucleic acid (LNA) allele-specific PCR (AS-PCR) primers and the cost-effective SYBR Green I detection chemistry.

One conceptually simple strategy to detect single-base substitutions is AS-PCR, which is based on positioning the 3′ base of a PCR primer to match one variant allele [1]. Over the years, a number of strategies have been developed to improve the specificity and reliability of primers in AS-PCR. Among these are the incorporation of additional mismatches near the 3′ end [2] and the use of high-affinity DNA analogues such as LNAs [3,4]. Although AS-PCR assays provide an elegant method to discriminate between alleles, accurate quantification of the variants is not attainable because of the intrinsic endpoint detection by conventional PCR methods. This limitation is fully addressed using the real-time qPCR method, whereby PCR product accumulation is monitored at each PCR cycle by means of fluorescent detection. In this article, we evaluate the discriminating power of different AS-PCR primers on a real-time qPCR platform. As a model, we use various tissues and cell types derived from a segmental neurofibromatosis type 1 (NF1, MIM 162200) patient.

To determine the *NF1* mutation underlying the segmental phenotype, selectively cultured Schwann cells [5] derived from peripheral nerve sheath tumors were screened by a highly sensitive *NF1* mutation detection cascade as described previously [6]. A nonsense mutation (c.2041C > T (p.R681X)) was revealed in *NF1* exon 13. The region spanning the point mutation was amplified, and PCR products were cloned in the pCR2.1-TOPO vector (Invitrogen). The wild-type and mutant inserts were confirmed by gDNA sequencing on an ABI3730XL using BigDye chemistry (Applied Biosystems). Cloned fragments were reamplified and purified (MSB Spin PCRapace, Invitek), and concentrations were determined using the PicoGreen double-stranded DNA (dsDNA) Quantification Reagent (Molecular Probes) on a TD-360 fluorometer (Turner Designs).

The mutant allele-specific qPCR assay was optimized to achieve a maximal discriminating power between mutant and wild-type alleles, evaluated as the difference in the cycle threshold (C_t) values of mutant amplification products between matched (mutant-specific) and mismatched (wild-type-specific) primers while preserving high amplification efficiency. Real-time qPCR reactions were performed on an iCycler iQ instrument (Bio-Rad) using a 1× SYBR Green I Master Mix (Eurogentec), 250 nM of primers, 10 nM fluorescein, and 5×10^3 molecules of mutant plasmid input. Primers were designed with the freely available Primer 3

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[1] *Abbreviations used:* FISH, fluorescent in situ hybridization; LOH, loss of heterozygosity; gDNA, genomic DNA; qPCR, quantitative PCR; LNA, locked nucleic acid; AS-PCR, allele-specific PCR; NF1, neurofibromatosis type 1; dsDNA, double-stranded DNA; C_t, cycle threshold.

web tool (Whitehead Institute, http://frodo.wi.mit.edu/cgi-bin/primer3) and consisted of a common forward primer (5′-tcttccacccttgactctca-3′) together with an AS-PCR reverse primer, resulting in an 82-bp amplicon. We evaluated the following six AS-PCR reverse primers for their discriminating power: a wild-type or mutant DNA primer with an additional 3′ subterminal mismatch (underlined) (5′-ctagtttggtctgggcttgttg/a-3′) and two wild-type or mutant 3′ LNA (bold) primers (5′-ctagtttggtctgggcttgtc**g/a**-3′ and 5′-ctagtttggtctgggcttgtt**g/a**-3′). Although the mutation itself dictates the choice of the 3′ terminal nucleotide in the primers, the nucleotide at the penultimate position was chosen based on the previously observed reduced amplification efficiency when thymidine occupies this primer position [7]. The thermal profile consisted of 1 cycle at 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s and at 61 °C for 1 min. After PCR amplification, a melting curve was generated to check the specificity of the PCR reactions (absence of primer–dimers or other nonspecific amplification products). Data acquisition and automated analysis were done by the iCycler iQ software (version 3.1, Bio-Rad). Real-time PCR results in Fig. 1 illustrate that the differences in C_t value (ΔC_t) between matched and mismatched primers are 0.3 for the DNA primer (with 3′ subterminal mismatch), 1.5 for the 3′ LNA primer, and 6.8 for the 3′ LNA primer with 3′ subterminal mismatch. Clearly, the introduction of the 3′ subterminal primer:template mismatch shifts C_t values toward higher values but also significantly increases the discriminating power of the 3′ LNA primer.

Equimolar dilutions of wild-type and mutant plasmid PCR fragments were used to generate standard curves of 5 log-10 orders of magnitude. To enhance linearity and reproducibility, dilutions were made in a 10-ng/μl λ DNA-containing carrier solution. Quantification of mutant alleles in the presence of the wild-type form was tested by mixing an excess (5×10^5 molecules) of wild-type allele with a 5-point 10-fold dilution series of the mutant form (5×10^5–50 molecules). As a control, a standard curve containing only the mutant allele was used. Ideally, both series should result in overlapping amplification plots. We observed that at low levels of the mutant allele (<2500 molecules), the excess of the wild-type allele (5×10^5 molecules) significantly impaired accurate quantification of the low-abundant alternative allele (Fig. 2). Therefore, the sensitivity of the quantitative assay is estimated at 1/200 (mutant allele vs. wild-type allele).

Comparative analysis revealed that the *bona fide NF1* mutation under study (c.2041C > T) is present as a variant in at least three *NF1* pseudogenes. To exclude interference of *NF1* pseudogenes with the quantification of the *bona fide NF1* mutation, all unknown samples were amplified with *NF1*-specific exon 13 primers prior to nested real-time qPCR. The first-round amplification was performed on a PTC-200 Thermal Cycler (MJ Research) using 10 ng gDNA input and a PCR touch-down program starting at 61 °C gradually reduced (1 °C /cycle) to 52 °C for an additional 30 cycles. Because both wild-type and mutant alleles are amplified from the same *NF1* exon 13 amplicon, the initial proportional representation of both alleles is preserved. The first-round amplification products were purified with Exonuclease I and Shrimp Alkaline Phosphatase (USB) and were diluted (1/100,000) in a 10-ng/μl λ DNA-containing carrier solution.

Nested real-time quantitative PCRs were performed on an iCycler iQ instrument. In each experiment, duplicates of a standard dilution series of specific PCR fragments for each allelic variant and triplicates of 2 μl of unknown sample template were amplified in a 15-μl reaction containing 1× SYBR Green I Master Mix and 250 nM of allele-specific primers. The thermal profile was as described above. Each experiment was performed twice, and data acquisition and automated analysis were done by the iCycler iQ software (version 3.1). The relative number of molecules of

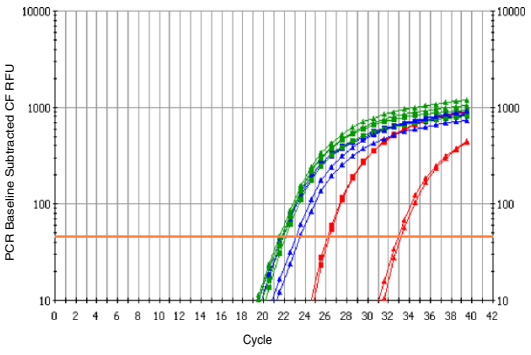

Fig. 1. Amplification plots of mutant plasmid using different AS-PCR primers: mutant (rectangle) and wild-type (triangle) DNA primer (green), 3′ LNA primer (blue), and 3′ LNA primer with 3′ subterminal mismatch (red) (replicates are shown). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

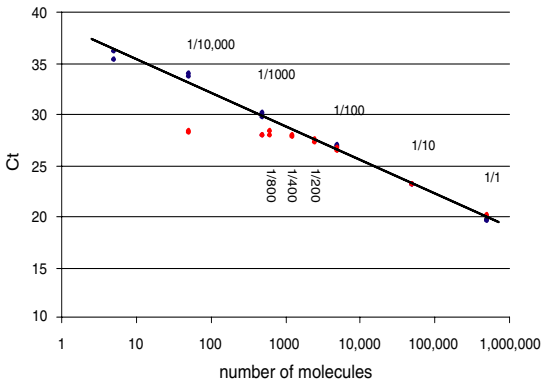

Fig. 2. Quantification of mutant alleles in the presence of the wild-type form. An excess (5×10^5 molecules) of wild-type allele was mixed with a dilution series of the mutant form (red dots). As a control, a standard curve containing only the mutant allele was used (blue dots). At low levels of the mutant allele (<2500 molecules or <1/200), the presence of the wild-type allele significantly impaired accurate quantification of the low-abundant alternative allele, as illustrated by lower C_t values for the red dots. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

each allele was determined by interpolating the C_t values of the unknown samples to each standard curve, followed by determination of the fraction of mutant alleles. Quantitative results of both experiments were highly concordant (see Table 1 in Supplementary material), showing mutant alleles in 1.41–47.40% of various tissues of the NF1 segmental patient.

In conclusion, we have described a real-time quantification strategy for the detection of low-percentage mosaic point mutations. We evaluated the discriminating properties of different AS-PCR primers and demonstrated that the use of a 3′ LNA primer with an artificial mismatch at the 3′ subterminal position had the largest discriminating power. To our knowledge, our method represents the first quantitative allele discrimination assay taking advantage of 3′ LNA AS-PCR primers and SYBR Green I detection chemistry, allowing accurate, sensitive, and cost-effective quantification of single-nucleotide changes. The presented methodology offers opportunities for research and molecular diagnostic applications where quantification of two DNA sequences that differ by only one nucleotide is desired. Examples include population SNP genotyping, mutation detection, and risk assessment of disease transmission to offspring by mosaic patients.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ab.2006.07.039.

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Molecular dissection of isolated disease features in mosaic NF1 patients

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In preparation

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Abstract

Mosaic neurofibromatosis type 1 (NF1) can present as mild generalized disease, segmental disease or gonadal mosaicism. Here we show that different clinical subtypes emerging within mosaic NF1 result from a postzygotic *NF1* mutation in neural crest derived cell types. The study of the affected cell types provides important insight into developmental concepts underlying particular NF1-related disease features. Four mosaic NF1 patients with different clinical manifestations (neurofibromas only, pigmentary changes only, and association of both symptoms) were investigated at the molecular level. For each patient various tissues and cell types were tested with quantitative assays capable of detecting low-percentage *NF1* mutations. This approach confirmed the bi-allelic inactivation of Schwann cells in neurofibromas and demonstrated for the first time bi-allelic *NF1* inactivation in melanocytes in NF1-related café-au-lait macules. Interestingly, both disease features appear to arise even within a background containing predominantly *NF1* wild-type cells. This report provides molecular evidence that the mosaic NF1 phenotype reflects the embryonic timing and accordingly the neural crest derived cell type involved in the somatic *NF1* mutation.

Introduction

Neurofibromatosis type 1 (NF1, [MIM 162200]) is a common autosomal dominant disorder caused by alterations in the *NF1* gene. The *NF1* encoded protein, neurofibromin, functions as a negative regulator of Ras mediated signaling [1, 2, 3]. The primary clinical features of NF1 are café-au-lait macules (CALMs), freckling and benign peripheral nerve sheath tumors or neurofibromas [4]. NF1 patients also have a predisposition to develop a wide spectrum of other symptoms, illustrating the critical function of neurofibromin in a variety of tissues and cell types. The biological context underlying the development of many NF1-related symptoms, however, remains largely unknown.

Mosaic NF1 is caused by a postzygotic *NF1* lesion [5, 6, 7] and can present as mild generalized disease, segmental disease or gonadal mosaicism [8]. The mosaic phenotype most probably reflects the timing of and tissues involved in the somatic mutation. Since segmental NF1 is characterized by the regionally limited distribution of NF1 diseases signs, it provides the opportunity to study cell populations differing only with regard to the mutation(s) giving rise to mosaicism. Determining when and in what cell types inactivation of the *NF1* gene occurs is critical for understanding the basic pathology of NF1-related symptoms.

In this report, we investigated one mildly affected and three segmental NF1 patients with different clinical manifestations, i.e. neurofibromas only, pigmentary changes only, and combination of both neurofibromas and pigmentary changes. In order to elucidate the involvement of particular cell types and mutational mechanisms in the respective phenotypes, we investigated various tissues and cell types derived from every mosaic patient with quantitative assays capable of detecting low-percentage *NF1* mutations.

Material and Methods

Patient material

Four mosaic NF1 patients with distinct clinical manifestations were included in the study.

Neurofibromas only

Patient SNF1-1 is a 46 years old female and has several small cutaneous neurofibromas within a limited body region on the trunk. Her two sons (20 and 23 years old) don't show any NF1-related symptoms. Peripheral blood from the three individuals, together with buccal smears, hair roots, urine and Schwann cells and fibroblasts cultured from three neurofibromas of the female patient were available for analysis.

Pigmentary defects only

Patient SNF1-2 is a 23-year old male presenting with several CALMs within a background of hyperpigmented skin involving the entire right leg, hip and lower back (Figure 1A). Peripheral blood, buccal smears, hair roots and urine together with fibroblasts and melanocytes cultured from normal skin, CALM and the hyperpigmentation area were available for analysis.

Patient SNF1-3 is a 37 year old female with freckling in the groin. Her both daughters (3 and 5 year old) present with full-blown NF1. Peripheral blood from the three individuals together with buccal smears, hair roots and urine of patient SNF1-3 were available for analysis.

Pigmentary defects and neurofibromas

Patient SNF1-4 is a 15-year old female with more than six CALMs scattered over the body and several small neurofibromas located on the right hand within a hyperpigmented background (Figure 1B). Peripheral blood, buccal smears, hair roots and urine, together with Schwann cells and fibroblasts derived from one neurofibroma and fibroblasts and melanocytes derived from the hyperpigmentation area (right hand), two CALMs (left thigh and right lower back) and normal skin (right buttock) were available for analysis. Magnetic resonance imaging (MRI) of the patient's right hand revealed a subcutaneous nodule with a few smaller surrounding subcutaneous satellite lesions. Histopathologically, the lesions arising within the hyperpigmentation area were determined as neurofibromas due to the absence of mitotic activity and the mixture of elongated spindle shaped Schwann cells and fibroblasts in a background of wavy collagenous fibers. This diagnosis was confirmed by immunohistochemistry (S100 positivity in the majority of spindle shaped cells).

Cell culture

Skin biopsy

A biopsy of normally pigmented and/or hyperpigmented skin was taken using 5 mm punch biopsy excision. To separate the epidermal layer (with melanocytes anchored to the basal membrane) from the underlying dermis (with fibroblasts), skin biopsies were incubated overnight at 4 °C in dispase II (Boehringer Mannheim). Melanocytes were cultured in Ham's F10 medium (Gibco, Invitrogen Ltd) supplemented with 2.5 % fetal calf serum (FCS), 1 % Ultroser, 5 ng/ml basic fibroblast growth factor, 10 ng/ml endohelin-1, 0.33 nM cholera toxin, 0.033 mM isobutyl-methyl-xanthine, 5.3 nM 12-

O-tetradecanoyl phorbol-13-acetate and 20 ng/ml stem cell factor. Dermal fibroblasts were grown in DMEM medium (Gibco, Invitrogen Ltd) supplemented with 10% FCS.

