GENES ON CHROMOSOME 22 INVOLVED IN THE PATHOGENESIS OF CENTRAL NERVOUS SYSTEM TUMORS

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GENEN, GELEGEN OP CHROMOSOOM 22, DIE BETROKKEN ZIJN BIJ DE PATHOGENESE VAN TUMOREN VAN HET CENTRALE ZENUWSTELSEL

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Een overwinning wordt niet in kilometers maar in centimeters behaald. Win nu een beetje, houd wat je hebt en win later nog een beetje meer.

Louis L'Amour

Voor Willeke

Contents

List of abbreviations

APC	Adenomatous polyposis coli
bp	basepair
DCC	Deleted in colorectal carcinoma
FAP	Familial polyposis coli
FISH	Fluorescent in situ hybridization
FMTC	Familial medullary thyroid carcinoma
GRAIL	Gene recognition and analysis internet link
HNPCC	Hereditary nonpolyposis colorectal cancer
kb	kilobase
LOH	Loss of heterozygosity
Mb	Megabase
MCC	Mutated in colorectal cancer
MEN	Multiple endocrine neoplasia
mRNA	messenger RNA
MTC	Medullary thyroid carcinoma
NF1	Neurofibromatosis type 1
NF2	Neurofibromatosis type 2
ORF	Open reading frame
Rb	Retinoblastoma
RFLP	Restriction fragment length polymorphism
RT-PCR	Reverse transciptase-polymerase chain reaction
SSCP	Single strand conformation polymorphism
VHL	von Hippel-Lindau
WAGR	Wilms' tumor, aniridia, genitourinary abnormalities, mental retardation
WT	Wilms' tumor
wt	wild-type
YAC	Yeast artificial chromosome

Chapter I

General introduction

1 Cancer is a genetic disease

It is nowadays generally accepted that cancer can be considered as a genetic disease. However, there are two clear differences between cancer and most other genetic diseases. First, most cancers are caused essentially by somatic mutations, whereas all other genetic diseases are caused solely by germline mutations. Second, for most cancer types at least two mutations are required before a tumor may arise. For most pediatric and some adult tumors only a limited number of mutations are supposed to be sufficient for tumor development (Knudson, 1971, 1986; Haber and Housman, 1992). In contrast, most adult cancers appear to require more than two genetic changes. This 'multiple-hit' concept of tumorigenesis stems from several lines of evidence. First, most adult cancers show an exponential increase in incidence with age, suggesting that the accumulation of different hits is involved in tumor development (Vogelstein and Kinzler, 1993). Second, a dramatically increased incidence of cancer has been observed in individuals with chromosome instability syndromes such as Bloom's syndrome (Vijayalaxmi et al., 1983; Seshadri et al., 1987). Third, repeated administration of mutagen to test animals is generally required before a tumor arises (Saffhill et al., 1985). Fourth, increasing evidence becomes available for the involvement of multiple genetic alterations in common human tumors. The most extensively studied example is the accumulation of genetic alterations observed in human colon tumors, in which mutations in *APC* and *ras* found in adenomatous polyps have been classified as early events and mutations in *p53* and *DCC* as later events that may contribute to tumor progression (Fearon and Vogelstein, 1990; section 3.3). In a specific human cancer, mutations in one particular gene appear to precede those in others (Vogelstein and Kinzler, 1993), although after this first hit the accumulation of changes rather than their specific order seems to be important for tumor progression (Marx, 1989).

2 Oncogenes and tumor suppressor genes

Molecular genetic analysis of tumorigenesis has revealed two fundamental mechanisms of neoplasia: 1) the activation of dominant oncogenes or 2) the inactivation of recessive tumor suppressor genes (anti-oncogenes). Accumulating evidence suggests that the progression of many tumors to full malignancy requires both types of changes in the tumor cell genome.

2.1 Oncogenes

First evidence for the existence of oncogenes came from the induction of sarcomas by the Rous sarcoma retrovirus, containing the *v-src* oncogene (Martin, 1970). Later, other retroviral oncogenes (*v-onc*) and their human homologues (*c-onc*) were discovered in various cancers. Also the dissection of chromosomal translocations, tumor associated DNA amplifications and the use of gene-transfer have resulted in the identification of more oncogenes. Until now 100 or more of them have been isolated. Under normal conditions proto-oncogenes are thought to be involved in the regulation of cell proliferation. They act as growth factors or their receptors. Within the cell, they function as downstream modulators in signal transduction pathways. Furthermore, the nuclear (proto-)oncoproteins may regulate gene transcription in response to these signals (Hunter, 1991). Different mechanisms (point mutation, amplification or translocation) are involved in the activation of a proto-oncogene into an oncogene. Mutations in the proto-oncogenes occur in one of the two alleles of the gene and they act in a dominant way relative to the wild-type allele. Therefore, they are called 'gain of function' mutations that give normal cells neoplastic properties (Weinberg, 1989).

2.2 Tumor suppressor genes

Three lines of evidence support the existence of growth-constraining tumor suppressor genes: 1) somatic cell hybrids 2) familial cancer and 3) loss of heterozygosity in tumors.

The first evidence that tumor suppressor genes are involved in neoplastic

transformation came from somatic cell fusion experiments, which showed that fusion of tumor cells with normal cells almost always results in the outgrowth of nontumorigenic hybrids (Sager, 1985; Harris, 1988). Sometimes these hybrid cells reverted back to a tumorigenic state, which could be correlated with the loss of specific 'normal' chromosomes (Stanbridge, 1990). These and other fusion experiments suggest that normal cells contain genetic information capable of suppressing the neoplastic growth in the tumor cells. The tumor cells have lost this information during their evolution from normal to tumor cells.

The second indication came from the existence of familial cancer. In 1971, Knudson proposed the two-hit model for the development of retinoblastoma and one year later also for Wilms' tumor (Knudson and Strong, 1972). This model was based on epidemiological analyses of age of onset of multifocal (hereditary) versus single (sporadic) cases. It implies that in heritable retinoblastoma the first hit is already present in the germline and only one somatic hit is required for tumor formation. In sporadic cases two somatic mutational events are needed, which explains the later age of onset in these patients. In 1973 Comings suggested that the mutations were in the two alleles of the same gene. Definite proof for the involvement of a recessive tumor suppressor gene was obtained when the nature of these germline and somatic mutations became clear. First indications came from karyotypic analyses of retinoblastoma tumor cells, which sometimes uncovered interstitial deletions that involved chromosomal band 13q14 (Yunis and Ramsay, 1978). Genetic analysis of blood and tumor DNA pairs and the isolation of the retinoblastoma gene (*Rb1*) eventually confirmed Knudson's theory (Cavenee et al., 1983; Friend et al., 1986; Fung et al., 1987; Lee et al., 1987). These studies support the idea that a recessive tumor suppressor gene can contribute to the development of a tumor when both alleles have been inactivated (Fig. 1). The study of other familial cancers have contributed towards the elucidation of more cancer susceptibility loci and genes. The isolated genes responsible for the small fraction of hereditary cancers are very useful for the study of these and the commoner sporadic cancers. Although the predisposing cancer genes are phenotypically dominant, at the genetic level they are recessive (both copies of the gene should be inactivated). The apparent dominant inheritance of these diseases reflects the high probability that the second allele is hit in a somatic cell.

The third clue came from consistent and specific chromosome deletions in tumor cells. Karyotyping and 'loss of heterozygosity' (LOH) studies clearly showed loss of

particular (parts of) chromosomes in human tumors. The latter approach uses the frequent occurrence of restriction fragment length polymorphisms (RFLPs) of some DNA probes or polymorphic microsatellites in the human genome. This technique takes advantage of the heterozygous state of a patient's normal (constitutional) DNA for a particular polymorphic marker, loss of that part of the chromosome is the case when tumor tissue of the same patient shows reduction or loss of one of the alleles (Fig. 1).

