Pathogenesis of Neurofibromatosis 1 Associated Neurofibromas

by

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ABSTRACT

Neurofibromatosis 1 (NF1) is an autosomal dominant disease. Neurofibromas, benign tumours that develop from peripheral nerves, are a hallmark feature of NF1. Malignant peripheral nerve sheath tumours (MPNSTs) are one of the leading causes of death in people with NF1. Clinical evidence suggests that most MPNSTs develop from pre-existing plexiform neurofibromas.

Most studies treat all NF1-associated neurofibromas as a single entity, ignoring important differences between pathological details, clinical presentation and natural history. I analysed clinical information on 476 probands with NF1 from the Henri Mondor database and found that individuals with subcutaneous neurofibromas were 3 times more likely to have internal plexiform neurofibromas and that individuals with internal plexiform neurofibromas were 20 times more likely to have MPNSTs than individuals without such tumours. These findings suggest that pathogenic differences in some neurofibromas may lead to different risks of progressing to malignancy.

I collected formalin-fixed paraffin-embedded samples from NF1 patients and classified them histologically as nodular or diffuse neurofibromas. By using histochemistry, I found that mast cells were absent in MPNSTs and significantly more abundant in diffuse neurofibromas than in nodular neurofibromas. Mast cells were located at the periphery of nodular neurofibromas but were evenly distributed throughout diffuse neurofibromas.

Double immunofluorescent staining of S100 (a marker of Schwann cells, the presumed tumour progenitor cell type) and neurofibromin (the protein product of NF1 gene) (Nf) showed that diffuse neurofibromas had significantly more S100+/Nf+ cells and fewer S100-/Nf- cells than nodular neurofibromas. Using laser microdissection of immunofluorescently stained slides, I found that some neurofibromas show evidence of clonal (presumably neoplastic) proliferation of S100+/Nf- cells while other neurofibromas appear to be neurofibromin haploinsufficient and polyclonal, and thus may be hyperplastic rather than neoplastic lesions.

The results presented in this thesis support the hypothesis that neurofibromas in people with NF1 are pathogenically heterogeneous and that some kinds of neurofibromas are associated with the development of MPNSTs. These findings have important implications for the surveillance and treatment of people with NF1.
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<td>4′6-diamidino-2-phenylindole-2HCl (DAPI)</td>
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<td>Applied biosystems (ABI)</td>
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<td>Fluorescence in-situ hybridization (FISH)</td>
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<td>Guanosine diphosphate (GDP)</td>
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<td>Magnetic resonance imaging (MRI)</td>
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<td>Malignant peripheral nerve sheath tumour (MPNST)</td>
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<td>Matrix metalloproteinases (MMP)</td>
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<td>Messenger ribonucleic acid (mRNA)</td>
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<td>Neurofibromatosis type 1 (NF1)</td>
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<td>Odds ratio (OR)</td>
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<td>Polymerase chain reaction (PCR)</td>
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<td>Sodium acetate (NaOAc)</td>
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<td>Tris (hydroxymethyl) methylamine (Tris)</td>
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<td>Tuberous sclerosis complex (TSC)</td>
<td></td>
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<td>Ultraviolet (UV)</td>
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<td>Vascular endothelial growth factor (VEGF)</td>
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<td>X-chromosome inactivation (XCI)</td>
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CO-AUTHORSHIP STATEMENT

In Chapter 2, "Association between benign and malignant peripheral nerve sheath tumors in NF1", I was responsible for the design and data analysis of the research presented. I was responsible for the preparation of the manuscript with input from all contributing authors. P Wolkenstein was the physician who collected the data on the patients and J Revuz and J Zeller designed the database that stores the patient information.

In Chapter 3, "Heterogeneity of mast cell distribution in NF1 neurofibromas", I was responsible for the design of the research. In addition I performed all the immunohistochemistry, data collection and data analysis. VM Riccardi and J Wechsler reviewed tissue sections for this study to characterize them histologically. Together, M Sutcliffe and I reviewed tissue sections to assess tumour characteristics. J Vielkind financially supported the pathological services necessary for this project. P Wolkenstein contributed some of the samples used in this study. I was responsible for the preparation of the manuscript with input from all contributing authors.

In Chapter 4, "Pathogenetic heterogeneity of NF1-associated neurofibromas", I was responsible for the design of the research. In addition, I performed all histochemistry, data collection and data analysis. The majority of the molecular work was performed in the lab of, and with partial financial support by, C Brown. J Fee assisted in trouble-shooting laser microdissection. VM Riccardi and J Wechsler reviewed tissue sections for this study to characterize them histologically. Together, M Sutcliffe and I reviewed tissue sections to assess tumour characteristics. P Wolkenstein contributed some of the samples used in this study. I was responsible for the preparation of the manuscript with input from all contributing authors.
1. INTRODUCTION

Background on Neurofibromatosis

Neurofibromatosis 1 (NF1) is one of the most common dominantly inherited diseases, with a birth incidence of 1 in 3500 people (1). NF1 was first clinically recognized by von Recklinghausen in 1882 (2), but it took more than 100 years for criteria for diagnosing NF1 to be established (3) (Table 1.1). The disease is characterised by multiple cutaneous neurofibromas, plexiform neurofibromas, malignant peripheral nerve sheath tumours, and optic and other central nervous system gliomas, as well as by café-au-lait spots and abnormalities of the skeletal, cardiovascular and central nervous systems.

Table 1.1 National Institutes of Health diagnostic criteria for NF1.

<table>
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<th>Cardinal Clinical Features (Any two or more are required for diagnosis)</th>
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<tr>
<td>Six or more café-au-lait spots over 5mm in greatest diameter in prepubertal individuals or over 15mm in greatest diameter in postpubertal individuals</td>
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<td>Two or more neurofibromas of any type, or one plexiform neurofibroma</td>
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<td>Freckling in the axillary or inguinal regions</td>
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<td>Optic glioma</td>
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<td>Two or more Lisch nodules (iris hamartomas)</td>
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<td>A distinctive osseous lesion such as sphenoid dysplasia or thinning of the long bone cortex with or without pseudarthrosis</td>
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<tr>
<td>A first degree relative (parent, sibling, or offspring) with NF1 by the above criteria</td>
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The NF1 gene is located in the pericentric region of the long arm of chromosome 17 and was identified in 1990 (4, 5). The gene has 60 exons and encompasses 350kb of genomic DNA. The large gene size may account for the high estimated mutation rate of $1 \times 10^{-4}$ per generation (6), with 50% of NF1 cases due to new mutations. There are 3 other genes embedded within intron 27b that are transcribed in the opposite direction of the NF1 gene; two ectopic viral integration site loci (EV12A, EV12B) and the locus for
oligodendrocyte-myelin glycoprotein (OMGP). EV12A is expressed in the brain and bone marrow, and EV12B is expressed exclusively in the bone marrow (7), while OMGP is expressed on the surface of oligodendrocytes (8). Mutations within NF1 may inactivate one or all of these genes, but their role in the pathogenesis of disease is not known.

Unprocessed pseudogenes that share most of the NF1 sequence have been identified at 2q21, 12q12, 14q11, 15q11, 18p11, 21q11, and 22q11. Six of these regions are closely associated with centromeres, consistent with their emergence by pericentric interchromosomal transposition (9). 24 exons (exons 7-9, 10b, 11-23 and 24-27b) of the functional NF1 gene have homologous counterparts in the pseudogenes. Only the pseudogene on chromosome 21 has an uninterrupted reading frame, although pseudogenes on chromosome 2, 15 and 21 have shown some transcriptional activity (10).

Neurofibromin, the protein product of the NF1 gene, is over 220kD in size (11) and is a ubiquitously expressed protein. The best characterised function of NF1 was deduced from a domain encoded by exons 21-27a that is homologous to the GTPase-activating (GAP) domain of human p120GAP and yeast IRA1 and IRA2 (12). The GAP domain functions as a negative regulator of Ras by accelerating the conversion of active Ras-GTP to inactive Ras-GDP. Ras functions as part of a signal transduction pathway involving epidermal growth factor, nerve growth factor and platelet-derived growth factor. Ras can stimulate cellular proliferation through the Raf-MAK (mitogen activated kinase) pathway (13) and can also inhibit apoptosis through the phosphoinositol 3' kinase (PI3 kinase) pathway (14).

Three major alternatively spliced and differentially expressed isoforms of NF1 mRNA have been identified in humans. The most common isoform includes exon 23a within the GAP-related functional domain (15) and results in a decrease in GAP activity (16) in differentiated cells (17). Another isoform contains exon 48a and is preferentially expressed in muscle (18), and a third isoform that includes exon 9a is found exclusively in the central nervous system during embryogenesis (19).

A second domain upstream of the NF1 GAP-related domain has a role in cAMP signaling as it contains cAMP-dependent protein kinase A (PKA) binding sites (15). The NF1 homologue in Drosophila melanaster acts as an activator of the cAMP pathway as
well as a negative regulator of Ras (20); moreover, \( NF1^{+/} \) mutant flies can be rescued by an \( NF1 \) transgene and expression of activated PKA (21). This suggests that PKA functions downstream of or parallel to neurofibromin in Drosophila. In rats, it was found that neurofibromin GAP activity was decreased upon binding and phosphorylation by PKA and subsequent interaction with 14-3-3, a protein known to modulate various target proteins (22).

**Types of Neurofibromas in NF1**

In this thesis, I use the words “tumour” and “neurofibroma” interchangeably as a matter of convenience. The question of whether neurofibromas are true neoplasms is a focus of my research, and my use of “tumour” in this context is not intended to imply anything about the nature of the process that produces these lesions. Neurofibromas are always benign; the malignant counterpart is the malignant peripheral nerve sheath tumour (MPNST). In this thesis any reference to malignancy will be explicit.

Neurofibromas are tumours that develop within a peripheral nerve, and sometimes individual axons are visible within the tumour. Within the peripheral nervous system, each axon is surrounded by a Schwann cell to form a nerve fibre. Several nerve fibres become encased within the perineurium, a tubular structure made up of perineurial cells and collagen, to form a nerve fascicle. The space between the Schwann cells that surround the individual axons and the perineurium is the endoneurium. In larger nerves, several nerve fascicles are bundled together and are encased by a dense fibrous sheath, the epineurium (Figure 1.1). The development of a neurofibroma results in an increase in the number of Schwann cells and disorganized nerve structure with possible breakdown of some structures (Figure 1.1).
Figure 1.1 Schematic drawing showing a normal nerve and a neurofibroma. Illustration courtesy of Dr Gottfried. Images published in Neurosurgery. 58(1), 1-16, 2006.

Neurofibromas vary in terms of their age of appearance, natural history, and malignant potential. Consequently, several different classifications of neurofibromas have been proposed (Table 3.1). None of these classifications has achieved universal acceptance, and the literature contains many examples of inconsistent use and confusion of various clinical and pathological descriptive terms.

All neurofibromas contain Schwann cells (the suspected progenitor cell type), but fibroblasts, mast cells, lymphocytes, perineurial cells, endoneurial fibroblasts and endothelial cells also occur in varying numbers (23). Despite the similar cell types, neurofibromas can be classified histopathologically into two categories of diffuse and nodular neurofibromas. Diffuse neurofibromas are predominantly located in the dermis and involve the thin terminal branches of peripheral nerves (Figure 1.2A). Diffuse neurofibromas are characterized by proliferation of cells that are not limited by the perineurium and can encircle normal structures (exocrine glands, hair follicles and
vessels) without destroying them. The proliferating cells are irregularly dispersed within a fibrous and/or myxoid background. Myelin fibrils are rare or absent.

Nodular neurofibromas usually involve deeper tissues, and the cellular proliferation is intraneural and enclosed within large hypertrophic nerves circumscribed by the perineurium (Figure 1.2B). Nodular tumours include both single and plexiform types. Single nodular tumours involve just one fascicle, while nodular plexiform tumours may involve several fascicles, nerve branches or a nerve plexus. Dispersed or fascicular myelin fibrils are usually found in the central area of the nodules. On transverse section, the central area is loose with a myxoid background substance, while the peripheral area is usually more cellular.

Some neurofibromas contain both diffuse and nodular components; such tumours are usually found in the deep dermis and subcutaneous tissue. The nodular component is surrounded by diffuse extraneural cell proliferation, infiltrating the fat tissue and other normal pre-existing structures.

**Figure 1.2 Examples of histologically defined types of neurofibromas that develop as part of NF1.**

A. H&E section at 10.25x magnification of a histologically-defined diffuse neurofibroma with an arrow pointing to the skin that overlays the neurofibroma. B. H&E section at 10.25x magnification of a histologically-defined nodular neurofibromas with an arrow pointing to perineurium that circumscribes the neurofibroma.

Riccardi (24) has distinguished 4 types of neurofibromas clinically that differ with respect to their age of appearance, natural history, and malignant potential. This clinical classification does not correlate completely with the pathological classification given above. Riccardi's cutaneous neurofibromas occur in the skin surface and develop from
the terminal branches of peripheral nerves (Figure 1.3A). Subcutaneous neurofibromas are firm and lie deeper in the subcutaneous layer of the skin (Figure 1.3B). They have discrete margins but may extend along a nerve. Nodular plexiform neurofibromas arise within major peripheral nerves and may lie just beneath the dermis or much deeper in the body (Figure 1.3C). They can be localized or extend the length of the nerve, involve an entire nerve plexus, or spinal nerve roots at multiple layers. The last type is diffuse plexiform neurofibromas, which are sometimes referred to as congenital plexiform neurofibromas because they are usually apparent at birth or within the first few years of life (Figure 1.3D). These neurofibromas can be superficial, involving the skin and subcutaneous tissue, or involve deeper tissue. The tumours do not have discrete borders but invade adjacent normal tissues. Subcutaneous, nodular plexiform and diffuse plexiform neurofibromas can progress to malignancy, resulting in MPNSTs.

**Figure 1.3 Examples of clinically defined types of neurofibromas that develop as part of NF1.** C is an MRI image of the pelvis in frontal section.

The histological and clinical approaches do not classify individual tumours into the same groups. Both clinically defined cutaneous and diffuse plexiform neurofibromas usually are diffuse neurofibromas histologically, although some diffuse plexiform neurofibromas are histologically-mixed diffuse and nodular neurofibromas. Tumours that are classified
clinically as subcutaneous or nodular plexiform are usually nodular neurofibromas on histopathological exam.

Most studies of neurofibroma pathogenesis treat all neurofibromas as a single class despite their clinical and pathogenic heterogeneity. This heterogeneity is important because some kinds of neurofibromas have little, if any, malignant potential, while other kinds may progress to malignancy. MPNSTs are one of the leading causes of death in NF1 patients (25). There is currently no effective treatment known for benign neurofibromas or MPNSTs except surgical removal in favourable cases.

The “two-hit hypothesis”

In the early 1970's Alfred Knudson suggested that two mutations or “hits” were required for the development of a retinoblastoma and that the inheritance of one of these mutations could account for the earlier onset and frequent bilateral occurrence of the hereditary form of this tumour (26). Subsequent molecular studies supported Knudson's hypothesis in both hereditary and sporadic retinoblastomas by demonstrating mutations of both alleles of a gene that was named “RB1” in both hereditary and sporadic retinoblastomas (27). In hereditary retinoblastoma, individuals begin life with a constitutional mutation that inactivates one allele of the RB1 gene. The “second hit” occurs somatically and usually involves all or part of the chromosome containing the normal RB1 allele, potentially affecting other genes and genetic markers in the region of the normal RB1 allele. If some of these genetic markers happen to be heterozygous in an individual, loss of one allele on the same chromosome as the normal RB1 allele produces loss of heterozygosity (LOH), a cell-specific phenotype that is relatively easy to test in tumour tissue.

The “two-hit” hypothesis has subsequently been shown to apply to many other genes involved in many other inherited and sporadic tumours, and functional loss of both alleles of such genes is considered to be a principal mechanism of tumourigenesis (28, 29). As a consequence, LOH for a particular gene within a specific tumour type has become a major criteria for the involvement of that gene in tumour pathogenesis (30).

Genes that permit tumour development when both alleles are inactivated or lost are known as “tumour suppressor genes” (29). NF1 is a confirmed tumour suppressor gene as most constitutional mutations in NF1 result in loss of function of the protein, and LOH
has been observed in pheochromocytomas (31), myeloid leukemia (32) and MPNSTs (33, 34) in NF1 patients.

**Loss of Heterozygosity**

LOH analysis is performed by screening paired blood and tumour samples with polymorphic genetic markers in the region of an established or suspected tumour suppressor gene. The detection of LOH exploits the presence of DNA polymorphisms throughout the genome to discriminate between the paternal and maternal alleles at a tumour suppressor locus. In practice, identification of LOH is somewhat arbitrary because a decrease in the relative intensity, rather than a total loss of one allele, is usually observed in tumours. This is referred to as "allelic imbalance", and different studies have used different cutoffs to diagnose LOH. Although allelic imbalance is usually considered to be evidence of LOH, the ratio of alleles in neoplastic tissue can also be disturbed by other mechanisms, including trisomy and local DNA amplification (29).

In some instances, however, inactivation of both alleles of a tumour suppressor gene may occur but LOH may not be detected. Failure to observe LOH may result from technical limitations of the assay, such as inactivation of the normal allele of the tumour suppressor gene by point mutations, small deletions or epigenetic mechanisms that do not produce loss of closely linked polymorphic markers. In other circumstances, LOH would not be expected because the neoplasm arises by a mechanism such as activating mutations of one or more oncogenes rather than inactivation of both alleles of a tumour suppressor gene.

Vogelstein *et al.* (35) explained the increasing incidence of cancer in the general population with age by means of a more complex multistep tumorigenesis model. This model describes neoplastic development as the expansion of a clone of cells that has accumulated several somatic mutations. The neoplasm may be either benign or malignant, depending on whether its cells possess the ability to metastasize. The process is seen as one of progressive selection for cells with an increasing proliferative advantage. The first mutation in a somatic cell provides a slight growth advantage to its progeny. One of these cells acquires a further proliferative advantage through a second mutation, and one of its cellular progeny acquires a third mutation, possibly forming a benign tumour. Subsequent mutations in the descendants of these cells may permit
them to escape apoptosis, cell cycle checkpoints, or other growth control mechanisms or to become genetically unstable, increasing the likelihood for a tumour to progress.

Colorectal cancer is a well characterized cancer that follows the multistep model of tumourigenesis (35). Tumour initiation occurs with a mutation in the tumour suppressor gene, \textit{APC} (adenomatosis polyposis coli), which causes a hyperproliferation of the normal epithelium resulting in a benign colon adenoma. Mutations of the \textit{APC} gene can occur somatically, causing a single tumour, or in the germline, resulting in a predisposition that may cause the development of thousands of adenomas. A subsequent mutation in the \textit{RAS} gene occurs, leading to a further expansion of the cells and an increase in the size of the benign adenoma. Additional mutations in the \textit{DCC} (deleted in colorectal carcinoma) and \textit{P53} genes result in an increase in the clonal expansion and progression from benign adenoma to malignant carcinoma. Other alterations occur that permit metastasis.

\textit{Clonality in Neoplastic Cells}

Neoplasia almost always arises through mutation, although examples of abnormal epigenetic changes have also been documented as likely contributing events (36, 37). A acquired mutational event in somatic cells arise randomly, giving the affected cell a growth advantage compared to the surrounding cells. The cell then gives rise to a genetically identical clone of cells that, over time, can form a tumour. All of the cells of the resulting tumour are clonal derivatives of the one common ancestor cell that underwent the mutation. Such tumours are said to be “clonal” (Figure 1.4).
Figure 1.4 Cartoon depicting neoplasia as a clonal expansion of one cell that underwent a stochastic somatic mutation.

Each dark blue circle represents a normal cell in any tissue. One cell (light blue) has a somatic mutation that gives it a proliferative advantage over all other surrounding cells. The light blue cell expands clonally to form a tumour.

Most normal tissues are said to be “polyclonal”, i.e., composed of a mixture of cells that arose from many different precursors, none of which had a significant growth advantage over the others. Clonality may be observed among adjacent cells in normal solid tissues because the two daughter cells produced by mitotic division tend to remain close to each other in solid tissues. However, the “patches” of clonality that occur in normal tissues are usually small. The finding of a large region of clonality within a tissue suggests the presence of a neoplastic process.