Neurofibroma

Culture conditions for neurofibroma-derived Schwann cells and fibroblasts were as previously described [9, 10]. The presence of forskolin in the Schwann cell medium (SCM) promotes proliferation of cells bearing only the first hit (*NF1*$^{+/-}$, SC F+). Replacement of proliferation medium by serum-free N_2 medium and subsequently by proliferation medium without forskolin promotes proliferation of cells containing both hits (*NF1*$^{-/-}$,SC F-). To estimate the purity of Schwann cell and fibroblast cultures derived from the neurofibroma of patient SNF1-4, immunofluorescence staining with rabbit S100 primary antibody (Dako) was performed as previously described [11]. For one SC F- culture (patient SNF1-1, neurofibroma 3), Schwann cells were separated from contaminating fibroblasts by using p75 (nerve growth factor receptor)-coupled MACS® Microbeads (Miltenyi Biotec) which magnetically label the Schwann cell target population. Subsequent growth of recovered cells in SCM resulted in a highly pure Schwann cell culture (>95%, estimated by S100 staining).

NF1 mutation screening

NF1 mutation analysis (GenBank reference sequence NM_000267.1) was performed essentially as described [12]. Briefly, genomic DNA (gDNA) was extracted from melanocyte cultures with the QiaAmp procedure (Qiagen) and from all other cell cultures with the Puregene procedure (Gentra). Cultures were treated with puromycin (200 µg/ml, 4-6 hours) before RNA extraction (RNeasy kit, Qiagen). The entire *NF1* complementary DNA (cDNA) was sequenced using the ABI3730XL genetic analyser (Applied Biosystems). All mutations found at the cDNA level were confirmed on gDNA by cycle sequencing. Multiplex ligation-dependent probe amplification (MLPA) analysis was performed using the SALSA NF1 area kit (MRC Holland) following the manufacturer's instructions to detect deletions.

Fine mapping *NF1* deletion breakpoints

The genomic deletions detected by MLPA analysis were evaluated in further detail. The location of the *NF1* multi-exon deletion breakpoints was determined by long-range PCR with primers residing in the segments adjacent to the deleted interval. Amplification products were subsequently sequenced by using the ABI3730XL genetic analyzer (Applied Biosystems). The location of the *NF1* microdeletion breakpoints was determined by aspecifically amplifying and sequencing paralogous sequence variants (PSVs) in the low copy repeats (LCRs) flanking the *NF1* microdeletion region (De Raedt et al., in press). By scoring the relative intensity of both nucleotides of the PSV, the location of the breakpoint was determined: centromeric (higher relative intensity of the nucleotide specific for the telomeric LCR) or telomeric (higher relative intensity of the nucleotide specific for the centromeric LCR) of the PSV investigated.

Loss of heterozygosity *NF1* region

Loss of heterozygosity (LOH) in the *NF1* gene region was evaluated by genotyping 2 microsatellite markers telomeric (3'NF1-3, 3'NF1-1) [13] and 4 within the *NF1* gene (Alu, IVS27AC33.1, IVS38GT53.0, IVS27TG24.8) [14, 15, 16, 17]. Genomic DNA from paired melanocyte/Schwann cell and lymphocyte cultures of the same patient was investigated by touch-down PCR for the microsatellite markers and subsequently analyzed on the ABI3130XL genetic analyser (Applied Biosystems) with the GeneMapper software Version 3.7 (Applied Biosystems). To determine the extent of LOH in different samples from patient SNF1-4, additional SNPs proximal (rs6505129, rs6505165 and rs8071580) and distal (rs753750 and rs9904537) to the *NF1* microdeletion region were evaluated as previously described [18]. LOH for a SNP was scored when the average ratio (SNP nucleotide/ control nucleotide) of the two alleles in the tested tissue fell outside the 95% confidence interval of the ratios observed in control blood DNA of the same patient and when the average ratios in tested tissue versus control blood were at least 20% different. The mechanism underlying LOH (deletion *versus* mitotic recombination) was evaluated by semi-quantitative PCR taking advantage of the amplification of *NF1* exon 22 (103 bp) together with the corresponding fragment of its pseudogene located on chromosome 15 (107 bp) as previously described [18].

Cloning *NF1* point mutations

In order to determine whether both *NF1* mutations detected in SCF- cultures derived from the neurofibromas of patient SNF1-1 resided on different alleles, cloning experiments were performed. For neurofibroma 1 (*NF1* c.2041C>T and c.1655T>G), a fragment containing both alterations and an additional SNP in exon 13 (rs2285892) was amplified (516 bp), cloned in the pCR2.1-TOPO Vector (Invitrogen) and sequenced. For neurofibroma 2 (*NF1* c.2041C>T and [c.603T>C; 604_621del]), a fragment containing the deletion and a SNP in exon 5 (rs1801052) was amplified (286 bp), cloned and sequenced. Since rs1801052 and rs2285892 are in complete linkage, information on the genotype of the SNP in exon 13 linked with the 1st hit (cloning experiment neurofibroma 1) and information on the genotype of the SNP in exon 5 linked with the 2nd hit (cloning experiment neurofibroma 2), provides information on the fact whether both mutations reside on the same (exon 5/exon13: A/G or G/A) or different (exon5/exon13: A/A or G/G) haplotypes.

Quantification of *NF1* mutations

Real-time quantitative PCR

In order to detect low-percentage mosaicism for *NF1* point mutations against a background of normal and pseudogene alleles, a nested real-time quantitative PCR (qPCR) assay was developed [19]. Briefly, the region spanning the *NF1* point mutation was amplified (primer sequences available upon request) and equimolar dilutions of cloned PCR fragments (wild-type and mutant alleles) were used to generate standard curves of 5 orders of magnitude. For actual quantification, allele-specific 3' locked nucleic acid (LNA) primers (Eurogentec) were used. Since both somatic *NF1* point mutations appeared to be present in several *NF1* pseudogenes, samples were amplified with *NF1* specific primer pairs and diluted prior to nested real-time qPCR. In order to detect low-percentage

mosaicism for *NF1* multi-exon deletions, a real-time qPCR assay using deletion-specific primers was developed. Briefly, a primer pair situated in the deletion specifically amplified the wild-type allele and a second breakpoint overlapping primer pair specifically amplified the mutant allele. Equimolar dilutions of both PCR fragments were used to generate standard curves of 5 orders of magnitude.

Real-time qPCR reactions were performed on an iCycler iQ instrument (Bio-Rad). In each experiment, duplicates of a standard dilution series of specific PCR fragments for each allele variant (wild-type and mutant) and triplicates of 10 ng DNA of unknown samples (different tissues from segmental NF1 patient under study and non-NF1 control sample) were amplified in a 15µl reaction containing 1 x SYBR Green I Master Mix (Eurogentec) and 250 nM of allele-specific primers. The thermal profile consisted of 1 cycle at 95°C for 10 minutes followed by 40 cycles at 95°C for 15 seconds and at 61°C (c.2042C>T), 63°C (c.2325+1G>A) or 60°C (c.1783_2000+205del422) for 1 minute. Each experiment was performed twice and data acquisition and automated analysis was done by the iCycler iQ software version 3.1 (Bio-Rad). The relative number of molecules of each allele was determined by interpolating the Ct values of the unknown samples to each standard curve, followed by the determination of the fraction of mutant alleles (number of mutant molecules divided by sum of number of wild-type and mutant molecules).

FISH analysis

To determine submicroscopic *NF1* deletions, dual color FISH was performed [20] using PAC clones 22 (926B9; 5'NF1) and 13 (1002G3; 3'NF1) [13]. To investigate mosaicism, at least 100 interphase nuclei were evaluated.

Results

Neurofibromas only (patient SNF1-1)

NF1 mutation screening on selectively cultured Schwann cells (SC F-) derived from two neurofibromas revealed an identical mutation (c.2041C>T (p.R681X)) in each tumor in addition to two tumor specific alterations (c.1655T>G (p.L552R) and [c.603T>C; c.604_621del], respectively). Cloning experiments demonstrated that, for the two tumors, both *NF1* mutations resided on different alleles. Quantification of mutant transcripts in the presence of the wild-type form was tested by mixing an excess ($5x10^5$ molecules) of wild-type allele with a 5 point 10-fold dilution series of the mutant form ($5x10^5$ – 50 molecules). As a control, a standard curve containing only the mutant transcript was used. Ideally, both series should result in overlapping amplification plots. We observed, however, that at low levels of mutant transcript (<2500 molecules), the presence of the wild-type transcript ($5x10^5$ molecules) significantly impaired accurate quantification of the low-abundant alternative transcript. Therefore, the sensitivity of the quantitative assay is estimated at 1/200. Real-time qPCR demonstrated the presence of the first hit (percentage mutant allele ± standard error of the mean (SEM)) in EBV transformed white blood cells (3.7±1.1%), hair follicles (1.8±0.5%), fibroblasts derived from both neurofibromas (8.4±1.6% and 19.4±7.9%, respectively) and selectively cultured Schwann cells from a third smaller neurofibroma (6.7±2.5%). Buccal smears, urine and fibroblasts derived from the third neurofibroma were negative as well as blood from both children of the patient and the control sample (Table 1).

Pigmentary defects only

Patient SNF1-2

Combined *NF1* cDNA sequencing and MLPA analysis revealed an *NF1* microdeletion exclusively present in the melanocytes derived from CALMs and background hyperpigmentation area. A second alteration (c.1226_1227del) was only detected in the melanocytes of the CALM. Further characterization of the microdeletion revealed an atypical deletion with the proximal breakpoint residing within the centromeric LCR flanking the *NF1* gene and the distal breakpoint located centromeric from the telomeric LCR flanking the *NF1* gene before *JJAZ1* exon 7 (minimum size of the deletion 1,30 Mb). FISH analysis of skin fibroblasts (CALM, hyperpigmented and unaffected skin) and blood lymphocytes with *NF1* specific probes revealed low percentages of mosaicism (<5%) for the *NF1* microdeletion (Table 2).

Patient SNF1-3

MLPA analysis on peripheral blood of one of the daughters of patient SNF1-3 pointed to an *NF1* multi-exon deletion (including exons 12a and 12b) at the genomic level. Long-range PCR confirmed the presence of a ~2kb deletion. Further characterization by sequencing localized the deletion breakpoints in exon 12a (c.1783) and intron 12b (c.2000+205) with additional bases at the deletion junction. Excess wild-type transcript did not influence quantification of low-abundant mutant transcript since identical amplification plots were obtained for mutant transcript and mutant transcript with excess wild-type fragment standard curves. Real-time quantitative PCR

demonstrated that the mutant allele was present in peripheral blood of both daughters with full-blown NF1 (44.9±12.7% and 47.8±15.0%, respectively). The *NF1* mutation was also detected in a low percentage of cells derived from different tissues from the mother: peripheral blood (1.5±0.6%), buccal smear (2.8±1.0%), urine (3.8±1.7%) and hair (7.2±2.9%), while the control sample was negative (Table 3).

Pigmentary defects and neurofibromas (patient SNF1-4)

Analysis of neurofibroma-derived Schwann cells (SC F+ and F-) and melanocytes derived from both the hyperpigmentation area and two CALMs remote from that zone revealed an identical *NF1* mutation (c.2325+1G>A) leading to out-of-frame skipping of exon 14 and deletion of the wild-type *NF1* allele. For all samples, the proximal deletion breakpoint resided between rs6505129 (chromosome 17 reference position 24777972, NCBI build 36.2) and rs6505165 (position 25598975), while the distal deletion breakpoint resided between the most distal PSV in the telomeric *NF1* LCR (position 27439522) and rs9904537 (position 27579216) (minimum/maximum size of deletion 1.84 Mb/2.80 Mb). To test the influence of excess wild-type transcript on detection of the mutant transcript (c.2325+1G>A), a 5 point 10-fold dilution series of the mutant form ($5x10^5$ – 50 molecules) was mixed with excess wild-type ($5x10^5$ molecules). Equal mounts of water (instead of wild-type transcript) were added to a second series of dilutions. Comparison of both amplification plots revealed that at low levels of mutant transcript (<1250 molecules), the presence of the wild-type transcript significantly impaired accurate quantification of the low-abundant alternative transcript. Therefore, the sensitivity of the quantitative assay is estimated at 1/400. Real-time qPCR demonstrated the presence of the intragenic *NF1* mutation (percentage mutant allele ± SEM) in fibroblasts derived from both the neurofibroma (12.3±6.9%) and hyperpigmented area (2.0±0.6%). Peripheral blood, melanocytes and fibroblasts derived from normal skin, fibroblasts derived from both CALMs, buccal smear, urine and the control sample were negative. As expected, the mutation could not be detected in peripheral blood from the parents and siblings of the patient (Table 4). In order to pick up low-percentage mosaicism for the *NF1* deletion, we are planning FISH experiments on skin fibroblasts (CALM, hyperpigmented and unaffected skin) and blood lymphocytes with *NF1* specific probes. Immunofluorescence staining revealed S100 positive cells in the SC F- (>95%), SC F+ (>95%) and fibroblast (~10%) cultures derived from the neurofibroma.

Discussion

In this report, four mosaic NF1 patients with different clinical manifestations were investigated at the molecular level to provide insight into the cell types and mutational mechanisms involved in the development of particular NF1-related disease features.

Neurofibromas only (patient SNF1-1)

In selectively cultured Schwann cells (SC F-) derived from two neurofibromas, an identical *NF1* mutation in addition to two tumor specific alterations on the other allele were detected clearly confirming the tumor-initiating properties of Schwann cells in neurofibroma development [9, 21, 22]. Quantitative mutation screening of fibroblasts derived from both neurofibromas revealed only low percentages of mutant -first hit- allele (8.4±1.6% and 19.4±7.9%, respectively), undetectable by conventional mutation detection techniques (i.e. PCR *NF1* exon 13 and subsequent sequencing). Similarly, Shultz et al. (2000) [23] could not demonstrate any *NF1* mutation in neurofibroma-derived fibroblasts from a segmental NF1 patient by use of the protein truncation test, enzymatic mutation detection and FISH. Remarkably, only a limited amount of mutant allele (6.7±2.5%) was detected in selectively cultured Schwann cells (purity estimated at >95%) from a third smaller tumor, further illustrating the high abundance of $NF1^{+/+}$ cells in the tumor microenvironment. A growing body of experimental evidence supports the idea that *NF1* haploinsufficiency in the tumor environment promotes (plexiform) neurofibroma formation in mice [24, 25, 26, 27, 28]. Haploinsufficient mast cells, for example, have been shown to be hypermigratory and to have increased survival and proliferation potential in response to stem cell factor (SCF), secreted by $Nf1^{-/-}$ Schwann cells [25, 27]. Whether the abundance of *NF1* wild-type cells in (dermal) neurofibromas from segmental NF1 patients reflects a slightly different pathogenesis or, alternatively, is associated with the small size of these patients' tumors (heterozygous neighboring cells may promote tumor growth more efficiently than wild-type neighboring cells) remains an important question.

