Development and Application of Microarray-Based Comparative Genomic Hybridization

Analysis of Neurofibromatosis Type-2, Schwannomatosis and Related Tumors

PATRICK BUCKLEY
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Abstract

Neurofibromatosis type-2 (NF2) is an autosomal dominant disorder with the clinical hallmark of bilateral eighth cranial nerve schwannomas. However, the diagnostic criterion is complicated by the presence of a variable phenotype, with the severe form presenting with additional tumors such as peripheral schwannoma, meningioma and ependymoma. We constructed a microarray spanning 11Mb of 22q, encompassing the NF2 gene, to detect deletions in schwannoma. Forty seven patients were analyzed and heterozygous deletions were detected in 45% of tumors. Using this array-based approach, we also detected genetic heterogeneity in a number of samples studied. Despite the high sensitivity and the comprehensive series of studied schwannomas, no homozygous deletions affecting the NF2 gene were detected (paper I). In order to detect more subtle deletions within the NF2 locus, a higher-resolution gene-specific array was developed, for the detection of disease-causing deletions using a PCR-based non-redundant strategy. This novel approach for array construction significantly increased the reliability and resolution of deletion-detection within the NF2 locus (paper II). To further expand the coverage of the 11 Mb microarray, we constructed the first comprehensive microarray representing a human chromosome for analysis of DNA copy number. This 22q array covers 34.7 Mb, representing 1.1% of the genome, with an average resolution of 75 kb (paper III). Using this array, we analyzed sporadic and familial schwannomatosis samples, which revealed two commonly deleted regions within the immunoglobulin lambda locus and the GSTTI/CABIN1 locus. These regions were further characterized using higher-resolution non-redundant arrays, bioinformatic tools, positional cloning and mutational screening. Missense mutations were detected in the CABIN1 gene, which may contribute to the pathogenesis of schwannomatosis and therefore requires further study (paper IV). Meningioma is the second most common NF2-associated tumor and loss of 1p has been previously established as a major genetic factor for disease initiation/progression and also correlates with increased morbidity. We analyzed 82 meningiomas using a chromosome 1 tiling-path genomic microarray. The distribution of aberrations detected supports the existence of at least four regions on chromosome 1, which are important for meningioma tumorigenesis (paper V).

Keywords: Genomic microarray, Array-CGH, DNA copy number variation, Neurofibromatosis type-2, Schwannomatosis, Schwannoma, Meningioma, NF2, Chromosome 22

Patrick Buckley, Department of Genetics and Pathology, Rudbecklaboratoriet, Uppsala University, SE-75185 Uppsala, Sweden

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To Sarah
List of Publications

This thesis is based on the following publications, which will be referred to in the text by their roman numerals:


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Abbreviations

ANILFR Average normalized inter-locus fluorescence ratio
Array-CGH Microarray based comparative genomic hybridization
BAC Bacterial artificial chromosome
bp Base pair
cDNA Complementary deoxyribonucleic acid
CGH Comparative genomic hybridization
CNP Copy number polymorphism
DFSP Dermatofibrosarcoma protuberans
DGCR DiGeorge critical region
dNTP Deoxynucleotidetriphosphate
ERM Ezrin, Radixin, moesin
EST Expressed sequence tag
kb Kilobase
LCL Lymphoblastoid cell line
LCR Low copy repeat (also referred to as segmental duplication)
LOD Logarithm of odds
Mb Megabase
mRNA Messenger ribonucleic acid
NF2 Neurofibromatosis type-2
NF2 Neurofibromatosis type-2 gene
NAHR Non-allelic homologous recombination
PAC P1-derived artificial chromosome
PCR Polymerase chain reaction
RCA Rolling circle amplification
RFLP Restriction fragment length polymorphism
SNP Single nucleotide polymorphism
STS-PCR Sequence tag site polymerase chain reaction
VNTR Variable number tandem repeat
YAC Yeast artificial chromosome
Introduction

Cancer

For a normal cell to divide, it must first duplicate its genome, which contains approximately 6 billion base pairs, and then partition the DNA equally between two daughter cells. This cellular division is under strict genetic regulation, however, through the accumulation of mutations a variant cell may develop that undermines this control and proliferates abnormally. This unrestricted growth potential leads to the clonal expansion of the abnormal or cancer cell which, in time, develops into a tumor.

More than 100 years ago, Theodor Boveri developed a number of theories that were proven to be fundamental corner stones of cancer research (1) (Figure 1). Through his research on the fertilization of sea-urchin eggs by two sperm instead of one, Boveri formulated many hypotheses some of which were: i) abnormal segregation of chromosomes to daughter cells may give rise to tumors; ii) each of the chromosomes carry different hereditary information; iii) a specific incorrect combination of chromosomes is the cause of abnormal cellular growth which in turn is transmitted to daughter cells. Boveri also made far-sighted observations about the effect of environment on the development of cancer which he summarized in a paper published in 1902:

“If I survey reports about the etiology of carcinoma and the many suggestions of physical and chemical insults, and if I consider on the other hand that pressure, shaking, narcotics and abnormal temperatures are precisely the agents with whose help we may produce multipolar mitoses in young eggs, then it appears possible to me that we have before us the entire causal sequence of certain tumours” (2).

In 1954, it was proposed that cancer was a multi-stage disease which correlated with age (3). Seventeen years later Knudson proposed the “two hit” hypothesis which aimed to address this process in a retinoblastoma model (4). This hypothesis was based upon the assumption that cancer was clonally derived (5) and had acquired hits or mutations within both copies of a critical gene leading to its biallelic inactivation. It is now generally accepted that cancer-related mutations primarily occur in two categories of genes, namely, tumor suppressor genes and oncogenes. Many tumor suppressor genes are key regulators of ubiquitous cell signaling pathways and are affected by
loss-of-function mutations. However, loss of function has different outcomes depending on the particular developmental time frame and the specific type of cell or tissue. There are three general features which define a tumor suppressor gene (6). First, inheritance of a single mutated allele predisposes the cell to disease, because only one additional mutation is required for complete loss of gene function. Therefore, inherited cancers are generally manifested at an earlier age (4, 7). Second, they are recessive and undergo biallelic inactivation in tumors (8-10). However, as in the case of the TP53 gene, tumor suppressors, once mutated, can also have a dominant negative effect, meaning that the mutated gene product can inhibit the function of the wild-type gene product in heterozygotes for the mutant form of TP53 (11-13). Thirdly, the same gene is commonly biallelically inactivated in a somatic cell which gives rise to the sporadic form of the cancer (14, 15).