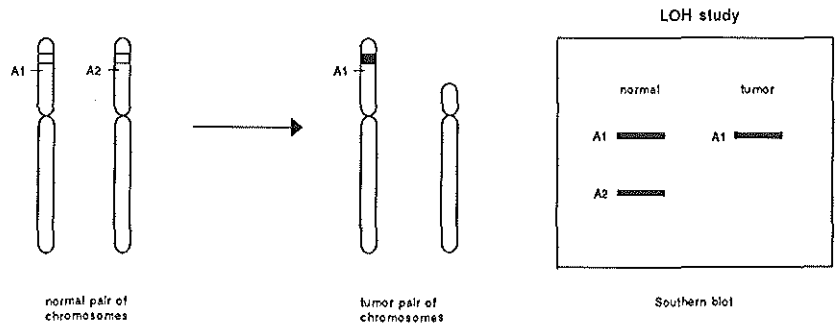

Figure 1. The most commonly observed inactivation scheme for a recessive tumor suppressor gene. The normal (wild-type) copies of the tumor suppressor gene are shown with the open boxes. In the tumor both copies of the gene are inactivated by a small mutation in one allele (black box) and loss of the portion of the chromosome that harbours the second allele. Loss of heterozygosity (LOH) of this chromosomal part can be detected on a Southern blot. This analysis uses the ability of a polymorphic DNA marker, specific for the relevant region of the chromosome, to detect both the maternal and paternal alleles (A1 and A2). The comparison of normal and tumor DNA from the same individual can distinguish whether one allele is lost in the tumor.

A comprehensive list of consistently observed allele losses in solid tumors has been described by Seizinger et al. (1991). A combination of linkage analyses in cancer families and LOH studies in both sporadic and familial tumors have led to the localization of a large number of (mostly) tumor suppressor gene loci. The 'positional cloning' (Collins, 1992) and/or candidate gene approach eventually resulted in the cloning of some of these genes. A number of examples are listed in Table 1. In the following sections these genes are briefly described.

The products of tumor suppressor genes may be involved in the negative regulation of cell growth, induction of terminal differentiation and/or apoptosis. The gene can contribute

to tumorigenesis if a mutation inactivates the gene (Weinberg, 1989). Thus, the mutations are 'loss of function' mutations. Possible mechanisms involved are: (partial) loss of a chromosome, point mutations, small deletions or insertions, translocations and genomic imprinting.

Table 1. Isolated tumor suppressor genes

Heritable cancer syndrome	Chromosomal location	Gene*	Reference
Retinoblastoma, Osteosarcoma	**13q14**	*Rb1*	Friend et al., 1986; Fung et al., 1987; Lee et al., 1987
Wilms' tumor	**11p13**	*WT1*	Call et al., 1990; Gessler et al., 1990
Li-Fraumeni syndrome	**17p13**	*p53*	Malkin et al., 1990
Familial adenomatous polyposis	**5q21**	*APC*	Groden et al., 1991; Joslyn et al., 1991; Kinzler et al., 1991b; Nishisho et al., 1991
	5q21	*MCC*	Kinzler et al., 1991a
	18q21.3	*DCC*	Fearon et al., 1990a
Hereditary non-polyposis colorectal cancer	**2p16**	*hMSH2*	Fishel et al., 1993; Leach et al., 1993
	3p21-23	*hMLH1*	Bronner et al., 1994
Neurofibromatosis type 1	**17q11**	*NF1*	Cawthon et al., 1990; Viskochil et al., 1990; Wallace et al., 1990
Neurofibromatosis type 2	**22q12**	*NF2*	Rouleau et al., 1993; Trofatter et al., 1993
von Hippel-Lindau	**3p25**	*VHL*	Latif et al., 1993
Multiple endocrine neoplasia 2	**10q11**	*RET*	Mulligan et al., 1993a

* All except the *RET* gene are examples of tumor suppressor genes. If a particular gene is thought to be the primary genetic event the gene and its chromosomal localization are written in bold letters.

3 Molecular cloning and function of tumor suppressor genes

In the last few years an enormous progress was seen in the cloning and/or identification of genes involved in hereditary cancer syndromes and tumor progression. This section gives an overview of some of these genes together with a short description of their putative function and possible involvement in other tumor types.

3.1 The Retinoblastoma (*Rb1*) gene

The *Rb1* gene was isolated in 1986, on the basis of its primary role in retinoblastoma development (Friend et al., 1986; Fung et al., 1987; Lee et al., 1987). The gene encompasses 200 kb on chromosome 13q14 and encodes a 105 kD nuclear phosphoprotein. Most alterations observed in the gene in retinoblastoma are deletions and nonsense mutations and involve both alleles. These changes result in a truncated or absent protein and are in agreement with the suspected loss of function mutations as was postulated for tumor suppressor genes. Inactivating mutations of both copies of the gene occur in many other tumor types including breast carcinoma, prostate carcinoma, osteosarcoma, soft tissue sarcoma and small-cell lung carcinoma (Bookstein et al., 1990a; Friend et al., 1987; Harbour et al., 1988; Hensel et al., 1990; Lee et al., 1988; T'Ang et al., 1988; Varley et al., 1989; Yokota et al., 1988). This indicates that loss of Rb1 function is important in the tumorigenesis of many tumors. In hereditary retinoblastoma 5-10% of the patients also develop osteosarcomas and soft tissue sarcomas. An explanation for the finding that they have no increased risk for other tumors might be that for the other tumors mutations in other genes must happen first. The growth suppressing role of the wild-type (wt) Rb1 protein was clearly illustrated by experiments in which the wt *Rb1* gene was introduced into Rb1-deficient tumor cells. These studies demonstrated suppression of cell-growth and tumorigenicity (Huang et al., 1988; Bookstein et al., 1990b; Sumegi et al., 1990; Takahashi et al., 1991; Goodrich et al., 1992a).

Insight into the function of Rb1 started with the finding that DNA tumor virus encoded oncoproteins (E1A, large T antigen and E7) form complexes with the host cell Rb1 protein (DeCaprio et al., 1988; Whyte et al., 1988; Dyson et al., 1989). The current idea

is that this interaction inactivates Rb1 function by removing it from the growth regulating machinery of the cell, equivalent to a deletion or mutation in the *Rb1* gene. Other experiments suggest that the Rb1 protein is involved in cell cycle regulation, probably by regulating the progression through the G1 phase of the cell cycle. Among others, the Rb1 protein is phosphorylated in a cell-cycle dependent manner: hypophosphorylated Rb1 protein predominates in G1, whereas heavily phosphorylated forms appear just prior to the G1 to S transition and persist during the S, G2 and M phase (Buchkovich et al., 1989; DeCaprio et al., 1989; Chen et al., 1989). The Rb1 protein is a substrate for different kinases involved in the cell cycle (Matsushime et al., 1992; Ewen et al., 1993; Kato et al., 1993). In addition, all three viral oncoproteins bind only to the hypophosphorylated state of the Rb1 protein (Ludlow et al., 1989). This suggests that the hypophosphorylated form of the Rb1 protein is active in cell growth control. Moreover, hypophosphorylated instead of hyperphosphorylated Rb1 protein is able to bind the transcription factor E2F (Chellappan et al., 1991; Shirodkar et al., 1992). The interaction of Rb with E2F probably blocks the transactivation of certain positively acting growth-regulating genes such as *c-myc* (Thalmeier et al., 1989). Further study showed physical interaction between the c-myc and Rb1 proteins and microinjection of cells with Rb1 and c-myc results in the inhibition of the ability of the Rb1 protein to arrest the cell cycle (Rustgi et al., 1991; Goodrich and Lee, 1992b). All these data together suggest that the Rb1 protein functions by negatively regulating the progression through the cell cycle by the inhibition of transcription factors involved in entry into the S phase.

3.2 The *p53* gene

The nuclear phosphoprotein p53 was originally discovered in extracts of SV40 transformed cells (Lane and Crawford, 1979). The protein was found in a complex with SV40 large T antigen. In the mid-1980s the corresponding gene was cloned (Matlashewski et al., 1984; Lamp and Crawford, 1986) and assigned to chromosome 17p13.1 (van Tuinen et al., 1988).

Originally p53 was classified as an oncogene because cotransfection of *ras* and *p53* genes transformed embryo fibroblasts. However, the *p53* cDNA clones used in these experiments were later observed to contain missense mutations. More recent experiments

with wild-type (wt) *p53* proved the opposite. From these studies wt p53 was found to be involved in growth suppression and inhibition of transformation (Finlay et al., 1989; Eliyahu et al., 1989; Baker et al., 1990). Therefore, the wt *p53* gene has clear characteristics of a tumor suppressor gene.