There are some exceptions to this rule, for example some neoplasms are virally induced (38, 39), arising from the infection of a polyclonal set of cells. There are also examples of benign tumours that do not display clonality, such as the cortical tubers that occur as part of tuberous sclerosis complex (TSC), a dominantly-inherited disease (40-42). However, tubers are considered to be hamartomas that arise through a process of dysplasia rather than true neoplasms.

The most frequently-used assay of tissue clonality in humans is based on the fact that normal tissues in females are mosaic, being comprised of a mixture of cells, some of which have an active X-chromosome inherited from the mother, and others of which have an active X-chromosome inherited from the father. Mary Lyon first hypothesized that one X-chromosome in each female cell is randomly inactivated (43), and this is true in almost all cells of human females. X-inactivation occurs in early embryogenesis, and
once one of the two X-chromosomes in an embryonic cell is inactivated, the same X-chromosome (maternal or paternal) remains inactivated in all of the clonal progeny of that cell. X-inactivation is a random process, and on average half of the cells in any woman would be expected to have an inactive paternal X-chromosome, and the other half to have an inactive maternal X-chromosome. However, the actual ratio of the two cell types in any normal tissue follows a Gaussian distribution with a mean of 50:50. Substantial variation occurs around this mean as a result of the relatively small size of the cell pool present at the time of X-inactivation (43). It is therefore not surprising that normal female tissues often have a more or less unequal proportion of cells with the maternal or paternal X-chromosome inactive.

The pattern of X-inactivation in a tissue can be estimated by analysis of polymorphic X-linked marker genes (e.g., AR, androgen receptor) that contain restriction sites that are methylated on the inactive X-chromosome but not on the active X-chromosome (44). The marker is amplified by PCR with and without preceding digestion by a methylation-sensitive restriction enzyme. Following digestion, only the allele on the methylated inactive X chromosome persists as an intact fragment that can be amplified by PCR (Figure 1.5).

**Figure 1.5 Schematic of X-Chromosome Inactivation (XCI) assay with Androgen Receptor.**

A. Blue box represents the AR locus polymorphic CAG repeat of allele A; red box represents allele B. The vertical lines represent HpaII restriction sites and black filed arrows represent the PCR primers. Alleles A and B represent two alleles in an individual heterozygous at the AR locus. B. After genomic digestion with HpaII, there are two different scenarios: random XCI and skewed XCI. HpaII sites that are not methylated will be cut (indicated by an open space between the restriction sites). Methylated sites will remain intact. In random XCI, allele A is cut half the time, as is allele B. In completely skewed XCI, only one allele is cut (allele B in the figure). C. Upon PCR of the digested samples, amplification from the AR locus will only occur if the allele has not been cut with HpaII. D. The alleles can then be separated by electrophoresis.
Normal polyclonal tissues in a heterozygous female have a mixture of cells expressing the maternal and paternal alleles on the active X-chromosome. Such tissues usually exhibit approximately equal expression of each allele of the majority of X-linked genes. The presence of the same inactive allele in all cells of a tumour from a woman whose normal tissues exhibit both inactive alleles in approximately equal amounts confirms the monoclonal origin of the neoplasm.

Demonstrating clonality within a tissue is consistent with it being neoplastic, but clonality analysis does not provide information on the specific molecular events that lead to tumour initiation or progression. Clonality only means that one or more rare events (usually presumed to be mutations) have occurred during the process of tumour development. A tumour that is of clonal origin may contain several distinct subclones as a result of subsequent mutations. In addition, a tumour sample may contain enough non-malignant cells that it appears to represent a polyclonal population.

Mouse Models of NF1

Mouse models of NF1 have been developed, but these models only partially recapitulate the human disease. Mice heterozygous for \textit{Nf1} are viable but do not develop neurofibromas of any kind, and homozygous \textit{Nf1} null mice die in utero of heart defects, pointing to a critical function of neurofibromin in mouse embryonic development (45). Mice with a conditional deletion of both \textit{Nf1} alleles restricted to Schwann cells survive with enlarged peripheral nerves, demonstrating that complete deficiency of \textit{Nf1} in Schwann cells is sufficient to initiate a proliferative abnormality of the Schwann cell (46). Interestingly, these mice develop neurofibromas of the dorsal root ganglia only if other cells in the mice are heterozygous for \textit{Nf1} – an \textit{Nf1} haploinsufficient tumour environment appears necessary for the pathogenesis of these histologically-mixed tumours (46). The neurofibromas that arise in this mouse model are histologically similar to nodular plexiform neurofibromas that occur in people with NF1.

Molecular Analysis in NF1 Associated Neurofibromas

It is widely assumed that all benign NF1 neurofibromas develop when a "second hit" occurs somatically in the normal \textit{NF1} allele. Schwann cells are the suspected progenitor in neurofibromas because they constitute a large proportion of the tumour, 40-80\%, and some cultures derived from NF1 patient neurofibromas display loss of the normal \textit{NF1} allele in Schwann cells but not in fibroblasts (47-49).
Studies have focused on uncovering somatic mutations or "second hits" in NF1-associated neurofibromas. Evidence of clonality or LOH has been observed in 26% of discrete dermal neurofibromas (Table 1.2). A dermal neurofibroma reported by Sawada et al. (50) was especially informative because the somatic mutation in the second NF1 allele was identified. However, both the mutant and non-mutant version of the second NF1 allele were found in a homogenous-appearing area of tumour cells, suggesting that these cells were not all clonal derivatives of the cell that had sustained the second pathogenic mutation. This interpretation is strongly supported by studies of cultured Schwann cells from 10 neurofibromas obtained from 6 NF1 patients (51) whose germ-line NF1 mutations had been identified. Two different populations of Schwann cells were demonstrated in cultures from 7 of these 10 tumours; one population had a "second hit" somatic NF1 mutation, and the other did not. This finding raises the possibility that the "second hit" mutation occurred as a secondary event within a neurofibromma that had already developed through some polyclonal process. Alternatively, these tumours may have arisen through a "two-hit" mechanism, but the proliferating neoplastic clone (presumably Schwann cells) stimulated invasion of the tumour by non-neoplastic Schwann cells, perineural cells, mast cells and fibroblasts.
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† Author classified neurofibromas as benign, no distinction between dermal or plexiform
‡ Author found LOH in Schwann cells but not in fibroblast cultures from patient neurofibroma samples
Plexiform neurofibromas occur in about half of NF1 patients and may be either diffuse or nodular. Although these two types of plexiform tumours are clinically distinct, reported studies of LOH and clonality do not distinguish between them. The majority of plexiform neurofibromas have not shown evidence of clonality or LOH in the NF1 region (Table 1.2). However, Schwann cell cultures derived from 4 of 6 plexiform neurofibromas showed karyotypic abnormalities, while cultures of dermal neurofibromas did not display any chromosomal abnormalities (66). Karyotypic heterogeneity was observed in the cultures from 3 of these plexiform tumours. The observation of karyotypic abnormalities in plexiform neurofibromas may indicate that such changes are frequent in these tumours or at least in those that require surgical removal. Alternatively, chromosomal abnormalities may not have been present in the tumours but may have arisen in culture.

The low percentage of observed NF1 LOH in benign neurofibromas is often attributed to technical limitations of the assays, such as their inability to identify point mutations or epigenetic mechanisms (e.g. promoter methylation) that may inactivate the normal NF1 allele. To date, there is no evidence that methylation plays a role in inactivating the remaining normal NF1 allele (57, 67, 68). Moreover, it is thought that LOH and, hence, clonality in Schwann cells often remain undetected because of the abundance of non-neoplastic stromal cells present in these heterogeneous tumours. A third possibility that is rarely considered is that LOH is not observed because some neurofibromas do not develop as a result of “two hits” but rather as non-neoplastic proliferations of the involved tissue.

Evidence of LOH or second somatic mutations does not preclude the possibility that mutant neurofibromin protein expressed from the NF1 gene could have residual function. There are several examples of expression of NF1 transcripts that are not full-length neurofibromin but appear to be stable. The N-isoform of neurofibromin lacks the GAP-related domain and is expressed in normal brain and brain tumours, but it is not known what function or role this isoform has (69). NF1 mRNA is subject to mRNA editing, and one such edit produces a stop codon in the 5' portion of the NF1 GAP-related domain, which can result in an incomplete transcript (70). Moreover, an NF1 patient has been reported who had a nonsense mutation that was expressed at the same level as the normal NF1 allele, suggesting that the mutated transcript was stable.
The function of the transcript is not known, but if it is not degraded by non-sense mediated decay it may function to some extent (70).

**Mast Cells within Neurofibromas**

Local trauma may play a role in the development of some types of neurofibromas (71), especially cutaneous neurofibromas, as the skin is prone to bumps and bruising. Local trauma results in an inflammatory response, a process that involves mast cells. Several studies have noted the presence of mast cells within neurofibromas (72, 73), but it is not clear what type of neurofibromas were analysed or how many of them were from individuals with NF1. Mast cells have long been thought to play a role in the development of neurofibromas, as neurofibromas often itch or burn as they develop, and individuals with NF1 have described instances in which forceful trauma to the skin was followed by intense itching and the subsequent development of a neurofibroma (71). In addition, the conditional *Nf1* knock-out mouse model has shown the importance of the mast cells in the neurofibroma microenvironment. Mast cell infiltration in the thickened nerves and neurofibromas of the dorsal root is more substantial when the *Nf1* null Schwann cells proliferate in an *Nf1* heterozygous background than in the wild type background (46), and cutaneous mast cells outside of the tumours are also moderately increased and demonstrate enhanced growth in vitro in *Nf1* heterozygous mice compared to wild type mice (74).

Mast cells synthesize and store several factors that are involved in angiogenesis and cellular proliferation. When stimulated to degranulate, mast cells can secrete all of these products or a selection of them. Mast cells synthesize and express c-Kit, a tyrosine kinase receptor that is the product of the *c-Kit* proto-oncogene. The ligand for c-Kit, which is called SCF or KitL, promotes proliferation, migration and survival of mast cells. KitL is also expressed from normal Schwann cells and MPNST cell lines derived from individuals with NF1, while c-Kit is not expressed in Schwann cells from normal individuals (75). The expression of KitL by Schwann cells could recruit mast cells to the area, and, if stimulated to degranulate by local trauma or other means, could promote the formation or subsequent growth of neurofibromas. The expression of c-Kit by abnormal Schwann cells raises the possibility of autocrine-stimulated growth of Schwann cells.
If they are involved in the pathogenesis of neurofibromas, it is not known whether mast cells act during tumour initiation or progression or if their involvement differs among different types of neurofibromas.

**Malignant Peripheral Nerve Sheath Tumours**

People with NF1 have an average decrease in life expectancy of 15 years (25). MPNSTs are one of the most frequent causes of death among people with NF1 (25). MPNSTs have a poor prognosis because metastases to the lung, liver, brain, soft tissue, bone, regional lymph nodes, skin or retroperitoneum are common (76). The lifetime risk for an MPNST is 8 to 13% in NF1 patients (77). The risk is even higher (16-25%) for individuals with NF1 whose pathogenic mutation is a deletion of the whole *NF1* gene (78).

MPNSTs are true neoplasms, resulting from the clonal expansion of a cell population that has sustained mutations of both *NF1* alleles and likely additional mutation(s) at other loci to allow for malignant progression. Studies performed on both fresh MPNST samples and cell lines derived from MPNSTs have demonstrated clonality and loss of *NF1* expression, as well as an increase in ras activity (79-81). Most, if not all, MPNSTs in NF1 patients appear to develop from pre-existing plexiform neurofibromas (82, 83). In contrast, cutaneous neurofibromas rarely, if ever, progress to malignancy. Given the fact that many NF1 patients develop hundreds or thousands of times more cutaneous neurofibromas than plexiform neurofibromas, this difference in malignant potential is striking. The number, size, and type of benign neurofibromas vary greatly among NF1 patients, but it is not known if an individual’s risk for developing an MPNST bears any relationship to the burden of benign neurofibromas or if having one particular type of neurofibroma increases this risk.

**Research Objectives**

1. Determine if an individual’s risk for an MPNST is dependent on tumour burden or burden of one particular tumour type.

2. Determine the proportion of Schwann cells and mast cells in histologically-defined diffuse and nodular neurofibromas.
3. Determine the presence or absence of the neurofibromin protein in Schwann cells and non-Schwann cells within histologically-defined diffuse and nodular neurofibromas.

4. Determine if Schwann cells expressing or not expressing neurofibromin and non-Schwann cells expressing neurofibromin are clonal in histologically-defined diffuse and nodular neurofibromas.

**Hypotheses:**

**Risk of developing MPNSTs**

As the number of neurofibromas that an individual has increases, the probability that one of them may become malignant may also increase. Since subcutaneous, nodular and diffuse plexiform neurofibromas can progress to malignancy, I hypothesize that individuals who have these types of neurofibromas have a higher risk of developing MPNSTs compared to individuals without these types of neurofibromas.

**Mast cell distribution in neurofibromas**

Individuals with NF1 complain of intense "itchiness" in areas before cutaneous neurofibromas develop and the improper tissue formation and invasiveness of diffuse plexiform neurofibromas are likely to trigger mast cell infiltration. Therefore, I hypothesize that histologically-defined diffuse neurofibromas will have a high proportion of mast cells present. The observation that mice null for *Nf1* exhibit enlarged peripheral nerves and nodular plexiform neurofibromas with a higher density of mast cells on an *Nf1* heterozygous background compared to a wild type background suggests that mast cells are involved in this type of neurofibroma development. Therefore, I hypothesize that the number of mast cells will be high in nodular neurofibromas because they will be drawn into the tumour by the *NF1* Schwann cells.

**Histologically-defined diffuse neurofibromas**

I hypothesize that histologically-defined diffuse neurofibromas develop as a result of excessive proliferation of haploinsufficient *NF1* progenitor cells (Figure 1.6). Therefore, neurofibromin expression should be observed in all cell types, including Schwann cells, in these tumours, and all cells should exhibit a polyclonal phenotype. The hypothesis that histologically-defined diffuse neurofibromas develop from non-neoplastic cellular proliferation is supported by knockout mouse studies in which *Nf1* Schwann cells...
show increased invasiveness and angiogenesis compared to Schwann cells from wild type litter-mates (84) and skin fibroblasts proliferate beyond the normal time of maturation in wound healing (85).

Histologically-defined diffuse neurofibromas include both clinically-defined cutaneous and most diffuse plexiform neurofibromas. The anecdotal evidence cited above that local trauma may be a factor in the development of cutaneous neurofibromas is consistent with their being proliferative rather than neoplastic lesions. In addition, fibroblasts obtained from scars of normal humans display an increase in neurofibromin signal intensity by immunohistochemistry, suggesting that increased neurofibromin activity may play a role in the normal response of fibroblasts to tissue injury (86) and that this response may be abnormal in people with NF1.

Clinically-defined diffuse plexiform neurofibromas almost always develop early in life. At least some diffuse plexiform neurofibromas are present before the 16th week of gestation because the hair and pigmentation patterns in skin overlying the tumours is altered in a manner that cannot occur later in fetal development (87). It is possible that haploinsufficiency for NF1 could disrupt the checks and balances that normally regulate the growth of fetal tissues. Normal fetal development entails sequential events dependent on the proper regulation of cellular proliferation, migration, differentiation and death. In most cases these processes require coordinated regulation of several factors within and between cell types. If the level of one or more of these factors is not optimal, a particular pathway may be disrupted.
Figure 1.6 Haploinsufficient model of tumour development in histologically-defined diffuse neurofibromas in NF1.

Schwann cells are represented by circles and all other cells are represented by squares. The blue colour represents allele A at the AR locus and red represents allele B. A. A normal tissue in a non-NF1 individual has two functional copies of the NF1 gene (+/+) in all cells. When there is a stimulus to proliferate the cells are kept in check by the two functional copies of NF1. B. In individuals with NF1, all cells are heterozygous for NF1 (+/-). When there is a stimulus to proliferate, all cells will expand in number because half the amount of neurofibromin is not sufficient to keep the cells in check, resulting in a tumour from this abnormal proliferation. The tumour will be polyclonal because there is a mixture of allele A and allele B at the AR locus in the cells that proliferated.

Histologically-defined nodular neurofibromas

I hypothesize that histologically-defined nodular neurofibromas develop by a "second hit" mutation of the NF1 gene in Schwann cells (Figure 1.7). Therefore, neurofibromin expression should not be observed in the majority of Schwann cells but will be observed in all other cells in this tumour. Some Schwann cells may demonstrate neurofibromin expression if they are drawn into the tumour, along with other cell types, by the neoplastic Schwann cells. Schwann cells that do not express neurofibromin should
exhibit a clonal phenotype, while Schwann cells expressing neurofibromin and all other cell types should exhibit a polyclonal phenotype.

**Figure 1.7 “Second hit” model of development for histologically-defined nodular neurofibromas in NF1.**

Schwann cells are represented by circles and all other cells are represented by squares. Alleles A and B are represented by blue and red, respectively. In an NF1 individual, all cells are heterozygous for *NF1* (+/-). In this model there is a mixture of allele A and allele B in Schwann cells and all other cell types. A second somatic mutation at the *NF1* locus occurs in one Schwann cell (-/-) that provides a growth advantage to that cell, which allows it to expand clonally to form a tumour. As all NF1~ Schwann cells were all derived from the one progenitor, these cells will display a clonal phenotype, while NF1~ Schwann cells and all other cells will be polyclonal because there was a mixture of both allele A and allele B in the tissue to begin with.

![Diagram of the second hit model](image)

**Relevance**

There is currently no way of predicting which individuals with NF1 will develop neurofibromas or MPNSTs. MPNSTs are one of the leading causes of death among people with NF1, and this is often because the MPNST has already metastasized widely by the time it is detected. It would be helpful to be able to predict which individuals are at high risk for developing MPNST in order to monitor them closely for early signs of malignancy, a strategy that may improve the chances of survival.

Since most MPNSTs develop from pre-existing neurofibromas, understanding how different types of neurofibromas develop could help treat them and prevent some from progressing to malignancy. The *NF1* gene is known to have a high mutation rate. LOH
and subtle “second hit” mutations have been observed in the NF1 gene, but this alone does not prove that a neurofibromin-like protein is not expressed. Looking at neurofibromin expression rather than LOH will provide information on the proportion of Schwann cells that express neurofibromin, and assessing clonality will show how the cells proliferated to form the tumour. This information could help in the development of treatments, since treating a tumour that has Schwann cells that do not express neurofibromin may involve different approaches than if the Schwann cells are haploinsufficient, retaining some neurofibromin expression.

Most studies treat all neurofibromas as a single group, failing to take into consideration histological and clinical differences that may be indicative of different pathogenic mechanisms. The inconsistency in LOH and clonality results that have previously been reported in NF1 might be explained by pooling the results of tumours with different pathogenesis. Consideration of differences in histological appearance, clinical features and natural history may help to illuminate an analysis of the mechanisms involved in neurofibroma development.
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2. ASSOCIATION BETWEEN BENIGN AND MALIGNANT PERIPHERAL NERVE SHEATH TUMOURS IN NF1

INTRODUCTION

Neurofibromatosis 1 (NF1) is an autosomal dominant disease affecting 1 in 3000 to 1 in 4000 people. NF1 is characterized by multiple dermal neurofibromas, plexiform neurofibromas, malignant peripheral nerve sheath tumours (MPNSTs), and optic and other CNS gliomas, as well as by café-au-lait spots and abnormalities of the skeletal, cardiovascular and central nervous systems. The NF1 gene is located on chromosome 17q11.2, and its protein, neurofibromin, functions as a tumour suppressor.

People with NF1 have a decrease in life expectancy of 15 years, with MPNSTs as a leading cause of death in young adults (1, 2). MPNSTs have a poor prognosis because metastases to the lung, liver, brain, soft tissue, bone, regional lymph nodes, skin or retroperitoneum are common (3). The frequency of MPNSTs in the general population is 0.001%, compared to 2-5% in people with NF1 (4). The lifetime risk for MPNST is 8 to 13% in NF1 patients (5). Most studies show that the peak incidence of MPNSTs is in the 7th decade of life in the general population but in the 3rd or 4th decade in people with NF1 (5), although these tumours may occur at a much younger age in either population (3).