In contrast to tumor suppressor genes, oncogenes are distinguished by acquisition of gain-of-function mutations that provide them with novel qualitative traits or increased quantitative function (16). These genes are altered forms of normal genes known as proto-oncogenes and are frequently located in the vicinity of translocation breakpoints (17). The cellular mechanisms
which these genes are involved in include the ordered progression through
the cell cycle, cell division and differentiation (16).

It has been suggested that increased genetic mutation rates must be a pre-
requisite for activation/inactivation of oncogenes and tumor suppressor
genes, respectively. It has also been proposed that the defining characteristic
or driving force behind this process is genomic instability (18, 19). A brief
history of cancer research discoveries is summarized in figure 1.

Chromosomal aberrations and disease

In the early 1980’s there were only 1,800 cases of diseases associated with
abnormal chromosomes reported in the literature (35). As of November
2004, this number has increased to 46,952 (36). Loss or gain of chromo-
somes or chromosomal segments is one of the hallmarks of cancer cells and
it has been hypothesized that it is a key component promoting further
mutagenesis in clonally expanding tumor cells (18). Chromosomal abnor-
malities can be categorized into numerical or structural aberrations.

Numerical abnormalities are thought to occur in 1-3% of pregnancies and
are lethal for the majority of cases (37, 38). Examples of numerical abnor-
malities include triploidy (69XXX), trisomy (47XX +21) and monosomy
(45X). Although trisomy has been observed in patients, for example in
Down syndrome, these abnormalities are only tolerated in gene-poor chro-
mosomes such as chromosome 21. The presence of one autosome in an oth-
erwise diploid cell, known as monosomy, is always lethal during develop-
ment, suggesting that loss rather than gain of genetic material has more seri-
ous consequences for the cell.

Structural aberrations account for a high proportion of human cancers.
These aberrations can be further subdivided into balanced and unbalanced
rearrangements. Balanced aberrations include reciprocal translocations and
inversions, whereas unbalanced rearrangements include deletions (interstitial
and terminal), insertions, duplications and unbalanced translocations. A
mechanism of misalignment and non-allelic homologous recombination
(NAHR) has been proposed to play a role in the generation of alterations in
chromosome structure both in meiosis and mitosis (39, 40). It is now known
that low copy repeats (LCRs) or segmental duplications, can mediate aber-
rant recombination, which results in the generation of balanced and unbal-
anced structural alterations (41-44). These segmental duplications are de-
fined as inter- and/or intra-chromosomal sequence stretches of 1 to 400 kb,
which display high sequence similarity to each other. The role of segmental
duplications in the generation of disease associated genomic aberrations has
been implicated in the development of numerous diseases such as Neurofi-
bromatosis type-1, Prader-Willi syndrome, DiGeorge and Velcardiofacial
Normal diploid genome

**Figure 2. Summary of major chromosomal abnormalities.** The top center schematic represents how two chromosomal pairs (one metacentric; green, and one acrocentric; blue) appear in a normal diploid genome. Metacentric chromosomes have the centromere in the middle of the chromosome with the p- and q-arms of approximately equal length. Acrocentric chromosomes have the centromere very close to one end of the chromosome, resulting in a short p-arm and long q-arm. Chromosomal alterations which have disease-causing or cancer-related effects are shown on the second half of the figure. Chromosomal imbalances such as polyploidy and aneuploidy are numerical whereas translocations, deletions and amplifications are known as structural aberrations. Adapted from Albertson *et al.* (54).

syndromes as well as Smith-Magenis syndrome (45-53). Figure 2 summarizes a number of the numerical and structural chromosomal abnormalities.

**Chromosome 22: a significant disease-related chromosome**

Historically, chromosome 22 has served as a model autosome in which results are inferred or scaled up to the rest of the genome (55-58). Figure 3 summarizes the genomic landmarks achieved to date on a full chromosomal level. Although this acrocentric chromosome accounts for only 1.5% (49.3 megabases (Mb)) of the genome, it contains approximately 600 genes (2% of all genes). Recent studies aimed at empirically identifying regions of high transcriptional activity across chromosome 22 have confirmed many of the transcripts already annotated in the public databases, but have also identified at least 2 times more transcripts expressed from this chromosome (57, 59). This gene density, coupled with the fact that chromosome 22 is one of the first chromosomes replicated during mitosis demonstrates the importance of this chromosome to the cell (58).
Chromosome 22 milestones

1991 - First comprehensive linkage map (74)
1995 - Complete Yac-based physical map (75)
1999 - Sequencing of the full chromosome (55)
2002 - Linkage disequilibrium map (56)
2002 - Transcriptional activity map (57, 59)
2002 - Chromosomal genomic microarray (paper III)
2003 - Reannotation of the chromosome (76)
2004 - Replication timing (58)

Associated diseases

Acral melanoma
Breast cancer
Colon cancer
Chronic myeloid leukemia
Dermatofibrosarcoma protuberans
DiGeorge syndrome
Ependymoma
Glioma
Meningioma
Neurofibromatosis type-2
Oral Cancer
Ovarian cancer
Pheochromocytoma
Schwannomatosis
Wilms tumour

In total, 695 chromosome 22 entries are sited in the Mitelman database with associated disease phenotypes (http://cgap.nci.nih.gov/Chromosomes/Mitelman). Some of these disorders have been described to have multiple aberrations across the chromosome, while others have been fine mapped to specific chromosomal regions. The scientific progress in the analysis of chromosome 22 over the last 15 years is presented to the right of the figure.

In addition, although chromosome 21 and 22 are of approximately the same size, trisomy 22 is not compatible with life (60), although mosaic patients has been reported (61, 62). These patients harbor three copies of chromosome 22 in a fraction of the cells of their bodies and usually present with growth and mental retardation as well as heart defects, kidney, and genital disorders (63). According to the literature there exists a wide spectrum of disorders that are associated with chromosome 22-specific aberrations (figure 3). These diseases range from the contiguous gene disorder of DiGeorge syndrome (64) to many forms of cancer (65-68), with a distinct bias towards cancers of the nervous system i.e. schwannoma, meningioma and ependymoma (69-73). One of the first disease-associated genes mapped to chromosome 22 and which has a role in the development of some of the above mentioned tumors is the neurofibromatosis type-2 gene (NF2).