Different mechanisms have been found in human tumors that alter p53 and in turn inactivate wt p53 function. These alterations can occur at either the protein or DNA level. At the protein level, the wt p53 protein was found in a complex with various DNA tumor virus encoded oncoproteins (E1B, large T antigen and E6). In addition, p53 was found in a complex with MDM2, a cellular protein with transforming properties (Momand et al., 1992). Furthermore, mutant p53 can bind with wt p53 (see below). The binding of these proteins most likely inactivates p53 and thereby contributes to oncogenesis. Alterations at the DNA level appear to be the most common mechanism. These alterations include the presence of a mutations in the *p53* gene and loss of the chromosomal region that bears this gene. Most tumors show a mutation in one copy of the gene and loss of the second allele because the *p53* containing portion of chromosome 17p is lost. In these cases p53 follows a recessive mechanism of tumorigenesis. Mutation analyses of the *p53* gene in human tumors revealed two remarkable findings. First, mutations in this gene were the most common genetic alteration yet identified in human cancer. These include cancers of the colon, lung, oesophagus, breast, liver, brain and hemopoietic tissues (Hollstein et al., 1991; Levine et al., 1991; Levine, 1992). Second, 80% of *p53* mutations involve single base changes (missense mutations). Most mutations are clustered in the central, highly conserved regions of the gene. However, the types and sites of the mutations can vary amoung tumor types and probably reflect exposure to specific etiologic agents (Harris and Hollstein, 1993). Many of the *p53* missense mutations, which lead to an amino acid change and a full-length mutant protein, show similar detrimental effects on protein conformation (Gannon et al., 1990) and function (Kern et al., 1992; Vogelstein and Kinzler., 1992). On the protein level an increase in half-life from minutes to hours has been observed (Bartek et al., 1991). Although most tumors show abnormalities of both alleles, some tumors show only one mutated copy. This indicates that in these cases p53 did not follow a recessive mechanism of tumor development. Instead a 'dominant negative' mechanism was proposed in which only one mutated copy of p53 is sufficient to contribute to tumor formation. One plausible explanation for this might be that the abundantly expressed mutated p53 protein competes with and blocks the activity of the

endogenous wt p53 by forming mixed tetrameric complexes (Kern et al., 1992; Harris et al., 1992; Hupp et al., 1992). Reports have also appeared in which certain missense *p53* mutations show some oncogenic properties as well (Wolf et al., 1984; Dittmer et al., 1993). Thus, these missense *p53* mutations can not only result in functional competition with wt p53 resulting in loss of function, but some of them have gained also new properties that result in a gain of function.

Mutations in p53 were not only found in sporadic human cancers but also in the germline of patients with the Li-Fraumeni cancer syndrome (Malkin et al., 1990). These patients are characterized by a high incidence of different cancers including early-onset breast carcinoma, childhood sarcomas, and other neoplasms. However, other cancers, for example colon and small cell lung carcinoma, with a high incidence of somatic mutations of p53 are hardly observed in Li-Fraumeni patients. This might be due to the capacity of a certain tissue to divide once a mutation in *p53* has occurred or is constitutionally present and thereby increasing the chance of undergoing a second mutation in the gene. For instance breast tissue displays an increased growth in adolescence, whereas colon and lung tissues have only a limited potential to divide under normal conditions (Knudson, 1993). Germline mutations in p53 are also, though very rarely, observed in childhood sarcomas (Toguchida et al., 1992) and among some women with breast cancer (Borresen et al., 1992). Transgenic p53 knock-out mice (Donehower et al., 1992) and mice expressing mutated p53 proteins (Lavigueur et al., 1989) showed that p53 is not important for embryonic development, because the mice were viable and appeared completely normal, although they displayed an increased risk for the development of some tumor types, particularly lymphomas.

Functional studies of the p53 protein have revealed that p53 plays a role in the control of the cell cycle (Bischoff et al., 1990; Stüzbecher et al., 1990), processes involving DNA repair, DNA replication, genomic instability (Kastan et al., 1992; Yin et al., 1992; Livingstone et al., 1992), and programmed cell death (Clarke et al., 1993; Lowe et al., 1993a; Lowe et al., 1993b). Although p53 occupies an important position in all the above mentioned processes this protein is probably not required in normal cells. However, when the cells are damaged, for instance by gamma-irradiation, the concentration of p53 increases. This results in an arrest of the cell cycle, which allows the repair of DNA damage. Thus, p53 is supposably acting as a kind of 'guardian of the genome' (Lane, 1992). The most likely way by which p53 executes these various activities is through its ability to act as a

transcriptional modulator. p53 can act directly or indirectly as a transactivator of transcription of different genes. Induction of transcription was observed in genes containing a p53 binding site after binding to p53 (Kern et al., 1991; El-Deiry et al., 1992; Kern et al., 1992). GADD45 (growth arrest and DNA damage inducible), an enzyme involved in DNA repair, contains such a binding site. p53 was found to be necessary for the induction of this enzyme (Kastan et al., 1992). Repression of transcription was found in genes containing a TATA promoter sequence (Ginsberg et al., 1991). This repression is probably the consequence of the ability of p53 to bind to a component of the transcription-initiation complex (Seto et al., 1992; Mack et al., 1993). Recently, a gene has been cloned that might at least in part explain the biological functions of p53. This gene, *WAF1/Cip1/Sdi1* (Wild-type p53-activated fragment 1; Cdk-interacting protein 1; Senescent cell-derived inhibitor), contains a p53-binding site, is highly induced by wild-type p53, can inhibit cell growth, is up-regulated in senescent cells, and is found in complexes important for cell cycle regulation (El-Deiry et al., 1993; Harper et al., 1993; Noda et al., 1994). All these features resemble those described for p53 and suggest that this gene is a meaningful key effector of p53.

3.3 The isolation of *APC*, *MCC*, *DCC*, *hMSH2* and *hMLH1* and their putative role in colorectal tumorigenesis

Colorectal tumorigenesis provides an excellent system to study and search for genetic alterations involved in the different well-defined stages of colon tumor development. The studies of both sporadic colorectal cancer and especially the familial polyposis coli (FAP) syndrome, have resulted in more insight in the accumulation of different genetic alterations during tumor progression. FAP patients are characterized by the appearance of hundreds or even thousands of benign adenomatous polyps in the colonic mucosa some of which eventually develop into carcinomas. The study of this tumor type resulted in the isolation of three different tumor suppressor genes: *DCC*, *MCC* and *APC* (Fearon et al., 1990a; Groden et al., 1991; Joslyn et al., 1991; Kinzler et al., 1991a; Kinzler et al., 1991b; Nishisho et al., 1991). Other hereditary conditions, which predispose to colorectal cancer, are known as hereditary nonpolyposis colorectal cancer (HNPCC). In HNPCC cases only diffuse polyps more proximal in the colon are observed, which also might degenerate into carcinoma.

Recent studies in these families have resulted in the isolation of two other genes: *hMSH2* and *hMLH1* (Fishel et al., 1993; Leach et al., 1993; Bronner et al., 1994).

The gene that predisposes to FAP, the adenomatous polyposis coli (*APC*) gene, has been isolated and is located at chromosome 5q21 (Groden et al., 1991; Joslyn et al., 1991; Kinzler et al., 1991b; Nishisho et al., 1991). Apart from the germline *APC* mutations in FAP patients, somatic mutations were detected in sporadic colorectal cancer (Powell et al., 1992). The mutations most often involve deletions and nonsense mutations (Nagase and Nakamura, 1993). The localization of APC mutations may explain at least in part the difference in phenotypic appearance (Nagase et al., 1992; Nagase and Nakamura, 1993; Olschwang et al., 1993; Spirio et al., 1993). One might speculate that a constitutional *APC* mutation might give the mucosal cells a growth advantage, which increases the change of getting a second mutation in the other allele that results in polyp formation. The APC gene encodes a large 2,843 amino acid protein with a coiled coil structure in the N-terminal part. No significant resemblance to any known gene was found in the databases, which makes predictions about its function difficult. However, recently immunoprecipitation experiments identified interactions between APC and alpha and beta catenin (Rubinfeld et al., 1993; Su et al., 1993). These experiments suggest that APC might interfere indirectly with cadherins, proteins that mediate cell-cell interactions. Therefore, APC might be involved in cell adhesion. Although most cases of familial polyposis have shown linkage to and mutations in the *APC* gene (Spirio et al., 1993), another family was recently described in which linkage to the *APC* gene was excluded (Stella et al., 1993).

About 150 kb proximal to the *APC* gene another gene was identified. This gene, *MCC* (Mutated in Colorectal Cancers), is thought to contribute to colorectal tumorigenesis because several sporadic colon carcinomas showed gross structural alterations or point mutations, mostly missense mutations, in this gene (Kinzler et al., 1991a; Nishisho et al., 1991). In addition, about 40% of the colorectal tumors revealed allelic loss of 5q21, which includes the *MCC* and *APC* genes (Kinzler et al., 1991a). No *MCC* mutations have been found in FAP patients. The gene encodes a protein of 829 amino acids, which shows a short region of homology with G protein-coupled receptors and contains a coiled coil structure. Further study is required to investigate the role of this gene in colorectal neoplasia.