Some patients with NF1 appear at greater risk than others of developing MPNSTs. For example, people with NF1 whose pathogenic mutation is a deletion of the whole NF1 gene are thought to have a lifetime risk of 16 to 25% for developing an MPNST (6). There are several reports of MPNSTs developing in the field after radiation treatment for another tumour, as might be expected for individuals who carry one constitutional mutation of the NF1 tumour suppressor gene (3, 7).

Most, if not all, MPNSTs in NF1 patients appear to develop from pre-existing plexiform neurofibromas (8, 9). The number, size, and type of benign neurofibromas vary greatly among NF1 patients, but it is not known whether an individual's risk for developing an MPNST bears any relationship to the burden of benign neurofibromas.

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One study found that the greatest risk factors associated with MPNSTs in NF1 patients were pain related to a mass and the presence of subcutaneous and cutaneous neurofibromas (10). In this study, the median ages of the NF1 patients with MPNSTs was 26.4 years and without MPNSTs was 14.7 years, but this difference was not considered in the analysis. The presence of both subcutaneous and cutaneous neurofibromas is dependent on age, with the majority of people above the age of 16 having at least one of these tumours (11). Therefore, many people in the group of patients with NF1 without MPNSTs would not be old enough to exhibit subcutaneous or cutaneous neurofibromas, whereas almost all of those with MPNSTs would be expected to have dermal tumours just on the basis of their age.

We used logistic regression to determine if associations exist between MPNST and cutaneous, subcutaneous, superficial plexiform or internal plexiform neurofibromas among patients with NF1.

**METHODS**

*Subjects and Data Description*

All patients included in this analysis were diagnosed with NF1 according to established clinical criteria (12, 13). Data were obtained on all NF1 patients seen in the NF clinic at the Henri-Mondor Hospital (Réseau NF-Mondor) between June 1988 and May 2004. At the time of the analysis, this database had extensive demographic and clinical data on 476 NF1 probands, 31 of their affected parents and 16 affected children. Only probands were included in the present analysis. The study protocol was approved by the hospital Ethics Committee.

We analyzed cross-sectional data on cutaneous neurofibromas, subcutaneous neurofibromas, and internal and superficial plexiform neurofibromas recorded at the most recent complete clinical assessment. All clinically-apparent neurofibromas were included, regardless of size. Discrete dermal tumours seen on physical exam were considered to be cutaneous neurofibromas if they moved with the skin and subcutaneous neurofibromas if the skin moved over the lesion (14). Superficial plexiform neurofibromas are more diffuse than the discrete tumours and involve the skin or subcutaneous tissue, which is thickened or raised, and the overlying skin is often hyperpigmented or hypertrichotic (14). Internal plexiform neurofibromas were not observed on physical examination but were identified by imaging studies.
Data were obtained during routine clinical assessment of these patients in the NF Clinic. The standard protocol was to perform a physical examination and clinical history on each patient yearly. Between 1988 and 1995, patients also received a screening ophthalmologic exam using a slit lamp, chest x-ray, abdominal ultrasound exam, cerebral CT or MRI, and 24-hour urinary catecholamine analysis every five years to look for common problems associated with NF1. Additional studies were done to investigate any symptoms of internal neurofibromas or MPNST that occurred. After 1995, the screening was discontinued and imaging studies were only done to investigate patient symptoms. All information was recorded using the standard format and consistent definitions of clinical features developed for the National Neurofibromatosis Foundation International Database (15).

The number of cutaneous neurofibromas present in a patient was counted or estimated on physical exam and recorded semi-quantitatively as none, 1-9, 10-100 or greater than 100. Subcutaneous and superficial plexiform neurofibromas were recorded as present or absent. The presence or absence of internal plexiform neurofibromas was determined by routine abdominal ultrasound and chest x-ray examinations. MRI or CT examinations were performed when evidence of an internal plexiform neurofibroma was seen on the ultrasound exam or chest radiograph or when the patient had symptoms such as pain or neurological deficits suggestive of an internal plexiform neurofibroma. Patients coded as ‘unknown’ for a particular feature were not considered in models involving that feature.

MPNSTs were suspected by clinical examination or by imaging studies and were confirmed by biopsy. Probands with MPNSTs were followed longitudinally every three months. If a patient did not attend the follow-up appointment, the status of the patient was determined by a telephone call to the individual or family.

**Statistical Analysis**

Non-parametric Kruskal-Wallis and Mann-Whitney U tests were calculated using SPSS (SPSS, Inc., Chicago, Illinois, 1998) to assess the relationship of age to each type of neurofibroma. A p-value less than or equal to 0.05 was considered to be significant.

Logistic regression models were developed using SPSS software (SPSS, Inc., Chicago, Illinois, 1998). Many NF1 features have a higher prevalence in older patients, so age
(coded to the nearest year) at exam was treated as a covariate in all analyses. The first set of models had the presence of internal plexiform neurofibromas as the response variable and either cutaneous, subcutaneous or superficial plexiform neurofibromas as the explanatory variable. The second set of models had the presence of an MPNST as the response variable and cutaneous, subcutaneous, superficial plexiform or internal plexiform neurofibromas as the explanatory variable. Associations with explanatory variables were considered to be significant if the 95% CI of the odds ratio excluded 1.0.

Survival rates from the time of MPNST diagnosis were calculated for all probands with MPNSTs who had died or were still living at the time of the analysis (May 2004). Five-year survival by gender was determined using Kaplan-Meier curves, and the significance of gender on survival was determined using a log rank test (SPSS, Inc., Chicago, Illinois, 1998).

RESULTS

Prevalence of Neurofibromas

We studied 476 probands. 93% of these NF1 patients are from France. Subject age at examination ranged from 1 to 77 years, with an average of 33 years (median = 31 years).

Semi-quantitative data on cutaneous neurofibromas were available for 443 NF1 probands. Cutaneous neurofibromas were recorded as absent in 66 (15%) of the individuals and present in the rest. Sixty-eight (15%) of the patients had 1-9 cutaneous neurofibromas, 170 (38%) had 10-100 cutaneous neurofibromas and 139 (31%) had more than 100 cutaneous neurofibromas. The average age (± 1 standard deviation) for individuals without cutaneous neurofibromas was 21 ± 10 years, for individuals with 1-9 neurofibromas was 27 ± 11 years, for individuals with 10-100 neurofibromas was 33 ± 12 years and for individuals with more than 100 cutaneous neurofibromas was 42 ± 12 years. These values were significantly different from each other (p<0.001). Of the 443 probands with information on cutaneous neurofibromas, 248 (56%) were female. 88% of the females had one or more cutaneous neurofibromas compared to 67% of males. The average age of females for each of the categories of cutaneous neurofibromas was higher than that of the males, but the differences are not significant.
Subcutaneous neurofibromas were present in 224 (48%) of the 468 individuals on whom information was available. The mean age of patients with (34 ± 14 years) and without (33 ± 13 years) subcutaneous neurofibromas was not significantly different.

Superficial plexiform neurofibromas were present in 194 (41%) of the 471 individuals on whom information was available. The mean age of patients with and without superficial plexiform neurofibromas was the same (33 ± 14 years).

There were 145 NF1 probands who received at least a routine abdominal ultrasound exam and chest radiograph to identify internal plexiform neurofibromas. Eighty-six of these individuals underwent imaging studies as part of a routine screening protocol, and the other 59 people had imaging studies to investigate symptoms suggestive of an internal tumour. There were 54 internal plexiform neurofibromas identified in 49 (34%) probands. Twenty-eight plexiform neurofibromas presented with pain, and 26 were asymptomatic. Forty-four of the 49 people who were found to have internal plexiform neurofibromas underwent CT or MRI exams. The CT or MRI was done because of symptoms of an internal tumour in 30 patients and for confirmation after an abnormal abdominal ultrasound or chest x-ray exam in the other 14 individuals.

The average age of patients with internal plexiform neurofibromas was 36 ± 14 years, compared to 34 ± 14 years for individuals without internal plexiform neurofibromas (p=0.48). Of the internal plexiform neurofibromas that were identified, 43 (80%) were of the spinal nerve roots or ganglia, nine (17%) were non-spinal in the abdomen or pelvis and two (4%) were non-spinal in the chest.

There were 19 spinal neurofibromas that did not present with symptoms to the 18 individuals affected. Twenty-four spinal tumours were symptomatic in 21 individuals – eight complained of pain, nine had neurological symptoms of spinal cord compression, five had both pain and symptoms of spinal cord compression, one complained of pain and had bone destruction, and another complained of symptoms of compression of the duodenum. One individual who had compression of the spinal cord experienced paralysis involving the region below the area of compression, and two other individuals had respiratory distress and subsequently died as a consequence of their spinal cord compression.
Two individuals had non-spinal chest neurofibromas; both were asymptomatic. Nine non-spinal tumours of the abdomen or pelvic region were identified in eight individuals, five of whom were asymptomatic. One patient who had a neurofibroma in the region of the pancreas developed cholestasis, another individual with intra-abdominal neurofibromas had gastrointestinal bleeding, and another had abdominal pain and was found to have a neurofibroma of the pelvis.

Of the 469 individuals with information on MPNST status, 25 (5%) had MPNSTs. Twenty-four of these 25 patients were symptomatic, with the majority of individuals complaining of pain in the area and enlargement of a previously-recognized tumour (Table 2.1). The individual who did not present with symptoms had an MPNST of a lower limb that was discovered during an operation for pseudarthrosis [Patient 24]. The average age of those with MPNSTs (31 ± 12 years) did not differ (p=0.35) from the average age of individuals without MPNSTs (33 ± 12 years). All 25 of these MPNSTs were shown pathologically to contain elements of benign plexiform neurofibroma and, therefore, are likely to have arisen from a pre-existing benign tumour. The MPNST arose in one of the internal benign plexiform tumours that had previously been imaged in eight of the 20 cases in which imaging studies has been performed. Seven more of these 20 MPNSTs may have arisen in relationship to a tumour that was imaged but cannot be confirmed to have done so. The remaining five MPNSTs arose in tumours that were apparently unrelated to the ones identified by imaging. The details of each are given in Table 2.1 and summarized below.
## Table 2.1 Summary of Patient Data for those with MPNSTs.

<table>
<thead>
<tr>
<th>Patient Number</th>
<th>Sex</th>
<th>Age at MPNST Diagnosis</th>
<th>Location of MPNST</th>
<th>Internal Plexiform Neurofibromas Seen on Imaging Studies</th>
<th>Survival (Months)</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>22</td>
<td>Face</td>
<td>ND</td>
<td>30</td>
<td>Dead</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>29</td>
<td>Face</td>
<td>None</td>
<td>84</td>
<td>Alive</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>32</td>
<td>Face</td>
<td>L3-S1</td>
<td>3</td>
<td>Dead</td>
</tr>
<tr>
<td>4</td>
<td>F</td>
<td>39</td>
<td>Cervical</td>
<td>ND</td>
<td>108</td>
<td>Alive</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>49</td>
<td>C2</td>
<td>C2-C7</td>
<td>15</td>
<td>Dead</td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td>49</td>
<td>C2-C3</td>
<td>C2-C6</td>
<td>60</td>
<td>Alive</td>
</tr>
<tr>
<td>7</td>
<td>F</td>
<td>17</td>
<td>Trunk</td>
<td>ND</td>
<td>12</td>
<td>Dead</td>
</tr>
<tr>
<td>8</td>
<td>F</td>
<td>18</td>
<td>Trunk</td>
<td>Thoracic</td>
<td>12</td>
<td>Dead</td>
</tr>
<tr>
<td>9</td>
<td>M</td>
<td>27</td>
<td>T1</td>
<td>T1-T3</td>
<td>4</td>
<td>Alive</td>
</tr>
<tr>
<td>10</td>
<td>M</td>
<td>30</td>
<td>Abdominal</td>
<td>ND</td>
<td>29</td>
<td>Dead</td>
</tr>
<tr>
<td>11</td>
<td>F</td>
<td>20</td>
<td>Lumbar Spine</td>
<td>ND</td>
<td>29</td>
<td>Alive</td>
</tr>
<tr>
<td>12</td>
<td>M</td>
<td>24</td>
<td>L2</td>
<td>L1-L2</td>
<td>0</td>
<td>Dead</td>
</tr>
<tr>
<td>13</td>
<td>F</td>
<td>25</td>
<td>Sacrum</td>
<td>T5-S1</td>
<td>48</td>
<td>Alive</td>
</tr>
<tr>
<td>14</td>
<td>M</td>
<td>31</td>
<td>L4-L5</td>
<td>L4</td>
<td>60</td>
<td>Alive</td>
</tr>
<tr>
<td>15</td>
<td>F</td>
<td>31</td>
<td>Pelvis</td>
<td>Lumbar &amp; Sacral</td>
<td>19</td>
<td>Dead</td>
</tr>
<tr>
<td>16</td>
<td>F</td>
<td>33</td>
<td>Sciatic Nerve</td>
<td>L5-S2</td>
<td>23</td>
<td>Dead</td>
</tr>
<tr>
<td>17</td>
<td>M</td>
<td>34</td>
<td>L2-L3</td>
<td>L2-L3</td>
<td>14</td>
<td>Dead</td>
</tr>
<tr>
<td>18</td>
<td>F</td>
<td>37</td>
<td>Upper Limb</td>
<td>Cervical</td>
<td>36</td>
<td>Alive</td>
</tr>
<tr>
<td>19</td>
<td>M</td>
<td>53</td>
<td>Upper Limb</td>
<td>S1-S2</td>
<td>12</td>
<td>Dead</td>
</tr>
<tr>
<td>20</td>
<td>M</td>
<td>56</td>
<td>Upper Limb</td>
<td>None</td>
<td>84</td>
<td>Alive</td>
</tr>
<tr>
<td>21</td>
<td>F</td>
<td>15</td>
<td>Lower Limb</td>
<td>L5-S1</td>
<td>52</td>
<td>Dead</td>
</tr>
<tr>
<td>22</td>
<td>F</td>
<td>19</td>
<td>Lower Limb</td>
<td>L2-L3 &amp; T9-T10</td>
<td>3</td>
<td>Dead</td>
</tr>
<tr>
<td>23</td>
<td>F</td>
<td>21</td>
<td>Lower Limb</td>
<td>None</td>
<td>55</td>
<td>Alive</td>
</tr>
<tr>
<td>24</td>
<td>M</td>
<td>21</td>
<td>Lower Limb</td>
<td>Lumbar &amp; Sacral</td>
<td>108</td>
<td>Alive</td>
</tr>
<tr>
<td>25</td>
<td>M</td>
<td>36</td>
<td>Lower Limb</td>
<td>Lumbar</td>
<td>16</td>
<td>Dead</td>
</tr>
</tbody>
</table>

ND = Imaging studies for internal plexiform neurofibromas not done
M = Male; F = Female
Three individuals [Patients 1, 2, and 3] had MPNSTs of the face that developed from previously-identified plexiform neurofibromas. Patient 3 also had a spinal neurofibroma at L3-S1. Patient 2 received a body CT scan only after his referral to the Henri Mondor Hospital for suspicion of an MPNST, and no internal plexiform neurofibromas were found. Patient 1 did not have imaging studies performed to look for internal plexiform neurofibromas.

Eight internal MPNSTs were confirmed pathologically to originate from a previously-identified internal plexiform neurofibroma; all were tumours of the spinal nerves [Patients 5, 6, 9, 12, 13, 14, 16 and 17]. A pelvic MPNST was identified in an individual who had internal plexiform neurofibromas of the lumbar and sacral spine [Patient 15]. Another individual was found to have an internal plexiform neurofibroma of the thoracic spine and developed an MPNST of the trunk [Patient 8]. Both of these malignancies were suspected to have originated from previously-unidentified internal plexiform neurofibromas, and pathology reports demonstrated benign components in the MPNST.

Three MPNSTs of the upper limb [Patients 18, 19 and 20] and 5 of the lower limb [21, 22, 23, 24, and 25] developed from previously-identified plexiform tumours outside of the area imaged. Six of these individuals [Patients 18, 19, 21, 22, 24 and 25] also had internal plexiform neurofibromas of the spine. Both individuals who did not have internal plexiform neurofibromas [Patients 20 and 23] had normal abdominal ultrasound and chest x-ray exam three years before diagnosis of the MPNST.

MPNSTs of the cervical spine, lumbar spine, abdomen and trunk were identified in four individuals [Patients 4, 7, 10 and 11] who had no previous imaging performed to identify internal plexiform neurofibromas. Pathology reports confirmed that there were benign components in each of these malignant tumours.

**Logistic Regression**

The logistic regression analysis of cutaneous and internal plexiform neurofibromas was limited to 139 NF1 probands who had information on the presence of internal plexiform neurofibromas as well as semi-quantitative data on cutaneous neurofibromas. There was no significant association between cutaneous and internal plexiform neurofibromas (Table 2.2).
Table 2.2 Summary of odds ratios and 95% confidence intervals for logistic regression models.

<table>
<thead>
<tr>
<th>Response Variable</th>
<th>Explanatory Variable*</th>
<th>Odds Ratio (95% Confidence Interval)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Internal Plexiform Neurofibromas</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cutaneous Neurofibromas 1-9</td>
<td></td>
<td>1.70 (0.49, 5.91)</td>
</tr>
<tr>
<td>10-100</td>
<td></td>
<td>0.55 (0.14, 2.14)</td>
</tr>
<tr>
<td>&gt;100</td>
<td></td>
<td>0.88 (0.36, 2.13)</td>
</tr>
<tr>
<td>Age (per year)</td>
<td></td>
<td>1.00 (0.97, 1.03)</td>
</tr>
<tr>
<td>Subcutaneous Neurofibromas</td>
<td></td>
<td>3.58 (1.68, 7.64)</td>
</tr>
<tr>
<td>Age (per year)</td>
<td></td>
<td>1.00 (0.98, 1.03)</td>
</tr>
<tr>
<td>Superficial Plexiform Neurofibromas</td>
<td></td>
<td>0.74 (0.36, 1.54)</td>
</tr>
<tr>
<td>Age (per year)</td>
<td></td>
<td>1.00 (0.98, 1.00)</td>
</tr>
<tr>
<td>MPNST</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cutaneous Neurofibromas 1-9</td>
<td></td>
<td>0.79 (0.15, 4.08)</td>
</tr>
<tr>
<td>10-100</td>
<td></td>
<td>1.20 (0.28, 5.13)</td>
</tr>
<tr>
<td>&gt;100</td>
<td></td>
<td>1.56 (0.51, 4.80)</td>
</tr>
<tr>
<td>Age (per year)</td>
<td></td>
<td>0.98 (0.94, 1.02)</td>
</tr>
<tr>
<td>Superficial Plexiform Neurofibromas</td>
<td></td>
<td>0.90 (0.62, 1.32)</td>
</tr>
<tr>
<td>Age (per year)</td>
<td></td>
<td>1.02 (1.01-1.04)</td>
</tr>
<tr>
<td>Subcutaneous Neurofibromas</td>
<td></td>
<td>2.81 (1.14, 6.94)</td>
</tr>
<tr>
<td>Age (per year)</td>
<td></td>
<td>0.99 (0.95, 1.01)</td>
</tr>
<tr>
<td>Internal Plexiform Neurofibromas</td>
<td></td>
<td>20.52 (5.3, 79.5)</td>
</tr>
<tr>
<td>Age (per year)</td>
<td></td>
<td>0.94 (0.90, 0.99)</td>
</tr>
<tr>
<td>MPNST</td>
<td>Internal Plexiform</td>
<td>18.06 (4.55, 73.4)</td>
</tr>
<tr>
<td>Neurofibroma</td>
<td>Subcutaneous Neurofibroma</td>
<td>2.08 (0.60, 7.24)</td>
</tr>
<tr>
<td>Age (per year)</td>
<td>Age (per year)</td>
<td>0.94 (0.89, 0.98)</td>
</tr>
</tbody>
</table>

* Comparisons were made with features that were absent in an individual.
The association between cutaneous neurofibromas and MPNSTs was tested on 439 individuals who had semi-quantitative data on numbers of cutaneous neurofibromas as well as information on the presence or absence of MPNSTs. There was no significant association between the number of cutaneous neurofibromas measured on a semi-quantitative scale and the presence of MPNSTs (Table 2.2).