Neurofibromatosis type-2

Neurofibromatosis type-2 (NF2) is an autosomal dominant disorder, which affects approximately 1 in 40,000 individuals (77) with a full penetrance by the age of 60 years. Affected individuals usually develop bilateral
Neurofibromatosis type-2

- Bilateral eighth-cranial nerve masses seen with appropriate imaging techniques
  OR
  - A first-degree relative with NF2 and either unilateral eighth nerve mass or
  two of the following
    - Meningioma
    - Schwannoma
    - Glioma (ependymoma)
    - Neurofibroma
    - Juvenile posterior subcapsular lenticular opacity

Figure 4. Diagnostic criteria for NF2. The clinical diagnostic criteria for NF2 are summarized to the left of the figure. The diagram to the right outlines the anatomical position of the characteristic schwannomas (red dot) commonly associated with NF2.

Schwannomas derived from the vestibular branch of the eighth cranial nerve, however, the diagnostic criteria is complicated by the presence of a variable phenotype (78-80). The mild Gardner type has a late onset (after 30-35 years) with slow-growing bilateral schwannomas of the eighth cranial nerve as the only feature. The severe Wishart type has an early onset, with the presence of eighth cranial nerve schwannomas before the age of 25 years and other additional tumors (figure 4 and 5) (77, 81). However, there are intermediately affected cases, which do not easily fall into either category. It is likely that this pronounced clinical heterogeneity reflects different genetic mechanisms underlying the development of the disease. In a fraction of NF2 families anticipation can also occur. Analysis of pedigrees suggests deterioration of the disease phenotype in successive generations, from a clear Gardner type to an intermediate or a Wishart type (77, 82). To date, there is no molecular explanation for this genetic feature in NF2. In 1990, a National Institute of Health clinical consensus meeting was held to determine the best criteria for the diagnosis of NF2 (83). Figure 4 summarizes the results of this meeting and illustrates the wide spectrum of phenotypic determinants which define the disease.

The NF2 gene and protein

In an attempt to identify the NF2 predisposition gene (or genes), genome-wide restriction fragment length polymorphism (RFLP) studies and linkage studies were carried out which initially localized the putative gene to chromosome 22q (84, 85). In 1991, a constitutional 700 kb deletion on chromosome 22q12 in an NF2 patient was first reported at the European Neurofibromatosis meeting in Vienna and later published (86). One and a half years later, the merlin or schwannomin (also known as the neurofibromin 2) gene (NF2) was identified and independently cloned by two groups (8, 9). The NF2 gene has since been characterized as a 17 exon gene spanning 100 kb of chromosome 22. This gene is highly conserved through evolution both in the
Figure 5. Magnetic Resonance Images of the spectrum of tumors associated with the NF2 variable phenotype. This figure illustrates the wide variation in tumor burden related to the mild and the severe form of NF2. The NF2 mild phenotype usually presents with only bilateral vestibular schwannomas, whereas severe NF2 patients usually present with bilateral vestibular schwannomas and at least one of the additional tumors displayed. The arrows indicate the location of each of the specific tumors. Adapted from The National Neurofibromatosis Foundation website (www.nf.org).

coding as well as the non-coding sequence (87). The NF2 ortholog in mouse is 98% identical on the protein level to human and maps to a locus on chromosome 11, which is a syntenic region to human chromosome 22 (88). The NF2 protein product known as merlin or schwannomin is a member of the protein 4.1 family of cytoskeletal-associated proteins, which includes protein 4.1, talin, moesin, ezrin and radixin. All family members share a homologous domain of approximately 270 amino acids at the N-terminus (89). Cellular localization studies show that endogenously expressed merlin mainly localizes to areas of membrane remodeling and is thought to play a major role in the regulation of cytoskeletal-associated events such as cell motility (90, 91). Although the tumor suppressor function of merlin remains largely unknown, some reports suggest that it acts as an inhibitor of Rac-induced cyclin D1 accumulation in the G1 phase of the cell cycle (92, 93).
Genotype - Phenotype correlations in NF2

Several studies have been reported, which aimed to characterize germline mutations in the NF2 gene in affected individuals (94-96). Previous studies of NF2 patients report large discrepancies in mutation frequencies (36-66%) (95-100). In the remaining fraction of NF2 patients, the genetic mechanism behind the development of NF2 is still unknown. The results obtained to date suggest that the correlation between genotype and phenotype in NF2 is not straightforward. Patients with truncating mutations have usually, although not exclusively, the severe form of NF2 (97, 99). The infrequent missense mutations occur in both mild and severe forms with no apparent correlation between the clinical form and type of mutation, however somatic mosaicism has been shown to be associated with the mild phenotype (98, 101). Several studies have also reported NF2 pedigrees with a wide phenotypic spectrum. Analysis of constitutionally transmitted mutations in these families has shown both protein truncating and missense mutations (96, 102, 103). In another study, 19 NF2 patients were comprehensively analyzed for various types of mutations (point mutations and deletions) in the NF2 gene, affecting its open reading frame (104). This study increased the frequency of mutation detection in NF2 patients to 84% and revealed a relatively high frequency of deletions of various sizes. The observed types of mutation from this study oppose the existence of a clear-cut genotype/phenotype correlation, when mutations in the NF2 gene are considered as the only variable. However, the number of analyzed patients was limited and the analysis should therefore be confirmed in a larger series of NF2 patients.

Deletion analysis of the NF2 locus has also indicated complications between genotype and phenotype correlations in NF2. However, in the literature, a total of 5 cases with relatively small deletions (up to 800 kb) which encompass the entire NF2 gene have been described (86, 104, 105). All of these patients had the mild form of the disease. The majority of these patients were members of NF2 kindreds, with other similarly affected family members with deletions, making somatic mosaicism for the disease-causing mutation an unlikely explanation for the mild phenotype. Furthermore, array-CGH based analysis of numerous deletion mutations in NF2 patients also suggests that larger deletions encompassing and extending beyond the NF2 locus are associated with the moderate or severe phenotype (106). This implies that larger deletions, while inactivating NF2, may also inactivate an additional gene(s) which may promote the development of the moderate/severe phenotype.