Another important gene involved in these carcinomas is the *DCC* (Deleted in Colorectal Carcinomas) gene. This gene was identified because LOH studies showed frequent

loss of markers on the long arm of chromosome 18 (Vogelstein et al., 1988; Fearon et al., 1990a). Only about 11% of early stage adenomatous polyps but over 70% of carcinomas show loss of 18q (Vogelstein et al., 1988; Cho et al., 1994). This suggests that *DCC* plays a role in advanced adenomas. The gene is expressed in most normal tissues, including colonic mucosa, though its expression was greatly reduced or absent in most colorectal carcinomas (Fearon et al., 1990a). So far, one missense mutation has been identified in the coding region of the gene (Cho et al., 1994). The gene encodes a 190-kD transmembrane phosphoprotein probably a cell surface receptor involved in adhesion to an extracellular matrix or basement membrane component (Fearon et al., 1990a). LOH for chromosome 18q is also seen in breast tumors (Devilee et al., 1991a) and gastric cancer (Uchino et al., 1992). To date germline mutations in this gene were not observed. This might indicate that DCC is involved in tumor progression rather than tumor initiation.

In addition to alterations in these tumor suppressor genes other changes (genes) are frequently involved such as *K-ras* mutations (Vogelstein et al., 1988), loss and/or mutations in the *p53* gene on 17p (Baker et al., 1989; Nigro et al., 1989; Vogelstein, 1989), loss of 22q (Okamoto et al., 1988; Vogelstein et al., 1989; Miyaki et al., 1990) and loss of the *nm23-H1* gene on 1p35 (Leister et al., 1990; Cohn et al., 1991). Loss of 1p35 was most frequently observed in the non metastatic colon tumors (Cohn et al., 1991). The model for colorectal tumorigenesis as developed by Fearon and Vogelstein (1990b) is illustrated in figure 2. One should keep in mind, however, that after the initiating APC mutation the depicted sequence of events is by no means obligatory.

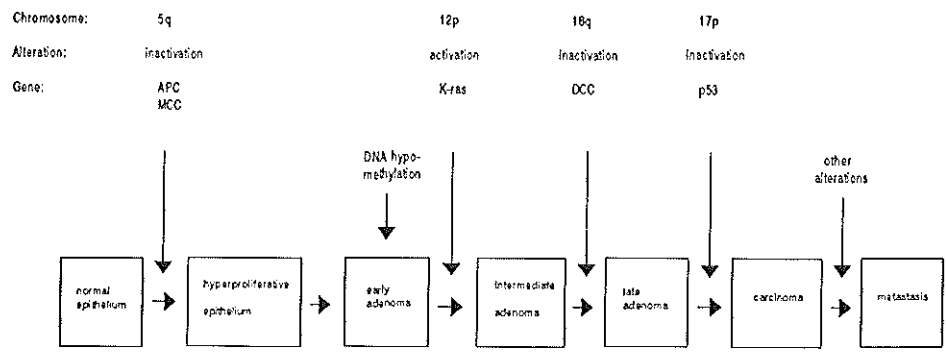

Figure 2. A genetic model for colorectal tumorigenesis. Modified from Fearon and Vogelstein (1990b).

In about 15% of all colon tumors another initiating mutation is likely. These tumors reveal widespread instability of microsatellite sequences, whereas loss of heterozygosity for chromosomal loci is mostly absent (Thibodeau et al., 1993). This implicates that we deal with a different group of colon tumors. Furthermore, these alterations were also found in tumors from HNPCC patients. Linkage analysis in HNPCC families suggested that at least three genes are involved: one at chromosome 2p16, another at chromosome 3p21-23, and a third at an unidentified locus (Aaltonen et al., 1993; Peltomäki et al., 1993; Lindblom et al., 1993). Both the candidate gene approach (Fishel et al., 1993) and the positional cloning approach (Leach et al., 1993) have resulted in the identification of the *hMSH2* gene at chromosome 2p16. Mutations in this gene were found in sporadic colon tumors that showed dinucleotide repeat instability and HNPCC patients. Both splice acceptor site mutations and mis- and non-sense mutations were observed. The human *hMSH2* gene shows high homology with the prokaryotic *MutS* gene, which is part of the mismatch repair pathway in E. coli. MutS mutants exhibit dinucleoide repeat instability like that observed in HNPCC patients (Levinson and Gutman, 1987; Strand et al., 1993). In vitro experiments with extracts of HNPCC tumor cells showed a profound defect in strand-specific mismatch repair (Parsons et al., 1993). In addition, very recently a candidate mismatch repair gene on chromosome 3p has been isolated because of its homology with *MutL*, another gene involved in the mismatch repair pathway in E. coli (Bronner et al., 1994). This gene was called *hMLH1*. In a chromosome 3-linked HNPCC family missense mutations were found in affected individuals. Mutations in *APC*, *K-ras* and *p53* are also observed in this group of tumors. It might be that mutations in mismatch repair genes result in an increase risk of generating mutations in other oncogenes and tumor suppressor genes. The *hMSH2* and *hMLH1* genes probably play a role in carcinogenesis by inducing a mutator phenotype. Yet, why would mutations in these genes only predispose to specific tumors? Future studies should shed light on this intriguing question.

3.4 The isolation of the *WT1* and *VHL* genes

Wilms' tumor is a pediatric kidney cancer which probably arises from blastemal kidney cells (Beckwith et al., 1990). Most tumors occur sporadically, however these tumors are also

found in association with the hereditary disorders WAGR, Denys-Dash and Beckwith-Wiedemann. A combination of genetic mapping, karyotype and DNA analyses have resulted in evidence supporting three distinct loci for Wilms' tumor, two on the short arm of chromosome 11 (11p13 and 11p15) and one still unknown (Haber and Housman, 1992). Apart from the involvement of the 11p15 locus in the development of Wilms' tumor, loss of this region has been found in breast cancer, lung cancer and acute myelogenous leukemia (Ali et al., 1987; Henry et al., 1989; Weston et al., 1989; Ahuja et al., 1990). However, the 11p13 locus is probably involved in only a very limited number of tumor types.

In 1990 two independent groups were able to isolate the 11p13 derived *WT1* gene, using the positional cloning approach (Call et al., 1990; Gessler et al., 1990). This 345 amino acid protein has the hallmarks of a transcription factor (among others four zinc finger domains). The binding of this protein to the EGR-1 and IGF-II promoter results in transcriptional repression (Drummond et al., 1992; Wang et al., 1992). Most mutations, both small mutations in the *WT1* transcript and (homozygous) deletions in the gene, resulted in inactivation of the *WT1* gene and some cases clearly support the recessive tumor suppressor gene model (Haber et al., 1990; Cowell et al., 1991; Huff et al., 1991; Ton et al., 1991). Not all tumors show mutations in (both alleles of) this gene suggesting that at least some altered *WT1* gene products can function in a 'dominant negative' way (Haber et al., 1990) or that in these cases another putative tumor suppressor locus plays a role too. Evidence exists that both 11p13 and 11p15 loci can play a role in a single tumor (Henry et al., 1989). Mutations in the *WT-1* gene are probably restricted to Wilms' tumors, as so far only a somatic homozygous missense mutation in the *WT1* gene was found in a mesothelioma (Park et al., 1993). The following findings indicate an important role of WT1 in urogenital development. Denys-Drash syndrome is a rare human developmental disorder of the genito-urinary system characterized by pseudohermaphroditism, renal failure and predisposition to Wilms' tumor. This syndrome is caused by specific point mutations in the zinc finger domain of the WT1 protein or other mutations involving this domain (Pelletier et al., 1991; Little et al., 1993). The expression of the *WT1* gene is limited to certain organs including the genitourinary tract (Pritchard-Jones et al., 1990). This probably explains the limited occurrence of *WT1* mutations in cancers other than Wilms'tumor. In addition, murine WT1 homozygous knock out mice are lethal with failure of kidney and gonad development (Kreidberg et al., 1993). The heterozygous mice did not develop Wilms' tumor. This might

be explained by assuming that the number of target-cells in mice is too low and therefore a second mutation in the other allele never occurs.

Another mechanism of inactivation which could be involved in the pathogenesis of Wilms' tumor is genomic imprinting, because in more than 90 percent of the tumors maternal allelic loss is observed. This imprinting is probably restricted to the 11p15 locus because this preferential loss is not the *WT1* gene on 11p13 (van Heyningen and Hastie, 1992; Koi et al., 1993). The preferential loss of the maternal allele might indicate that the paternal allele is imprinted, resulting in loss of expression of the susceptibility gene from this locus. Other mechanisms which might explain this finding are that imprinting could somehow enhance the rate of induction of small mutations in the paternal allele or protect for mutations in the maternally derived gene during gametogenesis. The net result in all of these models is inactivation of both maternal and paternal alleles as could be expected when the predisposing gene is a tumor suppressor gene. However, other mechanisms might be the outcome of this imprinting effect when we deal with an oncogene for instance. More details about this issue are provided in a very recent review about genomic imprinting (Tycko, 1994).