Pigmentary defects only (patient SNF1-2)

Combined *NF1* cDNA sequencing and MLPA analysis revealed an *NF1* microdeletion exclusively present in melanocytes derived from affected skin of patient SNF1-2 (Table 2). Although a high frequency of mosaic *NF1* microdeletions are caused by somatic recombination of the *JJAZ1* gene [29], the current *NF1* lesion does not represent a typical type II deletion. A second alteration (c.1226_1227del) was only present in melanocytes derived from the CALM, not in the hyperpigmented background area. Previously, Eisenbarth et al. (1997) [30] did not detect somatic *NF1* inactivation in melanocytes cultured from 10 CALMs of patients with classical NF1. The discrepancy with the current data can probably be explained by different melanocyte culture conditions and by the fact that, before, the cells were only analyzed for LOH at the *NF1* locus whereas we performed comprehensive *NF1* mutation screening. Available evidence suggests that paracrine cytokines interacting between epidermal melanocytes and non-melanocytic cells in the skin play a central role in epidermal hyperpigmentation (reviewed in [31]). Although the

etiopathogenesis of NF1-related pigmentary lesions remains largely unknown, it has been postulated that the mechanism of epidermal hyperpigmentation and mast cell infiltration in NF1 CALMs might be associated with increased secretion of cytokines such as SCF and hepatocyte growth factor by dermal fibroblasts [32]. In the same line, De Schepper et al. (2006) [33] reported increased levels of soluble SCF in fibroblast supernatant from NF1 CALM but, importantly, also in NF1 normal skin compared with control individuals. In other words, paracrine networks are at play in the NF1 skin, but are not sufficient for CALM development. Here, we demonstrate that bi-allelic *NF1* inactivation in the melanocyte may be required for CALM formation, while even in the haploinsufficient state the *NF1* gene presumably has an effect on skin color. Recently, a significant increase in melanocyte density was demonstrated in NF1 CALM skin compared with NF1 normal skin, control normal skin and control CALM skin [33]. These NF1 CALM melanocytes also display a higher melanin content and melanogenesis [34]. In light of our current findings, it is tempting to speculate that bi-allelic *NF1* inactivation in melanocytes might be the underlying molecular mechanism for the distinct properties of these NF1-related pigmentary lesions. In a next step, it will be essential to determine the dysregulating consequences of neurofibromin loss and *NF1* haploinsufficiency on signaling pathways in melanocytes. Previous studies on segmental pigmentary lesions revealed *NF1* mutations in 9% and 18% of fibroblasts [6, 7] and in 20% of keratinocytes [7] cultured from CALMs. Also in this report, low percentages of the *NF1* mutation were detected in the fibroblasts derived from the hyperpigmentary and CALM skin lesions of patient SNF1-2 (3% and 4%, respectively). Therefore, we speculate that pigmentary lesions can arise within an environment consisting of predominantly *NF1* wild-type cells.

Although a definite association between malignant melanoma and NF1 has not yet been established, one clinical report describes the occurrence of malignant melanoma arising from a pre-existing CALM in an NF1 patient [35]. This may imply a model of multi-step tumor progression, a genetic scenario previously demonstrated for the plexiform neurofibroma – malignant peripheral nerve sheath tumor sequence. Stepwise transformation from melanocytes to melanoma has been described previously for non-NF1 related melanomas [36]. While *BRAF* and *NRAS* mutations occur at the stage of the benign nevus, additional molecular lesions in *CDKN2A* and *PTEN* are required for the progress towards malignancy. Interestingly, *BRAF* and *NRAS* mutations activate the MAP-Kinase pathway, an effect which can also be caused by *NF1* inactivation. Therefore, the question emerges whether, instead of *BRAF* and *NRAS* mutations, *NF1* inactivation in NF1-related CALMs might act as an alternative mechanism to activate the MAP-Kinase pathway. Interestingly, double inactivation of the *NF1* gene was recently demonstrated in an early disease stage melanoma arising in a 15-year old NF1 patient [37].

Pigmentary defects and neurofibromas (patient SNF1-4)

In patient SNF1-4 an identical *NF1* mutation (c.2325+1G>A) was revealed in neurofibroma-derived Schwann cells and melanocytes derived from both the hyperpigmentation area and two CALMs remote from that zone. Moreover, all samples showed deletion (1.84 Mb-2.80 Mb) of the wild-type *NF1* allele. Currently, we are further pinpointing the deletion breakpoints in these samples by using

high resolution techniques. Given the fact that melanocytes and Schwann cells can arise from a bipotent glial-melanocyte precursor [38], it is tempting to speculate that one or both *NF1* mutations occurred in a common neural crest precursor (Figure 2). The clinical picture of neurofibromas arising within a hyperpigmented background might be compatible with this hypothesis. In other words, loss of heterozygosity for the underlying *NF1* mutation in an early developmental stage (e.g. bipotent glial-melanocytic precursor [38]) might be responsible for the segmental involvement of epidermis (melanocyte) and several nerve branches (Schwann cell). Unexpectedly, the *NF1* point mutation was also detected in neurofibroma-derived fibroblasts (culture passage 2), albeit at a significant lower frequency (12.3±6.9%). Immunofluorescence staining demonstrated that the latter observation most probably can be explained by the presence of S100 positive cells (Schwann cells and/or melanocytes) in the fibroblast cultures. It is well known that different neurofibromas from one NF1 patient bear different second hits (patient SNF1-1 and [11]) and arise during the second decade of life [4]. Strikingly, both inactivating *NF1* mutations in the melanocytes derived from the hyperpigmentation zone (right hand) and two CALMs remote from that area (left thigh and right lower back) seem to be identical. This finding is particularly intriguing with respect to the typical congenital appearance of many NF1 CALM lesions. It remains to be explored whether the early presentation of pigmentary NF1 signs could be attributed to bi-allelic *NF1* inactivation in melanocytic precursors (melanoblasts) during embryonic development (Figure 2). Melanoblasts migrate in mice from the neural crest dorsolaterally and enter the skin where they proliferate clonally and finally differentiate into mature skin melanocytes [39, 40]. One might assume that neurofibromin loss in melanoblasts will result in enhanced proliferation and, hence, increased melanocyte density [33] in NF1-related CALM lesions. It will be essential to molecularly dissect more CALM lesions derived from different areas of the body and to determine the (presumably different) second hit in NF1 clinical features arising only later in life.

Mosaic transmission (patient SNF1-3)

A somatic mutation may affect somatic cells only (somatic mosaicism), gonadal cells only (gonadal mosaicism) and a proportion of both cell types (gonosomal mosaicism). The clinical picture of patient SNF1-3 (freckling only) and her both daughters (full-blown NF1) together with the quantitative data (Table 3) illustrate that this patient is a gonosomal mosaic. Clearly, and as opposed to previous animal work [41], the risk of transmitting disease to offspring is not proportional to the percentage of body area involved. The molecular data obtained from patient SNF1-3, and all other mosaic patients described in this report, irrefutably indicate that the percentage of the *NF1* mutation in non-neural crest derived cells is often so low that it would be missed by routine *NF1* screening. This implies that an accurate diagnosis for mosaic NF1 can only be established by comprehensive screening of those cell populations giving rise to mosaicism, i.e. Schwann cells in neurofibromas and/or melanocytes in pigmentary lesions. This new insight will incontestably facilitate genetic counseling of individuals with mosaic NF1. Screening the relevant cell type in the mosaic NF1 patient can provide a molecular marker useful in the prenatal and presymptomatic diagnostic setting.

In conclusion, we provide for the first time molecular evidence that different NF1 mosaic phenotypes result from a postzygotic *NF1* mutation in neural crest derived cell types. While *NF1* deficiency in Schwann cells is essential for neurofibroma development, bi-allelic *NF1* inactivation in melanocytes seems to be required for NF1-related CALM development. Both disease features arise even within a background containing predominantly $NF1^{+/+}$ cells. These findings provide important insight into developmental concepts underlying NF1-related disease features and open avenues for improved genetic counseling of individuals with mosaic NF1.

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Table 1. Overview of *NF1* mutations revealed by routine mutation detection techniques (Routine) and real-time quantitative PCR (qPCR) in different tissues derived from segmental patient SNF1-1 presenting with neurofibromas only.

(SC F-: Schwann cells grown without forskolin, Fibro: fibroblasts, +: mutation detectable, -: mutation not detectable, qPCR: percentage mutant allele ± standard error of the mean determined by real-time quantitative PCR)

Patient	Sample		*NF1* mutation		
			c.2041C>T (p.R681X)		Second hit
			Routine	qPCR	
SNF1-1	Neurofibroma 1	SC F-	+	41.4±15.1%	c.1655T>G (p.L552R)
		Fibro	-	8.4±1.6%	-
	Neurofibroma 2	SC F-	+	47.4±15.6%	[c.603T>C; c.604_621del]
		Fibro	-	19.4±7.9%	-
	Neurofibroma 3	SC F-	-	6.7±2.5%	-
		Fibro	-	-	-
	Blood		-	3.7±1.1%	-
	Buccal smear		-	-	-
	Urine		-	-	-
	Hair		-	1.8±0.5%	-
Child 1	Blood		-	-	-
Child 2	Blood		-	-	-

Table 2. Overview of *NF1* mutations revealed by routine mutation detection techniques (Routine) and quantitative assays (FISH) in different tissues derived from segmental patient SNF1-2 presenting with pigmentary defects only.

(MC: melanocytes, +: mutation detectable, -: mutation not detectable, FISH: percentage of interphase nuclei with *NF1* microdeletion determined by FISH analysis, NA: no data available)

Patient	Sample		*NF1* mutation		
			NF1 microdeletion		Second hit
			Routine	FISH	
SNF1-2	Café-au-lait spot	MC	+	NA	c.1226_1227del
		Fibro	-	4%	-
	Hyperpigmentation	MC	+	NA	-
		Fibro	-	3%	-
	Unaffected skin	MC	-	NA	-
		Fibro	-	0%	-
	Blood		-	2%	-
	Buccal smear		-	NA	-
	Urine		-	NA	-
	Hair		-	NA	-

Table 3. Overview of *NF1* mutations revealed by routine mutation detection techniques (MLPA) and real-time quantitative PCR (qPCR) in different tissues derived from segmental patient SNF1-3 presenting with freckling only.

(+: mutation detectable, -: mutation not detectable, qPCR: percentage mutant allele ± standard error of the mean determined by real-time quantitative PCR)

Patient	Sample	*NF1* mutation	
		c.1783_2000+205del422	
		MLPA	qPCR
Child 1	Blood	+	44.9±12.7%
Child 2	Blood	+	47.8±15.0%
SNF1-3	Blood	-	1.5±0.6%
	Buccal smear	-	2.8±1.0%
	Urine	-	3.8±1.7%
	Hair	-	7.2±2.9%

Table 4. Overview of *NF1* mutations revealed by routine mutation detection techniques (Routine) and real-time quantitative PCR (qPCR) in different tissues derived from mosaic patient SNF1-4 presenting with neurofibromas and pigmentary defects.

(SC: Schwann cells grown with (F+)/ without (F-) forskolin, Fibro: fibroblasts, MC: melanocytes, +: mutation detectable, -: mutation not detectable, qPCR: percentage mutant allele ± standard error of the mean determined by real-time quantitative PCR, NA: no data available, S100: S100 immunofluorescence data available)

Patient	Sample		*NF1* mutation			
			c.2325+1G>A		deletion *NF1* allele	
			Routine	qPCR		
SNF1-4	Neurofibroma (hand, right)	SC F-	+	88.8±37.5%	+	S100
		SC F+	+	67.2±32.8%	+	S100
		Fibro	+	12.3±6.9%	-	S100
	Hyperpigmentation (hand, right)	MC	+	96.9±75.1%	+	
		Fibro	-	2.0±0.6%	-	
	Café-au-lait spot (thigh, left)	MC	+	71.8±20.8%	+	
		Fibro	-	-	-	
	Café-au-lait spot (lower back, right)	MC	+	54.9±33.6%	+	
		Fibro	-	-	-	
	Normal skin (buttock, right)	MC	-	-	-	
		Fibro	-	-	-	
	Blood		-	-	-	
	Buccal smear		-	-	-	
	Urine		-	-	-	
	Hair		-	NA	-	
Mother	Blood		-	-	-	
Father	Blood		-	-	-	
Brother	Blood		-	-	-	
Sister	Blood		-	-	-	

Figure 1. Illustration clinical subtypes of mosaic NF1 patients

A. Patient SNF1-2 presents with several CALMs (illustrated by asterisks) within a pigmented background involving the entire right leg, hip and lower back.
B. Patient SNF1-4 presents with more than six café-au-lait macules (one depicted on the left) scattered over the body and several small neurofibromas (illustrated by asterisks) located on the hand within a hyperpigmented background (depicted on the right).