In addition, the most conclusive evidence for the presence of additional disease predisposition genes comes from the analysis of a clinically related but genetically distinct disorder known as schwannomatosis. Schwannomatosis patients develop multiple peripheral schwannomas in the absence of eighth cranial nerve schwannomas and therefore do not satisfy the diagnostic
criteria for NF2 (107). A recent study has been described, in which linkage analysis of familial schwannomatosis patients verifies a candidate locus on chromosome 22q (with a LOD score of 6.6) and also excludes the NF2 locus as the germ-line event in these patients (108). This candidate locus is the subject of intense study, aiming at the identification and characterization of a gene which is responsible for both the development of schwannomatosis as well as the phenotypic variation seen in NF2.

NF2-associated tumors

Schwannoma

Schwannoma is a benign nerve sheath tumor originating from Schwann cells, which form myelin sheaths around nerve axons (figure 6). Intracranial schwannomas account for an estimated 8% of all primary tumors within the skull, whereas spinal schwannomas represent 25% of all primary adult spinal tumors. The presence of bilateral eighth cranial nerve schwannoma is virtually a universal feature of NF2. Eighth cranial nerve schwannomas originate within the auditory canal, and initial symptoms include tinnitus, impaired

**Figure 6. Schwann cell.** A schwann cell is a specialized glial cell that wraps around axons providing protection and electrical insulation. Myelinated axons are surrounded by a lipid-rich layer called the myelin sheath, which is produced by the Schwann cell.
hearing and balance dysfunction. If left untreated, eighth cranial nerve schwannomas can compress the brain stem and eventually cause hydrocephalus. Over 95% of diagnosed schwannomas are sporadic and unilateral, with no association to NF2 (109, 110). Sporadic schwannomas develop as solitary slow growing tumors and it has been suggested that they differ from NF2-associated schwannomas with respect to their growth pattern as well as proliferative potential (111). Schwannomas may also develop on other cranial nerves, with sensory nerves more affected than motor nerves (112, 113). A common fundamental genetic aberration in both sporadic and NF2-associated schwannomas is the biallelic inactivation of the \( \text{NF2} \) tumor suppressor gene (114). Sporadic schwannomas inactivate both \( \text{NF2} \) alleles somatically, whereas NF2-associated schwannomas harbor a germline \( \text{NF2} \) gene mutation as well as an acquired somatic mutation. Although regions of DNA copy number imbalance have been identified, such as 9q, loss of chromosome 22 is the most common consistent finding in schwannoma (115). The frequency of reported 22q deletions, using different methodological approaches, range between 30-80% (69, 84, 114-117). Because of this ambiguous deletion frequency, further study is required to assess the true frequency of chromosome 22 loss in a large series of schwannomas.

**Meningioma**

Meningioma is usually a slow growing, benign tumor attached to the dura mater section of the brain and is composed of neoplastic meningothelial or arachnoidal cells. This tumor type accounts for an estimated 13-26% of all intracranial tumors (118). Epidemiological studies indicate that >90% of meningiomas are not diagnosed during the normal life-time (119). The vast majority of these tumors are sporadic, however 50% of neurofibromatosis type-2 (NF2) patients present with this tumor, making it the second most frequent tumor type associated with NF2 (112, 113). In spite of this high frequency, molecular analyses of sporadic tumors have only revealed aberrations of the \( \text{NF2} \) gene in approximately half of the tumors studied (71, 120-123). More recent unpublished work from our group has failed to detect mutations in conserved non-genic sequences within the \( \text{NF2} \) gene in meningiomas (Hansson *et al.*, unpublished). Furthermore, epigenetic analysis of the \( \text{NF2} \) gene has demonstrated aberrant methylation as a mechanism of gene silencing in one quarter of the sporadic meningiomas studied (124). A number of studies also report the complete or partial loss of chromosome 22 as the primary consistent aberration in sporadic meningiomas, thus implicating other chromosome 22-located genes which may be related to the initiation and/or progression of this tumor (125, 126). In addition to chromosome 22, other sites of the genome have been implicated in meningioma development (127). Among these, deletions of the short arm of chromosome 1 have been established as the second major genetic factor for the initiation/progression of meningioma and which also correlate with increased morbidity (128, 20).
Although previous studies have proposed candidate loci on chromosome 1, they have been limited by the low resolution of techniques applied (128-130).

**Ependymoma**

Ependymoma arises from the ependymal lining of the cerebral ventricles and from the remnants of the central spinal canal (131). Approximately 6% of patients with the severe form of NF2 develop spinal ependymoma (112). Mutations in the NF2 gene and loss of heterozygosity around the NF2 locus have been reported in spinal ependymoma (132-135). Although chromosome 22 is frequently deleted in ependymoma, no other 22q genes have yet been assigned to the development of this tumor (136, 137). Further study is therefore required to link chromosome 22 genes to the initiation and/or progression of this tumor type.

The three tumor types described above account for the majority of suffering of NF2 severe phenotype patients. It is therefore important to study their relationship to the NF2 gene and identify currently unknown genes which may play a part in the development of these associated tumors.

**Clinical outcome and treatment of NF2 patients**

NF2 is a clinically heterogeneous disease ranging from a mild to severe phenotype (figure 5). Because of this clinical heterogeneity, treatment and survival varies depending on the severity of the disease phenotype. An NF2 clinical study carried out in the UK established the mean age of first symptom as 22 (range 2-52) years and of diagnosis as 28 (range 5-66) years with no patients presenting after the age of 55. Common symptoms included deafness (44%; mostly unilateral), tinnitus (10%) and vertigo or balance dysfunction (8%) (112). As the disease advances, patients develop a progressive loss of hearing and sight, physical disability and chronic pain associated with the tumor burden (138). Most patients die from complications of the disease and in the severe phenotype, spinal tumors may cause severe muscular weakness (112).