The von Hippel-Lindau (VHL) disease is a dominantly inherited cancer syndrome in which in addition to renal cell carcinoma, also hemangioblastomas of the central nervous system and retina and pheochromocytomas occur. However, Wilms' tumors are never found. Recently, the *VHL* gene on chromosome 3p25-p26 was isolated and both large (nonoverlapping) deletions and small mutations were observed in VHL kindreds and sporadic renal cell carcinomas (Latif et al., 1993). The gene probably encodes a 284 amino acid protein without any significant homology to already isolated genes in the databases. However, an acidic tandemly repeated pentamer is observed in the putative protein, which suggests a role in signal transduction or cell adhesion (Latif et al., 1993).

3.5 The *RET* proto-oncogene

The *RET* proto-oncogene, located on chromosome 10q11.2, was originally isolated because the gene became activated by rearrangement during NIH3T3 cell transformation (Takahashi et al., 1985). Later, it also appeared to play a role in human cancer because in 25% of

human papillary thyroid carcinomas this gene showed rearrangements (Grieco et al., 1990). The *RET* gene encodes a transmembrane tyrosine kinase protein with an as yet unknown ligand.

In the dominantly inherited cancer syndromes multiple endocrine neoplasia type 2 (MEN 2A and B), familial medullary carcinoma (FMTC), and the dominantly inherited Hirschsprung's disease, which is a developmental disorder, specific mutations have been found in the *RET* proto-oncogene (Donis-Keller et al., 1993; Mulligan et al., 1993a; Edery et al., 1994; Hofstra et al., 1994; Romeo et al., 1994). MEN 2 and FMTC are characterized by the development of bilateral medullary thyroid carcinoma (MTC). In both MEN 2A and B pheochromocytomas are observed and in MEN 2B a distinct more aggressive and complex phenotype is apparent (Nanes and Catherwood, 1992). Hirschsprung's disease is characterized by congenital absense of parasympathetic innervation in the lower intestinal tract and cancer-association has never been described. The mutations observed in the *RET* gene might explain the distict clinical phenotypes. The MEN2A and FMTC mutations were all missense mutations involving the 4 conserved cysteines in the extracellular region and probably interfere with ligand binding (Donis-Keller et al., 1993; Mulligan et al., 1993a,b). In MEN2B all mutations found so far were specific threonine to methionine substitutions in codon 918 in the intracellular tyrosine kinase domain of RET. The same mutation was also observed in 6 sporadic MTC tumors (Hofstra et al., 1994). The nature of the mutations found in Hirschsprung's disease however are different. These include deletions, nonsense and missense mutations and are presumably loss of function mutations.

The *RET* mutations observed in the cancer syndromes suggest that the gene is an oncogene rather than a tumor suppressor gene because: 1) very specific heterozygous amino acid substitutions were observed and 2) chromosome 10 allelic loss was hardly ever found in these tumors (Landsvater et al., 1989; Nelkin et al., 1989; Mulligan et al., 1993a,b). However, the observation that the RET protein dimerizes (Bongarzone et al., 1993; Rodriguez and Park, 1993) suggests that a dominant-negative mechanism can not be ruled out. The presumed loss of function mutations found in Hirschsprung's disease indicate that haplo-insufficiency (dosage-effect) is the underlying mechanism. Homozygous *RET* gene knockout mice die soon after birth and show impairment in kidney development and lack enteric neurons throughout the digestive tract (Schuchardt et al., 1994).

3.6 The *NF1* gene

Neurofibromatosis type 1 (NF1), also known as von Recklinghausen neurofibromatosis, is an autosomal dominant disorder with a heterogeneous clinical manifestation (Riccardi, 1981). The hallmarks of NF1 are: cutaneous or subcutaneous neurofibromas, café au lait spots and Lisch nodules. The *NF1* gene, located on chromosome 17q11.2, was isolated in 1990 and encodes a 2,818 amino acid protein (Cawthon et al., 1990; Viskochil et al.,1990; Wallace et al., 1990). The gene product, neurofibromin, shares high homology with GTPase activator proteins (Buchberg et al., 1990; Xu et al., 1990a; Xu et al., 1990b). The protein may function as a negative regulator of the $p21^{ras}$-mediated signal transduction pathway and/or down stream of $p21^{ras}$ (Bollag and McCormick, 1992). The possible interaction of neurofibromin with microtubules suggests a role in mitosis and cell division (Seizinger, 1993). *NF1* gene mutations were observed in sporadic tumors that are also found in NF1 patients (neurofibroma, neurofibrosarcoma, pheochromocytoma and astrocytoma), but in addition in neuroblastoma, malignant melanoma, a sporadic colon carcinoma and myelodysplastic syndrome, diseases that do not occur in NF1 patients (Li et al., 1992). Recent data suggest that the *NF1* gene functions at least in some of the tumors as a recessive tumor suppressor gene, because homozygous deletions in the NF1 locus have been found in a neurofibrosarcoma from a NF1 patient, some melanomas and some neuroblastomas (Andersen et al., 1993; Johnson et al., 1993; Legius et al., 1993).

3.7 The *NF2* gene

Neurofibromatosis type 2 displays an autosomal dominant inheritance and is genetically linked to chromosome 22 (Rouleau et al., 1990). The hallmark for this disease is the development of bilateral schwannomas of the eighth cranial nerve. Apart from this, multiple spinal and intracranial schwannomas and meningiomas may appear in these patients (Evans et al., 1992a). Recently two independent groups have isolated the *NF2* gene, which encodes a 595 amino acid protein, called merlin (moesin-ezrin-radixin like protein, Trofatter et al., 1993) or schwannomin (Rouleau et al., 1993). High homology of the N-terminal 340 residues of this protein to the cytoskeleton associated proteins moesin, ezrin and radixin suggests a

role in mediating interactions between the cell membrane and the cytoskeleton. Mutations were found in both the germline of NF2 patients and sporadic variants of tumors associated with NF2 (Rouleau et al., 1993; Trofatter et al., 1993; Bianchi et al., 1994; Jacoby et al., 1994; Ruttledge et al., 1994; Chapter VIII). Most mutations result in frameshifts, which lead to truncation of the protein, and presumably a loss of function. Almost all meningiomas with complete or partial loss of chromosome 22 revealed mutations in the *NF2* gene. These findings support the view that the *NF2* gene functions as a recessive tumor suppressor gene. Mutations in the *NF2* gene transcript were also observed in breast carcinoma and melanoma, neoplasms unrelated to NF2. However, no mutations were observed in pheochromocytomas and colon carcinomas in which chromosome 22q loss has frequently been observed (Bianchi et al., 1994).

4 MENINGIOMA

4.1 Cells of origin

In 1922 the name 'meningioma' was first introduced by Harvey Cushing for a benign tumor of the meninges of the central nervous system. The meninges, the coverings of the brain and spinal cord, are composed of three layers with at the outside the pachymeninx or dura mater and at the inside the leptomeninges composed of the arachnoid and pia mater. Embryogenesis of the meninges, at least in humans, is still not very well elucidated. Experimental work in lower vertebrates indicates that the dura mater and probably also the arachnoid are mesodermal in origin, whereas the pia mater probably is ectodermal in origin and is largely derived from neural crest cells (Kepes, 1982; Russell and Rubinstein, 1989). Different lines of evidence suggest that meningiomas arise from arachnoid cells (Kepes, 1982; Upton and Weller, 1985; Russell and Rubinstein, 1989).

4.2 Frequency, incidence and occurrence

Intracranial meningiomas represent approximately 13-19% of all primary intracranial tumors that are treated by surgery and an incidence of 12% was recorded for the spinal meningiomas (Russell and Rubinstein, 1989). The actual incidence of meningiomas is probably much higher because these tumors are slowly growing and may stay asymptomatic in a large number of cases. An estimate of the overal incidence of meningiomas could be calculated from a Swedish study in which during a 10-year period 172 meningiomas were found in 11,793 autopsies: a prevalence of 1.46% (Rausing et al., 1970). However, in only 11 of these cases meningiomas were the main cause of death.