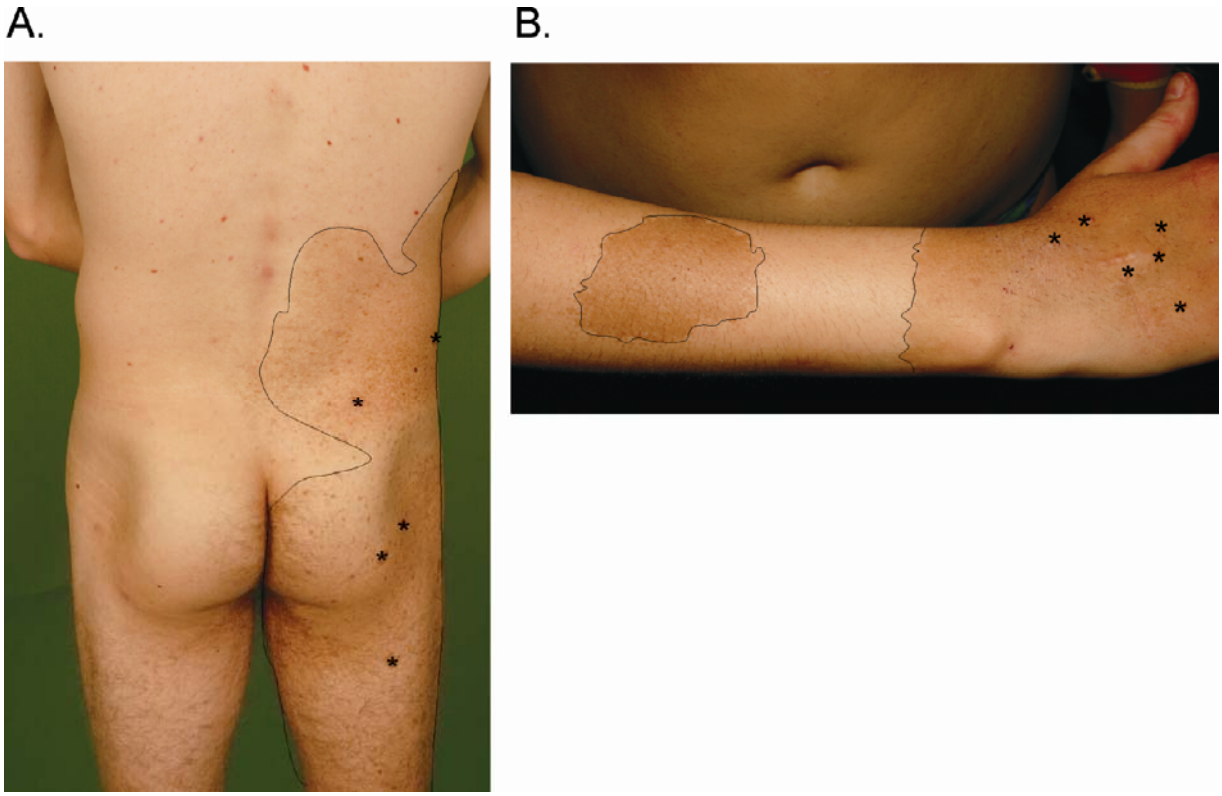
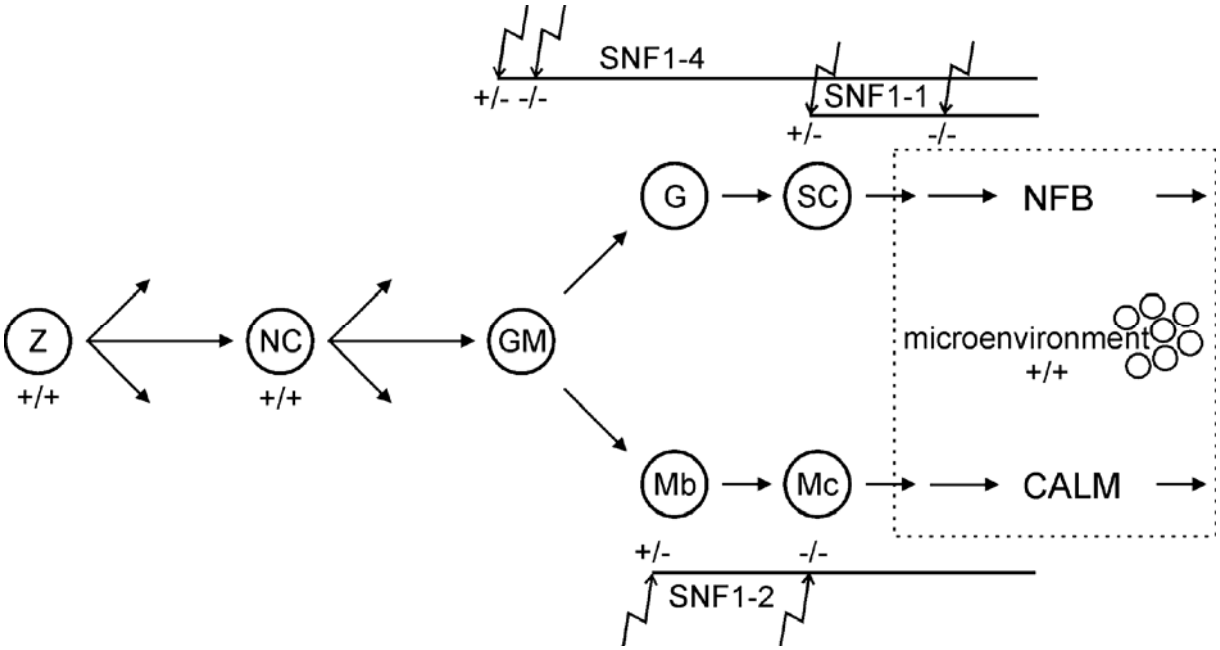


Figure 2. Proposed genetic model for neurofibroma and café-au-lait macule development in mosaic NF1 patients.

The clinical mosaic phenotype reflects the timing and accordingly the cell type involved in the somatic mutation. While mutations occurring in terminally differentiated cells (i.e. Schwann cells or melanoblasts/melanocytes) will give rise to solitary symptoms (i.e. neurofibromas only (patient SNF1-1) or pigmentary lesions only (patient SNF1-2)), somatic alterations earlier in embryonic development (i.e. common precursor of both cell types) will result in associated symptoms (i.e. neurofibomas and pigmentary defects (patient SNF1-4)). Interestingly, both disease features appear to arise even within a background containing predominantly *NF1*$^{+/+}$ cells.

(Z: zygote, NC: neural crest stem cell, GM: glial-melanocytic precursor, G: glial cell, SC: Schwann cell, Mb: melanoblast, Mc: melanocyte, NFB: neurofibroma; CALM: café-au-lait macule)



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Molecular pathogenesis of multiple gastrointestinal stromal tumors in NF1 patients

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Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal tumors of the gastrointestinal tract. *KIT* and *PDGFRA* activating mutations are the oncogenic mechanisms in most sporadic GISTs. In addition to sporadic occurrences, GISTs are increasingly being recognized in association with neurofibromatosis type 1 (NF1), yet the underlying pathogenic mechanism remains elusive. To gain an insight into the mechanisms underlying GIST formation in NF1 patients, we studied seven GISTs from three NF1 patients with a combination of different techniques: mutation analysis (*KIT*, *PDGFRA* and *NF1*), western blotting, array CGH and *ex vivo* imatinib response experiments. We demonstrate that (i) the NF1-related GISTs do not have *KIT* or *PDGFRA* mutations, (ii) the molecular event underlying GIST development in this patient group is a somatic inactivation of the wild-type *NF1* allele in the tumor and (iii) inactivation of neurofibromin is an alternate mechanism to (hyper) activate the MAP-kinase pathway, while the JAK-STAT3 and PI3K-AKT pathways are less activated in NF1-related GIST compared with sporadic GISTs. In conclusion, we report for the first time the molecular pathogenesis of GISTs in NF1 individuals and demonstrate that this type of tumor clearly belongs to the spectrum of clinical symptoms in NF1.

INTRODUCTION

Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal tumors of the gastrointestinal tract (1). They occur predominantly in the stomach (70%) and small intestine (10–20%) and originate from primitive cells with characteristics of the interstitial cells of Cajal (ICCs), the autonomic pacemaker cells which regulate peristalsis in the digestive tract (1–3). Most sporadic GISTs contain gain-of-function mutations in *KIT* (80–85%) (4,5) and strongly express the constitutively activated protein (6). Some GISTs contain gain-of-function *PDGFRA* mutations as an alternate oncogenic mechanism (7,8). Both receptor tyrosine kinases transduce their signals through the downstream PI3K-AKT (9), MAP-kinase (10,11) and JAK-STAT3 signaling cascades (12–16). Therapeutic targeting with the tyrosine kinase inhibitor imatinib (Gleevec, Novartis, Basel, Switzerland) shows a clinical benefit in up to 80% of patients with sporadic GIST (17,18).

GISTs are increasingly recognized in adults with neurofibromatosis type 1 (NF1), an autosomal dominant genetic disorder caused by alterations of the *NF1* gene and affecting 1/3500 individuals worldwide. Neurofibromin, the protein encoded by *NF1*, functions as a GTPase-activating protein for Ras by catalyzing the hydrolysis of active Ras-GTP to an inactive Ras-GDP (19–23). The primary clinical features of the disease are café-au-lait spots, freckling and benign peripheral nerve sheath tumors or neurofibromas (24).

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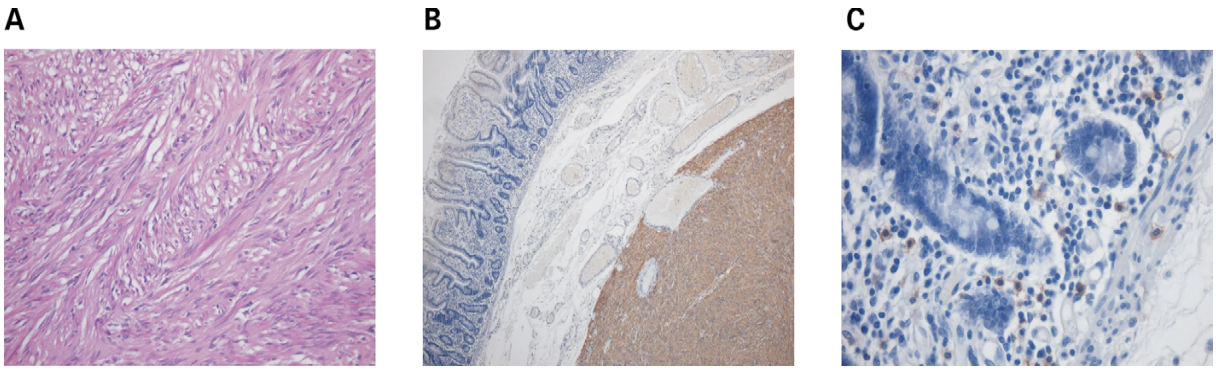

Figure 1. Histopathological analysis of NF1-related GISTs. (**A**) Low power view, showing the spindle cell character of the NF1-related GIST (H&E stain, ×200). (**B**) KIT staining shows strong expression in the tumor (immunoperoxidase stain for CD117, ×50). (**C**) Detail of the mucosa. Only the mast cells are KIT positive (immunoperoxidase stain for CD117, ×350).

Neurofibromin deficiency in neurofibromas results in hyperactivation of the MAP-kinase pathway. On the basis of a single Swedish study of 70 NF1 patients, it is estimated that adults with NF1 might have a risk for GISTs as high as 7% (25). GISTs in NF1 patients tend to be multiple and are located predominantly within the small intestine (26–28). Morphologically and immunohistochemically, GISTs occurring in the NF1 patients are similar to sporadic GISTs (26–29). Little is known about the molecular basis underlying GIST formation in NF1, and whether there is a difference in the molecular pathogenesis of sporadic and NF1-related GISTs. It remains an intriguing question why NF1 patients are at an increased risk for this type of tumor.

RESULTS

Histopathological analysis

The NF1-related intestinal tumors corresponded to bona fide spindle cell type GISTs (Fig. 1A). The tumor cells were strongly positive for CD117 (Fig. 1B and 1C). The mast cells served as positive control for CD117, whereas the other tissue elements of the mucosa and submucosa were negative (Fig. 1B and 1C). There was a variable positivity for alpha smooth muscle actin, CD34 and S-100 protein, whereas desmin was negative.

Mutation analysis

KIT/PDGFRA. Analysis of the entire *KIT* and *PDGFRA* coding region did not reveal any activating mutation in the NF1-related GIST specimens (Table 1). One single nucleotide polymorphism (SNP) in the 3′-UTR region of *KIT* (rs2213181, http://www.ncbi.nlm.nih.gov/SNP) was revealed in all GISTs derived from patient NF1-1. In the GIST of patient NF1-3, one silent polymorphism was found in *KIT* (p.K546K) and five alterations were found in *PDGFRA*: three silent mutations (p.N204N, p.G313G and p.A603A), one 3′-UTR SNP (rs7680422, http://www.ncbi.nlm.nih.gov/SNP) and one missense mutation [c.1825T>C (p.S478P)]. The missense mutation was not predicted to have an impact on protein activity (Polyphen algorithm, http://tux.embl-heidelberg.de/ramensky) and was found in 19/98 (19%) control individuals. All detected variants were also present in DNA extracted from peripheral white blood cells of the respective patients. The detection of heterozygous polymorphisms indicates the presence of both *KIT* alleles in one out of seven tumors and both *PDGFRA* alleles in four out of seven tumors.

NF1. Mutation screening of peripheral blood revealed *NF1* germline mutations in all three patients (Table 1). The somatic alterations in the *NF1* gene region detected in six of the seven GISTs (Table 1) were three nonsense mutations, one donor splice site mutation, a multi-exon deletion encompassing *NF1* exons 10c–21 and one loss of heterozygosity (LOH) event including the *NF1* gene. All *NF1* mutations are assumed to have an inactivating effect (Table 1). Analysis of the nonsense mutation p.R2616X in different cell types derived from tumor II of patient NF1-2 demonstrated that the somatic *NF1* mutation was exclusively present in the cells cultured with the medium selective for ICC culture and not in fibroblasts grown from the same tumor.

Western blotting

All seven NF1-related tumors showed only rudimentary neurofibromin expression in comparison with the control GIST882 cell line harboring the *KIT* p.K642E mutation and the sporadic GIST with the *KIT* p.557–558delWK mutation (Fig. 2). NF1-related GISTs demonstrated normal total KIT expression but low levels of constitutive KIT autophosphorylation in comparison to the control GIST882 and sporadic GIST cells (Table 2). Total MAPK and MAPK phosphorylation was ubiquitously more pronounced in NF1-related GIST tissue. On the basis of the densitometric quantification of autoradiographic signals, the relative p-MAPK expression in NF1-related GIST was 3–18-fold higher than in sporadic GIST (Table 2). Expression of total AKT was variable in NF1-related GISTs and not associated with protein phosphorylation. Moreover, NF1 tumors did not express STAT3 protein.

Table 1. Molecular analysis of seven GISTs derived from three NF1 patients

Patient	Tumor	Material	*KIT*	*PDGFRA*	*NF1*		
					Germline	Somatic	Mutation effect
NF1-1	I	F	−	−	c.4269+1G>T	c.5546+2T>A	Frameshift leading to PTC
	II	F	−	−		c.5242C>T (p.R1748X)	PTC
	III	F	−	−		ND	
NF1-2	I	F	−	−	c.6791dupA	c.279T>A (p.C93X)	PTC
	II	ICC + F	−	−		c.7846C>T (p.R2616X)	PTC
	III	ICC + F	−	−		del [ex10c−21]	Truncating
NF1-3	I	F	−	−	c.7807delG	LOH	Loss wild-type *NF1* allele

F, frozen; −, negative; ND, no mutation detected; PTC, premature termination codon; ICC, interstitial cells of Cajal.

Array CGH experiments

Array CGH experiments on NF1-related GISTs showed similar losses of chromosome regions as seen in sporadic GISTs (i.e. losses of chromosomes 11, 14, 22 and 1p) in addition to several extra alterations (Table 3). These additional chromosomal abnormalities seem not to be recurrent in the different tested NF1-related GISTs. Array CGH confirmed LOH of the *NF1* gene in the large tumor of patient NF1-3 [loss of the clones containing the *NF1* gene (RPCI5-926B9 and RP11-229K15)] while no other aberrations were observed (Table 3 and Fig. 3).

Phenotype of cultured cells

After 3 days in culture, the primary cells of tumor III from patient NF1-2 exhibited spindle shape type of growth and 95% were KIT immunopositive. The Ki-67 labeling index was 0.5 and 8.7% in ligand unstimulated and ligand stimulated cultures, respectively (Fig. 4).