The primary treatment for NF2 is removal of tumors by micro-surgery (139). Stereotactic radiosurgery, usually with a gamma knife, is an alternative technique, however this method is less efficient in the treatment of bilateral compared to unilateral vestibular schwannomas (140-142). Because the tumors associated with NF2 are relatively slow growing, surgical intervention may cause disability sooner than the detrimental effects of tumor growth. It is therefore advised to closely monitor such tumors with respect to growth rate before considering surgery (138). The mean overall survival from date of diagnosis in the 150 UK NF2 cases studied was 15 years (112). However, it has been shown that diagnosis and treatment of patients in specialized NF2 multidisciplinary clinics leads to lower overall mortality rates.
(143, 144). Future advances in genetic-based early diagnostics, imaging techniques and surgery will lead to improvements in the quality of life and survival of NF2 patients.

Schwannomatosis

Schwannomatosis is characterized by multiple cutaneous and spinal schwannomas, in the absence of bilateral eighth cranial nerve schwannomas (figure 7) (145). Because schwannomatosis and NF2 share the same histological tumor type, namely schwannoma, several reports have previously classified this disease as a clinical variant of NF2. However, there are important clinical and genetic features which distinguish these two disease entities.

Schwannomatosis patients present with schwannomas on the peripheral nerves in the absence of bilateral eighth cranial nerve schwannomas, which is the main clinical diagnostic criterion of NF2. Previous genetic analysis of schwannomas derived from these patients has shown a wide spectrum of NF2 gene truncating mutations. However, in contrast to NF2 patients, different tumors from the same patient do not share mutations and more importantly these mutations are not present in the constitutional DNA of the schwannomatosis patients (146, 147). In addition, a detailed molecular study of the NF2 gene in schwannomatosis families has excluded germline inactivation, while linkage analysis has defined the schwannomatosis locus on the centromeric side of the NF2 gene on 22q (108).

Schwannomatosis

- At least two non-intradermal schwannomas
- No 8th cranial nerve schwannomas on MRI scan
- No constitutional NF2 mutation
- Age older than 30 years
  OR
- One schwannoma plus a first degree relative who meets the above criteria

Figure 7. Diagnostic criteria for schwannomatosis. The clinical diagnostic criteria for schwannomatosis are summarized to the left of the figure. The diagram to the right exemplifies some anatomical positions where schwannomas (red dot) may arise.

Recently, a clinical consensus meeting was held to establish the genetic and clinical features of schwannomatosis. Figure 7 outlines the diagnostic criteria for definite schwannomatosis as proposed by MacCollin et al. (107). There are, however, complications in the diagnosis, which the authors have
addressed by outlining clinical criteria for both possible as well as segmental schwannomatosis. The possible schwannomatosis criteria include all of the features of the definite criteria with the exception that the patient is under 30 years of age. This requirement takes into account the possible growth of bilateral eighth cranial nerve schwannomas, which would then classify the disease as severe NF2. The segmental form of the disease must meet the definite or possible criteria but should be restricted to one limb or less than five contiguous segments of the spine, in the absence of an affected first degree relative (107).

**Methods for assessing DNA copy number variation**

The year 1956 is considered to mark the beginning of modern human cytogenetics as it was discovered that the normal human karyotype consisted of 46 chromosomes (148, 149). Further advances in technology led to banding techniques, which by exploiting differential staining intensities of chromosomes, allowed the identification of each chromosome and the characterization of structural abnormalities associated with specific genetic syndromes (150, 151).

The first widespread molecular technique for the analysis of DNA was based on the detection of RFLPs. This method relies on the comparison of band profiles generated after restriction enzyme digestion of target DNA. RFLP analysis in combination with methods developed by Ed Southern (152) was used to directly detect deletions and duplications in genomic DNA (153-155). The next development in DNA-based genetic markers was the identification of variable number tandem repeats (VNTRs). The first type of VNTRs discovered were known as minisatellites and are comprised of approximately 10-100 bp repeating sequence units (156, 157). Originally called DNA finger-printing, this method used restriction endonucleases to cut sites flanking the polymorphic sequence. Differences in size are based on the variable number of repeats within the digested fragment which could be measured by Southern blot (157-163). In 1989, three groups reported the identification of di-, tri and tetra-nucleotide repeats, which were highly polymorphic with respect to the number of repeats within the genome (164-166). These DNA microsatellites were also shown to be abundantly dispersed throughout the genome. The advent of the polymerase chain reaction (PCR) (167, 168) allowed these polymorphic repeats to be efficiently amplified and used as the next generation of genetic markers for linkage and DNA copy number analysis (169-171).

At the same time as the discovery of microsatellites, the field of cytogenetics was advancing rapidly, with the further development of fluorescent in
Figure 8. Microarray based comparative genomic hybridization methodology.

*situ* hybridization (FISH) (172-174) and the invention of the metaphase-based form of comparative genomic hybridization (metaphase-CGH) (175). The latter conceptually novel technique used differentially fluorescent labeled test and reference DNA, competitively hybridized to metaphase chromosomes immobilized on a glass slide. The detection of altered fluorescent ratios between test and reference therefore indicated a gene copy number imbalance. This method allowed significant questions related to disease-associated gene dosage alterations to be addressed (176-178). However, the main limitation of metaphase-CGH is the low resolution of analysis. The sensitivity of deletion and amplification detection is 10 and 2 Mb, respectively (179).

The limited resolution of metaphase-CGH was overcome when chromosomes were replaced by genomic clones, derived from the human genome sequencing project, as targets for hybridization on the glass slide (180, 181). First published in 1997, matrix-based comparative genomic hybridization or
Figure 9. Construction of clone based minimal tiling-path genomic microarrays. The above diagram summarizes the coverage of a 500 kb segment of a chromosome for microarray construction. There are two genes within the 500 kb sequence and their exons are denoted by dark blue. Five large insert BAC clones cover the genomic sequence in an overlapping fashion. The genomic clone DNA is prepared and printed on a glass slide. The five enlarged and highlighted spots represent the clones which cover the 500 kb segment.

array-CGH allowed the resolution of analysis to increase by more than an order of magnitude. This method, similar to metaphase-CGH, is based on the dual colored competitive hybridization of both test and reference DNA to a printed microarray (182, 183, paper v) (figure 8). The first large scale arrays which applied the array-CGH methodology were composed of cDNA clones (184). In this study, breast cancer-associated deletions and amplifications were mapped using 3,660 clones across the genome. The use of cDNA clones as array targets has the added advantage of assessing both DNA copy number as well as gene expression using the same platform. It is therefore possible to correlate the expression pattern of genes encompassed within regions affected by DNA copy number loss or gain. However, this study also
demonstrated the limitations of cDNA clones as targets for detecting aberrations in the relatively more complex genomic DNA, compared to mRNA.