Between 2-3 times as many females develop meningiomas as males and the highest incidence is observed in the fifth and sixth decade of life (Zang, 1982). During childhood meningiomas are distinctly rare (Perilongo et al., 1992). There are three patterns of occurrence (Butti et al., 1989; Domenicucci et al., 1989; Russell and Rubinstein, 1989; McDowell, 1990; Sieb et al., 1992):

1) sporadic solitary cases;

2) as part of the hereditary syndrome neurofibromatosis type 2 (NF2);

3) multiple or familial aggregation of meningiomas.

The first group involves the majority of the cases and consists of tumors occurring randomly (not associated with a hereditary syndrome) in the general population. The second one involves meningiomas found in individuals with NF2. The diagnostic criteria for NF2 are depicted in table 2 (Evans et al., 1992b).

Table 2. Diagnostic criteria for NF2

Bilateral vestibular schwannomas **or** family history of NF2 **plus**
1) unilateral vestibular schwannoma **or**
2) any two of: meningioma, glioma, neurofibroma, schwannoma, posterior subcapsular lenticular opacities.

This disorder may be divided in at least two distinct forms. One is called Wishart type and has an early onset, rapid course and multiple tumors in addition to bilateral vestibular schwannomas. The other, Gardner type, is characterized by a later age of onset, a more benign course and is usually limited to bilateral vestibular schwannomas (Evans et al., 1992b). In both categories meningiomas can occur. The third group consists of patients with multiple or familial aggregation of meningiomas without evidence of NF2. In this group, at least in some of the cases, a predisposing mutation is probably involved (Chapter VII). It remains to be established whether these different groups arise as a result of different mutations in one gene or that different genes or combination of genes are involved.

4.3 Anatomic localization

According to Russell and Rubinstein (1989) meningiomas can be found at seven different sites in the body: 1) intracranial; 2) spinal; 3) intraventricular; 4) orbital; 5) intrapetrous; 6) (extra-) calvarial and 7) ectopic. The intracranial and spinal meningiomas are the most common ones. These tumors are often further subdivided in base (sphenoid ridge, tuberculum sellae, olfactory grooves, pontocerebellar angle, petrous ridge of the temporal bone, posterior

fossa and foramen magnum), convexity (parasagittal region and free convexity), falx cerebri, intraventricular and spinal cord meningiomas (Casalone et al., 1990). Total surgical resection is dependent on the anatomic localization of the tumor and influences the recurrence rate (Black, 1991).

4.4 Histopathology

Microscopic examination of meningiomas by many neuropathologists resulted in 1979 in the following WHO classification scheme (Zülch, 1979):

1. meningotheliomatous (endotheliomatous, syncytial, arachnotheliomatous)
2. fibrous (fibroblastic)
3. transitional (mixture of type 1 and 2)
4. psammomatous
5. angiomatous
6. haemangioblastic
7. haemangiopericytic
8. papillary
9. anaplastic (malignant)

Other subtypes are also known but less common such as lipoblastic and xanthomatous meningiomas (Kepes, 1982). Most tumors show features corresponding with a mixture of different histological subtypes, from which the first four occur most predominantly (de la Monte et al., 1986). From a histopathological point of view, most of these tumors are considered to be benign and only very rarely truly malignant invasive meningiomas occur. However, different degrees of anaplasia, which may to some extent influence biological behaviour, can be identified in all classes of benign tumors (Jääskeläinen et al., 1985; de la Monte et al., 1986; Vagner-Capodano et al., 1993). A significant association was observed in this respect between the in vivo growth rate of meningiomas and the histopathological grade of the tumors (Jääskeläinen et al., 1985). The grading system according to increasing anaplasia used in this thesis involves the following six histological parameters: loss of architecture, increased cellularity, nuclear pleomorphism, mitotic figures, focal necrosis and brain infiltration (Jääskeläinen et al., 1985; Vagner-Capodano et al., 1993). Three different

grades can be distinguished using these criteria: grade I: benign; grade II: atypical and grade III: anaplastic. It remains to be established whether this grading system is of clinical relevance.

4.5 Etiology

From the literature different studies suggest that the following factors may be involved in the etiology of meningioma. These include: 1) genetic predisposition (section 4.2) 2) steroid hormones and their receptors 3) radiation 4) head injury 5) SV-40-related papova viruses.

The postulated role of the steroid hormones and their receptors came from different lines of evidence. First, a higher incidence of these tumors is observed in women and an accelerated growth has been observed during pregnancy (Zang, 1982; Roelvink et al., 1987). In other studies this association was not found (Schlehofer et al., 1992; and cited references). Second, the majority of meningiomas and normal leptomeningeal tissue contain progesterone receptors. The presence of oestrogen receptors is still a matter of discussion. The significance of the progesterone receptors in these tumors was further investigated with in vitro experiments using progesterone and progesterone inhibitors. The results of these experiments are not very conclusive, although they might point to a slight growth promoting role for progesterone (Koper et al., 1990).

Radiation is nowadays a well established causative factor in meningioma development. Meningiomas have occurred with increased frequency and at an earlier age in people receiving high-dose irradiation for the treatment of an intracranial growth or after low-dosage scalp irradiation for fungal infections (Russell and Rubinstein, 1989).

A large number of reports going back as far as 1813 suggest the involvement of previous head injury and the development of meningioma years later (Al-Rodhan and Laws, 1990). Some investigators found a statistically significant association between meningioma occurrence and a history of head trauma, others observed site-specific head injuries with subsequent tumor development at the same site years later (Russell and Rubinstein, 1989).

Different reports reviewed by Zang (1982) might support the role of SV40-related papova viruses and the formation of meningiomas. In about 35% of histological sections or

early cell cultures the presence of SV40 related T antigens was described (Weiss et al., 1975; Scherneck et al., 1979; Krieg et al., 1981). However, these findings were not confirmed by others (Merletti et al., 1975).

4.6 Cytogenetic and molecular genetic studies of meningiomas

As early as 1967, cytogenetic studies showed non-random loss of one G-group chromosome in meningiomas. This was the first specific chromosomal aberration observed in solid tumors (Zang and Singer, 1967). The subsequent development of banding techniques and molecular genetic studies revealed that monosomy of chromosome 22 is the hallmark for these tumors (Zang, 1982; Seizinger et al., 1987a; Dumanski et al., 1990a). In a considerable number of tumors loss of chromosome 22 is the only cytogenetically visible chromosomal alteration (Al Saadi et al., 1987; Maltby et al., 1988; Vagner-Capodano et al., 1993). These results indicate that loss of genetic material from this chromosome is a primary and specific event in the development of these tumors, which is reminiscent of the involvement of a tumor suppressor gene(s) as is mentioned in section 2. In addition, other less common non random chromosomal changes occur. The most frequent ones are loss or rearrangements of chromosomes 1, 7, 14, 18 and 19 and these are thought to play a role in tumor progression (Katsuyama et al., 1986; Al Saadi et al., 1987; Maltby et al., 1988; Rey et al., 1988; Casalone et al., 1990). The karyotypic evolution observed in meningiomas appears to be associated with more anaplastic features (Vagner-Capodano et al., 1993).

As mentioned earlier the majority of meningiomas are sporadic cases but they are also found in patients with NF2 or in familial or multiple meningioma patients. Genetic linkage studies in NF2 families, tumor deletion mapping in both sporadic and hereditary meningioma cases resulted in the identification of at least three different loci on the long arm of chromosome 22, which might be involved in the pathogenesis of meningioma. In figure 3 the three chromosome 22 loci are depicted.

Evidence for the involvement of the most proximal locus in band 22q11 came from cytogenetic and molecular genetic findings in two independent patients with meningiomas. The meningioma from the first patient revealed a reciprocal t(4;22)(p16;q11), which resulted

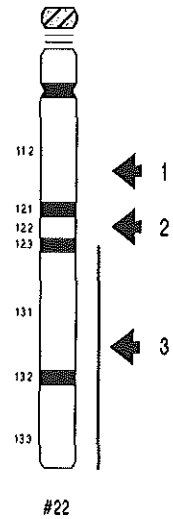

Figure 3. Localization of the three putative loci on the long arm of chromosome 22 involved in meningioma development.

in the disruption of a subsequently isolated *MN1* gene at the translocation breakpoint (Chapter VI). The second patient, who developed multiple meningiomas, was the carrier of a constitutional interstitial deletion of 1,5 kb. This deletion was mapped about 10 kb proximal to the *MN1* gene (Chapter VII). In both cases mutations in the *NF2* gene (see below) were not observed (Chapter VIII).