The logistic regression analysis of subcutaneous neurofibromas and internal plexiform neurofibromas was limited to 144 individuals who had information on the presence or absence of both internal plexiform and subcutaneous neurofibromas. Seventy-six of these patients had subcutaneous neurofibromas, 35 (46%) of whom also had internal plexiform neurofibromas. In contrast, of the 68 patients who did not have subcutaneous neurofibromas, only 13 (20%) had internal plexiform neurofibromas. There was a significant association between the presence of subcutaneous and internal plexiform neurofibromas, with individuals having subcutaneous neurofibromas being 3.58 times more likely to have internal plexiform neurofibromas than individuals without subcutaneous neurofibromas (Table 2.2).

There were 464 individuals who had information on presence or absence of both subcutaneous neurofibromas and MPNSTs. Seventeen (8%) of the 222 patients with subcutaneous neurofibromas also had MPNSTs, compared to seven (3%) of the 242 NF1 individuals without subcutaneous neurofibromas. There was a significant association between the presence of subcutaneous neurofibromas and MPNSTs (Table 2.2). Individuals with subcutaneous neurofibromas were almost three times more likely to have an MPNST as individuals who did not have subcutaneous neurofibromas.

The association between superficial and internal plexiform neurofibromas was tested on 143 individuals who had information on presence or absence of both kinds of plexiform neurofibromas. There was no significant association between the presence of superficial and internal plexiform neurofibromas (Table 2.2).

There were 465 individuals who had information on presence or absence of both superficial plexiform neurofibromas and MPNSTs. There was no significant association between superficial plexiform neurofibromas and MPNSTs (Table 2.2).

The analysis of internal plexiform neurofibromas and MPNSTs was limited to 141 NF1 probands who had information on the presence or absence of both internal plexiform neurofibromas and MPNSTs. There was no significant association between these variables (Table 2.2).
neurofibromas and MPNSTs. Forty-nine individuals had internal plexiform neurofibromas, 17 (35%) of whom also had MPNSTs. In contrast, only three (3%) of the 92 individuals without internal plexiform neurofibromas had MPNSTs. The association between the presence of internal plexiform neurofibromas and MPNSTs was very strong, with individuals who have internal plexiform neurofibromas being over 20 times more likely to have an MPNST than individuals without internal plexiform neurofibromas. Age was also negatively associated with the development of MPNSTs in this analysis, the rate decreasing about 6% per year (Table 2.2).

When logistic regression was done with both subcutaneous and internal plexiform neurofibromas as explanatory variables, the association of subcutaneous neurofibromas with MPNSTs was no longer significant, but the association with internal plexiform neurofibromas remained very strong (Table 2.2). After adjustment for age and the presence of subcutaneous neurofibromas, NF1 probands with internal plexiform neurofibromas were over 18 times more likely to have MPNSTs as probands without internal plexiform neurofibromas.

**Survival Analysis**

Survival analysis in NF1 patients with MPNSTs was limited to the 25 subjects who had MPNSTs. Fourteen individuals died as a result of their MPNSTs. The mean survival after diagnosis for individuals who died was 1.4 ± 1.1 years. The average length of follow-up for individuals still alive at the time of analysis was 5.3 ± 2.5 years. The five-year survival was 41% (95% CI= 21%- 62%) (Figure 2.1) for the 25 patients with MPNSTs and was similar for males and females (p=0.79).
DISCUSSION

We sought to identify features of NF1 that characterize patients who are at the highest risk for developing MPNSTs. We found that individuals with internal plexiform neurofibromas were 18 times more likely to develop MPNSTs than patients without internal plexiform neurofibromas, after adjustment for age and the occurrence of subcutaneous neurofibromas. In addition, individuals with subcutaneous neurofibromas were more than three times as likely to have internal plexiform neurofibromas as patients without subcutaneous neurofibromas.

The association observed between subcutaneous and internal plexiform neurofibromas may reflect shared pathogenic mechanisms and is consistent with the histopathological similarities among cutaneous, subcutaneous and plexiform neurofibromas. In addition, loss of the normal NF1 allele has been observed in at least some cases in all three types of neurofibromas (16-23). It is somewhat surprising, given the histopathological similarities between these three types of benign tumours, that cutaneous neurofibromas are not associated with internal plexiform neurofibromas. However, cutaneous tumours have a different natural history than plexiform neurofibromas because plexiform tumours may undergo malignant transformation while cutaneous neurofibromas almost never do. This difference in clinical behavior may reflect a difference in pathogenesis (24). A weak association between cutaneous, subcutaneous and plexiform neurofibromas has been observed in one other study (25). However, the majority of plexiform tumours examined in that study were superficial plexiform neurofibromas (25). We did not observe any
association of superficial plexiform neurofibromas with either internal plexiform neurofibromas or MPNSTs in the present study, which included a subset of the data analyzed in the previous study (25).

MPNSTs in people with NF1 usually arise within a preexisting plexiform neurofibroma (26). Our finding that all 25 of the MPNSTs included in this study contained elements of benign plexiform neurofibroma on histopathological examination is consistent with this interpretation. We found internal plexiform neurofibromas in 34% of the patients who had undergone imaging studies. We observed internal plexiform neurofibromas most frequently in the spinal nerves, followed by other locations in the abdomen/pelvis, and then by other locations in the chest. Only five individuals had two clearly distinct internal plexiform neurofibromas, which is a lower proportion of multiplicity than found in studies of plexiform neurofibromas that were apparent on clinical exam (12.5-21%) (27, 28).

The prevalence of various clinical features among the NF1 patients in this dataset may not reflect the prevalence among individuals with NF1 in general because our data were obtained from patients seen at a specialized clinic, and the analysis was restricted to probands. The patients included may, therefore, be more severely affected than the NF1 population in general. It is unlikely that patient selection for imaging studies biases the results, as an equal proportion of individuals with neurofibromas was identified among those selected by routine examination (33%) compared to those identified by symptoms (35%). Moreover, the prevalence and distribution of internal plexiform neurofibromas observed in this study is similar to that found in a study that performed CTs on 91 NF1 patients over 16 years old (mean age = 35 years). Among these 91 NF1 patients, 20% had internal neurofibromas on chest CT, and 40% had internal neurofibromas on abdominal/pelvic CT. Forty paraspinal tumours were identified, 17 on chest and 23 on abdominal/pelvic scan, constituting 72% of all abnormal scans (29). This report did not state how many tumours each patient had or how many of the patients had a single tumour that was seen on both the abdominal and chest scan.

A study that performed spinal MRIs on 54 NF1 patients aged 5 to 56 years observed spinal neurofibromas in 64% of all individuals examined, with more than five tumour masses in 79%. Among 24 individuals who complained of sensory impairment or paralysis, spinal tumours were identified in 23 (93%). Spinal tumours were also identified in 12 (40%) of the 30 individuals who did not have symptoms of such a
tumour. Spinal tumours were seen in almost half of the asymptomatic individuals over the age of 15 but in only 25% of asymptomatic individuals under age 15 (30).

With the exception of the information on survival after diagnosis of MPNST, the data analyzed from the Henri Mondor Database are cross-sectional and, therefore, cannot be used to assess temporal relationships. This means that the associations we observed cannot be used to predict the risk of an individual NF1 patient to develop an MPNST. A large-scale longitudinal study of the natural history of internal plexiform neurofibromas is necessary to develop predictive models.

A recent study examined MRI from 50 NF1 patients who had previously-identified plexiform neurofibromas. Ten patients had inhomogeneous-appearing tumours on MRI, and seven of these presented with pain or swelling. All 10 were diagnosed pathologically as MPNSTs. The ages of these 10 patients ranged from 13 to 56 years, with a mean age of 28 years. A high internal tumour burden was observed in five of the patients, two had a parent with NF1 who died from an MPNST and two others had had a previous MPNST (31).

MPNSTs have a poor prognosis, and in most studies the prognosis appears to be worse in NF1 patients than in those who do not have NF1 (3, 32). A recent investigation found a five-year survival of only 21% in people with NF1 who had an MPNST (5). In the current study, the five-year survival was 41% in both males and females. This is not in agreement with the suggestion that the prognosis of MPNST is worse in males than females with NF1 (5, 7).

A study using the Neurofibromatosis Institute Database to identify clinical features that may be associated with mortality in patients with NF1 looked at 703 patients with a mean follow-up time of 2.4 years. Forty individuals died during the follow-up, 27 of whom where adults. Of the 40 individuals who died, 16 (40%) had an MPNST. Using age-adjusted logistic regression, the study found that subcutaneous neurofibromas (OR= 3.6, 95% CI= 1.2, 11.3) and male gender (OR=5.6, 95% CI= 1.5, 20.9) were independent predictors of mortality in adults with NF1 (33). In the present study, the finding that individuals with NF1 who have subcutaneous neurofibromas are at increased risk of developing an MPNST, one of the leading causes of death among NF1 patients (34), supports the previous finding that subcutaneous neurofibromas are associated with mortality (33).
The 1997 consensus guidelines for management of NF1 patients state that imaging studies should not be done unless the clinical exam identifies problems that require such studies (13). However, patients with NF1 who have symptoms of an underlying tumour are not the only ones at risk for developing internal plexiform neurofibromas, and identifying an MPNST after it becomes symptomatic is often too late to provide a surgical cure. We found that NF1 patients with internal plexiform neurofibromas were much more likely to develop MPNSTs than NF1 patients without internal plexiform neurofibromas, and over half of patients with internal plexiform neurofibromas were not symptomatic. This raises the question of whether adults with NF1 should have routine imaging for internal plexiform neurofibromas to identify those at high risk of developing MPNSTs. The association between internal plexiform neurofibromas and subcutaneous neurofibromas is not strong enough to be clinically useful to identify individuals who have a greater risk of internal neurofibromas.

Eleven of the 25 individuals with MPNSTs in this study [Patients 1,5,7,8,14,15,17,20,21,22 and 24] have been tested for a constitutional NF1 microdeletion by either marker analysis, specific break-point PCR, semi-quantitative PCR or FISH (6). Three of the eleven were found to carry deletions of the whole NF1 gene [Patients 8, 21, and 22]. In the current study, it was not possible to determine whether individuals with microdeletions and internal plexiform neurofibromas are at an even greater risk of developing MPNSTs compared to those with other pathogenic mutations and internal plexiform neurofibromas.

If routine imaging studies of adults with NF1 were advocated to look for internal plexiform neurofibromas or MPNSTs, it is not clear how such screening should be done. We do not know which type of imaging study would be most appropriate for routine screening, how often such screening should be performed, or whether all NF1 patients or only a subset defined by age or other clinical features should be screened. Several imaging methods are available, including ultrasound examination, X-ray examination, CT, and MRI, each of which was used on at least some of the patients in the present study.

Each of these techniques has advantages and disadvantages. Ultrasound examination is a safe and relatively inexpensive imaging procedure, but it has both low spatial resolution and poor soft tissue contrast compared to other imaging techniques.
Conventional radiographic examination is also inexpensive and under optimal conditions has good spatial resolution, but soft tissue contrast and thus detection of internal plexiform neurofibromas is very poor. CT provides better visualization of soft tissues and better spatial resolution but is more expensive. In addition, CT involves greater exposure to ionizing radiation, which is a particular concern in patients who carry a constitutional mutation of a tumour suppressor gene. MRI provides high spatial resolution and excellent soft tissue contrast without exposure to ionizing radiation but is expensive and more time consuming for the patient. However, it is probably the best approach to routine screening if the cost-benefit ratio can be shown to justify it. There is an urgent need to address the excess mortality and morbidity associated with NF1 more effectively, and rigorous prospective studies with detailed phenotypic analysis and imaging studies are necessary to determine the clinical utility of routine body MRI in affected adults.
REFERENCES


3. HETEROGENEITY OF MAST CELL DISTRIBUTION IN NF1 NEUROFIBROMAS²

INTRODUCTION
Neurofibromatosis 1 (NF1) is an autosomal dominant disorder with an incidence of 1:3500 (1). Benign neurofibromas are the hallmark feature of NF1. All neurofibromas contain Schwann cells (the suspected progenitor cell type), but fibroblasts, mast cells, lymphocytes, perineurial cells, endoneurial fibroblasts and endothelial cells also occur in varying numbers (2). Neurofibromas also vary in terms of their age of appearance, natural history, and malignant potential. Consequently, several different classifications of neurofibromas have been proposed (Table 3.1).

Table 3.1 Examples of different classifications for NF1-associated neurofibromas.

<table>
<thead>
<tr>
<th>Source</th>
<th>Types of Neurofibromas</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Clinical</strong></td>
<td></td>
</tr>
<tr>
<td>Crowe, Schull &amp; Neel (3)</td>
<td>Cutaneous</td>
</tr>
<tr>
<td></td>
<td>Subcutaneous</td>
</tr>
<tr>
<td></td>
<td>Plexiform</td>
</tr>
<tr>
<td>Riccardi (4)</td>
<td>Cutaneous</td>
</tr>
<tr>
<td></td>
<td>Subcutaneous</td>
</tr>
<tr>
<td></td>
<td>Nodular plexiform</td>
</tr>
<tr>
<td></td>
<td>Diffuse plexiform</td>
</tr>
<tr>
<td>Huson &amp; Hughes (5)</td>
<td>Dermal</td>
</tr>
<tr>
<td></td>
<td>Nodular</td>
</tr>
<tr>
<td></td>
<td>Plexiform</td>
</tr>
<tr>
<td>Korf &amp; Rubenstein (6)</td>
<td>Dermal</td>
</tr>
<tr>
<td></td>
<td>Plexiform</td>
</tr>
<tr>
<td><strong>Histopathological</strong></td>
<td></td>
</tr>
<tr>
<td>Harkin and Reed (7)</td>
<td>Plexiform</td>
</tr>
<tr>
<td></td>
<td>Non-plexiform</td>
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<tr>
<td>Masson (8)</td>
<td>Encapsulated</td>
</tr>
<tr>
<td></td>
<td>Diffuse</td>
</tr>
<tr>
<td>Woodruff (9)</td>
<td>Localized cutaneous</td>
</tr>
<tr>
<td></td>
<td>Localized subcutaneous</td>
</tr>
<tr>
<td></td>
<td>Plexiform</td>
</tr>
<tr>
<td></td>
<td>Diffuse (skin or subcutaneous layer)</td>
</tr>
<tr>
<td></td>
<td>Massive soft tissue</td>
</tr>
</tbody>
</table>

² A version of this chapter has been submitted for publication. Tucker T, Riccardi VM, Sutcliff M, Vielkind J, Wechsler J, Wolkenstein P, Friedman JM. Heterogeneity of mast cell distribution in NF1 neurofibromas.
None of these classifications has achieved universal acceptance, and the literature contains many examples of inconsistent use and confusion of various clinical and pathological descriptive terms. Most studies of neurofibroma pathogenesis treat all neurofibromas as a single class despite their clinical and pathogenic heterogeneity. This heterogeneity is important because some kinds of neurofibromas have little, if any, malignant potential, while other kinds may progress to malignancy. The resulting neoplasms, malignant peripheral nerve sheath tumours (MPNSTs), are one of the leading causes of death in NF1 patients (10). There is currently no effective treatment known for benign neurofibromas or MPNSTs except surgical removal in favourable cases.

Although the Schwann cell is the prominent cell type in neurofibromas, mast cells may play an important role in neurofibroma development (11), and mast cell infiltration is a prominent feature of some neurofibromas (12). In the present study, we used histological and immunohistochemical techniques to study the occurrence and distribution of mast cells in neurofibromas from individuals with NF1. Neurofibromas were classified histopathologically as diffuse or nodular (single or plexiform). Nodular neurofibromas are circumscribed by the perineurium while diffuse neurofibromas are not.

We found that diffuse neurofibromas had a higher proportion and more uniform distribution of mast cells than nodular neurofibromas. Mast cells that were present within a tumour region showed no obvious clustering around blood vessels and were not seen in the surrounding normal tissue. Mast cells were rarely seen in NF1-associated MPNSTs.

**MATERIALS & METHODS**

**Sample Collection**

We collected formalin fixed-paraffin embedded samples from Creteil Hospital (Paris, France), The Neurofibromatosis Institute (La Crescenta, California), Vancouver General Hospital (Vancouver, Canada) and Children’s and Women’s Hospital (Vancouver, Canada). All individuals had a confirmed diagnosis of NF1 according to established criteria (13). In total we studied 49 benign neurofibromas from 37 individuals and 4 MPNSTs from 4 individuals. The study protocol was approved by the University of British Columbia Research Ethics Committee.
Histopathological Classification of Neurofibromas

Each neurofibroma was classified histopathologically on the basis of its appearance in hemotoxylin and eosin (H&E) stained sections as diffuse or nodular according to the definitions used by one of the authors (J.W.). The diffuse neurofibromas were predominantly located in the dermis and involved the thin terminal branches of peripheral nerves. Diffuse neurofibromas were characterized by proliferation of cells that are not limited by the perineurium and can encircle normal structures (exocrine glands, hair follicles and vessels) without destroying them. The proliferating cells were irregularly dispersed within a fibrous and/or myxoid background. Myelin fibrils were rare or absent.

Nodular neurofibromas usually involved deeper tissues, and the cellular proliferation is intraneural, enclosed within large hypertrophic nerves circumscribed by the perineurium. The nodular tumours included both single and plexiform types. Single nodular tumours involve just one fascicle, while nodular plexiform tumours may involve several fascicles, nerve branches or a nerve plexus. Dispersed or fascicular myelin fibrils are usually found in the central area of the nodules. On transverse section, the central area is loose with a myxoid background substance, while the peripheral area is usually more cellular.

Some neurofibromas contain both diffuse and nodular components; such tumours are usually found in the deep dermis and subcutaneous tissue. The nodular component is surrounded by diffuse extraneural cell proliferation, infiltrating the fat tissue and other normal pre-existing structures. The diagnosis of all 4 MPNSTs was obtained from the histopathology reports.

Identification of Granulated Mast Cells

Serial sections were cut from each neurofibroma. One section was stained with H&E, and a second section was stained with 0.1% toluidine blue, a metachromatic dye that uniquely identifies normal granulated mast cells. Sections were deparaffinized in xylene, hydrated through graded alcohols (100%, 95%, and 70%) and stained with toluidine blue for 30 seconds and rinsed in water. The slides were then dehydrated through graded alcohol (70%, 95%, 100%) and cleared in xylene.
Identification of Mast Cells and Schwann Cells

Immunohistochemistry using a c-Kit antibody was performed to identify all mast cells, regardless of whether they contain toluidine blue-positive granules. Serial sections were cut from 9 benign neurofibromas (5 diffuse and 4 nodular) and from 3 MPNSTs. One section of each for c-Kit, and one was dual labeled for c-Kit and S100 (a Schwann cell marker). After deparaffinization, heat antigen retrieval was performed at 70°C in EDTA buffer pH 6.0. Endogenous peroxidase activity was blocked with 3% hydrogen peroxidase solution. The tissue sections were incubated with 1:400 mouse anti-human c-Kit antibody (Zymed, San Francisco, CA) over-night at room temperature. The sections were incubated with a biotin-labeled goat anti-mouse IgG secondary antibody and visualized using ABC Elite and Nova Red (Vector Laboratories, Canada Inc, Burlington, ON). Nuclei were identified using hemotoxylin. Dual immunohistochemistry was performed as above with the addition of 1:400 rabbit anti-human S100 antibody (Dako Cytomation, Mississauga, ON) and mouse anti-human c-Kit antibody. Secondary antibodies were conjugated with Alexa 488 or Alexa 568 (Invitrogen Canada Inc., Burlington, ON).

Tumour Characteristics

H&E and toluidine blue-stained tumours were independently reviewed by two of the authors (M.S. and T.T.). Each tumour was graded as a whole on a 3-point Likert scale for cellularity and vascularity and on a 4-point Likert scale for mast cell density and lymphocytic infiltration. For cellularity and vascularity, scaling was determined at 200x magnification on the H&E section. Mast cell density was determined at 400x magnification on toluidine blue-stained slides and rated as 0, 1-5, 6-10 and >10 mast cells per field. Lymphocytic infiltration was determined at 400x magnification as a percentage of the total cells. Five to eight fields chosen at random were taken to represent the tumour as a whole. In addition, the total number of mast cells per section was determined by manual counting of one section on both the toluidine blue and c-Kit stained sections. The samples were scanned to determine if there was any clustering of mast cells in particular regions or around particular tumour features, and the adjacent normal tissue, when present, was compared to the tumour tissue to determine if there were differences in cellularity, vascularity or mast cell density.
Statistical Analysis

Kendall's correlation was calculated using SPSS (SPSS, Inc. Chicago, Illinois, 2001) to determine the relationship between vascularity, cellularity and mast cell density. The Mann-Whitney U rank test was performed using SPSS (SPSS, Inc. Chicago, Illinois, 2001) to determine if there was a significant difference in the mast cell density between the two types of neurofibromas. A p-value less than or equal to 0.05 was considered to be significant.