In 2001, the first DNA clone-based genome scanning array containing 2,400 large insert BACs was published, which had an average resolution of 1.2 Mb across the genome (185). This type of scanning array has been applied to address a wide variety of questions ranging from evolutionary aspects of the human genome to disease predisposition (186-190). The production of these arrays has been streamlined through the development of efficient strategies for the amplification of genomic clone DNA from minute amounts of an initial bacterial culture (185, 191, paper III). These approaches allow the large-scale amplification of genomic clones for the construction of arrays which cover larger chromosomal distances with a higher density.

The next advancement in genomic array technology was the construction of minimal tiling-path microarrays. These arrays are constructed using overlapping clones, which provides a more comprehensive and higher resolution analysis than the scanning genomic microarrays (106, paper III) (figure 9). The power of array-CGH in combination with tiling-path genomic microarrays has been demonstrated by the localization of a gene responsible for CHARGE syndrome (192). Using this approach, the authors identified a de novo 2.3 Mb constitutional deletion from the analysis of three patients. The 9 annotated genes within this region were screened for mutations and one gene (CHD7) was found to be heterozygously mutated in 10 of 17 cases. Although the authors commented that other genetic factors are likely to be involved, this particular example demonstrates the power of array-CGH technology to go virtually from disease phenotype to an aberrant genotype in a limited number of experiments. It is clear that studies such as this mark a promising beginning to a field that has yet to reach its full potential. To further capitalize on the power of tiling-path resolution genomic arrays, a full-genome tiling-path array has been developed which contains 32,433 (32 K) BAC clones and covers approximately 98% of the genome with an average resolution of 100 kb (193).

Another future direction in the array-CGH field is the use of oligonucleotides as array targets instead of cloned DNA fragments. Although several studies have reported initial success with this method (194-196), they lack the robustness offered by the use of genomic clone-based arrays, when studying total genomic DNA. It is only a matter of time, however, when a whole genome oligonucleotide array will be available to assess DNA copy number imbalances at a resolution as high as 100 bps.
Normal and disease-associated genetic variation

During the past decade, the study of human genetic variation at the DNA level has received considerable attention (197-199). The dominating type of variation explored so far in the genome has been at the level of single nucleotide polymorphisms (SNPs) (200), overshadowing the issue of copy number polymorphisms (CNPs) (201, paper i). Recently, array-CGH has been applied to identify regions of the genome which are polymorphic with respect to DNA copy number. These large-scale CNPs have not been previously identified due to the lack of a reliable high-throughput technique. Two landmark studies have reported the presence of CNPs in the human genome using different genome-wide array-CGH based strategies (196, 202). Iafrate et al. have used commercially available BAC genome scanning arrays, while Sebat et al. have developed and applied a custom made oligonucleotide array for the assessment of copy number variation. Both studies convincingly demonstrated the presence of genomic imbalances among normal individuals, which overlap with genes and segmental duplications in the genome and may contribute to phenotypic variation as well as disease susceptibility. In these reports, Sebat et al. and Iafrate et al. identified 76 and 255 loci, respectively, that display copy number variation in the human genome.

An important aspect of this type of variation is the mechanism by which it occurs. It is likely that segmental duplications, through the process of meiotic non-allelic homologous recombination, play an important role in the generation of this type of DNA copy number variation. However, a significantly more detailed level of analysis is required to determine the impact of these repetitive elements on the generation of CNPs. The knowledge of CNPs in the human genome is likely to increase in accordance with advancements in technologies. However, the analysis of CNPs is considerably more difficult to assess than SNPs, due to the complexity of variation and costs involved in their detailed assessment (paper i). It is not only the presence or absence of the CNP which is important, but the type and size of the copy number variation must also be taken into account. It is only when the “normal baseline” of CNPs has been established that DNA copy number imbalances detected in pathological specimens can truly be correlated with disease predisposition.
Aims of the present study

- To determine the deletion frequency of an 11 Mb region of chromosome 22, encompassing the NF2 locus, in sporadic schwannoma.

- To develop a diagnostic tool for the rapid, high-resolution detection of deletions within the NF2 locus, using array-CGH.

- To increase the resolution of analysis of array-CGH for the detection of subtle DNA losses and gains.

- To construct, validate and apply a tiling-path genomic microarray of chromosome 22 to analyze chromosome 22-associated disorders.

- To identify regions outside the NF2 locus which may have a role in the predisposition to schwannomatosis and development of schwannoma.

- To perform detailed DNA copy number profiling of meningioma using a chromosome 1 tiling-path genomic microarray.
Results and discussion

Array-CGH profiling of sporadic schwannoma using a genomic array covering 11 Mb of chromosome 22 (Paper I)

This study was the first array-CGH project which we undertook at the Rudbeck Laboratory, Uppsala. It was important therefore to ensure that the array-CGH methodology was firmly established in the group for the reliable assessment of DNA copy number. To achieve this, we constructed an array which covered approximately 11 million base pairs of chromosome 22, encompassing the NF2 tumor suppressor gene, for the DNA copy number analysis of paired schwannoma and constitutional DNA. The frequency of previously reported loss of genetic material from 22q differs greatly, ranging between 30-80%. Using array-CGH, we detected heterozygous deletions of chromosome 22 in 21 out of 47 cases (45%). The NF2 locus was deleted in all but 2 of these 21 cases. The deletion patterns observed in the tumors could be broadly summarized into three types. The predominant profile was a continuous deletion encompassing all measurement points on the array and thus extending beyond the 11 Mb segment of chromosome 22 (consistent with monosomy). This profile was observed in 12 tumors out of 21 deletion cases. The second type of profile was similar to the first category, i.e. findings suggesting a continuous heterozygous deletion of the full 11 Mb segment. However, the level of fluorescence ratios, for the clones scored as deleted, was higher than expected for a single copy. The third class of findings was interstitial deletions of various sizes.