The second locus at band 22q12 is positioned about 1.7 Mb distal to the *MN1* gene. This locus harbours two genes, which both showed alterations in meningiomas. The first gene is the *NF2* gene recently isolated by two independent groups (Trofatter et al., 1993; Rouleau et al., 1993). In both NF2 associated and sporadic meningiomas mutations in the *NF2* gene have been observed (section 3.7 and Chapter VIII). This implies that mutations in the *NF2* gene are important in the development of sporadic meningiomas. As yet it is not known if a mutation in this gene is a prerequisite for the development of meningiomas. The second gene is the *MEN* gene (candidate meningioma gene) located 200 kb centromeric to the *NF2* gene. This gene has been isolated from a region which was homozygously deleted in one meningioma and codes for a ß-adaptin protein. The deletion did not include the *NF2*

gene (Dumanski et al., NNFF consortium on gene cloning, Ann Arbor, april 24-26, 1993). However, Sanson et al (1993) observed a germline deletion in a NF2 family in which both the *NF2*- and the *MEN* gene were lost. In this family, however, meningiomas were not found. Therefore, further study is required to find out if this gene is involved in the development of (a subset of) meningiomas.

The most distal locus on chromosome 22 was defined because in two meningiomas terminal deletions were observed telomeric to the *NF2* gene (Dumanski et al., 1990a; Rey et al., 1993). Since the most distal breakpoint was located telomeric to *MB* at 22q12.3 this locus was mapped on 22q12.3-qter. In addition, other reports describe chromosome 22 abnormalities restricted to this distal region. Two patients with multiple meningiomas were described with a constitutional ring chromosome 22, resulting in loss of chromosome 22 sequences distal to 22q13 (Arinami et al., 1986; Petrella et al., 1993). This might suggest that a gene is located in the deleted region or that the ring chromosomes lead to somatic instability of chromosome 22. Unfortunately, in neither case cytogenetic analysis was carried out on the tumor cells. Apart from this, in a sporadic vestibular schwannoma a reciprocal translocation was found with the breakpoint at 22q13. No mutation in the *NF2* gene was observed in these tumor cells (Wolff et al., NNFF consortium on gene cloning, Ann Arbor, april 24-26, 1993).

Furthermore, linkage analysis in a family with multiple meningiomas and ependymomas suggests that another meningioma locus distinct from NF2 is involved that might be located on another chromosome (Pulst et al. 1993). In this study they excluded a region of 15 cM on chromosome 22, which includes the *NF2* gene, as the site for the predisposing mutation.

All together these results indicate that different genes may be involved in the development of vestibular schwannoma and meningioma and that further studies are required to establish the importance of the individual genes.

Three groups have investigated whether genomic imprinting of chromosome 22 occurs in sporadic meningiomas and schwannomas and NF2 schwannomas by examining the parental origin of chromosome 22 loss. No preferential parental loss of this chromosome could be observed. This suggests that genomic imprinting is not a common mechanism of gene inactivation in these tumors (Fontaine et al., 1990; Sanson et al., 1990; Fontaine et al., 1991).

4.7 Other tumors with loss or rearrangements of chromosome 22

In addition to meningiomas and vestibular schwannomas, other tumors also show non random loss of chromosome 22. Medullary thyroid carcinoma, an endocrine tumor also found in MEN 2, showed LOH for chromosome 22q in approximately 10% of the cases. However, in 40% of both sporadic and familial pheochromocytomas (MEN 2), deletions of (parts of) the long arm of chromosome 22 have been observed, with the smallest region of overlap between D22S10 and D22S22 (Khosla et al., 1991; Tanaka et al., 1992). This region includes all three putative meningioma susceptibility loci (Fig 3, Delattre et al., 1991). Reviewing the data on 31 ependymomas, deletions and translocations of chromosome 22 were observed in 13 of them (Rey et al., 1987; Bown et al., 1988; Dal Cin and Sandberg, 1988; Griffin et al., 1988; Jenkins et al., 1989; Savard and Gilchrist, 1989; James et al., 1990; Ranson et al., 1992; Sainati et al., 1992; Weremowicz et al., 1992). One ependymal tumor showed a der(22)t(22;?)(q11.2;?) and another one revealed loss of one chromosome 22 and a balanced translocation at q13.3 in the remaining 22 homologue (Stratton et al., 1989; Weremowicz et al., 1992). This might suggest that a gene at 22q13.3 is involved. Monosomy and structural changes of chromosome 22 are the most consistent specific chromosomal alterations observed in malignant mesothelioma. The structural abnormalities all revealed breakpoints at 22q11 (Fletjer et al., 1989; Hagemeijer et al., 1990). This is in the region where we found the *MN1* gene. In leiomyoma, a small tumor derived from smooth muscle most often of the uterus, also non random loss of chromosome 22 was found (Sreekantaiah and Sandberg, 1991). In addition, chromosome 22 deletions have been observed in gliomas and predominate in the higher malignancy grades. LOH studies in these tumors have identified terminal deletions due to breakpoints at 22q13, suggesting that this region might be the location of a glioma suppressor gene (Rey et al., 1993). A few reports about rhabdoid tumors describe monosomy 22 as the only cytogenetic change in two cases and a der(22)t(9;22(p13;q11) with a deletion of 22qter distal to BCRL2 in another (Biegel et al., 1990; Biegel et al., 1992). Recently a reciprocal t(11;22)(p15.5;q11.23) was identified in such a tumor (Newsham et al., 1994). The breakpoint was mapped proximal to the NF2 locus and might be in the region where we mapped the *MN1* gene. It would be very interesting to find out if this gene is involved. LOH studies in breast cancer revealed specific deletions of chromosome 22 sequences, with a preference in the lobular carcinomas (Devilee

et al., 1991b; Larsson et al., 1990). In colon cancer Vogelstein et al. (1989) found in about 30% of the tumors loss of chromosome 22q. Miyaki et al.(1990) showed that chromosome 22 loss in colorectal cancer is a relatively late event. Less than 5% of the adenomas from FAP patients showed loss of chromosome 22, whereas 33% of invasive carcinomas revealed this loss. So far, mutations in the *NF2* gene were observed in 1/69 breast tumors, 6/20 primary and melanoma metastases, and 2/64 colorectal carcinomas (Bianchi et al., 1994; Arakawa et al., 1994) and 1/8 ependymomas (Rubio et al., 1994). However, 5 pheochromocytomas and 30 astrocytomas did not reveal mutations in this gene (Bianchi et al., 1994). Further study is required to elucidate whether the above mentioned genes and/or other chromosome 22 specific genes are involved in the pathogenesis these tumors.

4.8 Chromosome 22

Chromosome 22 is the second smallest acrocentric chromosome, comprising approximately 56 Mb or 1.9% of the haploid genome (Morton, 1991). The short arm (22p) only harbours the ribosomal genes of the nucleolar organizer region, while the long arm (22q) represents the bulk of the chromosomal DNA. The long arm of the chromosome is involved in a number of diseases. These include Di George syndrome, velo-cardio-facial syndrome, cat eye syndrome and a number of cancers such as chronic myelocytic leukemia, Burkitt lymphoma, Ewing sarcoma, meningiomas and vestibular schwannomas (McDermid et al., 1993). The past few years a lot of effort has been put into the genomic characterization of chromosome 22. This was facilitated by the development of high-resolution panels of somatic cell hybrids, detailed linkage maps and the isolation of a large number of chromosome 22 specific DNA markers (Dumanski et al., 1990b; Budarf et al., 1991; Delattre et al., 1991; Dumanski et al., 1991; Fiedler et al., 1991; Emanuel et al., 1991; Frazer et al., 1992; van Biezen et al., 1993; Emanuel et al., 1993). Very helpful in the construction of a genomic contig of this chromosome is the availability of chromosome 22 specific cosmid libraries (de Jong et al., 1989). These cosmid and genomic YAC libraries have resulted in the isolation of a contig of about 1100 kb on human chromosome 22q12 (Xie et al., 1993). Very recently a physical map became available for 22q11-q12 using pulsed-field gel electrophoresis. This long-range restriction map spans approximately 11 Mb of DNA (McDermid et al., 1993).

5 Scope of the thesis

Cytogenetic analysis and LOH studies of meningioma tumors points to the localization of (a) tumor suppressor gene(s) on the long arm of chromosome 22 because:

- in about 50% of the tumors one copy of chromosome 22 is lost and this is often the sole chromosomal abnormality and
- in a few percent of the cases additional aberrations concerning chromosome 22, such as translocations (all in band q11,q12) and partial loss of the long arm are found.