RESULTS

Mast cells are present in many neurofibromas, but it is not known if the quantity or distribution of mast cells differs between different types of neurofibromas. We studied the cellularity, vascularity, and density and distribution of mast cells in 49 benign neurofibromas from 37 NF1 patients and in 4 MPNSTs from 4 NF1 patients. Each of the benign tumours was classified histopathologically as a diffuse or nodular neurofibroma. 32 of the neurofibromas we studied (from 27 individuals with NF1) were classified histopathologically as diffuse neurofibromas. 15 of the neurofibromas (from 9 individuals with NF1) were classified as nodular tumours. Two neurofibromas (from 2 individuals) were a mix of both diffuse and nodular components and were not included in the statistical analysis due to the small number.

Figure 3.1 shows examples of diffuse and nodular neurofibromas with typical patterns of cellularity, vascularity, and lymphocytic infiltration on H&E staining. The mast cell distribution in each of these same neurofibromas as determined by toluidine blue staining is also shown.
Figure 3.1 Images of typical neurofibromas in the current study that represent the majority of the tumour.

A. H&E section of a diffuse neurofibroma at 100x magnification. B. Serial section to (A) stained with toluidine blue to identify mast cells (*) at 100x magnification taken at the periphery of the tumour. C. H&E section of a nodular neurofibroma at 100x magnification. D. Serial section to (C) stained with toluidine blue to identify mast cells at 100x magnification. Note the higher cellularity and abundance of smaller blood vessels (arrow) in A compared to C and the lymphocytic infiltration (L) in C, some of which is indicated. Also note the presence of the reddish-purple mast cells (*) in the diffuse neurofibroma (B) and the absence of mast cells in the nodular neurofibroma (D).

**Cellularity**

Nodular neurofibromas tend to be less cellular than diffuse neurofibromas (Figure 3.1), with almost 80% of nodular tumours falling into category 1 for cellularity (Figure 3.2). These tumours displayed more dense fibrotic tissue and areas of degeneration in the central portion of the neurofibroma but not at the periphery. In contrast, the diffuse tumours had higher cellularity with a more uniform distribution of cells throughout the tumour. There was a significant correlation between cellularity and histopathological tumour type (Kendall's tau = -0.384, p=0.007).
Figure 3.2 Distribution of pathological features of neurofibromas classified histopathologically.

A. Cellularity estimated on a 3-point Likert scale with 3 being the highest cellularity; B. Vascularity estimated on a 3-point Likert scale with 3 being the highest vascularity; and C. Mast cell density estimated on a 4-point Likert scale with 4 being the highest mast cell density.

![Graph A: Cellularity](image)

![Graph B: Vascularity](image)

![Graph C: Mast Cell](image)

There was often a higher proportion of lymphocytes within nodular neurofibromas than within diffuse neurofibromas. Lymphocytic infiltration was generally distributed evenly within the nodular neurofibromas (Figure 3.1), while lymphocytes were more localized when they were observed in diffuse tumours.
Vascularity

Nodular neurofibromas typically had fewer large vessels than diffuse tumours, which had more smaller vessels (Figure 3.1). Vessels were evenly distributed throughout the tumour in diffuse and nodular neurofibromas. Overall, vascularity was not significantly correlated with tumour type (Kendall's tau = -0.243, p=0.085). However, vascularity was significantly correlated with cellularity (Kendall's tau =0.510, p<0.001.), as more cellular neurofibromas had a higher number of blood vessels.

Mast Cell Distribution Within Neurofibromas and MPNSTs

The majority of mast cells were located within the tumour component, with only a few located in the surrounding normal tissue. Nodular neurofibromas had significantly fewer mast cells than diffuse neurofibromas (p=0.021) (Figures 3.1 & 3.2). The few mast cells seen within nodular neurofibromas were located at the periphery of the tumours (Figure 3.3A &3.3B). The mast cells were evenly distributed throughout diffuse neurofibromas, with no obvious mast cell clusters forming around particular structures (Figure 3.1). Areas of nodular neurofibromas seen within a mixed nodular and diffuse tumour contained very few mast cells, although the surrounding diffuse tumour tissue contained many mast cells (Figure 3.3C & 3.3D).
Mast cells did not appear to cluster around blood vessels or within areas of lymphocytic infiltration, but mast cell density was correlated with overall tumour vascularity (Kendall’s tau =0.513, p<0.001) and cellularity (Kendall’s tau =0.456, p=0.001).

Four MPNSTs were also stained with toluidine blue. Two had no observable mast cells, and another had very few mast cells. The fourth MPNST included a benign-appearing component from which the malignancy may have arisen. In this sample, mast cells were frequent in the benign region but absent from the malignant portion (Figure 3.4).
Figure 3.4 Mast cell distribution in MPNSTs.

A. H&E section of a histologically benign neurofibroma component adjacent to a MPNST at 50x magnification. B. Serial section of a histologically benign neurofibroma adjacent to an MPNST stained for toluidine blue showing mast cells (*) at 50x magnification. C. H&E section of a malignant component of the same MPNST showing high cellularity and mitoses at 100x magnification. D. Serial section of the malignant component stained for toluidine blue showing the absence of mast cells at 100x magnification.

Distribution of Granulated and Non-granulated Mast Cells

To ensure that the differences we found in mast cell density were not biased by observing only the granulated mast cells shown with toluidine blue, we used c-Kit immunostaining to identify all mast cells, both granulated and degranulated. The number of c-Kit positive cells was higher than the number of toluidine blue positive cells in each of the tumours studied. To ensure that this increase was not due to neoplastic Schwann cells as well as mast cells expressing c-Kit, we dual immunolabeled an adjacent section of each tumour with antibodies to c-Kit and S100. No colocalization of the c-Kit and S100 signals was seen with confocal imaging (Figure 3.5). We conclude that the greater frequency of c-Kit-positive than toluidine blue-positive cells in these
tumours probably indicates that some mast cell degranulation occurs in both types of neurofibromas.

Figure 3.5 Confocal image of a diffuse neurofibroma stained with S100 and c-Kit.

Confocal image of a diffuse neurofibroma at 400x magnification showing staining for both S100+ (red) Schwann cells and c-Kit+ (green) mast cells. The Schwann cells do not express c-Kit.

There was no relationship between the increase in the number of c-Kit positive-cells in comparison to the number of toluidine blue-positive cells and neurofibroma type; however, there was a strong correlation between toluidine blue counts and c-Kit staining (Kendall’s tau =0.894, p<0.001). As expected from the toluidine blue results, the total number of c-Kit-positive cells was significantly lower in nodular neurofibromas than in diffuse neurofibromas (p=0.014).

DISCUSSION

Mast cells are a consistent finding in neurofibromas in people with NF1. However, the role of the mast cell in the development of neurofibromas in NF1 individuals is poorly understood. The purpose of this study was to determine if the density and distribution of mast cells differs between neurofibromas classified by their histopathological appearance and if mast cell infiltration is related to other histological characteristics of these tumours, which may indicate different pathogenesis for each type of neurofibroma. We found that nodular neurofibromas have significantly fewer mast cells than diffuse neurofibromas and that MPNSTs show little or no mast cell infiltration.

Mast cells are formed in the bone marrow. They are released into the blood as undifferentiated precursors and do not differentiate until they leave the vasculature and
enter the target tissue. Mast cells can be found in endoneurial and perineurial spaces of peripheral nerves (14), particularly when the nerve is damaged and in need of repair.

Previous studies that have investigated mast cells within neurofibromas found results that are generally similar to those of our study. Some, but not all, neurofibromas were found to have a high density of mast cells (12, 15, 16), but it was not clear what types of neurofibromas were examined in those studies or how many of the tumours came from individuals with NF1. Isaacson (12) described 4 neurofibromas that were densely hyalinised with an appearance similar to some of the dense fibrous nodular neurofibromas in the current study. Fewer than 5 mast cells per 400x field were observed in these tumours; this is similar to our findings in the current study. In addition, Isaacson noted that an entrapped thickened nerve within what was called a “plexiform neurofibroma” displayed fewer mast cells than the tumour tissue; this was also observed in the present study in 2 neurofibromas that were a mix of both nodular and diffuse tissue (Figure 3.3).

Johnson and coworkers noted the abundance and even distribution of mast cells throughout a large number of neurofibromas but did not observe mast cells in the majority of MPNSTs examined (15). One neurofibroma was classified as subcutaneous, and it had a greater concentration of mast cells at the periphery of the tumour (15), as observed in some of the nodular neurofibromas in the present study.

Patients with NF1 neuropathy also have enlarged nerves, but sural nerve biopsies from such individuals showed a disorganized perineurium and hypercellular epineurium with mast cells primarily located close to blood vessels within the epineurium and endoneurium (17). The location of mast cells within these thickened nerves contrasts to the location observed in nodular neurofibromas in the present study, where mast cells were found primarily at the perineurium and/or epineurium border. The restriction of mast cells to the endoneurium or perineurium may distinguish neuropathy and frank neurofibroma development.

Zhu et al used a unique system to study neurofibroma development in a mouse model. Using a Cre transgene under the control of the Schwann cell Krox20 promoter, these investigators produced a homozygous knockout of Nf1 in Schwann cells while maintaining an Nf1 heterozygous state in all other cells (11). The peripheral nerves of these mice are enlarged, and some of the animals develop dorsal root neurofibromas
that resemble nodular plexiform neurofibromas in NF1 patients. Interestingly, formation of neurofibromas occurs only if other cells in these mice are heterozygous for \textit{Nf1} – an \textit{Nf1} haploinsufficient tumour environment appears necessary for the pathogenesis of these histologically-mixed tumours (11). The thickened nerves in these mice show infiltration of mast cells, and this infiltration is more substantial when the \textit{Nf1} null Schwann cells proliferate in an \textit{Nf1} heterozygous background than in the wild type background. However, it is not clear where the mast cells were found within the thickened nerves. Mast cells are normally found in close proximity to the Schwann cells that myelinate these nerves (7). Moreover, cutaneous mast cells outside of these tumours are also moderately increased and demonstrate enhanced growth in vitro in \textit{Nf1} heterozygous mice compared to wild type mice (18). These observations suggest that mast cells are involved in neurofibroma formation in this mouse model, but the pathogenic process may differ somewhat from that which occurs naturally in humans with NF1.

Riccardi (4) has distinguished 4 types of neurofibromas clinically that differ with respect to their age of appearance, natural history, and malignant potential (Table 3.1). This clinical classification does not correlate with the pathological classification used in the present study. Riccardi’s “cutaneous neurofibromas” occur in the skin surface and develop from the terminal branches of peripheral nerves. “Subcutaneous neurofibromas” are described as firm and lying deeper in the subcutaneous layer of the skin. They have discrete margins but may extend along a nerve. “Nodular plexiform neurofibromas” (which may or may not exhibit the pathological features used to make this diagnosis in the present study) arise within major peripheral nerves and may lie just beneath the dermis or much deeper in the body. They are often found in the spinal nerves, nerve roots or ganglia. Riccardi also describes “diffuse plexiform neurofibromas” that do not correspond to the pathological classification used in the present study. Riccardi’s “diffuse plexiform neurofibromas” usually are apparent at birth or within the first few years of life. These neurofibromas can be superficial, involving the skin and subcutaneous tissue, or involve deeper tissue. The tumours do not have discrete borders but invade adjacent normal tissues. Most of the tumours in our study were not characterized using this clinical classification, and the histological and clinical approaches do not classify individual tumours into the same groups. Both cutaneous and “diffuse plexiform” tumours usually are diffuse neurofibromas histologically,
although some congenital or early onset tumours are mixed diffuse and nodular neurofibromas. Tumours that are classified clinically as subcutaneous or nodular plexiform neurofibromas are usually nodular neurofibromas on histopathological exam. However, the only way to determine the histological type of a neurofibroma is to examine it histologically.

In the present study, the number of mast cells was lower in histologically-defined nodular neurofibromas than in histologically-defined diffuse neurofibromas. Nodular neurofibromas differ from diffuse neurofibromas in that nodular tumours are surrounded by an intact epineurium and perineurium, although the organization of the endoneurium is disrupted with abnormal accumulation of cells (8). It is tempting to speculate that the epineurium and/or perineurium acts as an anatomical barrier that excludes mast cells from nodular neurofibromas. In contrast, mast cells might have direct access to the tumour tissue in diffuse neurofibromas, which develop distal to the end of the epineurium and perineurium or in which the epineurium and perineurium are broken down (8). The striking difference between the number of mast cells in neurofibromas of different types may indicate that somewhat different mechanisms underlie the development of these different kinds of neurofibromas. However, we cannot exclude the possibility that the difference in mast cell number and distribution we observed are largely a result of the differences in overall tumour cell number and distribution seen in nodular and diffuse neurofibromas.

Mast cells have been observed in a number of malignant neoplasms including breast cancer (19), squamous epithelial carcinoma (20), colorectal cancer (21), and uterine cancer (22). In melanoma, mast cells are located at the margin of tumour and normal tissue (23), and mast cell counts within the tumour correlate strongly with microvascular density and shorter patient survival (24). In our study we found an abundance of mast cells in the histopathologically-defined diffuse neurofibromas, which include clinically-defined cutaneous neurofibromas that almost never progress to malignancy, and we found fewer mast cells in MPNSTs and histologically-defined nodular neurofibromas, which can progress to malignancy. However, we cannot rule out the possibility that the nodular neurofibromas we examined might subsequently have progressed to malignancy or that the MPNSTs developed from neurofibromas that were infiltrated by mast cells at an earlier point in the tumorigenic process. In fact, the conjunction of mast
cell-infiltrated histologically-benign neurofibroma tissue and mast cell-free MPNST in one NF1 patient (Figure 3.4) is compatible with the malignancy developing from a benign neurofibroma that is heavily infiltrated with mast cells.

We found mast cells to be evenly distributed in the tumour component of diffuse neurofibromas but not in the surrounding normal tissue. While there was no clustering of mast cells around blood vessels, there was a significant correlation between the density of mast cells and vascularity within a tumour (p<0.001). This is consistent with mast cells promoting angiogenesis by releasing factors such as vascular endothelial growth factor (VEGF), matrix metalloproteinases (MMP2, MMP9) and chymase. Similarly, there was a correlation between mast cell density and cellularity, suggesting that mast cells release factors that either directly or indirectly (e.g., by promoting angiogenesis) lead to increased cellular proliferation.

Also of interest is the abundance of lymphocytes we observed in nodular neurofibromas compared to diffuse neurofibromas. Johnson and coworkers studied lymphocytic infiltration in a variety of Schwann cell tumours and found that the number of B-lymphocytes varied greatly within neurofibromas (15). 18/32 neurofibromas studied by Johnson et al. showed fewer than 50 B-lymphocytes per section, and another 5 tumours had more than 150 B-lymphocytes. Only two of the nine MPNSTs studied showed B-lymphocytic infiltration. It is not clear what types of neurofibromas were examined in the Johnson study or if the MPNSTs were from individuals with NF1. The abundance of lymphocytic infiltration in the nodular neurofibromas we studied suggests that these tumours may be triggering an immune response.

Mast cells synthesize and express c-Kit, a tyrosine kinase receptor that is the product of the c-Kit proto-oncogene. The ligand for c-Kit, which is called SCF or KitL, promotes proliferation, migration and survival of mast cells. Neurofibromin and c-Kit are involved in overlapping cell signaling pathways. Neurofibromin acts as a negative regulator of the Ras pathway, converting active Ras-GTP to inactive Ras-GDP, while c-Kit activation by KitL induces the phosphorylation of Akt via the PI-3K pathway (25). In culture, it has been shown that Schwann cells express membrane-bound KitL (26) and secrete KitL (27), both of which would attract mast cells.

Cultured Schwann cells from Nf1−/− mice secrete five to six times more KitL than Nf1+/− or wild type Schwann cells. This increased KitL secretion increases migration of Nf1−/−.
mast cells towards $Nf1^{-/-}$ Schwann cells (27). In addition, $Nf1^{-/-}$ mast cells exhibited a 2-fold increase in migration and a 2-fold increase in proliferation compared with wild type cells in response to a given dose of KitL (27). The migration of the $Nf1^{-/-}$ mast cell was reduced to wild type levels when $Nf1^{-/-}$ mast cells were transduced with a retrovirus carrying $Nf1$, but little effect was observed when a Nf1 transgene harbouring a pathogenic mutation was introduced (27). Greater accumulation of mast cells occurred at the site of KitL cutaneous infusion in $Nf1^{-/-}$ mice than in wild type mice (28).

Normal adult Schwann cells do not express c-Kit (25), but Badache and coworkers demonstrated a correlation between an increase in the level of c-Kit synthesis and a decrease in neurofibromin synthesis in Schwann cell lines derived from 3 MPNSTs from NF1 individuals (29). In the present study, we did not observe c-Kit staining of Schwann cells in any of the three MPNSTs examined, and we only found mast cells in low numbers in one MPNST. None of these MPNSTs expressed neurofibromin (data not shown). The difference in results could be because we assessed c-Kit expression in primary tumours while Badache and coworkers used cell lines derived from NF1 MPNSTs. Additionally, we assessed c-Kit expression using immunohistochemistry while Badache used a different method of detection, Western blotting. The benign neurofibromas we studied did not show any evidence of S100+ Schwann cells expressing c-Kit when slides were dual labeled with antibodies for both S100 and c-Kit antigens (Figure 3.5).

The samples used in this study were archival material from surgical specimens that had been submitted for clinical pathology. Therefore, it was assumed that the sample was representative of the whole tumour and that the sections used to study tumour characteristics and mast cell density and distribution represent the tumour as a whole, although some variability among different portions would be expected because of the heterogeneous nature of neurofibromas.

In summary, we found that mast cells are significantly less frequent in histologically-nodular neurofibromas than in diffuse neurofibromas. This difference is consistent with clinical observations that different kinds of neurofibromas have different natural histories and different risks of malignant degeneration. The difference in mast cell infiltration and distribution suggests that mast cells may play different roles in the pathogenesis of different kinds of neurofibromas.
REFERENCES


4. PATHOGENETIC HETEROGENEITY OF NF1 ASSOCIATED NEUROFIBROMAS

INTRODUCTION
Neurofibromatosis 1 (NF1) is a common dominantly inherited autosomal disease with an incidence of 1 in 3500 (1). NF1 is caused by mutations in the NF1 gene that encodes neurofibromin, a ubiquitously expressed protein. Most constitutional mutations in NF1 result in a loss of function of the protein. Loss of the normal NF1 allele (Loss of Heterozygosity or LOH) has been observed in pheochromocytomas (2), juvenile myeloid leukemia (3) and malignant peripheral nerve sheath tumours (MPNSTs) (4) in NF1 patients, confirming NF1 as a tumour suppressor gene.

The hallmark feature of NF1 is the neurofibroma, a benign tumour composed of Schwann cells, fibroblasts, perineural cells, mast cells and endothelial cells. Schwann cells constitute a large proportion (40-80%) of neurofibromas and are thought to be the progenitor cell type because cultures derived from some NF1 patient neurofibromas display loss of the normal NF1 allele in Schwann cells but not in fibroblasts (5-7).

Neurofibromas are thought to arise according to the "two-hit" hypothesis, with an inactivating somatic mutation in the normal NF1 allele as the rate-limiting step. LOH or second somatic mutations of the NF1 locus have been observed in 26% of dermal neurofibromas and 36% of plexiform neurofibromas studied (8-24). The low percentage of observed LOH is usually attributed to technical limitations of the assay or to "contamination" of the neoplastic Schwann cell clone by an abundance of non-neoplastic stromal cells present in these heterogeneous tumours.