The inactivation of the NF2 tumor suppressor gene is an important event in the development of sporadic as well as NF2-related schwannomas. In spite of the high sensitivity and the comprehensive series of studied schwannomas, no homozygous deletions affecting the NF2 gene were detected. Thus, the biallelic inactivation of the NF2 gene in schwannomas has so far only been documented by either deletion of one allele and point mutation on the other, or a combination of two independent point mutations on both alleles. Our previous report suggested that there are sporadic schwannomas, which do not harbor deletions in the NF2 locus (116). We confirmed this by using a more reliable methodological approach, as we found new cases with interstitial deletions on 22q not involving the NF2 locus. This reinforces the
issue that other 22q-located genes might contribute to tumorigenesis of sporadic schwannoma.

Development of a strictly sequence-defined microarray for detection of diagnostically significant deletions within the \textit{NF2} gene (Paper II)

The \textit{NF2} tumor suppressor gene on chromosome 22 is deleted in approximately 15-20\% of NF2 patients. These deletions are difficult to detect due to the lack of a sensitive, rapid assay for the analysis of DNA copy number variation in the locus. To develop such a tool, we initially prepared a dense tiling-path array consisting of 25 cosmids spanning the \textit{NF2} locus for the construction of a high resolution array, which was extensively tested using a number of previously characterized patients with known \textit{NF2} deletions. However, the data obtained from these cosmids, as hybridization targets, was inaccurate. We determined that the inconsistent results were due to variation in batches of Cot-1 DNA, a commercial reagent used to block repetitive sequence. Increasing the quantity of Cot-1 DNA in the hybridization mixture had only a marginal effect on the suppression of repeats. These results clearly indicated that the repeats within the \textit{NF2} locus were problematic and we therefore focused on masking the repetitive sequence. The locus was divided into 10 kb intervals, and these were masked for repeats using the RepeatMasker program. We then excluded all common repeats/redundant sequences and designed PCR primers for the amplification of unique sequence. The unique sequences were PCR amplified from cosmids covering the locus. Each repeat-free pool (6 in total) was designed using 20 kb of original genomic sequence, with the exception of one 40 kb segment.

We performed validation experiments using a number of previously characterized patients with known \textit{NF2} deletions, which yielded highly reproducible results. This is the first study reporting a disease-associated gene for which a high-resolution genomic array has been developed. The main advantage of this method is that even the most difficult segments of the genome, which are practically impossible to approach, using the genomic clone-based approach, can be reliably analyzed. The presented example of the \textit{NF2} gene specific array will likely serve as a model for the development of similar tools for many other disease-related genes.

Development of a full-coverage, high-resolution genomic microarray representing chromosome 22 for clinical and research applications (Paper III)

In order to analyze chromosome 22 aberrations related to NF2 in further detail, we constructed the first human chromosomal genomic microarray. In
this study, we fully covered known segments of chromosome 22q (34.7 Mb) with genomic clones from the minimal tiling path without gaps larger than 100 kb. In total, 460 clones were prepared which resulted in an average resolution of 75 kb. The array was validated using normal and previously characterized cases. We also applied the microarray for the detection of homozygous and heterozygous deletions, amplifications and gains as well as IGLV/IGLC locus instability. An example of one of the more exciting results from this paper was the identification of a homozygous deletion identified in an aggressive brain tumor known as glioblastoma. This single homozygously deleted cosmid clone contains the 14-3-3 eta gene (acc. Q04917). The 14-3-3 eta gene may have a role in cancer development as other isoforms such as 14-3-3 sigma are aberrantly inactivated in breast cancer. This example illustrates the power of this technique to identify potential disease-related genes.

We also applied this array as a diagnostic tool in several well-studied disorders, such as NF2, DiGeorge syndrome and dermatofibrosarcoma protuberans (DFSP). The latter is a cutaneous tumor, the cytogenetic features of which include the translocation t(17;22)(q22;q13), resulting in a fusion between the collagen type I, alpha 1 gene (COL1A1) from chromosome 17 and the platelet-derived growth factor, B-chain gene (PDGFB) from chromosome 22. Array-CGH was carried out on two DFSPs containing the COL1A1/PDGFB fusion gene, and in both cases we detected the breakpoint at clone ID325. This clone contains the entire PDGFB gene, which is truncated as a consequence of the translocation. We also applied the strictly sequence-defined and PCR-based strategy described in paper II, for the preparation of measure points within genomic regions of high repeat content. The other successful development was the application of phi29 DNA polymerase for the amplification of genomic clone DNA used for printing genomic microarrays.

Evidence for genetic aberrations of chromosome 22 outside the NF2 locus in schwannomatosis and neurofibromatosis type 2 (Paper IV)

The genetic factors underlying the differences between schwannomatosis and NF2 are poorly understood, although available evidence implicates chromosome 22 as the primary location of gene(s) of interest. To investigate this, we performed DNA copy number analysis on samples derived from sporadic and familial schwannomatosis, NF2 as well as a large cohort of normal controls. Using the chromosome 22 tiling-path genomic array (Paper III), we identified two candidate regions of copy number variation. The first locus consisted of a 252 kb overlapping deletion region within the immunoglobulin lambda locus (IGL) detected in DNA derived from a sporadic and familial schwannomatosis case as well as an NF2 patient. The second region
of interest as defined by the 22q array analysis was at the GSTT1/CABIN1 locus. Both of these candidate loci map to a region of positive LOD score as previously determined by linkage analysis of schwannomatosis families.

To analyze these candidate loci in more detail we constructed region-specific PCR-based higher resolution microarrays, using the strategy outlined in paper II. Using this array, we identified 8 overlapping deletions within the IGL locus in NF2 and schwannomatosis samples derived either from lymphoblastoid cell lines or peripheral blood. In one peripheral blood-derived sample from a schwannomatosis patient, we detected IGL rearrangements, which cannot be exclusively attributed to B-cell-specific somatic recombination of IGL. Analysis of normal controls indicated that these IGL rearrangements were restricted to schwannomatosis patients. To further characterize this locus we carried out extensive bioinformatic analysis followed closely by positional cloning strategies. This analysis revealed that only IGL-related gene transcripts were expressed within this locus and the importance of this region for the development of schwannomatosis remains an open question.