Imatinib assay

As shown in Figure 5, KIT phosphorylation of primary NF1-related GIST cells seeded in culture is ligand-dependent as shown by very limited KIT phosphorylation in the absence of stem cell factor (SCF). SCF-dependent KIT phosphorylation of NF1-related GIST cells is totally inhibited by exposure to imatinib at concentration of 0.5 μM. The same inhibition is observed in the control autophosphorylated GIST882 cell line harboring the *KIT* p.K642E mutation. The level of MAPK phosphorylation of primary NF1-related GIST cells was moderately decreased after exposure to imatinib but the inhibition was neither complete nor dose-dependent. Imatinib decreased in a dose-dependent way the level of MAPK autophosphorylation at concentrations lower than 1.0 μM in the GIST882 cell line but not at any tested doses in control fibroblasts. The relative p-KIT and p-MAPK expressions, based on the densitometric quantification of autoradiographic signals, are shown in Table 4.

DISCUSSION

Although activating mutations of *KIT* and *PDGFRA* are known to be the most frequent genetic events in the development of sporadic GISTs, the underlying pathogenic mechanism leading to GIST formation in the NF1 setting remains elusive. In this study, total screening of *KIT* and *PDGFRA* in seven GISTs from three NF1 patients did not reveal any activating mutation. Several sequence variants were detected: three silent mutations in *PDGFRA* (p.N204N, p.G313G and p.A603A), 1 silent mutation in *KIT* (p.K546K), one SNP in the 3′-UTR region of both *KIT* and *PDGFRA* and one missense mutation in exon 10 of *PDGFRA* (p.S478P) that was not predicted to have an impact on protein activity (Polyphen algorithm) and that is a frequent polymorphism (19/98 controls, 19%) in the examined population. All variants were also present in normal control tissue (white blood cells) of the respective patients. In conclusion, the alterations do not correspond to known mutation hot spots in sporadic GISTs, are not tumor specific and have not been demonstrated to result in activation of the tyrosine kinases. This finding is in line with published reports (27–31). Of the 113 NF1-related GISTs previously published, only eight showed *KIT/PDGFRA* alterations. Takazawa *et al*. (2005) (30) reported three *KIT* (p.L558L, p.P627L and p.I653T) and two *PDGFRA* (p.P589S and p.R822S) mutations in 34 NF1-related GISTs. None have been demonstrated to result in activation of the protein nor have they been previously reported in sporadic or familial GIST. Yantiss *et al*. (2005) (29) detected an identical point mutation in *KIT* exon 11 in three tumors from one patient. As control tissue was not analyzed, this patient may have had a germline *KIT* mutation. Taken together, these results indicate that *KIT* or *PDGFRA* mutations are not playing a role in the development of GISTs in NF1 patients.

In accordance with Knudson's two-hit hypothesis (32), somatic inactivation of the *NF1* tumor suppressor gene leads to tumor formation in NF1 (33–36). In line with this concept, we detected somatic *NF1* alterations in six of seven NF1-related GISTs (Table 1). Analysis of different cell types derived from tumor II of patient NF1-2 demonstrated that the somatic *NF1* mutation was only present in tumoral cells cultured using specific conditions for ICCs (37). This is the first time that somatic *NF1* inactivation is demonstrated

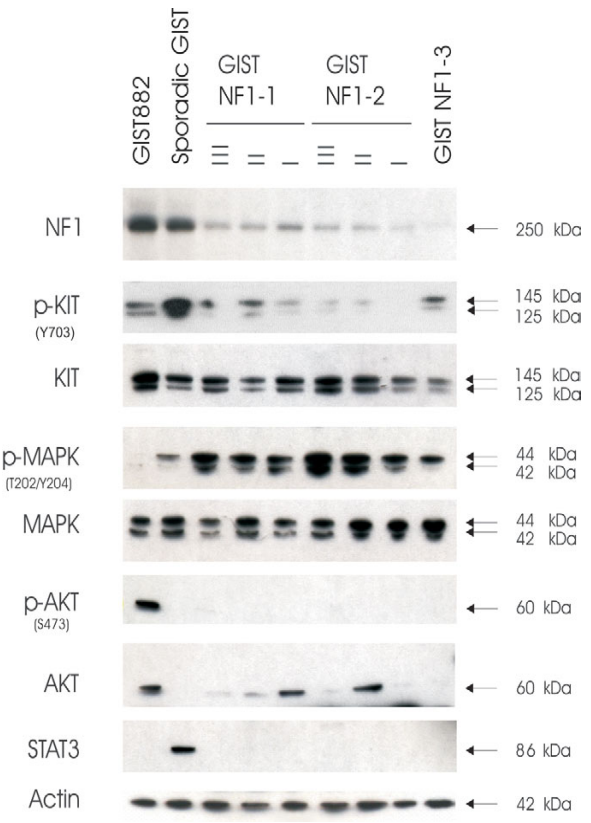

Figure 2. Analysis of KIT/PDGFRA downstream signaling pathways in two sporadic [GIST882 cell line (*KIT* p.K642E) and sporadic GIST (*KIT* p.557–558delWK)] and seven NF1-related GISTs by western blotting. Equal protein loading was demonstrated with anti-actin staining. On the basis of the densitometric quantification of autoradiographic signals, the relative p-MAPK expression in the NF1-related GISTs was 3–18-fold higher than in the sporadic GIST. Because of short exposure time, phosphorylation of MAPK of GIST882 is less visible.

in neoplastic GIST cells and it illustrates that the lack of functional neurofibromin, in the absence of *KIT* or *PDGFRA* mutations, can lead to GIST formation.

Neurofibromin functions as a negative regulator of the MAP-kinase pathway by catalyzing the hydrolysis of active Ras-GTP to an inactive Ras-GDP. In this light, inactivation of neurofibromin in NF1-related GISTs is the mechanism leading to tumor formation. To further elucidate this hypothesis, we evaluated the expression of KIT, neurofibromin and several downstream signaling molecules. Clear differences were observed between sporadic and NF1-related GISTs (Fig. 2). First, NF1-related GISTs demonstrated comparable total KIT expression but low levels of constitutive KIT autophosphorylation. Secondly, in contrast to sporadic tumors, NF1-related GISTs showed a dramatically reduced level of neurofibromin, confirming the inactivating effect of the *NF1* somatic mutations. Thirdly, strong expression and activation of MAPK in NF1-related GISTs illustrated increased signaling through the MAP-kinase pathway when compared with sporadic GIST cells. Fourthly, the JAK-STAT3 and PI3K-AKT pathways were shown to be less active in NF1-related GISTs. These data suggest that activation of the MAP-kinase cascade is a common cause of GIST development in both NF1 and non-NF1 patients, although there are important differences in the mechanism of activation (inactivation of *NF1* versus activation of *KIT/PDGFRA*).

Recurrent cytogenetic aberrations in sporadic GISTs include deletion of 14q, 22q, 1p, 11p and 9p and gain of 8p and 17q (reviewed in 5). These chromosomal changes can be viewed as secondary events acquired after the oncogenic mutations in the receptor tyrosine kinases and are associated with clinical progression. While deletion of chromosome arms 14q (38–43) and 22q (39,42–45) are frequent findings in sporadic GISTs and likely represent sites for tumor suppressor genes that play a role early in GIST formation, losses on chromosomes 1p, 9p and 11p are more significantly associated with malignancy (40,46–48). Gains on chromosomes 8q and 17q are associated with metastatic behavior (41,42,49). The array CGH experiments on NF1-related GISTs showed similar gains and losses of chromosome regions as seen in sporadic GISTs, i.e. losses of chromosomes 11, 14, 22 and 1p, in addition to several extra alterations (Table 3). These additional chromosomal abnormalities seem not to be recurrent in the different tested NF1-related GISTs. Remarkable is the array CGH profile of the very large tumor from patient NF1-3 where only the clones containing the *NF1* gene are deleted while no other aberrations are observed (Fig. 3). Taken together, the genomic changes observed in the analyzed NF1-related GISTs were similar to those described in sporadic GISTs and did not indicate any change that could be associated particularly with NF1-related GISTs, except for the deletion of the *NF1* gene region. It remains elusive why clinically symptomatic GISTs arise in only a minority of NF1 patients and why, in contrast to sporadic tumors, they tend to be multiple and predominantly located in the small intestine. The presence of multiple GISTs in NF1 might reflect a distinct rate-limiting step in oncogenesis compared with sporadic GIST. While a broad spectrum of inactivating genetic mechanisms might lead to suppression of the wild-type *NF1* allele and hence GIST formation in NF1 patients, only a limited set of specific activating mutations in *KIT/PDGFRA* will result in sporadic GIST. Moreover, genotypes at modifying loci, not detectable by array CGH, might trigger the development, location and number of multiple GISTs in NF1 patients.

On the basis of the array CGH profiles of the three tumors from patient NF1-2 and tumors I and II from patient NF1-1 (Table 3), one might argue that clustered GISTs are clonal. In this light, the identical chromosomal alterations in the tumors of each patient (loss 1pter→1p12 and 14 for NF1-2; loss 1pter→1p12, 14 and 22 for NF1-1) would reflect a common precursor lesion, whereas the tumor-specific alterations (cytogenetic aberrations and *NF1* somatic mutation) could be seen as secondary events. However, as illustrated by the same alterations in the tumors from two different patients, deletion of chromosomes 1p, 14 and 22 are recurrent cytogenetic aberrations in GISTs. Moreover, the apparently identical breakpoints at chromosome band 1p12 can be explained by the poor clone coverage at the centromere [21 Mb gap between RP11-418J17 (1p12) and RP11-417J8 (1q12)]. Overall, different tumors derived from the same

Table 2. Relative p-KIT and p-MAPK expression based on densitometric quantification of autoradiographic signals obtained by western blotting (data corresponding to Fig. 2)

	Sporadic GIST		NF1-related GIST						
	GIST882	Sporadic GIST	GIST NF1-1			GIST NF1-2			GIST NF1-3
			III	II	I	III	II	I	
p-KIT/KIT	0.44	3.05	0.64	0.67	0.30	0.22	0.18	0	0.81
p-MAPK/MAPK	0.10	0.11	1.78	0.82	1.50	1.73	1.46	0.98	0.32

Table 3. Summary of array CGH experiments on seven GISTs derived from three NF1 patients

Patient	Tumor	Array CGH	
		Gain	Loss
NF1-1	I	No gain	1pter→1p12, 2pter→2p11.2, 6q12→qter, 11pter→11p11.2, 13, 14, 18, 22
	II	10q25.1→10qter	1pter→1p12, 3q11.2→3qter, 11, 14, 19q13.11→19qter, 22, X
	III	6, 10, 20, 20	no loss
NF1-2	I	9q21.13→9qter	1pter→1p12, 14
	II	9q21.11→9qter	1pter→1p12, 14, 15, 22
	III	Xq22.1→Xqter	1pter→1p12, 14, 15
NF1-3	I	No gain	17q11.2 only RPCI5-926B9 and RP11-229K15 (=*NF1*)

patient bear different *NF1* somatic mutations and, with the exception of the typical GIST-related chromosomal alterations, array CGH profiles are quite diverse. Therefore, it is most likely that multiple GISTs in NF1 patients are independent tumors.

The tyrosine kinase inhibitor imatinib (Gleevec, Novartis) has tremendously improved the treatment of advanced sporadic GISTs, showing a clinical benefit in up to 80% of patients (17,18). To determine whether this drug could also be effective in NF1-related GISTs, we performed an *ex vivo* imatinib response experiment. As illustrated in Figure 5, ligand-dependent KIT autophosphorylation of primary NF1-related GIST cells and the control GIST882 cell line is totally inhibited by the exposure to imatinib at a concentration of 0.5 μM. In contrast to control fibroblasts, the level of MAPK autophosphorylation of primary NF1-related GIST cells was lowered after exposure to imatinib, although the inhibition was neither complete nor dose-dependent as clearly illustrated in the GIST882 cell line. These experiments thus suggest that imatinib might only have a mild effect on NF1-related GISTs. It will be important to determine whether this *ex vivo* observation can be translated into clinical benefit for NF1 patients with GISTs. As it was proven by *in vitro* studies (18), wild-type KIT is equally sensitive to imatinib as mutated KIT protein. Nevertheless, this observation does not translate to clinical response of patients with tumors that do not harbor *KIT* mutations (who poorly respond to the treatment). Therefore, *in vitro* assays may have only restricted predictive value.

In conclusion, we report for the first time the molecular pathogenesis of GISTs in NF1 individuals. Somatic *NF1* inactivation in the ICCs seems to be the molecular event underlying GIST development in the NF1 setting. Inactivation of neurofibromin in the absence of *KIT* or *PDGFRA* mutations is an alternate mechanism leading to GIST formation. Moreover, NF1-related GISTs show an increased signaling through the MAP-kinase pathway when compared with sporadic GIST cell lines. These findings clearly position GISTs in the range of clinical symptoms seen in NF1. It will be important to determine whether the tyrosine kinase inhibitor Gleevec used in the treatment of sporadic GIST also has an effect on NF1-related GIST.

MATERIAL AND METHODS

Patient material

Studies were performed on seven GISTs derived from three consenting NF1 patients. Peripheral blood was available for all patients as well as frozen tissue from the seven tumors and two tumor cell cultures from patient NF1-2. Control GIST cells included a GIST882 cell line harboring a *KIT* p.K642E mutation (50) and a sporadic GIST with a *KIT* p.557–558delWK mutation.

Phenotype NF1 patients

Patient NF1-1 is a 72-year-old female patient with widespread cutaneous neurofibromas. At the age of 59 years, a carcinoid tumor originating from the duodenum was resected. A GIST in the small intestine was diagnosed at the age of 68 and another in the retroperitoneal area adjacent to the bladder 4 years later. During surgery, both tumors were resected as well as a third not previously noted. The patient died postoperatively due to pulmonary complications. Her mother had had NF1 and had died due to complications related to a GIST at the age of 40.