There is also evidence that Schwann cells comprising an NF1-associated neurofibroma are themselves heterogeneous. Immunohistochemical analyses of some dermal and plexiform neurofibromas have shown both neurofibromin-negative and neurofibromin-positive Schwann cells (25). The former are usually interpreted as the neoplastic clone that has lost both functional copies of the NF1 gene, and the latter as normal "contaminants" of the tumour that have proliferated in response to the neoplastic...
process. A similar interpretation has been made of observations that S100-positive (presumably Schwann cell) lines grown from NF1-associated neurofibromas may contain a mixture of neurofibromin-positive and neurofibromin-negative cells or a mixture of cells with and without NF1 LOH (6, 25, 26)

The “two hit” hypothesis explains the development of some neurofibromas, but does not readily explain all of the clinical data regarding neurofibroma development. For example, the following observations in NF1 patients are not easily explained without invoking ad hoc modifications of the “two hit” hypothesis:

- The apparent association of trauma with the subsequent development of a neurofibroma at the same site (27).
- Eruptive neurofibromatosis, in which innumerable dermal neurofibromas develop over a period of a few months in a person with NF1 (28).
- Rapid increase in the development of neurofibromas during pregnancy, and subsequent regression of some tumours after delivery (29).
- The failure to find evidence of LOH or a “second hit” NF1 mutation in many neurofibromas, despite extensive molecular genetics studies (8-24).

We have proposed an alternative interpretation of these data: that NF1-associated neurofibromas, which are clinically heterogeneous in appearance, natural history, and propensity to progress to MPNSTs, (30) are also pathogenically heterogeneous (31). We suggest that in people with NF1 some neurofibromas arise through inactivation of the second (normal) NF1 allele in a Schwann cell and expansion of the resulting neoplastic clone, while other neurofibromas arise as polyclonal proliferations of NF1 haploinsufficient cells.

We used immunohistochemistry to determine the expression of neurofibromin (the protein product of the NF1 locus) in Schwann cells and non-Schwann cells of NF1-associated neurofibromas classified histopathologically as diffuse or nodular. We observed a significantly lower proportion of S100+/Nf+ cells and a significantly higher proportion of S100-/Nf- in nodular neurofibromas than in diffuse neurofibromas.

We then used laser microdissection to isolate S100+/Nf+, S100+/Nf- and S100-/Nf+ cells from tumours that had been removed from females with NF1. We tested for
clonality in each cell type using X-linked AR polymorphisms. We found clear evidence of clonality among the S100+/Nf- cells in some tumours, as would be expected if the neurofibromas arose through clonal expansion of neoplastic Schwann cells after a "second hit" eliminated all neurofibromin expression. In contrast, other tumours showed no evidence of clonality in any of the cell types examined, as would be expected if these neurofibromas arose as polyclonal proliferations of NF1 haploinsufficient cells.

METHODS

Sample Collection

We collected formalin fixed-paraffin embedded samples from Creteil Hospital (Paris, France), The Neurofibromatosis Institute (La Crescenta, California), Vancouver General Hospital (Vancouver, Canada), and Children's and Women's Hospital (Vancouver, Canada). We studied a total of 38 benign-neurofibromas from 29 people who all had a confirmed diagnosis of NF1 according to established criteria (32). The tumours used in this study are a subset of those used in Chapter 3. The study protocol was approved by the University of British Columbia Research Ethics Committee.

Histopathological Classification of Neurofibromas

Each neurofibroma was classified histopathologically on the basis of its appearance in hemotoxylin- and eosin- (H&E) stained sections as diffuse or nodular according to definitions used by one of the authors (J.W.). Diffuse neurofibromas were predominantly located in the dermis. They involved the thin terminal branches of peripheral nerves and were characterized by proliferation of cells that were not limited by the perineurium. The proliferating cells were irregularly dispersed within a fibrous and/or myxoid background and sometimes encircled normal structures (exocrine glands, hair follicles and vessels) without destroying them. Myelin fibrils were rare or absent.

Nodular neurofibromas usually involved deeper tissues. The cellular proliferation in these tumours was intraneural, enclosed within large hypertrophic nerves circumscribed by the perineurium. Single nodular tumours involved just one fascicle, while nodular plexiform tumours involved several fascicles, nerve branches or a nerve plexus. On transverse section, the central area of nodular tumours was loose, with a myxoid background substance, usually containing dispersed or fascicular myelin fibrils. The peripheral area of these tumours was usually more cellular.
Some neurofibromas contained both diffuse and nodular components; such tumours were usually found in the deep dermis and subcutaneous tissue. The nodular component was surrounded by a diffuse extraneural cellular proliferation that infiltrated the fat tissue and other normal structures.

**Tumour Characteristics**

H&E sections of each neurofibroma were independently reviewed by two of us (M.S. and T.T.) Each tumour was graded at 200x magnification on a 3-point Likert scale for cellularity and vascularity. Lymphocytic infiltration was determined at 400x magnification as a percentage of the total cells. Only neurofibromas with ≤30% lymphocytic infiltration were selected for this study. Lymphocytic infiltration was then rated on a 3-point Likert scale of ≤5%, 6-20%, 21-30%. Five to eight fields chosen at random were taken to represent the tumour as a whole. The section was then scanned to identify regions that represented the full range of cellularity within the specimen. These regions were marked for subsequent imaging and analysis after staining for S100 (a Schwann cells marker) and neurofibromin.

**Immunofluorescent Staining for S100 and Neurofibromin**

Four serial 5µm sections were cut after the H&E section from each of the neurofibromas and stained respectively for S100 only, neurofibromin (Nf) only, S100 and neurofibromin together or with no primary antibody as a negative control. Sections of a traumatic neuroma from a person who does not have NF1 were used as a positive control, and sections of an MPNST from a person with NF1 were used as a negative control for the specificity of neurofibromin staining. Traumatic neuromas are composed of similar cell types as neurofibromas, mainly Schwann cells and fibroblasts, both of which express neurofibromin. An average of 92% of cells in the traumatic neuroma were neurofibromin positive. There was no evidence of S100 or neurofibromin expression in the MPNST, although Schwann cells in an adjacent histologically benign component were S100 and neurofibromin positive.

All sections were deparaffinized in xylene, hydrated through graded alcohols (100%, 95%, and 70%) and equilibrated in phosphate-buffered saline. Heat antigen retrieval was performed in 10mM sodium citrate pH 6.0 at 70°C for 10 minutes. Samples were treated with fresh 1mg/ml sodium borohydride for 20 minutes at room temperature to
reduce autofluorescence and then blocked with 5% normal goat serum for 30 minutes. Primary antibody to S100 (polyclonal rabbit anti-cow; DAKO Canada Inc.; Mississauga ON, Canada) was added at a concentration of 1:400 and neurofibromin (monoclonal mouse anti-human; McNFN27; Novus Biologicals Inc.; Littleton, CO) at a concentration of 1:100. The sections were incubated in antibody solution overnight at room temperature. Goat anti-mouse and anti-rabbit IgG antibodies labeled with Alexa 488 and 568 fluorochromes, respectively, were then added, and the sections were incubated for 90 minutes at room temperature. Nuclei were identified by staining with DAPI. Slides were cover-slipped with VECTASHIELD© mounting medium (Vector Laboratories Canada Inc.; Burlington, ON, Canada).

**Image Acquisition**

Slides stained for S100 only or for neurofibromin only were scanned to ensure that the staining worked. Blood vessels within the tumour served as an internal positive control for neurofibromin staining. On the section that had been dual labeled for both S100 and neurofibromin, two images were taken using each of three filters (DAPI, FITC, RHOD) within each region that had been selected for examination on the H&E stained slide. Areas that had few blood vessels were chosen to avoid including S100-/Nf+ endothelial cells in the counts. Additional images were taken in areas where there was a change in the staining patterns for S100 and neurofibromin. All samples were imaged on a Zeiss AxioPlan 2 microscope equipped with a 14 bit AxioCam HRm camera at 400x magnification. Image resolution was 1388 X 1040 pixels, and images were saved in TIF format.

**Image Analysis**

Cellomics (Cellomics Inc, Pittsburg PA) bioimaging software was used to quantify the total number of cells within an image and the number of cells that were S100+/Nf+, S100+/Nf-, S100-/Nf+ and S100-/Nf-. The background pixel intensity was measured from five random areas for each of the images on each fluorescent channel. A cell was considered to be positive when its pixel intensity was two standard deviations above the mean background pixel intensity. This analysis was performed on each of the images, and averages were calculated for each tumour.
Statistical Analysis

Kendall's correlation was calculated using SPSS (SPSS, Inc. Chicago, Illinois, 2001) to determine the relationship between vascularity, cellularity, lymphocytic infiltration, tumour type and staining patterns. The Mann-Whitney U test was performed using SPSS (SPSS, Inc. Chicago, Illinois, 2001) to determine if there were significant differences between the S100+/Nf+, S100+/Nf-, S100-/Nf+, and S100-/Nf- cells as percentages of the total number of cells within nodular and diffuse neurofibromas. A p-value less than or equal to 0.05 was considered to be significant.

Method Development for Immunofluorescently Labeled-formalin Fixed Samples for Laser Microdissection

Metal cassettes and normal glass slides were found to be incompatible with the staining procedure described above. Glass slides with polyethylene naphtholate (PEN) membrane mounted on top were, therefore, used for this study.

Laser microdissection requires a dry sample, both for cutting and capturing. Formalin-fixed samples have high overall levels of tissue autofluorescence, which is further heightened when the samples are visualized dry. Xylene, 100% ethanol, and 95% ethanol were tested to determine if they could be used to wet the sample for visualization but would dry quickly enough to allow microdissection of the desired cells. Xylene provided the best visual picture of the tissue and evaporated quickly. However, xylene could only be applied to the tissue three times because it degraded the PEN membrane to which the tissue was attached.

After applying xylene to the tissue, tiled images of 120-140 fields at 600x were taken. Selected cells were microdissected on a Veritas microdissector using the ultraviolet (UV) laser and collected using the infrared (IR) laser. There are several parameters within the UV cutting that can be adjusted. These include the strength of the laser, the number of times the selection is cut, and the “tabs”, which are spaces on the specimen that are left uncut to help prevent tissue curling. Many experiments were required to develop conditions that provided satisfactory recovery of these formalin-fixed tissues with single cell microdissection when hundreds of cells were being selected in a small area. It was determined that the best option was to eliminate the “tabs” for cutting, which permitted good collection with a single cut.
The last parameter tested was the number of selections that could be collected within a given tiled image. This varied between samples. A greater number of samples could be collected from tissue that was more cellular than from tissue that was more fibrous. Assessment of the tissue was, therefore, required before beginning the microdissection.

**Tissue Preparation for Laser Microdissection**

Serial sections were cut from 3 diffuse neurofibromas from 3 females with NF1 and 3 nodular neurofibromas from 1 female with NF1. The first section was cut at 5μm, stained with H&E, and used as a reference slide. All other sections were cut at 8μm and mounted on PEN glass membrane slides (Molecular Devices, Sunnyvale, CA). One section was immunofluorescently stained (as described above) with rabbit anti-human smooth muscle actin (SMA) (AnaSpec, Inc., San Jose, California) at a concentration of 1:400 to identify blood vessels within the neurofibromas. The DNA purified from the blood vessels within the neurofibromas served as a control for molecular analysis because peripheral blood was not available. Subsequent sections were stained for both neurofibromin and S100 as described above, with the exception that Alexa 594 was used instead of Alexa 568 as the label on the secondary antibody.

**Sample Collection by Laser Microdissection**

Tiled images were taken using two filters (FITC, RHOD) on the Veritas microdissector. The images were compared to identify cells that were S100+/Nf+, S100+/Nf- and S100-/Nf+. Cells of each class were selectively cut with a UV laser and attached to a collection cap using an infrared laser. Based on the initial DNA assessment of the whole tumour, enough cells were microdissected to yield 20ng of DNA for each cell type.

**DNA Purification**

DNA was extracted from microdissected samples by overnight incubation at 42°C in 50μl of DNA extraction buffer per microdissection cap. The buffer contained 10mM Tris-HCl pH 8.0, 1mM EDTA, 1% Tween 20 and 0.1% Proteinase K. Phenol/Chloroform extraction was performed followed by ethanol precipitation with 4μg/μl of glycogen added to each 50μl digestion as a carrier. Ethanol precipitation was carried out at -20°C for a minimum of 2 hours in 0.1M sodium acetate and 95% ethanol pH 5.2 solution. Samples were resuspended in 10μl of water.
**XCI Clonality Assay**

Skewing of X-chromosome-inactivation (XCI) was used to assess clonality in each of the microdissected samples by means of a DNA methylation-sensitive assay using polymorphic variants of the androgen receptor (AR) gene. 5μl of purified genomic DNA was digested with *Hpall* and *Rsal*, and an undigested control with 5μl of genomic DNA was digested with *Rsal* alone. Both digestions were carried out in a total volume of 10μl. Nested PCR was performed on the DNA digests with the following primers for 35 cycles in each round: First round -- ARN1F (5' CTGCAGCGACTACCGCATC 3'), ARN2R (5' GCTCTGGGACGCAACCTC 3'); second round: ARF (5' TCCAGAATCTGTTCCAGAGCGTGC 3'), ARR (5' GCTGTGAAGGTTGCTGTTCCTCAT 3'). The second round of PCR was performed with a forward primer labeled with the HEX ABI Prism Dye, and products were sized using capillary electrophoresis on an ABI Prism 310 genetic analyzer (Applied Biosystems, Foster City, CA). Fluorescence was detected by ABI Prism data-collection software and analysed with GeneScan software. The peak area for each allele was used to determine XCI skewing. To account for preferential amplification, the areas of the peaks of the digested sample were normalized in relation to measurements from the undigested sample.

The degree of skewing was calculated as \((d1/u1)/(d1/u1+d2/u2)\), where \(d1\) and \(d2\) represent the peak areas of the two alleles from the digested samples, and \(u1\) and \(u2\) represent the peak areas of the two alleles from the undigested samples. Samples with \(\geq 90\%\) skewing were considered to be clonal.

**RESULTS**

We studied the expression of neurofibromin and S100, a Schwann cell marker, in 38 benign neurofibromas from 29 NF1 patients. 28 of the tumours (from 23 patients) were classified histopathologically as diffuse neurofibromas, 8 tumours (from 5 individuals) were classified as nodular neurofibromas, and two tumours (from 2 individuals) had a mix of both diffuse and nodular components. The mixed tumours were not included in the statistical analysis. Figure 4.1 shows examples of each type of neurofibroma with characteristic patterns of cellularity, vascularity, and lymphocytic infiltration.
Figure 4.1 Images of typical neurofibromas in the current study that represent the majority of the tumour.

A. H&E section of a nodular neurofibroma at 400x magnification. B. Serial section to (A) stained for neurofibromin and counterstained with DAPI at 400x magnification. C. Serial section to (A) stained for S100 and counterstained with DAPI at 400x. D. Merged image of (B) and (C). E. H&E section of a diffuse neurofibroma at 400x magnification. F. Serial section to (E) stained for neurofibromin and counterstained with DAPI at 400x magnification. G. Serial section to (E) stained for S100 and counterstained with DAPI at 400x. H. Merged image of (F) and (G). Note the higher cellularity in E compared to A and the lymphocytic infiltration (L) in A. Also note the presence of blood vessels marked with an arrow and stained positive in (B) and (F) for neurofibromin.
Cellularity

Nodular neurofibromas tended to be less cellular than diffuse neurofibromas (Figure 4.1, Figure 4.2), with almost 75% of nodular tumours falling into the lowest cellularity category. In comparison to diffuse tumours, nodular neurofibromas displayed more dense fibrotic tissue and areas of degeneration in the central portion, but not at the periphery. In contrast, the diffuse tumours had higher overall cellularity with a more uniform distribution of cells throughout the tumour. There was a significant correlation between cellularity and histopathological tumour type (Kendall’s tau = -0.474, p=0.003) (Table 4.1).

Lymphocytic Infiltration

Only neurofibromas with less than 30% lymphocytic infiltration were chosen for this study, as lymphocytes would contribute to the overall cellularity as determined by DAPI staining and cause an over-estimation of the percentage of cells within a neurofibroma that were neurofibromin positive and an under-estimation of those that were S100-positive. All neurofibromas that were excluded from this study were nodular neurofibromas. For the 38 tumours included in the study, the average lymphocytic infiltration for nodular neurofibromas was 13% compared to 9% in diffuse neurofibromas. The correlation of lymphocytic infiltration and tumour type could not be tested statistically because the most extreme cases were eliminated in the tumour selection process.

Lymphocytic infiltration was generally distributed evenly within the nodular neurofibromas, while lymphocytes were more localized in small clusters when they were observed in diffuse neurofibromas. There was a significant inverse correlation between lymphocytic infiltration with both cellularity (Kendall’s tau = -0.353, p=0.022) and vascularity (Kendall’s tau = -0.510, p=0.001) (Table 4.1).

Vascularity

Nodular neurofibromas typically had fewer large vessels than diffuse tumours, which had more smaller vessels (Figure 4.1, Figure 4.2). Vessels were evenly distributed throughout both diffuse and nodular neurofibromas. Vascularity was not significantly correlated with tumour type (Kendall’s tau = -0.187, p=0.254) but was significantly
correlated with cellularity (Kendall’s tau =0.479, p=0.002), as more cellular neurofibromas had a higher number of blood vessels (Table 4.1).

**Figure 4.2** Distribution of pathological features of neurofibromas classified histopathologically.

A. Cellularity estimated on a 3-point Likert scale with 3 being the highest cellularity; B. Vascularity estimated on a 3-point Likert scale with 3 being the highest vascularity; and C. Lymphocytic infiltration estimated on a 3-point Likert scale with 3 being the highest lymphocytic infiltration.

- **Diagram A**: Proportion (%) of diffuse and nodular neurofibromas for cellularity.
- **Diagram B**: Proportion (%) of diffuse and nodular neurofibromas for vascularity.
- **Diagram C**: Proportion (%) of diffuse and nodular neurofibromas for lymphocytic infiltration.
Table 4.1 Summary of Kendall's tau correlations for pathological features in histologically-defined nodular and diffuse neurofibromas from patients with NF1.

<table>
<thead>
<tr>
<th>Tumour Type</th>
<th>Cellularity</th>
<th>Vascularity</th>
<th>Lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumour Type Coefficient</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Significance</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cellularity Coefficient</td>
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<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Significance</td>
<td>P=0.003</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vascularity Coefficient</td>
<td>-0.187</td>
<td>0.479</td>
<td></td>
</tr>
<tr>
<td>Significance</td>
<td>P=0.254</td>
<td>P=0.002</td>
<td></td>
</tr>
<tr>
<td>Lymphocytes Coefficient</td>
<td>0.204</td>
<td>-0.353</td>
<td>-0.510</td>
</tr>
<tr>
<td>Significance</td>
<td>P=0.209</td>
<td>P=0.022</td>
<td>P=0.001</td>
</tr>
</tbody>
</table>

**Neurofibromin & S100 Staining within Benign Neurofibromas**

Figure 4.1 shows examples of typical staining patterns for S100 and neurofibromin in histologically-defined diffuse and nodular neurofibromas. S100-positive (Schwann) cells were scattered throughout all neurofibromas, often separated by S100-negative cells. There were areas that showed compact S100-positive cells, indicating trapped axons or nerve fibers.

When slides were dual labeled for S100 and neurofibromin, 25.4±9.6% (mean ±1SD) of cells within nodular neurofibromas and 41.9±14.2% of cells within diffuse neurofibromas stained positively for both S100 and neurofibromin (the phenotype expected for normal Schwann cells) (Figure 4.3). This difference was highly statistically significant (p=0.005). The proportion of S100-positive cells that were also neurofibromin-positive was somewhat lower in nodular neurofibromas (61.1±20.0%) than in diffuse neurofibromas (74.4±12.7%), but this difference did not reach statistical significance (p=0.071). Fifteen of the 38 tumours showed neurofibromin expression in more than 75% of S100-positive cells. Three tumours, one nodular and two diffuse, showed neurofibromin staining in more than 90% of S100-positive cells. In one of these diffuse neurofibromas, 100% of S100-positive cells also expressed neurofibromin.
Figure 4.3 Boxplot summarizing S100 and neurofibromin staining in nodular and diffuse neurofibromas.