In the second candidate region spanning the GSTT1 and CABIN1 genes, we observed the frequent copy number polymorphism at the GSTT1 locus. The allele frequencies for 0, 1 and 2 copies in a mixed population of Asian and Caucasian controls were 20%, 40% and 40%, respectively. We further detected missense mutations in the CABIN1 gene that are specific to samples from schwannomatosis and NF2, making this gene a plausible candidate, which may contribute to the pathogenesis of these disorders.

Comprehensive DNA copy number profiling of meningioma using a full-coverage chromosome 1 microarray identifies novel candidate tumor suppressor loci (Paper V)

Meningioma is a tumor of the meningeal tissue which lines the brain. This tumor type is the second most common NF2-associated tumor. Inactivation of the NF2 gene has been implicated as an important event during tumorigenesis in up to 50% of meningioma. However, additional genetic events linked to the inactivation of not yet characterized tumor suppressor genes from chromosome 1 are thought to be involved in the initiation and progression of these tumors. Therefore, this chromosome serves as a primary site in the genome where one should search for genes involved in both the initiation and progression of this tumor. The rationale of this study was to characterize copy number imbalances of chromosome 1 in meningioma, using a full-coverage genomic microarray containing 2118 distinct measurement points. In total, DNA from 82 meningiomas was analyzed. We detected a broad range of aberrations, such as deletions and/or gains of various sizes. Deletions were the predominant finding and ranged from monosomy to a 3.5 Mb
1p homozygous terminal deletion. Although multiple aberrations were observed across chromosome 1, every meningioma in which imbalances were detected harbored 1p deletions. The distribution of observed aberrations supports the existence of at least four loci on chromosome 1 (three on 1p and one on 1q), which are important for meningioma tumorigenesis. In a large series of cases we observed an association between the presence of segmental duplications and deletion breakpoints, which suggests their role in the generation of these tumor-specific aberrations. As 1p is the site of the genome most frequently affected by tumor-specific aberrations, our results may indicate loci of general importance for cancer development and progression.

Detection of genetic heterogeneity in tumor samples using array-CGH (Paper I, III and V)

One common aspect observed in three papers of this thesis and other tumor studies carried out in the group (paper iv), was the detection of genetic heterogeneity in a number of studied samples. The first type, detected in paper I and II, suggested analysis of a mixture of tumor DNA and normal contaminating tissue DNA. This is evident when analyzing tumors derived from male patients (XY) versus a female reference (XX) which display, using the X chromosome controls, an internal standard for the haploid fluorescent ratio. In tumor samples which displayed cellular heterogeneity, the fluorescent ratio for the detection of one DNA copy was higher than that of the X controls. It is reasonable to consider how the contamination of diploid (normal) cells could skew the fluorescent ratio of a one copy loss in a tumor. Although this point is made clearly in paper I, with approximately 25% of the deleted cases displaying this profile, we also detected it in Paper II in the context of glioblastoma and DFSP. One cannot, however, rule out the possibility of analysis of a mixed population of tumor cells in some of these samples. It is feasible that DNA copy number loss of chromosome 22 is not the primary event and therefore a proportion of the tumor cells may not have deleted chromosome 22, leading to a heterogeneous genotype within the tumor cells studied. Whether this heterogeneity represents an oligoclonal nature of the tumor or a progressive loss of chromosomal material as the tumor develops is subject to future investigation.

This type of mixed cell population was also convincingly shown in paper V in the context of chromosome 1 array-CGH profiling of meningioma. In three of the 82 tumors studied we detected fluorescent ratios indicating 1, 1.5 and 2 DNA copies. In the case of the detection of 1.5 copies, it is clear that this intermittent fluorescent ratio indicates the presence of a second tumor cell population which has deleted larger regions of chromosome 1. As mentioned above, this may be due to oligoclonality of the tumor or a tumor pro-
gression event. It is interesting to note, however, that all three tumors which displayed this profile were recurrent tumors. The detection of such profiles may identify regions of chromosome 1 which correlate with tumor growth and might therefore be of some prognostic significance. This ability to assess DNA copy number in a mixed cell population highlights yet another advantage of array-CGH when compared to other techniques used for detection of genomic aberrations.
Future perspectives

As this thesis is primarily based on the development and application of a microarray-based method for examining numerous questions related to NF2, it is my opinion that the key to unlocking the answers to biologically related questions in this case is through the advancement of the technology used. Genomic coverage and resolution of analysis are the two aspects which are essential for the future development of array-CGH. The application of the 32 K genome array offers the advantage of genome-wide coverage with a good resolution of analysis at a reasonable cost. However, in order to properly identify disease-associated DNA copy number imbalances, one should first assess the extent of such normal variation. Although a number of CNPs have already been identified, the use of the 32 K genome array to study hundreds of phenotypically “normal” individuals would provide a baseline of common DNA copy number variation. When such a baseline of CNP’s has been established and integrated with the data generated from SNP analysis, disease-specific variation may be identified and further studied.

There are many aspects of the work presented in this thesis, which would be very interesting to investigate further using such a genome-wide tiling-path array, some of which include profiling of i) constitutional DNA of mild and severe NF2, which may identify specific genomic regions which are responsible for the phenotypic modification of NF2; ii) tumors associated with the severe NF2 phenotype and their sporadic counterpart. Whole genome array-CGH profiling of both NF2-related tumors such as schwannoma, meningioma and ependymoma and the sporadic forms of these tumors may shed light on the mechanism of association to the NF2 phenotype. If the NF2-associated tumors share common genetic aberrations and if one could identify loci solely responsible for the generation of the tumor sporadically, remains an interesting future aspect of this work. Once regions have been identified using the whole genome approach, further analysis should be carried out on these regions by constructing a non-redundant high-resolution array across the candidate loci. This strategy may be used to detect more subtle aberrations, which would further refine the candidate loci with an aim to screen genes for mutations. The development of genome-wide microarrays based on oligonucleotides, however, would integrate a more extensive coverage than the 32 K genome array and a higher resolution than non-redundant arrays. It is only a matter of time when such microarrays will become routine for the assessment of DNA copy number alterations.
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patrick.buckley@gmail.com
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