Patient NF1-2 is a 59-year-old male with family history of neurofibromatosis type 1 and multiple cutaneous neurofibromas. Multiple GISTs of the duodenum were diagnosed by endoscopy following massive upper gastrointestinal tract bleeding. The episode of bleeding was preceded by the ingestion of a non-steroidal anti-inflammatory drug (nimesulide). During surgery, tumors were palpated at the serosal side of the duodenum and the proximal jejunum. No tumors could be palpated in the rest of the intestinal tract. Twelve small tumors (diameters of 3–22 mm) were removed from the duodenum and the proximal jejunum. A large lesion was localized in D2 and protruded at the anti-mesenterial side (diameter 20 mm). Two other large lesions (diameters 20 and 22 mm)

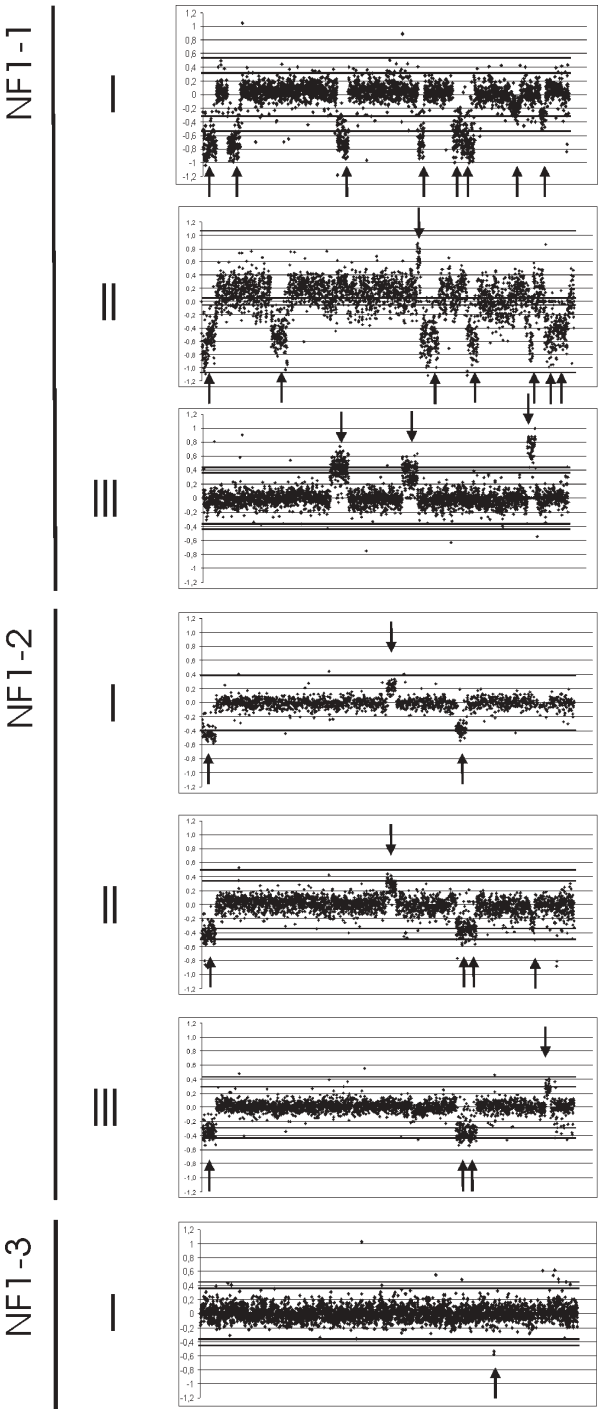

Figure 3. Array CGH profiles of seven NF1-related GISTs. Profiles are composed of numerically ordered autosomes (1–22) followed by X and Y. Arrows below and above profiles mark losses and gains, respectively.

showed mucosal ulcerations and were located in D2–D3. Pathological examination showed very few mitoses (less than 5 per 50 high-power fields). There were no post-operative complications, and post-operative follow-up at 2 years was uneventful. He did not receive adjuvant therapy.

Patient NF1-3 is a male with a severe thoraco-lumbar kyphoscoliosis and multiple cutaneous neurofibromas. At the age of 55, surgery was performed for a growing pelvic mass with nycturia and frequent micturition. The large tumor fills the pelvic cavity completely and is connected to the ileum over a distance of 4 cm. The large tumor was removed from the peritoneum, the bladder and the mesenterial side of the rectum and subsequently resected together with 7 cm of jejunum. The tumor measured $110 \times 110 \times 50\ mm^3$ and showed central necrosis. Pathological examination showed only one mitosis per 50 high-power fields. In the resected fragment of the jejunum, a tumor with a diameter of 20 mm was present (no mitoses detected) and three additional smaller GISTs (diameter 3–5 mm, no mitoses). The post-operative evolution was uneventful and the patient did not receive adjuvant therapy. At follow-up 2.5 years later, he is free of symptoms and a CT-scan of the abdomen did not reveal any mass.

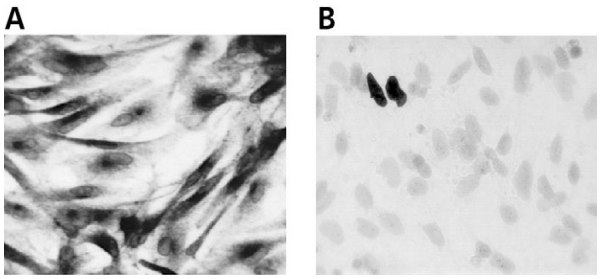

Figure 4. Phenotype of cultured cells. (**A**) After 3 days in culture, the primary cells of tumor III from patient NF1-2 exhibited spindle shape type of growth and 95% were KIT immunopositive. (**B**) The Ki-67 labeling index was 0.5 and 8.7% in ligand unstimulated and ligand stimulated cultures, respectively.

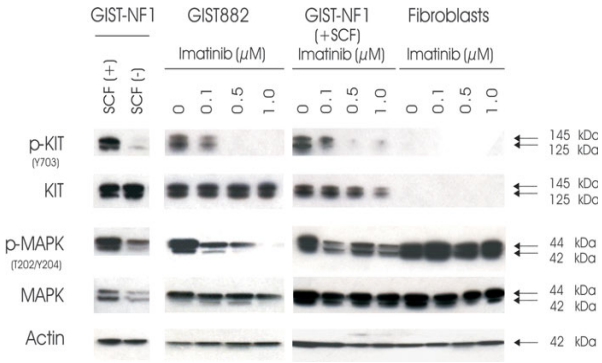

Figure 5. *Ex vivo* effect of imatinib on the phosphorylation of KIT Y703 and MAPK T202/Y204 residues in control GIST882 cell line (*KIT* p.K642E), primary NF1-related GIST cells cultured in medium supplemented with stem cell factor (SCF) and control human fibroblasts determined by western blot assay. Equal protein loading was demonstrated with anti-actin staining. The relative p-MAPK expression was evaluated on the basis of the densitometric quantification of autoradiographic signals. In contrast to control GIST882 cells with activating *KIT* mutation, the phosphorylation of wild-type *KIT* in primary NF1-related GIST cells under *in vitro* condition is ligand-dependent. Imatinib treatment resulted in only partial and dose-independent inhibition of MAPK posphorylation of NF1-GIST cells cultured under ligand stimulation.

Histopathology

Histopathological sections (5 μm) were cut from tumor paraffin blocks for routine H&E staining. Immunohistochemical

Table 4. Relative p-KIT and p-MAPK expression based on densitometric quantification of autoradiographic signals obtained by western blotting (data corresponding to Fig. 5)

	GIST-NF1		GIST-882				GIST-NF1 (+SCF)				Fibroblasts			
	SCF(+)	SCF(−)	Imatinib (μM)				Imatinib (μM)				Imatinib (μM)			
			0	0.1	0.5	1.0	0	0.1	0.5	1.0	0	0.1	0.5	1.0
p-KIT/KIT	1.01	<0.01	0.90	0.21	0	0	1.02	0.33	<0.1	<0.1	/	/	/	/
p-MAPK/MAPK	1.79	0.45	1.13	0.37	0.12	0	1.05	0.76	0.84	0.78	1.10	1.15	1.14	1.10

/: not applicable.

staining was conducted using the avidin–biotin–peroxidase complex method. Commercially available monoclonal (mc) or polyclonal (pc) antibodies specific for CD117 (pc, 1:250; DAKO, Glostrup, Denmark), CD34 (mc, 1:10; Becton Dickinson, San Jose, CA, USA), α-Smooth Muscle Actin (mc, 1:100; DAKO), desmin (mc, 1:20; ICN, Aurora, OH, USA) and S-100 (pc, 1:300; DAKO) were used. The CD117 immunostaining was performed without antigen retrieval and the presence of mast cells served as an internal control.

KIT/PDGFRA mutation analysis

Genomic DNA was extracted from snap-frozen tumor tissue of NF1-related GISTs using the High Pure PCR Template Preparation Kit (Roche). The entire *KIT* and *PDGFRA* coding region and the splice junctions were amplified by PCR. All amplicons were bidirectionally cycle sequenced using the ABI3730XL machine (Applied Biosystems).

NF1 mutation analysis

Germline *NF1* mutation analysis was performed (51). An improved strategy for *NF1* somatic mutation detection was developed (Maertens *et al*., manuscript in preparation) and applied specifically to the primary GIST cell cultures. Briefly, the second passage of ICC cell cultures from tumors II and III from patient NF1-2 was treated with puromycin before RNA extraction (RNeasy kit, Qiagen). Genomic DNA was extracted with the PureGene DNA purification kit (Gentra). The entire *NF1* cDNA was sequenced. Mutations found at the cDNA level were confirmed on gDNA. Detection of LOH was performed by genotyping microsatellite markers telomeric (3′NF1-3, 3′NF1-1) (52) and within (Alu, IVS27AC33.1, IVS38GT53.0, IVS27TG24.8) (53–56) *NF1*. Genomic DNA from paired frozen tissue or primary GIST cell cultures and lymphocyte cultures was investigated by multiplex PCR and analyzed on the ABI3100 genetic analyzer (Applied Biosystems) with the Genescan software (Applied Biosystems). Loss of the wild-type *NF1* allele was confirmed by at least one of the following techniques: FISH, array CGH, MLPA or SNP analysis. Frozen tumor specimens negative for LOH were subsequently submitted to PCR amplification and cycle sequencing of all *NF1* exons.

Western blotting

Proteins were isolated from snap-frozen tumor specimens and western blotting was performed as described in Debiec-Rychter *et al*. (57). In short, equivalent amounts of protein (30 μg) from clarified lysates were resolved with SDS–PAGE, transferred to PDVF membranes and immunoblotted sequentially with antisera specific for anti-NF1 (sc-67; 1:200; Biotechnology, Santa Cruz, CA, USA), anti-phospho-KIT (Y703) (1:250; Zymed, San Francisco, CA, USA), anti-KIT (1:500; DAKO), anti-phospho MAPK (T202/Y204) (1:1000; Cell Signaling Technology Inc., Beverly, MA, USA), anti-MAPK (1:1000; Zymed), anti-phospho AKT (S473) (1:500; Cell Signaling Technology Inc.), anti-AKT (1:500; Cell Signaling Technology Inc.), anti-STAT3 (1:500; Zymed) and anti-actin (1:500; Sigma, Saint Louis, MI, USA). After washes, membranes were probed with anti-rabbit or anti-goat immunoglobulin-HRP conjugate and incubated with ECL substrate (Pierce, Rockford, IL, USA). On the basis of the densitometric quantification of autoradiographic signals, the relative p-MAPK expression was determined in NF1-related GISTs.

Array CGH

Array CGH was performed as published previously (58). Tumor DNA was compared with DNA extracted from blood lymphocytes of a sex-matched control individual. Every case was analyzed twice and tumor DNA was once labeled with Cy3 and once with Cy5. Clones that gave the same result twice were taken into account.

Primary GIST cell culture and imatinib assay

The GIST specimen used for this experiment was tumor III of patient NF1-2. Primary cells were obtained from a disaggregated tumor specimen seeded at 60–70% confluence in 100 mm cell culture dishes and grown for 3 days in DMEM supplemented with 10% FBS, 20 ng/ml SCF, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 0.5 mM 3-isobutyl-1-methylxanthine (Sigma). As a control, the same NF1-2 primary GIST cells, GIST882 cell line and normal human fibroblasts were grown in similar conditions but without the SCF ligand. For evaluation of the phenotype, NF1-2 primary GIST cells from tumor III were seeded at 60–70% confluence in slide chambers and grown for 3 days in the same conditions as above. After fixation in 4%

paraformaldehyde, immunohistochemical staining using the polyclonal rabbit antihuman antibody against CD117 (1:250; DAKO) and Ki-67 antibody (1:250; DAKO) was performed by the avidin–biotin–peroxidase complex method. The proliferation index was determined as the percentage of Ki-67 positive cells of the total number of cells. Imatinib mesylate was provided by Novartis Pharmaceuticals Corporation. The compound was dissolved at 10 mM in 100% DMSO (Sigma). Controls were performed with solvent (DMSO) dilutions. The effect of imatinib on the autophosphorylation of the KIT Y703 and the MAPK T204/Y202 residues in cultured primary NF1-related GIST cells (either ligand stimulated or unstimulated), the control GIST882 cell line and human fibroblasts was determined by western blot assay. In short, cells were exposed to either vehicle alone or to imatinib mesylate within a range of doses (0.1, 0.5 and 1.0 μM) for 90 min, washed with ice-cold PBS and lysed. Lysates were incubated for 30 min at +4°C and then centrifuged for 20 min. Supernatants were removed and used for SDS gel electrophoresis and immunoblotting as described previously (57). Equivalency of loading intracellular proteins was demonstrated by stripping the membrane and re-blotting using anti-actin antibody (1:500; Sigma).

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CHAPTER 3

Discussion and summary

CHAPTER 3. DISCUSSION AND SUMMARY

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

Neurofibromatosis type 1 (NF1), one of the most common autosomal dominant genetic disorders worldwide, is caused by defects in the *NF1* gene. The *NF1* encoded protein, neurofibromin, functions as a negative regulator of Ras mediated signaling[1-3]. The wide spectrum of clinical manifestations observed in NF1 patients clearly illustrates the critical function of neurofibromin in a wide variety of tissues and cell types. Given the central role of Ras signaling pathways in so many biological processes, perhaps this is not surprising. Nevertheless, the basic pathology of many of the NF1-related symptoms, and thus the exact role of neurofibromin in different cellular contexts, remains largely unknown.

Elucidating the biological framework underlying the development of NF1-related symptoms has proven to be difficult. Aggravating factors include the large size of the gene, the presence of several *NF1* pseudogenes, the complex interactions between cell types, the *NF1* haploinsufficient state of all cells in the body, and the involvement of modifier genes. Despite this complexity, important progress has been made in deciphering the molecular and biochemical events that underlie NF1-associated tumor development in NF1 mouse models[4-7]. However, the concern that the mechanisms and signaling pathways by which Ras effects its biological consequences might exhibit significant species-specific differences[8], emphasize the value of dissecting tumorigenic as well as non-tumorigenic lesions in human cells. Determining when and in what cell type the crucial molecular defect occurs, is critical for understanding the basic pathology of any phenotype. Moreover, this information is essential for elucidating the normal function of the *NF1* gene and developing tailored treatment protocols in the long term. In this thesis, we exploited improved somatic mutation detection strategies on a diverse spectrum of cellular entities in order to gain insight into the developmental concepts underlying various NF1-related symptoms.