The boxes represent the interquartile range, with the top of the box representing the 75th percentile and the bottom the 25th percentile for the data. The line within the box represents the median. The whiskers on the boxes display the spread of the data. The circles represent outliers that are between 1.5 and 3 box lengths from the upper or lower edge of the box. The stars represent outliers that are greater than 3 box lengths from the upper edge of the box.

<table>
<thead>
<tr>
<th>Diffuse neurofibromas</th>
<th>Nodular neurofibromas</th>
</tr>
</thead>
</table>

Overall, there did not appear to be large aggregations of S100+/Nf- Schwann cells in any of the tumours. Rather, S100+/Nf- cells and S100+/Nf+ cells were both found throughout the tumours. There was no significant difference between nodular and diffuse neurofibromas in the mean percentage of cells that was S100-positive and neurofibromin-negative (the phenotype predicted for a Schwann cell neoplasm that arises by complete inactivation of the NF1 locus) (Figure 3). Cells negative for both S100 and neurofibromin were scattered throughout both types of neurofibromas. The mean percentage of these double negative cells was significantly higher in nodular neurofibromas (31.3±9.9%) than in diffuse neurofibromas (15.7±10.4%) (p=0.003, Figure 4.3). Five nodular neurofibromas and two diffuse neurofibromas had more than 35% of cells negative for both S100 and neurofibromin. Nodular neurofibromas usually
have central areas of degeneration or necrosis that tend to be negative for both S100 and neurofibromin, but particular care was taken to avoid these areas for imaging because they may not represent the tumour as a whole.

There was no difference between nodular and diffuse tumours in the percentage of cells that was S100-negative and neurofibromin-positive (the phenotype expected for normal stromal cells) (Figure 4.3). These cells were also distributed among the S100-positive cells.

Nine neurofibromas, 7 diffuse and 2 nodular, showed areas of non-uniform staining for either S100 or neurofibromin. Six of the diffuse neurofibromas showed little variability in the percentage of any of the cell types throughout the specimen, but one diffuse tumour showed a drop in percentage of all cell types with one area that was completely negative for both S100 and neurofibromin. Of the two nodular neurofibromas that showed variation in staining, one showed a drop in the percent of all cell types and the other showed a drop in the percent S100+/Nf+ cells in one particular area of the slide.

Four neurofibromas, all diffuse, showed variations in tumour appearance according to the H&E, and more images were taken in these differing areas. Three of these neurofibromas did not show any difference in the percentage of each of the cell types in comparison to the other areas of the tumour, while one neurofibroma showed some variation in the number of cells that were positive for both neurofibromin and S100.

**Correlations**

There was a significant inverse correlation between the number of S100+/Nf+ cells and the number of S100-/Nf- cells in a tumour (p<0.001) (Table 4.2). S100+/Nf+ cells were also inversely correlated with S100-/Nf+ cells (p=0.018) but not with S100+/Nf- cells (Table 4.2). S100-/Nf+ and S100+/Nf- cells were negatively correlated with each other (p<0.001). The number of lymphocytes in a neurofibroma was directly correlated with the number of S100-/Nf- cells (p=0.034) and inversely correlated with the number of S100+/Nf+ cells (p= 0.01) (Table 4.2).
Table 4.2 Summary of Kendall’s tau correlations for S100 and neurofibromin staining in histologically-defined nodular and diffuse neurofibromas from patients with NF1.

<table>
<thead>
<tr>
<th></th>
<th>S100+/Nf+</th>
<th>S100+/Nf-</th>
<th>S100-/Nf+</th>
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<td><strong>S100+/Nf+</strong></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Coefficient</td>
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<td></td>
<td></td>
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<tr>
<td>Significance</td>
<td></td>
<td></td>
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<tr>
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<td>1.0</td>
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<tr>
<td>Coefficient</td>
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<tr>
<td>Significance</td>
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<tr>
<td><strong>S100-/Nf+</strong></td>
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<td>-0.544</td>
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<tr>
<td>Coefficient</td>
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<tr>
<td>Significance</td>
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<td>P&lt;0.001</td>
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<tr>
<td><strong>S100-/Nf-</strong></td>
<td>-0.595</td>
<td>-0.124</td>
<td>0.142</td>
<td>1.0</td>
</tr>
<tr>
<td>Coefficient</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Significance</td>
<td>P&lt;0.001</td>
<td>P=0.299</td>
<td>P=0.238</td>
<td></td>
</tr>
</tbody>
</table>

**Clonality of S100+/Nf+, S100+/Nf- and S100-/Nf+ Cells**

If a neurofibroma develops from an S100-positive (Schwann) cell that sustains a "second hit" mutation of the *NF1* locus, the neoplastic cells should be clonally related to each other and should not express neurofibromin. Other cells within the tumour that are not part of the neoplastic clone would be expected to continue to express neurofibromin and to be polyclonal. In contrast, if neurofibromas arise by proliferation of *NF1* haploinsufficient cells, all cell types, including Schwann cells, should be polyclonal and should continue to express neurofibromin.

Six neurofibromas, 3 diffuse and 3 nodular, that had been removed from female NF1 patients were selected for microdissection based on DNA quality. After sections of each tumour were double stained for S100 and neurofibromin, and S100+/Nf+ cells (putatively normal Schwann cells), S100+/Nf- cells (putatively neoplastic Schwann cells that had sustained a "second hit" *NF1* mutation), and S100-/Nf+ cells (putatively normal non-Schwann cells) were isolated by laser capture microdissection (Figure 4.4).
Figure 4.4 Images showing selective capture of single cell microdissection of S100+/Nf-negative cells from heterogeneous tissue.

A. Diffuse neurofibroma stained for neurofibromin. Red circles indicate cells that are S100-/Nf+ when compared side-by-side to images of S100 staining at 600x magnification. B. Same tissue in (A) stained for S100. Green circles indicate cells that are S100+/Nf- and blue circles indicate cells that are S100+/Nf+ when compared side-by-side to images of neurofibromin staining at 600x magnification. C. Image of tissue after cutting all of the selected cells types. Red arrows indicate S100-/Nf+ cells cut with a UV laser, green arrows indicate S100+/Nf- cells cut with a UV laser, and the blue arrows indicate S100+/Nf+ cells cut with a UV laser at 600x magnification. After cutting, only the S100+/Nf- cells were captured using the infrared laser onto the collection cap. D. Images of collection cap showing specific removal of only S100+/Nf- cells from heterogeneous tissue at 400x magnification.
The X-chromosome inactivation (XCI) ratio of DNA isolated from each group of cells was then measured to determine clonality. Blood vessels were also microdissected from each tumour and tested to determine the XCI ratio in normal tissue because blood samples from these patients were not available (Figure 4.5). The results are summarized in Table 4.3.

**Figure 4.5 Images of Blood Vessel Microdissection.**

A. Tissue section stained with smooth muscle actin (SMA) to identify blood vessels within a diffuse neurofibroma at 200x magnification. B. Tissue following tissue cutting with a UV laser. Arrows indicate vessels that have been cut at 200x magnification. C. Tissue following selective collection of samples onto a collection cap using an infrared laser at 200x magnification. D. Image of collection cap showing specific removal of blood vessels at 200x magnification.
Table 4.3 Summary of clonality results from histologically-defined nodular and diffuse neurofibromas and the proportion of each cell type within the neurofibromas.

<table>
<thead>
<tr>
<th>Tumour</th>
<th>Staining Pattern (%)</th>
<th>XCI Ratios</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S100+/Nf+</td>
<td>S100+/Nf-</td>
</tr>
<tr>
<td>D1</td>
<td>38</td>
<td>30</td>
</tr>
<tr>
<td>D3</td>
<td>57</td>
<td>14</td>
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</tr>
<tr>
<td>N2</td>
<td>21</td>
<td>22</td>
</tr>
<tr>
<td>N3</td>
<td>20</td>
<td>18</td>
</tr>
</tbody>
</table>

D = Diffuse neurofibroma  
N= Nodular neurofibroma  
+ = positive staining  
- = negative staining  
Nf = neurofibromin
Tumour D1 is a diffuse neurofibroma with 30% S100+/Nf- cells. Normal blood vessels from this patient showed an XCI ratio close to the expected 50:50 (56:44). In contrast, the S100+/Nf- cells demonstrated clonality with an XCI ratio of 90:10 (Table 4.3). As expected, the XCI ratios of S100+/Nf+ and S100-/Nf+ cells were similar to that of the blood vessels. The proportion of S100-/Nf- cells in this tumour was very low (3%). These findings are consistent with this neurofibroma arising through clonal proliferation of a Schwann cell precursor that sustained a "second hit" NF1 mutation that abrogated neurofibromin expression.

D2 was the second diffuse neurofibroma studied. This neurofibroma had 25% S100+/Nf- cells. The XCI ratio of this cell population was opposite (21:79) that of the blood vessels (80:20). S100+/Nf+ and S100-/Nf+ cells both had XCI ratios close to the 50:50 expected for random XCI, although this was somewhat different from the ratio found in the blood vessels (Table 4.3). Although the XCI ratio in the S100+/Nf- cells did not meet our definition of clonality (90:10), it seems likely that this neurofibroma also arose through clonal proliferation of a Schwann cell precursor that sustained a "second hit" NF1 mutation.

The third diffuse neurofibroma analysed (D3) had 14% S100+/Nf- cells, and these cells did not show any evidence of clonality. The XCI ratio was the same as in the blood vessels (44:56) (Table 4.3). This tumour showed a high proportion (57%) of S100+/Nf+ cells, which also had an XCI ratio close to that of the blood vessels. The findings in this neurofibroma are consistent with the tumour arising as a polyclonal proliferation of NF1 haploinsufficient cells.

The three nodular neurofibromas (N1, N2 and N3) examined for clonality were all spinal tumours removed from the same patient. The normal blood vessels in all three of these neurofibromas showed similar XCI skewing (91:9, 95:5 and 87:13, respectively) (Table 4.3). All three of these tumours also showed high proportions of S100-/Nf- cells (32-38%). Only 13% of the cells in N1 stained positively for S100 but negatively for neurofibromin. The XCI ratio for this cell type was highly skewed (7:93) to the opposite allele than in the blood vessels. S100+/Nf+ cells and S100-/Nf+ cells both had XCI ratios similar to that of the normal blood vessels. The findings in this neurofibroma are consistent with
clonal proliferation of a Schwann cell precursor that sustained a "second hit" $NF1$
mutation.

Nodular neurofibroma N2 had 22% S100+/Nf- cells. This cell type was not clonal (23:77) but the XCI ratio differed greatly from that of the blood vessels, which was highly skewed in the opposite direction (95:5) (Table 4.3). The S100+/Nf+ cells and S100-/Nf+ cells in this sample both had XCI ratios close to that expected for random X-inactivation and thus different from the skewed XCI ratio observed in the blood vessels. The interpretation of this case is less clear, but the neurofibroma may also have arisen through clonal proliferation of a Schwann cell precursor that sustained a "second hit" $NF1$ mutation.

The third nodular neurofibroma tested (N3) had 20% of cells that were S100+/Nf-. The XCI ratio of these cells is compatible with random X-inactivation (46:54) but somewhat different from that of the normal blood vessels (87:13) (Table 4.3). The S100-/Nf+ cells in this tumour had an XCI ratio similar to that of the blood vessels, but the XCI ratio of the S100+/Nf+ cells resembled that of the S100+/Nf- cells and not that of the normal blood vessels. The interpretation of these data with respect to clonality is unclear.

DISCUSSION

NF1 neurofibromas are thought to develop according to Knudson's "two-hit" hypothesis, as a result of inactivation of both alleles of the $NF1$ locus (33-35). People with neurofibromatosis 1 have a constitutional mutation of $NF1$ in every cell of their bodies, and somatic mutation of the second (normal) allele in a Schwann cell is assumed to be the rate-limiting step in neurofibroma formation. Although the "two-hit" mechanism is widely accepted as the basis of neurofibroma formation in NF1, the development of all neurofibromas as a result of "second hit" mutations does not readily explain all of the available data.

We have suggested that some neurofibromas arise through inactivation of the second (normal) $NF1$ allele in a Schwann cell and subsequent clonal expansion to form a neoplasm, but other neurofibromas arise as polyclonal proliferations of $NF1$ haploinsufficient cells. The development of some neurofibromas as polyclonal proliferations of $NF1$ haploinsufficient cells could explain the exceptional observations listed above and others that are not easily reconciled with the "two-hit" model of tumour development. There is increasing evidence that haploinsufficiency of other tumour
Suppressor genes such as TSC1, TSC2, and P57 can also lead to the formation of tumours without a “second hit” (36-39).

The purpose of this study was to quantify the percentage of cells with immunohistological features of normal Schwann cells (S100+/Nf+ cells), neoplastic Schwann cells that have sustained a “second hit” NF1 mutation (S100+/Nf- cells), and normal non-Schwann cells (S100-/Nf+ cells) and to determine if the proportions of these cell types differ in histologically-defined nodular and diffuse neurofibromas. We also wanted to determine if Schwann cells that no longer express neurofibromin were clonal, as would be expected for a neoplasm that arose as the result of a “second hit” NF1 mutation. We found a significant difference between the mean percentages of S100+/Nf+ cells and of S100-/Nf- cells in nodular and diffuse neurofibromas. The findings of our clonality studies support the interpretation that some neurofibromas are clonal Schwann cell neoplasms while other neurofibromas are polyclonal proliferations of NF1 haploinsufficient cells.

Haploinsufficiency and loss of NF1 are thought to lead to tumour formation because neurofibromin, the protein product of the NF1 gene, functions to down-regulate Ras, a key cell cycle control protein. Neurofibromin enhances the conversion of active Ras-GTP to inactive Ras-GDP. Ras modulates several important signal transduction pathways, one of which is the MAP-kinase pathway. Inactivating mutations in one or both copies of NF1 lead to higher Ras concentrations and an increase in cellular proliferation (40-42).

There is substantial evidence that NF1 functions as a tumour suppressor gene, and LOH or second somatic mutations for NF1 have been observed in some neurofibromas, in accordance with the “two-hit” hypothesis. Overall, LOH or second somatic mutations have been observed in 26% of dermal neurofibromas and 36% of plexiform neurofibromas studied to date (8-24). The low percentage of LOH or second somatic mutations observed in NF1 neurofibromas has been attributed to detection methods that cannot identify all mutations, mutations outside of the region of the NF1 gene that was tested, or the frequent inactivation of the second (normal) NF1 allele by an epigenetic mechanism. However, no evidence of epigenetic inactivation of the NF1 gene has been observed (14, 43, 44).
To better understand NF1 neurofibroma development, Zhu and coworkers (45) used a cre/lox system to delete \(Nf1\) in Schwann cells while maintaining \(Nf1\) heterozygosity in all other cell types in mice. These animals showed thickened peripheral nerves and developed both cranial and spinal nerve neurofibromas. Interestingly, these features were only observed when other cells were heterozygous for \(Nf1\), pointing to the importance of the \(Nf1\) haploinsufficient background in neurofibroma development in mice. Similarly, embryonic day 12.5 \(Nf1^{-/-}\) Schwann cells implanted into the sciatic nerve of \(Nf1^{+/+}\) mice develop into tumours that resemble plexiform neurofibromas (46). Human \(NF1^{-/-}\) Schwann cells derived from neurofibromas of NF1 patients exhibit a growth advantage in culture and produce tumours that resemble plexiform neurofibromas when transplanted into the peripheral nerves of scid mice (25). The tumours produced in these experimental systems are models of nodular neurofibromas that develop in people with NF1, but the relevance of these models to other types of neurofibromas that occur as part of NF1 is unknown. It is interesting to note that multiple cutaneous neurofibromas, the feature that gives NF1 its name, and congenital diffuse neurofibromas do not develop in any of the NF1 mouse models described to date (47-50). Moreover, there are important differences between human nodular neurofibromas, which are presumed to arise as clonal outgrowths from a single mutant precursor that has sustained a “second hit” at the \(NF1\) locus, and these mouse models, in which tumours develop from a large number of neurofibromin-deficient Schwann cells.

Muir et al (25) also stained benign neurofibromas from people with NF1 for neurofibromin and S100. They found four basic patterns of neurofibromin staining in S100-positive regions: neurofibromin-negative, predominantly neurofibromin-negative with focal areas of distinct positive staining, predominantly neurofibromin-positive with focal areas of distinct negative staining, and neurofibromin-positive. We also observed two different populations of Schwann cells – those that expressed neurofibromin and those that did not – but we rarely saw large aggregates of S100-positive cells that did not express neurofibromin. Our tumours almost always showed mingling of neurofibromin-negative and neurofibromin-positive Schwann cells. Our findings are similar to those obtained by molecular analysis of a dermal neurofibroma in which the “second hit” somatic mutation of the \(NF1\) locus was identified (10). In this tumour, both the mutant and non-mutant version of the second \(NF1\) allele were found in a homogenous-appearing area of tumour cells.
S100-positive (presumably Schwann) cell cultures grown from neurofibromas removed from NF1 patients contain both neurofibromin-positive and neurofibromin-negative cell populations (6, 25, 26). It is not known how the heterogeneity of neurofibromin expression seen in these cultures relates to the tumours from which the cultures were grown. In the present study, we found that S100+/Nf- cells (i.e., cells with the phenotype expected for Schwann cells that have sustained an inactivating mutation of their normal NF1 allele) made up only 18% of the neurofibromas studied, and the proportion was similar in diffuse and nodular tumours. However, the proportion of S100+/Nf+ cells (the phenotype expected for normal Schwann cells) was significantly higher (42%) in diffuse neurofibromas than in nodular neurofibromas (24%). The percentage of S100-positive (presumably Schwann) cells that expressed neurofibromin averaged 61% (range, 25-100%) in nodular neurofibromas and 74% (range, 39-100%) in diffuse neurofibromas. These values are consistent with two other studies that estimated the proportion of Schwann cells with an inactive NF1 gene, i.e., the reciprocal fraction, as 16-62% in cultures derived from NF1 neurofibromas (8) and as 30-60% as estimated from LOH on sequence analysis of a whole tumour (19).

One possible explanation for the difference observed in the proportion of S100+/Nf+ cells between diffuse and nodular neurofibromas is that the diffuse neurofibromas were much more likely to have been obtained from individuals with constitutional whole NF1 gene deletions while the nodular neurofibromas were more likely to have been obtained from individuals with constitutional intragenic mutations. The "second hits" in neurofibromas from individuals with constitutional whole gene deletions are usually subtle mutations that might not completely abrogate neurofibromin expression (51), while "second hits" in neurofibromas from individuals with other constitutional mutations are more likely to cause LOH and thus lack neurofibromin expression (19, 23). This explanation is unlikely to account for the difference in frequency of S100+/Nf+ cells that we observed between nodular and diffuse neurofibromas because whole gene deletions account for only about 5% of constitutional mutations in people with NF1(52-54).

We found that about 31% of the cells in nodular neurofibromas and about 15% of cells in diffuse neurofibromas did not express either S100 or neurofibromin. We cannot rule out the possibility that many of the S100-/Nf- cells may actually be neurofibromin-positive cells that did not stain for neurofibromin. However, the sensitivity of the
antibody should be equal in both diffuse and nodular neurofibromas, and an average of 92% of cells in traumatic neuroma, which were used as a positive control in our study, were neurofibromin-positive.

We do not know what kind of cells the S100-/Nf- population represents, and Muir et al. (25) did not comment on this cell population in their study. One intriguing possibility is that the S100-/Nf- cells are dedifferentiated Schwann cells or, alternatively, immature Schwann stem cells that are part of a neoplastic clone that has suffered a "second hit" NF1 mutation. This is consistent with studies showing that increased signaling through the Ras/Raf/Erk pathway can drive Schwann cells to a dedifferentiated state (55). It is not clear why such cells should be more common in nodular than diffuse neurofibromas when S100+/Nf- cells are observed in a similar proportion in both kinds of tumour.

cAMP has been shown to induce redifferentiation of Schwann cells (55) and could be present in higher levels in diffuse than nodular neurofibromas. S100-/Nf- cells are often observed in MPNSTs (56), and the higher proportion S100-/Nf- cells in nodular neurofibromas raises concern about the risk of this tumour type progressing to malignancy. It is important to define the nature of the S100-/Nf- cells and to determine if they are more tumorigenic than other cell types within neurofibromas.

The correlation we observed between lymphocytic infiltration and the proportion of S100-/Nf- cells might indicate that this cell type is triggering an immune response, but it is also possible that the prevalence of both lymphocytes and S100-/Nf- cells both reflect some other underlying feature. In any case, we may not have completely defined the relationship between these two cell types because our study was restricted to neurofibromas in which lymphocytes constituted less than 30% of the total cell population.

Riccardi (30) has distinguished four types of neurofibromas – cutaneous, subcutaneous, nodular plexiform and diffuse plexiform – on the basis of their clinical features. A major benefit of Riccardi’s classification is that tumours with different natural histories are placed into different groups, and neurofibromas with a high potential for malignant degeneration (nodular plexiform and diffuse plexiform tumours) are distinguished from those with a much lower risk of malignancy (cutaneous and subcutaneous tumours). The tumours in our study were not all classified by this clinical method, and the histological and clinical approaches do not place all tumours into the same groups. Both
cutaneous and diffuse plexiform tumours usually are diffuse neurofibromas histologically, but some diffuse plexiform neurofibromas are mixed diffuse and nodular neurofibromas. Tumours that are classified clinically as subcutaneous or nodular plexiform neurofibromas are usually nodular neurofibromas on histopathological exam. However, the only way to determine the histological type of a neurofibroma is to examine it histologically.

Three previous studies have assessed clonality in NF1 neurofibromas. All of these studies tested the XCI ratio of whole tumours, which are made up of several different cell populations, rather than looking at each particular cell type separately. In total, 35 neurofibromas, 30 dermal and 5 plexiform neurofibromas, were previously assessed (8, 11, 57). One study did not find clonality in any of the 14 neurofibromas tested (57). In another study, eight dermal neurofibromas were found to have a monoclonal origin, although none of these tumours displayed LOH for NF1 (8). The last study found clonality in 3 dermal neurofibromas and 1 plexiform neurofibroma (11). The whole tumour XCI ratios in the current study are consistent with a polyclonal phenotype.

Using laser microdissection, we assessed the XCI ratio in three different cellular populations defined by S100 and neurofibromin staining, as well as in blood vessels (as normal tissue controls), from six NF1-associated neurofibromas. Our findings are compatible with a heterogenous origin of neurofibromas, with some tumours developing through clonal proliferation of S100+/Nf- cells, and other neurofibromas developing through a polyclonal process. There was evidence for both mechanisms in both histologically-defined diffuse and histologically-defined nodular neurofibromas. Clear evidence for clonality was seen in one nodular (N1) and one diffuse (D1) neurofibroma. N1 was defined clinically as a nodular plexiform neurofibroma, and D1 was defined clinically as a cutaneous neurofibroma. In both of these cases, it is likely that the S100+/Nf- cells represent a neoplastic clone of Schwann cells that sustained a “second-hit” mutation of the NF1 locus. It is interesting that this population made up only 30% of the cells in diffuse neurofibroma D1 and only 13% in the cells in nodular neurofibroma N1. Cells negative for both S100 and neurofibromin made up the greatest proportion of cells in neurofibroma N1. Unfortunately, we could not isolate these cells by microdissection without a specific marker, and we do not know if they were clonal or
It is interesting to speculate that they might be dedifferentiated (or just more primitive) Schwann cell precursors that are part of the neoplastic clone.

Two other neurofibromas (N2 and D2), which were classified clinically as a nodular plexiform neurofibroma and a cutaneous neurofibroma, respectively, probably also arose through clonal proliferation of a Schwann cell (or Schwann cell precursor) that sustained a "second hit" inactivating mutation at the NF1 locus. The XCI ratio in S100+/Nf- cells in these two tumours did not meet our formal definition of skewing (90:10 or greater), but both showed a predominance of the NF1 allele that was less frequent among cells in the normal blood vessels. The most parsimonious explanation is that these tumours were clonal but the S100+/Nf- cells tested were "contaminated" by non-clonal tissue of some type.

There was no evidence for clonality among the S100+/Nf- cells in one diffuse neurofibroma (D3). This tumour was classified clinically as a diffuse plexiform neurofibroma. All cell types within this tumour had an XCI ratio similar to the blood vessels. S100+/Nf- cells made up only 14% of the total in this tumour, and S100-/Nf- cells only 10%. We cannot rule out the possibility that the double negative population was clonal in this tumour, but if it was it represents an unusually small proportion of the total cell population, and it does not seem to have contributed substantially to the S100-positive population.

Interpretation of the clonality data in neurofibroma N3, which was classified clinically as a nodular plexiform neurofibroma, is less clear. A polyclonal origin may be most likely. A larger fraction (34%) of the cells in this tumour were negative for both S100 and neurofibromin, and the possibility that this population represents dedifferentiated or more primitive Schwann cell precursors that have sustained a "second-hit" inactivating mutation of the NF1 locus cannot be ruled out.

There are several possible explanations for the skewing observed in blood vessels from the individual from whom all three nodular neurofibromas examined for clonality were obtained. The first possibility is that the individual herself has skewed X inactivation throughout her body. 10% of women have a greater than 90% expression of one X chromosome by chance in blood (58). A second possibility is that the mesodermal stem cell pool from which the blood vessels arose was small and that skewed X chromosome inactivation occurred in derivatives of this pool by chance as a result of sampling. A third
possibility is that the tumour arose focally within a small mixed population of cells and the cells that produced neovascularature of the tumour all arose from a tiny initial pool within the focus of involvement. Growth of the tumour vasculature from a small population of cells could result in skewed XCI which, by chance, happened to be in the same direction in all three tumours. Without testing other tissues from this individual, we can not distinguish which of these possibilities is correct.

Another possibility is that the skewing observed in normal tissues such as blood vessels may be due to NF1 haploinsufficiency. Therefore, a study assessing XCI ratios in normal tissues of females with NF1 should be explored to see if the proportion of extreme (>90%) XCI skewing is greater than the proportion (10%) observed in the general population.

In summary, we found that a high proportion of S100-positive (presumably Schwann) cells in NF1-associated neurofibromas express neurofibromin. Although some neurofibromas appear to develop as clonal neoplasms as a result of "second hit" mutations of the normal NF1 allele in a Schwann cell, the origin of some other neurofibromas appears to be different. We favour the possibility that some neurofibromas arise as polyclonal proliferations of NF1 haploinsufficient cells, but it is also possible some neurofibromas arise from more primitive or de-differentiated cells of the Schwann cell lineage, rather than from mature Schwann cells. These observations need to be confirmed in a larger sample set to ensure they are not unique to the few tumours examined. Further studies are needed to clarify the nature of the S100-/Nf-cells that often make up a substantial fraction of the total cell population within neurofibromas and to determine the relationship of these and other cellular components to the development of MPNST in people with NF1.
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5. GENERAL DISCUSSION

Summary

Neurofibromas are the hallmark feature of NF1. Almost all individuals with NF1 develop neurofibromas in their lifetime, and some individuals develop hundreds or thousands of these tumours. Cutaneous neurofibromas can have a great effect on quality of life, and other kinds of neurofibromas may cause pain, deformity, functional impairment, or bleeding because of erosion into adjacent structures. Congenital diffuse and nodular plexiform neurofibromas may give rise to MPNSTs, which are a major cause of premature death in people with NF1 (1).

Mouse models of benign neurofibromas and MPNST have provided valuable insights into tumour development, but these models do not fully recapitulate the human situation. Cultures derived from NF1 neurofibromas also provide valuable information but cannot be used to study some critical host factors or the role of the tumour environment.

In order to understand tumour development in people with NF1 fully, it is necessary to study how neurofibromas arise in vivo and to take into consideration the different clinical features and natural histories of each type of neurofibroma. I used histology and immunohistochemistry to determine the cellular composition and expression of neurofibromin in diffuse and nodular neurofibromas and assessed clonality to define the cell populations that give rise to these tumours. I also investigated associations of benign and malignant peripheral nerve sheath tumours in a large series of NF1 patients in order to identify a group of patients who are at highest risk for developing MPNSTs.

Risk of MPNST Development

The strong association I observed between the occurrence of internal neurofibromas and MPNSTs in people with NF1 (Chapter 2) suggests that such individuals are at higher risk of developing MPNSTs than NF1 patients without internal neurofibromas. The large size and older age of subjects in the clinical database I used in this study are unique in NF1 research and allowed me to assess this association for the first time. My investigation was largely cross-sectional, but if the association I found with the development of malignancy can be confirmed in longitudinal studies of NF1 patients
with internal neurofibromas, such individuals may benefit from closer monitoring for signs and symptoms of malignant progression. Closer monitoring may permit earlier diagnosis and more effective treatment of MPNSTs, which currently account for the death of about 10% of people with NF1 (1).

The optimal approach to clinical monitoring of NF1 patients for MPNST development is unknown. Screening for symptoms is probably inadequate because most MPNSTs are metastatic at the time of clinical diagnosis (2). Routine whole-body imaging studies using CT or MRI would permit recognition of most internal neurofibromas and MPNSTs but cannot reliably distinguish benign and malignant peripheral nerve sheath tumours. PET scanning can distinguish benign neurofibromas from MPNST in most cases (3), but PET and MRI scans are expensive, and PET or CT imaging would require exposing NF1 patients to ionizing radiation, which itself may increase their risk of developing an MPNST (2, 4). A thorough cost-benefit analysis is needed to determine the clinical value of and optimal protocol for screening selected NF1 patients for malignancy.

My observation that cutaneous neurofibromas were not associated with the development of MPNST differs from the findings in one study that found a weak association with cutaneous neurofibromas. However, the median age of individuals without MPNSTs was lower than those with MPNSTs in that study (5), and the patients with MPNSTs were of the approximate age when cutaneous neurofibromas begin to appear. Therefore, the majority of individuals without MPNSTs would be expected to have few if any cutaneous neurofibromas based on age alone. Our observation is consistent with the clinical observation that cutaneous neurofibromas have not been reported to progress to malignancy, even though individuals with NF1 may have thousands of these benign tumours (6).

The association I observed between internal and subcutaneous neurofibromas could point to a common mechanism of tumour development. In contrast, the lack of association between cutaneous neurofibromas and either internal or subcutaneous neurofibromas suggests that more than one mechanism of neurofibroma pathogenesis may exist in people with NF1. The results from Chapters 3 and 4 are consistent with this interpretation.
**Benign Neurofibroma Development**

The classification of benign neurofibromas used for Chapters 3 and 4 differs from that used in Chapter 2. Chapter 2 classified externally-visible neurofibromas according to their clinical features, while Chapters 3 and 4 used a histopathological classification. Clinically-defined cutaneous and diffuse plexiform neurofibromas are generally classified as diffuse neurofibromas histopathologically, and clinically-defined subcutaneous and nodular plexiform neurofibromas are usually classified as nodular neurofibromas histopathologically. However, exceptions do occur, and the clinical classification of a neurofibroma cannot always be determined from its histopathological appearance or the histopathological classification from the clinical appearance.

The internal neurofibromas diagnosed by MRI or CT scan in Chapter 2 were not classified clinically, but most are probably nodular plexiform neurofibromas in Riccardi's clinical classification (7) or nodular neurofibromas in the histopathological classification used in Chapters 3 and 4. Thus, the lack of association between internal (predominantly nodular) neurofibromas and cutaneous (generally diffuse) neurofibromas found in Chapter 2 is consistent with the histological findings presented in Chapters 3 and 4, which are suggestive of pathogenetic differences between nodular and diffuse neurofibromas.

In Chapter 4, I found that S100-/Nf- cells were abundant in nodular neurofibromas but much less common in diffuse neurofibromas. I do not know what cell population the S100-/Nf- cells represent. It is unlikely that these cells are really S100-/Nf+ cells (normal stromal cells) that did not stain for neurofibromin because the sensitivity of the anti-neurofibromin antibody was 92% in the control and should be equal in nodular and diffuse neurofibromas. Two interesting possibilities are that the S100-/Nf- cells are 1. neoplastic Schwann cells that have sustained a "second-hit" of the NF1 locus and lost S100 expression through dedifferentiation or 2. neoplastic primitive Schwann cell precursors (stem cells) that sustained a "second hit" NF1 mutation. Either possibility could provide a population of neoplastic cells that drives tumour formation and leads to a high risk of malignant degeneration, accounting for the association observed in Chapter 2. We were not able to assess clonality in the S100-/Nf- population because we did not have a cell marker or nuclear stain to identify these cells. The use of nuclear stains lowered the quality of DNA in the samples that were already compromised from
the formalin-fixation process. However, the abundance of S100-/Nf- cells in nodular neurofibromas suggests that these cells have an important role in the development of this tumour type. It is important to assess the clonality of these cells to determine if they are the neoplastic clone that gave rise to the tumour.

I found fewer mast cells in histologically-defined nodular than diffuse neurofibromas (Chapter 3). The low numbers of mast cells observed in nodular neurofibromas could simply reflect anatomical differences that exist between nodular and diffuse tumours. Diffuse tumours are in direct contact with adjacent tissues, while nodular tumours are surrounded by the perineurium, which may serve as an anatomical barrier to invasion by mast cells. Alternatively, the relative abundance of S100-/Nf- cells and the relative paucity of S100+/Nf+ cells in nodular neurofibromas may provide less stimulus to mast cell invasion than in diffuse neurofibromas. Most of the histologically-defined nodular neurofibromas that I studied were classified clinically as nodular plexiform tumours. The histological and clinical similarities between these neurofibromas and subcutaneous tumours suggest that they share a common pathogenesis, but more subcutaneous neurofibromas need to be studied to assess this possibility.

Although clinically-defined cutaneous and diffuse plexiform neurofibromas have similar histological characteristics, the pathogenesis of these two kinds of tumours is likely to differ because they arise at different times of life and have very different natural histories. Two of the diffuse neurofibromas I studied for clonality were classified clinically as cutaneous tumours (Chapter 4). One showed definite clonality, and the other, possible clonality of S100+/Nf- cells. These tumours had few S100-/Nf- cells, in contrast to nodular plexiform neurofibromas. My observation that cutaneous neurofibromas often show abundant mast cell infiltration may reflect strong attraction or stimulation of mast cells by S100+/Nf- Schwann cells, as has been observed in the mouse model (8).

Only one of the diffuse neurofibromas examined for clonality in this study was classified clinically as a congenital diffuse plexiform tumour (Chapter 4). It had few neurofibromin-negative cells (either S100-positive or S100-negative) and demonstrated polyclonality in all cell types. This observation is consistent with the hypothesis that diffuse plexiform neurofibromas arise during fetal development through excessive proliferation of poorly-regulated NF1 haploinsufficient cells rather than through expansion of a neoplastic
Schwann cell clone that has sustained a “second-hit” that annuls neurofibromin expression. The abundance of mast cells in this tumour might be explained by recruitment or proliferation of mast cells in response to excessive cellular proliferation. However, this neurofibroma was located superficially, and the presence of many mast cells might simply reflect its proximity to the skin, where many mast cells normally reside. More diffuse plexiform neurofibromas from both deep and superficial locations need to be examined to confirm my findings regarding the numbers, distribution and clonality of various cell types.

**How this Study fits into current NF1 research**

The function of the *NF1* gene has not yet been fully elucidated. The best characterized function of neurofibromin is as a negative regulator of the Ras signaling pathway (9), but *NF1* is also involved in other signaling pathways, including cAMP (10). Haploinsufficiency or complete absence of neurofibromin has been shown to affect the proliferation of a variety of cell types (11-13), but research into the responsible mechanisms is ongoing. The ubiquitous expression of neurofibromin, its interactions with numerous other cellular pathways, and the variety and variability of phenotypes observed in people with NF1 show that NF1 is a complex disease. It is, therefore, not surprising that more than one pathogenic mechanism may lead to the development of neurofibromas, and trying to cut too deeply with Occam’s razor may not be the best way to understand the disease.

There is no cure for neurofibromas except surgical removal in favourable cases. Small discrete tumours can usually be removed successfully, but plexiform neurofibromas, especially diffuse plexiform tumours, usually cannot be removed in their entirety without substantial neurological compromise caused by surgical damage to nerves that traverse the tumour. Incompletely removed neurofibromas usually grow back.

Most studies of neurofibromas either lump all of these tumours together as a single benign group or categorize them as “dermal” or “plexiform”, with the “dermal” group containing both cutaneous and subcutaneous neurofibromas, as defined by Riccardi’s clinical classification, and the “plexiform” group containing both nodular and diffuse plexiform neurofibromas. The results of my work show that these groupings are insufficient. At the very least, clinically-defined cutaneous neurofibromas should be analysed separately from subcutaneous neurofibromas, and clinically-defined nodular
and diffuse plexiform neurofibromas should be analysed separately because their histological appearance, cellular composition, and natural history are different.

The differences I observed between histologically-defined diffuse and nodular neurofibromas help clarify how different kinds of neurofibromas develop and may provide insight into why some neurofibromas progress to malignancy, while most do not. My results support the current theory that neurofibromas can develop by clonal expansion of neoplastic Schwann cells that have sustained a “second-hit” NF1 mutation. However, I also found evidence that other neurofibromas develop by a different mechanism – polyclonal proliferation of NF1 haploinsufficient cells. It is also possible that some neurofibromas, particularly nodular neurofibromas, develop from a Schwann cell precursor rather than from mature Schwann cells. Further clinical, pathology, cell biology, and molecular genetic studies of neurofibromas are necessary to determine why individuals with NF1 and certain kinds of neurofibromas are more likely to develop MPNSTs than NF1 patients without such tumours.

**Future Directions**

The technique that I developed for laser microdissection of immunofluorescently-labeled cells in formalin-fixed paraffin-embedded samples is novel and has not previously been described. Fixed tissue of this kind is readily available, and the ability to perform molecular genetic studies on cells isolated from such tissue on the basis of immunofluorescent features should facilitate molecular genetic studies of a variety of tumours without the need for additional sample collection. This technique can also be applied to other kinds of tumours associated with NF1 as well as to bony and vascular lesions that occur in some patients with this condition.

The association I found between internal neurofibromas and MPNSTs is striking, but because this study was based on cross-sectional data, the results can not be used to assess individual risk. Nevertheless, my findings show the need for a longitudinal study of neurofibroma growth and MPNST development in young adults with NF1. Current clinical standards for care of NF1 patients recommend imaging studies for internal neurofibromas only if they are symptomatic. My data show that individuals with asymptomatic internal neurofibromas may also be at risk for developing MPNSTs. These findings raise important questions about whether routine whole-body imaging should be considered for all NF1 patients and whether those found to have internal...
plexiform tumours should be followed more closely for the development of an MPNST. Clinical research and cost-benefit studies of available imaging techniques for people with NF1 are clearly needed.

Most internal neurofibromas included in my association study of MPNST risk would be classified clinically as nodular plexiform neurofibromas. The relatively high proportion of S100-/Nf- cells I observed in histologically-defined nodular neurofibromas may be evidence of a previously-unappreciated dedifferentiated or precursor Schwann cell population that is of particular importance in the pathogenesis of these tumours and in their malignant progression. The techniques used in the current study precluded further molecular analysis of this cell type, but future studies of the origin, clonal nature, and genetic stability of the S100-/Nf- cells may provide critical insight into processes that lead to the formation and malignant progression of nodular plexiform neurofibromas.

My observations suggest that cellular interactions play a key role in neurofibroma development and imply that modifying genes may be of importance. Individuals with NF1 whose constitutional mutation is a micodeletion of the entire NF1 locus and its adjacent genomic region are characterised by unusual facial features, a high tumour burden and an unusually high risk of developing MPNST (14, 15). There are 17 genes contained within the 1.4Mb region that is usually deleted in these patients. The products of these other genes may interact with the NF1 gene, with neurofibromin, or with other factors in the signaling pathways that neurofibromin regulates. Proteins produced by genes elsewhere in the genome also appear to interact with neurofibromin (16, 17).

Studies of factors that influence neurofibromin activity and function are a major area of study at NF research laboratories throughout the world.

NF1 is a very complex disease, and my studies show that neurofibroma development is not simply a matter of a Schwann cell sustaining a “second hit” of the NF1 gene. In addition to having different clinical characteristics, different kinds of neurofibromas exhibit clear differences in cellular composition and gene expression. My results prove that the clinical and pathological heterogeneity of neurofibromas needs to be taken into account by researchers who are trying to understand how these tumours develop and, in some instances, progress to malignancy.
References