The requirement for second hit mutations to develop benign neurofibromas has been controversial. Early work in this area concluded that such mutations were not present in this tumor type[9]. More recently, investigators have reported second hit mutations or loss of heterozygosity (LOH) in human neurofibromas, albeit at low frequency (15-40%)[10-15]. Based on dual-label immunofluorescence data, one group speculated lately that neurofibromas develop in haploinsufficient tissues without the need for a second hit at the *NF1* tumor suppressor locus[16, 17]. In our opinion, it is risky to draw conclusions on the mutation status of a particular gene based on immunohistochemical data only. We hypothesized that the relatively low detection rates reported may be due to (1) the cellular heterogeneity of neurofibromas, i.e. the fraction of Schwann cells carrying the second hit may be too low to reveal the somatic mutation, and (2) the low sensitivity of the applied mutation detection strategies. In Paper 1, we present an improved *NF1* somatic mutation detection strategy. Extensive screening of the *NF1* gene in selectively grown Schwann cells (SC F-) derived from a large panel of neurofibromas resulted in the highest *NF1* somatic mutation detection rate described up to

now (76%). Moreover, our data strongly suggested that the acquired second hit underlies reduced *NF1* expression in Schwann cell cultures. Therefore, our work shows for the first time irrefutable evidence that the occurrence of two inactivating *NF1* mutations in a subpopulation of the Schwann cells is required for neurofibroma formation, which is in line with Knudson's two-hit hypothesis[18]. Moreover, the high somatic mutation detection rate emphasizes that genetic alterations, and not epigenetic events, constitute the prevailing mechanism for *NF1* inactivation in neurofibroma development.

In a next step, we compared the germline versus the somatic mutation spectrum. For several familial cancer syndromes, similar studies have provided important insights into the underlying mechanisms of tumorigenesis and mutagenesis. In familial adenomatous polyposis (FAP), for example, the non-random distribution of mutations in the *APC* gene reflects an optimal level of β-catenin signalling in colorectal tumors[19, 20]. A significant difference in the somatic inactivation mechanism in neurofibromas derived from *NF1* microdeletion patients and the general NF1 population was initially observed in Paper 1 and firmly established in Paper 2. While LOH is responsible for the somatic inactivation of *NF1* in a quarter of the neurofibromas from non-microdeletion patients, this mechanism was never observed in neurofibromas derived from *NF1* microdeletion patients. Compared to the general NF1 population, individuals with an *NF1* microdeletion frequently show a phenotype with more dermal neurofibromas at an earlier age. Now it becomes clear that both patient groups not only differ at the phenotypic and constitutional level, but also at the somatic level. This important new insight may open new avenues for a better understanding of the genetic basis underlying the high tumor burden of *NF1* microdeletion patients. Whether the wild-type *NF1* allele in microdeletion patients is, for an as yet undetermined reason, more vulnerable to other types of somatic inactivation remains an important question.

Compared to the age-matched NF1 population, most individuals included in Paper 1 suffered from a severe to excessive neurofibroma burden. Strikingly, the somatic mutation spectrum consisted predominantly of *NF1* minor lesion mutations, especially frameshift mutations. To our opinion, this mutation signature might reflect slightly reduced DNA repair efficiency as a trigger for *NF1* somatic inactivation preceding tumorigenesis. Several lines of evidence strengthen this hypothesis. First, there is increasing evidence that mild reductions in DNA repair capacity, assumed to be the consequence of common genetic variation, affect cancer predisposition[21]. Second, the *NF1* gene has been shown to be a mutational target in cells deficient for the mismatch repair process[22]. Third, epidemiologic studies suggest that the molecular basis underlying the phenotypic variability in NF1 is determined to a large extent by the genotype at modifying loci[23-26]. Future large and well-designed candidate gene association studies are needed to help further illuminate the putative role of common variants in DNA repair genes as contributing factors in NF1 tumor predisposition.

In Paper 4, four mosaic NF1 patients with different clinical manifestations (neurofibromas only, pigmentary changes only, and association of both symptoms) were investigated at the molecular

level. For this purpose, a real-time quantitative PCR approach capable of detecting low-percentage mosaic point mutations against backgrounds of normal and pseudogene alleles was optimized (Paper 3). For every mosaic patient, various tissues and cell types were tested to gain insight into the developmental concepts underlying particular NF1-related disease features. This approach elegantly confirmed the tumorigenic properties of Schwann cells in neurofibroma development. Moreover, we demonstrated for the first time that bi-allelic *NF1* inactivation in melanocytes seems to be the underlying trigger for NF1-related café-au-lait macule (CALM) development. Clearly, this finding provides an important stimulus for further research. First of all, additional NF1-related CALMs will need to be tested to confirm our initial observations. Screening different CALMs derived from the same NF1 patient will provide insight into the developmental timing of the second hit. In the context of the early onset and frequent congenital manifestation of CALMs, identical *NF1* second hits in different CALMs might point to inactivation of the *NF1* wild-type allele in melanocyte precursors (melanoblasts) during embryonic development. Besides NF1-related CALMs, it will also be of particular interest to screen the *NF1* mutation status in CALMs from healthy control individuals. The next step will be to determine the dysregulating consequences of neurofibromin loss on signaling pathways in melanocytes. In this respect, it will be of fundamental importance to study whether *NF1* inactivation in melanocytes triggers particular autocrine and paracrine networks resulting in increased melanocyte density[27], and a higher melanin content and melanogenesis[28], features which have been shown to be specific for NF1 CALMs.

Interestingly, both neurofibromas and CALMs in mosaic NF1 patients appear to arise even within a background containing predominantly *NF1* wild-type cells. Deciphering the complex interactions between the different cell types within the neurofibroma microenvironment is currently a major topic and challenge in NF1 research. Previous findings in mouse models have led to the emerging view that *NF1* haploinsufficiency in the tumor environment actively contributes to, or is even necessary for, neurofibroma formation[4-6]. However, considering the recurrent discrepancies in the tumor phenotypes between human and mice, especially in the context of Ras-related neoplasms[29], caution must be exercised in extrapolating these observations to humans. Moreover, the tumors that have been observed in the murine model to date have been plexiform neurofibromas derived from dorsal root ganglia and cranial nerves. Whether this is a consequence of the expression pattern of the Cre transgene used to generate the conditional mouse model or alternatively reflects a slightly different pathogenesis from the dermal neurofibromas remains an important question. At this moment, it is unclear whether the abundance of *NF1* wild-type cells in neurofibromas from segmental NF1 patients reflects a slightly different pathogenesis in humans or, alternatively, is associated with the small size of these patients' tumors (heterozygous neighboring cells may promote tumor growth more efficiently than wild-type neighboring cells). In this respect, it will be of particular importance to evaluate the involvement of *NF1* haploinsufficient mast cells in human neurofibroma development. Currently, we are undertaking efforts to isolate mast cells from neurofibromas of segmental patients in order to determine their *NF1* mutational status.

Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal tumors of the gastrointestinal tract and are increasingly being recognized in association with NF1. In contrast to the sporadic tumors where *KIT* and *PDGFRA* activating mutations are the oncogenic mechanisms, the molecular basis underlying GIST formation in NF1 remained elusive at the start of this thesis. Using a combination of mutation analysis, Western blotting and array CGH we demonstrated in Paper 5 that (1) the NF1-related GISTs do not have *KIT* or *PDGFRA* mutations, (2) the molecular event underlying GIST development in this patient group is a somatic inactivation of the wild-type *NF1* allele specifically in the interstitial cells of Cajal and (3) inactivation of neurofibromin is an alternate mechanism to (hyper) activate the MAP-Kinase pathway, while the JAK-STAT3 and PI3K-AKT pathways are less activated in NF1-related compared to sporadic GISTs. These findings clearly position GISTs in the spectrum of clinical symptoms seen in NF1. It will be important to determine if the tyrosine kinase inhibitor Gleevec used in the treatment of sporadic GIST also has an effect on NF1-related GIST.

The results of this thesis offer several future perspectives for guiding impending research efforts. First of all, the presented data demonstrate that a growing body of NF1-related symptoms, as well tumoral as non-tumoral lesions, require somatic inactivation of the wild-type *NF1* allele. This observation emphasizes the value of dissecting additional, as yet unexplored, NF1 clinical manifestations at the somatic level. Such studies are essential for identifying the cell types, the cellular processes and the molecular pathways altered by *NF1* defects. The analyses reported in this thesis mainly focused on pinpointing the cells and molecular defects underlying the development of various NF1-related symptoms. Accurately identifying the biochemical and cellular consequences of these cell-autonomous molecular defects, however, requires more functionally oriented analyses. As we enter into an era of targeted cancer therapeutics, an improved understanding of the signaling pathways dysregulated as a consequence of neurofibromin loss will ultimately provide additional molecular targets for drug design.

Identifying modifying genes is an important challenge in NF1 research. Such genes may be indispensable determinants of risk prediction, the current lack of which substantially contributes to the psychological burden where NF1 patients and their families are suffering from. Also motivating this search is the hypothesis that genetic modifiers might control rate-limiting steps during disease development, and as such may represent good therapeutic targets. To identify these modifiers, National Institute of Health (NIH) funded NF1 Consortia are in the process of collecting material and detailed phenotypic information from thousands of NF1 patients. Indeed, only genetic association studies on large well-defined patient groups offer a powerful approach for mapping causal genes with modest effects. In this thesis, we suggested a putative role for DNA repair genes as contributing factors in NF1 tumor predisposition. To help further test this hypothesis, well-designed candidate gene association studies on patient cohorts that can only be gathered by well-coordinated collaborative efforts will be of fundamental importance.

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SUMMARY

Neurofibromatosis type 1 (NF1; OMIM 162200) is one of the most common autosomal dominant genetic disorders, affecting approximately 1 in 3500 individuals worldwide. The most common clinical manifestations are pigmentary abnormalities together with the development of benign peripheral nerve sheath tumors or neurofibromas. In addition, NF1 patients are prone to a pleiotropy of other phenotypic features. Determining when and in what cell type the crucial molecular defect actually occurs has proven to be surprisingly difficult for many NF1-related symptoms. Impeding factors include the large size of the *NF1* gene, the presence of several *NF1* pseudogenes, the complex interactions between cell types within affected tissues, and the *NF1* heterozygous state of all cells in the body. The major goal of this thesis was to gain insight into the developmental concepts underlying various NF1-related symptoms by exploiting improved somatic mutation detection strategies on a diverse spectrum of cellular entities.

First, we investigated thoroughly the status of the somatically affected *NF1* allele in cultured Schwann cells derived from neurofibromas (Paper 1). The high somatic mutation detection rate together with the observation that the acquired second hit underlies reduced *NF1* expression, irrefutably demonstrated that two inactivating mutations in this particular cell type are required for neurofibroma formation. Given the high occurrence of somatic frameshift mutations, a putative role of reduced DNA repair efficiency as a trigger for *NF1* somatic inactivation preceding tumorigenesis was suggested. In a next step, we compared the germline versus the somatic mutation spectrum. A significant difference in the somatic inactivation mechanism in neurofibromas derived from *NF1* microdeletion patients and the general NF1 population was observed in Paper 1 and firmly established in Paper 2. This important new insight may open new avenues for a better understanding of the genetic basis underlying the high tumor burden of *NF1* microdeletion patients.

As opposed to classic NF1 where all cells of the body bear at least one mutated *NF1* allele, NF1 segmental phenotypes provide the opportunity to study cell populations differing only with regard to the mutation(s) giving rise to mosaicism. For this purpose, we developed a real-time quantitative PCR assay capable of detecting low-percentage mosaic point mutations against backgrounds of normal and pseudogene alleles (Paper 3) and molecularly dissected different clinical subtypes emerging within mosaic NF1 (Paper 4). This approach elegantly confirmed the tumorigenic properties of Schwann cells in neurofibroma development. Moreover, we demonstrated for the first time that bi-allelic *NF1* inactivation in melanocytes seems to be the underlying trigger for NF1-related café-au-lait macule development. Clearly, this finding provides an important stimulus for further research aiming to understand the etiopathogenesis of pigmentary lesions in NF1.

Finally, we unraveled the molecular pathogenesis of gastrointestinal stromal tumors (GISTs) arising in the NF1 setting (Paper 5). We demonstrated that, in comparison with sporadic GISTs, NF1-related

tumors do not bear *KIT* or *PDGFRA* mutations, but show somatic inactivation of the wild-type *NF1* allele in the interstitial cells of Cajal. In this respect, inactivation of neurofibromin constitutes an alternate mechanism to activate the MAP-Kinase pathway, while the Jak-Stat3 and PI3K-Akt pathways are less activated in NF1-related GIST compared to sporadic tumors. It will be important to determine if the tyrosine kinase inhibitor Gleevec used in the treatment of sporadic GIST also has an effect on NF1-related GIST.

In conclusion, we have provided significant new insights in the cell types and molecular defects underlying the development of various NF1-related symptoms. Accurately identifying the biochemical and cellular consequences of these cell-autonomous molecular defects will be of fundamental importance to elucidate the exact role of neurofibromin in different cellular contexts and may ultimately provide additional targets for tailored drug design.

SAMENVATTING

Neurofibromatose type 1 (NF1; OMIM 162200) is één van de meest voorkomende autosomaal dominante genetische aandoeningen, die wereldwijd ongeveer 1 op 3500 individuen treft. De meest voorkomende klinische kenmerken zijn pigmentaire afwijkingen en de ontwikkeling van perifere zenuwschede tumoren of neurofibromen. Daarnaast kunnen NF1 patiënten ook een wijde waaier aan andere symptomen vertonen. Voor veel NF1 gerelateerde klinische kenmerken is het verrassend moeilijk gebleken om te bepalen wanneer en in welk cel type het cruciale moleculaire defect plaatsvindt. Bemoeilijkende factoren omvatten de grootte van het *NF1* gen, de aanwezigheid van meerdere *NF1* pseudogenen, de complexe interacties tussen verschillende cel types in aangetaste weefsels, en de heterozygotie voor *NF1* in alle cellen van het lichaam. De belangrijkste doelstelling van deze thesis was om inzicht te verwerven in de ontwikkeling van verschillende NF1 gerelateerde symptomen, en dit door de toepassing van verbeterde somatische mutatie detectie strategieën op een divers spectrum van cellulaire entiteiten.

Eerst en vooral onderzochten we, in gekweekte Schwann cellen afkomstig van neurofibromen, de status van het somatisch aangetaste *NF1* allel (Artikel 1). Het hoge somatische mutatie detectie cijfer en de bevinding dat de verworven tweede hit aanleiding geeft tot een verlaagde *NF1* expressie, toonde ontegensprekelijk aan dat twee inactiverende mutaties in dit specifieke cel type noodzakelijk zijn voor de vorming van neurofibromen. Wegens het hoge percentage aan somatische mutaties met een effect op het leesraam, werd vooropgesteld dat een verlaagde efficiëntie van het DNA herstel mechanisme mogelijk een katalysator zou kunnen zijn voor somatische inactivatie van het *NF1* gen, en bijgevolg tumorvorming. In een volgende stap vergeleken we het germinale met het somatische mutatie spectrum. Een significant verschil in het mechanisme van somatische inactivatie in neurofibromen afkomstig van *NF1* microdeletie patiënten en de algemene NF1 populatie werd opgemerkt in Artikel 1, en vervolgens uitgebreid bevestigd in Artikel 2. Dit belangrijk inzicht kan nieuwe wegen openen naar een beter begrip van de genetische basis voor het verhoogd voorkomen van tumoren bij *NF1* microdeletie patiënten.

In tegenstelling tot klassieke NF1, waar in alle cellen van het lichaam tenminste één gemuteerd *NF1* allel aanwezig is, bieden NF1 segmentaire fenotypes de mogelijkheid om cel populaties te bestuderen die enkel verschillen in de mutatie die aanleiding geeft tot mosaïcisme. Voor deze reden ontwikkelden we een *real-time* kwantitatieve PCR techniek die in staat is om lage percentages aan mosaïsche puntmutaties op te pikken tegen een achtergrond van normale en pseudogen allelen (Artikel 3) en ontleedden we op moleculair niveau verschillende klinische subtypes die voorkomen binnen mosaïsche NF1 (Artikel 4). Deze aanpak bevestigde op elegante wijze de tumorigene eigenschappen van Schwann cellen in de ontwikkeling van neurofibromen. Bovendien toonden we voor de eerste keer aan dat bi-allelische *NF1* inactivatie in melanocyten de onderliggende oorzaak

zou kunnen zijn voor de ontwikkeling van NF1 gerelateerde *café-au-lait* vlekken. Onmiskenbaar verschaft deze bevinding een belangrijke stimulans voor verder onderzoek.

Tenslotte analyseerden we de moleculaire pathogenese van gastrointestinale stromale tumoren (GISTen) die voorkomen binnen de NF1 setting (Artikel 5). We toonden aan dat, in tegenstelling tot sporadische GISTen, NF1 gerelateerde tumoren geen *KIT* of *PDGFRA* mutaties dragen, maar een somatische inactivatie van het wild-type *NF1* allel vertonen in de interstitiële cellen van Cajal. Inactivatie van neurofibromine vormt een alternatief mechanisme om de MAP-Kinase signaal transductie cascade te activeren, terwijl de Jak-Stat3 en PI3K-Akt signaal transductie cascades minder geactiveerd zijn in NF1 gerelateerde dan in sporadische GISTen. Het zal belangrijk zijn om na te gaan of de tyrosine kinase inhibitor *Gleevec*, die gebruikt wordt bij de behandeling van sporadische GISTen, ook een effect zou kunnen hebben op NF1 gerelateerde GISTen.

Samenvattend kunnen we stellen dat we een significante bijdrage hebben geleverd in de identificatie van de cel types en de moleculaire defecten die verantwoordelijk zijn voor de ontwikkeling van verschillende NF1 gerelateerde symptomen. Het nauwkeurige bepalen van de biochemische en cellulaire gevolgen van deze cel autonome moleculaire defecten zal van cruciaal belang zijn om de exacte functie van neurofibromine in verschillende cellulaire omgevingen te ontrafelen. Uiteindelijk kan dit inzicht leiden tot bijkomende doelwitten voor op maat gemaakte geneesmiddelen.

RESUME

La neurofibromatose de type 1 (NF1; OMIM 162200) est une des maladies génétiques les plus communes qui affecte environs 1 individu sur 3500 mondialement. Les manifestations cliniques les plus caractéristiques sont les anomalies pigmentaires et les tumeurs des etuis de nerfs périphériques ou neurofibromes. En plus, les individus affectés peuvent présenter un éventail d'autres symptômes. Pour beaucoup de manifestations cliniques, il est apparu difficile de déterminer quand, et dans quel type de cellule le défaut moléculaire se situe. Des éléments qui compliquent cette analyse comprennent la grande taille du gène *NF1*, la présence de plusieurs pseudogènes *NF1*, les interactions complexes entre les différents types de cellules dans les tissus affectés, et l'état hétérozygote de *NF1* dans toutes les cellules du corps. Le but principal de cette thèse était d'obtenir une compréhension plus profonde du développement de différents symptômes relatés à la NF1, par appliquer des stratégies de détection des mutations somatiques améliorées à un éventail diverse des entités cellulaires.

D'abord, nous avons examiné profondément l'état de l'allèle *NF1* somatiquement affecté, spécifiquement dans les cellules de Schwann cultivées de neurofibromes (Article 1). Le taux élevé de détection de mutations somatiques et l'observation que la mutation seconde aboutit à une expression abaissée du gène *NF1*, a indiqué incontestablement que deux mutations inactivantes dans ce type de cellule spécifique est nécessaire pour le développement de neurofibromes. A cause du taux élevé de mutations somatiques avec un effet sur le cadre de lecture, il a été proposé qu'un efficience abaissé du mécanisme de réparation de l'ADN pourrait catalyser l'inactivation du gène *NF1*, et ainsi le développement de tumeurs. Ensuite, nous avons comparé le spectre des mutations germinales et somatiques. Une différence significative entre le mécanisme d'inactivation somatique des neurofibromes dérivés des patients avec une microdélétion de *NF1* et la population NF1 generale a été remarquée dans l'Article 1 et profondément examinée dans l'Article 2. Cette notion pourrait ajouter à une compréhension améliorée de la cause génétique de la présentation surélevée de tumeurs chez les patients avec une microdélétion de *NF1.*

Contrairement à la NF1 classique, où toutes les cellules du corps portent au moins un allèle *NF1* muté, les phénotypes segmentaires de la NF1 offrent la possibilité d'étudier des populations de cellules qui diffèrent seulement au niveau de la mutation causant le mosaïcisme. Pour cette raison, nous avons développé un essai de PCR quantitatif capable de détecter des pourcentages très bas de mutations ponctuelles mosaïques dans un contexte d'allèles normaux et de pseudogènes (Article 3) et nous avons analysé au niveau moléculaire de différentes manifestations cliniques qui se présentent dans la NF1 mosaique (Article 4). Cette approche a conformé avec élégance les caractéristiques tumeurigènes des cellules de Schwann dans le développement des neurofibromes. En plus, nous avons démontré pour la première fois que l'inactivation bi-allèlique du gène *NF1*

pourrait être la cause principale du développement des taches de café-au-lait relatées à la NF1. Indéniablement, cette constatation comporte un stimulant important pour la recherche d'avenir.

Finalement, nous avons analysé la pathogenèse moléculaire des tumeurs stromales gastro-intestinales (GISTs) qui se présentent dans le contexte de la NF1 (Article 5). Nous avons démontré que, contrairement aux GISTs sporadiques, les tumeurs relatées à la NF1 ne présentent pas de mutations dans les gènes *KIT* ou *PDGFRA*, mais montrent une activation somatique de l'allèle normal de *NF1* dans les cellules interstitielles de Cajal. L'inactivation de la neurofibromine compose un mécanisme alternatif pour activer les voies de signalisation de Map-Kinase, tandis que les voies de signalisation de Jak-Stat3 et PI3K-Akt sont moins activées dans les GISTs relatées à la NF1 que sporadiques. Il sera important de vérifier si l'inhibiteur de tyrosine kinase *Gleevec*, utilisé pour le traitement des GISTs sporadiques, aurait aussi un effet sur les GISTs relatées à la NF1.

En conclusion, nous avons apporté à la perspicacité des types cellulaires et défauts moléculaires responsables pour le développement des manifestations cliniques différentes de la NF1. Déterminer consciencieusement les répercussions biochimiques et cellulaires de ces défauts moléculaires et cellulaires autonomiques sera fondamental pour établir la fonction de la neurofibromine dans des contextes cellulaires différents. Finalement, cette perspicacité pourrait aboutir à l'identification des cibles additionnelles pour le développement des médicaments sur mesure.

LIST OF ABBREVIATIONS

AA	amino acid
aCGH	array comparative genomic hybridisation
APP	amyloid precursor protein
AC	adenylate cyclase
bp	base pair
CALM	café-au-lait macule
cAMP	cyclic adenosine monophosphate
cDNA	complementary DNA
CSRD	cysteine / serine rich domain
DNA	deoxyribonucleic acid
F	forskolin
FAP	familial adenomatous polyposis
FSNF	familial spinal neurofibromatosis
FTI	farnesyl transferase inhibitor
EGFR	epidermal growth factor receptor
ERK	extracellular signal-regulated protein kinase
EVI2A	ecotropic viral insertion site 2 protein A
EVI2B	ecotropic viral insertion site 2 protein B
GAP	GTPase activating protein
GEF	guanine nucleotide exchange factor
GIST	gastrointestinal stromal tumor
GM-CSF	granulocyte / macrophage colony-stimulating factor
GPCR	G-protein coupled receptor
GRD	GAP related domain
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
HNPCC	hereditary non-polyposis colorectal cancer
IBMX	3-isobutyl-1-methylxanthine
JMML	juvenile myelomonocytic leukaemia
kb	kilobase
KitL	kit ligand
kDa	kilodalton
LOH	loss of heterozygosity
MAPK	mitogen activated protein kinase
MPNST	malignant peripheral nerve sheath tumor
MRI	magnetic resonance imaging
mRNA	messenger RNA
Min	murine intestinal neoplasia
miRNA	micro RNA
Mom	modifier of Min
mTOR	mammalian target of rapamycin
NF1	neurofibromatosis type 1
NIH	National Institute of Health
OMGP	oligodendrocyte-myelin glycoprotein
OMIM	online mendelian inheritance in man
PCR	polymerase chain reaction
PDGFRA	platelet derived growth factor alpha
PI3K	phosphatidylinositol-3-kinase
PKA	cAMP-dependent protein kinase A
qPCR	quantitative PCR
RNA	ribonucleic acid
RT-PCR	reverse transcriptase PCR
SC	Schwann cell
SCF	stem cell factor
TSG	tumor suppressor gene

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Maertens O and De Raedt T. Somatic mutation analysis in neurofibromatosis type 1. IUAP meeting. UCL, Brussels, Belgium. 13/12/2004

Maertens O, Brems H, De Schepper S., De Paepe A., Legius E., Speleman F., Messiaen L. Comprehensive *NF1* screening on the selected tumorigenic cell population of dermal neurofibromas: towards an extensive study of the somatic mutation spectrum in neurofibromatosis type 1. Landelijk overleg klinische genetici (LOG) meeting. Utrecht, The Netherlands. 16/12/2004

Maertens O, Brems H, De Schepper S., De Paepe A., Legius E., Speleman F., Messiaen L. Comprehensive *NF1* screening on the selected tumorigenic cell population of dermal neurofibromas: towards an extensive study of the somatic mutation spectrum in neurofibromatosis type 1. First NF1 workshop: scientific research community FWO Flanders, molecular and cellular mechanisms in NF1, Leuven, Belgium, 10-11/01/2005

Maertens O, Debiec-Rychter M, Wozniak A, De Paepe A, Speleman F, Messiaen L, Legius E. Neurofibromatosis type 1 and gastrointestinal stromal tumors. The 11th European Neurofibromatosis meeting. Göteborg, Sweden, 7-10/07/2005

Maertens O, Debiec-Rychter M, Wozniak A, De Paepe A, Speleman F, Messiaen L, Legius E. Neurofibromatosis type 1 and gastrointestinal stromal tumors. Second NF1 workshop: scientific research community FWO Flanders, molecular and cellular mechanisms in NF1, Leuven, Belgium, 21-22/12/2005

Maertens O, De Schepper S, Vandesompele J, Janssens S, Speleman F, Legius E, Messiaen L. Molecular dissection of NF1-related disease features in segmental NF1 patients. The 56th annual meeting of the American Society of Human Genetics (ASHG). New Orleans, Louisiana, USA. 12/10/2006

Maertens O. New insights in the molecular pathogenesis of neurofibromatosis type 1. NF seminar. University of Alabama at Birmingham. Birmingham, Alabama, USA. 17/10/2006

Congresses, workshops and meetings

Contextualizing the Genome: the role of epigenetics in Genetics, Development and Evolution. Het Pand, Gent, Belgium, 25-28/11/2001

Bioinformatics training course in the Belgian EMBnet node. VUB, Brussels, Belgium, 2002.

Second annual meeting of the Belgian society of human genetics. Vrije Universiteit Brussel (VUB), Brussels, Belgium. 22/2/2002

NNFF International consortium for the molecular biology of NF1 and NF2. Aspen, Colorado, USA. 1-4/6/2002

Scientific day department internal Medicine, Ghent University Hospital, Ghent, Belgium, 17/01/2003

The 10th European neurofibromatosis meeting. Turku, Finland. 24-27/7/2003

Fourth annual meeting of the Belgian society of human genetics. Ghent University, Gent, Belgium. 19/3/2004

NNFF International consortium for the molecular biology of NF1, NF2 and schwannomatosis. Aspen, Colorado, USA. 23-25/5/2004 (oral presentation)

Stay at the neurofibromatosis research lab Leuven in the context of collaborative project 'Somatic loss of wild type *NF1* allele in Neurofibromas: Comparison of *NF1* Microdeletion and Non-Microdeletion Patients'. Supervision: Prof. Dr. Legius, Catholic University Leuven, Leuven, Belgium. 7/2004-8/2005

The first Marie-Curie Conference on arrayCGH and molecular Cytogenetics. Sanger Center, Hinxton, UK. 28/9-2/10/2004

The 54th Annual Meeting of the American Society of Human Genetics (ASHG), Toronto, Ontario, Canada, 26-30/10/2004 (oral presentation neurofibromatosis satellite symposium)

Arcturus Microgenomics workshop, Westburg, Leusden, The Netherlands, 23/11/2004.

Scientific day department internal Medicine, Ghent University Hospital, Ghent, Belgium, 20/01/2005 (poster)

The Children's tumor foundation international consortium for the molecular and cell biology of NF1, NF2 and schwannomatosis. Aspen, Colorado, USA, 5-8/06/2005 (poster)

The 11th European Neurofibromatosis meeting. Göteborg, Sweden, 7-10/07/2005 (oral presentation)

Sixth annual meeting of the Belgian society of human genetics. Antwerp University, Belgium. 17/3/2006 (poster)

Special Colloquium of the Royal Netherlands Academy of Arts and Sciences and the Erasmus Research School Molecular Medicine. The role of DNA Polymorphisms in Complex Traits and Diseases. Amsterdam, The Netherlands, 14-17/03/2006 (attendance of Master Classes given by John Blangero, Lon Cardon, Debby Nickerson and Henry Ehrlich)

Scientific day department internal Medicine, Ghent University Hospital, Ghent, Belgium, 30/04/2006 (poster)

The Children's tumor foundation International neurofibromatosis consortium. Aspen, Colorado, USA, 4-6/06/2006 (poster)

The 56th annual meeting of the American Society of Human Genetics (ASHG). New Orleans, Louisiana, USA. 9-13/10/2006 (oral presentation)

Miscellanea

Voluntary worker NF Kontakt Flemish lay group. November 2002-current

Belgian representative NF Europe laygroup network. November 2002-current