The purpose of this thesis was to identify and characterize (a) tumor suppressor gene(s), which (is) are involved in the pathogenesis of meningioma. To reach this goal we started with the isolation and characterization of single-copy probes for chromosome 22 (Chapter II). Cytogenetic analysis and genotype analysis of the tumors were carried out in order to identify the chromosomal aberrations in the tumors. Furthermore, other patient and tumor characteristics were investigated and mutually compared (Chapter III). The same approach was used to study the involvement of chromosome 22 in a patient with familial anaplastic ependymoma (Chapter IV). In order to localize a meningioma tumor suppressor gene on chromosome 22 we took advantage of a meningioma (MN32) in which a reciprocal t(4;22)(p16;q11) had occurred. We hypothesized that this translocation disrupts a potential tumor suppressor gene. To map this translocation breakpoint on chromosome 22 we made hybrid cell lines in which both reciprocal translocation products were segregated in different hybrids (Chapter V). Long-range restriction mapping was used to further characterize the breakpoint. One probe (D22S193) recognized the translocation breakpoint on pulsed field gels. Chromosome walking towards the translocation breakpoint and searching for transcribed sequences in this region resulted in the isolation of the *MN1* gene (Chapter VI). Probes from this region were used to investigate DNA from other meningiomas and blood of a patient with multiple meningiomas. Southern blot analyses observed aberrant restriction fragments in DNA isolated from a patient with multiple meningiomas (patient 55). The characterization of these alterations are described in chapter VII. Findings from other groups (see 4.6), the isolation of the *MN1* gene and the recent cloning of the *NF2* gene (Rouleau et al., 1993; Trofatter et al., 1993) raise a question about the relative contribution of these genes/loci in the development of meningiomas. To investigate the extent in which the *NF2* is involved we performed mutation analysis of the *NF2* gene transcript in our series of (mostly) sporadic meningiomas, including tumors 32 and 55 (Chapter VIII).

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Chapter II

Isolation and characterization of 25 unique DNA markers for human chromosome 22

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SHORT COMMUNICATION

Isolation and Characterization of 25 Unique DNA Markers for Human Chromosome 22

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Twenty-five single-copy anonymous DNA markers for human chromosome 22 were isolated. These markers were assigned to four different regions on the chromosome. Six markers recognize restriction fragment length polymorphisms. The relative positions of five of these polymorphic markers on the framework map of chromosome 22 were determined by linkage analysis. The sizes of the *Not*I fragments recognized by 22 markers were determined by pulsed-field gel analysis. The total length of the *Not*I fragments identified is at least 12 Mb, which represents about 20% of the entire chromosome. © 1993 Academic Press, Inc.

The total length of human chromosome 22 is estimated to be about 57 Mb (7). Loss or alterations in this chromosome suggesting the presence of a tumor suppressor gene are seen in sporadic meningioma and acoustic neuroma and in the same tumors when associated with the hereditary disorder neurofibromatosis type 2 (NF2) (15). In addition, the gene predisposing to NF2 has been mapped to chromosome 22 (14). To identify specific alterations in chromosome 22 in these tumors, we have isolated single-copy DNA sequences for chromosome 22.

Total DNA was isolated from two chromosome 22-specific phage libraries (ATCC No. 57733 and ATCC No. 57714). *Eco*RI–*Hin*dIII fragments were subcloned into pUC9. Colonies positive for hybridization with λ DNA and total human DNA were discarded, and the remaining 59 inserts were used for hybridization to total genomic DNA. Of these, 13 contained nonhuman inserts and 21 contained less frequent repeats. Twenty-five inserts could be unambiguously assigned to chromosome 22 by hybridization to DNA from hybrid cell line PgMe-25Nu, which contains chromosome 22 as the only human chromosome (5). Regional localization was established by hybridization to a panel of somatic cell hybrids. This divides chromosome 22 into four regions: region I, pter–q11; region II, q11–q12; region III, q12–q13; and region IV, q13–qter. The translocation breakpoints that divide these four regions were the Philadelphia translocation t(9;22)(q34;11) (5), the t(11;22)(q24;12) from Ewing sarcoma, and a t(1;22)(42;13) (4). Of the 25 probes, 5 map to region I, 8 to region II, 7 to region III, and 5 to region IV (Table 1).

Five inserts recognized RFLPs that have been previously described (10, 11). In addition, we observed that D22S205 detected two *Taq*I alleles of 4.4 kb (A1) and 2.9 kb (A2). The frequencies of the alleles, as estimated from 31 unrelated Caucasians, were 93.5% for A1 and 6.5% for A2. We typed five of the six polymorphic RFLP markers described in this paper (D22S181, D22S182, D22S183, D22S193, and D22S201) and five markers (D22S10, BCR, D22S1, D22S29, and IGLC) that were previously used for a linkage map of chromosome 22 (13). Markers were typed on 13 extended pedigrees consisting of a total of 386 individuals. Linkage analyses were performed using the LINKAGE program version 5.03 (8, 9). To establish the relative positions of five RFLPs described in this paper, four-point linkage analyses were performed using the recombination fractions from the chromosome 22 linkage map anchor points BCR, D22S1, and D22S29 (3, 13). Figure 1 summarizes our findings. Marker D22S201 could not be linked to any of the markers used. This is probably due to the telomeric localization of this probe (22q13–qter, Table 1). According to the linkage map, D22S193 is placed telomeric to D22S29 with odds of 1.8:1. However, we place D22S193 centromeric to D22S29, because D22S29 is located telomeric to the t(11;22) in Ewing sarcoma (13), whereas D22S193 is located centromeric to this translocation breakpoint (this paper).

As a first step toward a long-range restriction map of chromosome 22, the probes were further analyzed by pulsed-field gel electrophoresis and Southern blotting. To this end, DNA from human cell lines HeLa and T24 were digested with the enzyme *Not*I. The total size of the identified *Not*I fragments is over 12 Mb, approximately 20% of the entire chromosome. Table 1 shows the characteristics of all markers, together with the size of the observed *Not*I fragments. With some of the probes more than one hybridizing band was observed, probably due to methylation of CpG islands, and thus *Not*I sites, in some of the cells. From the pulsed-field experiments it ap-

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TABLE 1

Characteristics of 25 Single-Copy Probes for Chromosome 22

D no.	Lab name	Size[a]	RFLP	*Not*I fragment length (kb) HeLa	*Not*I fragment length (kb) T24
Region I (pter–q11)					
D22S181	NB17	0.7	*Bgl* II/*Taq*I	ND[b]	NRZ[c]
D22S182	NB35	0.8	*Bgl* II	ND	ND
D22S183	NB84	1.0	*Pst*I	270	270 + 310[d]
D22S184	NB85	1.1	—	360 + 580 + 630 + NRZ	NRZ
D22S185	NB103	0.7	—	210 + NRZ	NRZ
Region II (q11–q12)					
D22S186	NB14	0.5	—	1000	1000
D22S187	NB20	0.9	—	1000	1000
D22S188	NB21	1.3	—	NRZ	NRZ
D22S189	NB43	1.6	—	NRZ	670 + NRZ
D22S190	NB62	0.6	—	NRZ	NRZ
D22S191	NB66	2.1	—	190 + NRZ	190 + NRZ
D22S192	NB97	0.7	—	680 + NRZ	NRZ
D22S193	NB129	0.6	*Pst*I	265 + 410 + 630 + NRZ	265 + 630 + NRZ
Region III (q12–q13)					
D22S194	NB7	0.3	—	500	500
D22S195	NB44	1.1	—	420 + 500 + NRZ	420 + 500 + NRZ
D22S196	NB74	1.6	—	NRZ	NRZ
D22S197	NB108	0.3	—	420 + 500	420 + 500
D22S198	NB116	1.0	—	750	750
D22S199	NB117	1.9	—	350	350
D22S200	NB119	1.8	—	560	560
Region IV (q13–qter)					
D22S201	NB5	0.5	*Taq*I	190	ND
D22S202	NB8	0.8	—	ND	ND
D22S203	NB60	1.9	—	240 + 400	240 + 400
D22S204	NB70	0.3	—	ND	ND
D22S205	NB127	0.3	*Taq*I	ND	150

[a] Size insert in kb.
[b] ND: not determined.
[c] NRZ: nonresolution zone.
[d] Two hybridizing bands were observed.

peared that markers D22S1 (1, 6) and D22S186 and D22S187 (both this paper) are located on the same *Not*I fragment. The same is presumably the case with D22S15 (12) and D22S199 (this paper) and with D22S195 and D22S197 (both this paper). Comparison of the *Not*I fragments of the region centromeric to the Ewing sarcoma translocation as recently described by Budarf *et al.* (2) with those from the same region described here shows no obvious relations, although it should be mentioned that especially in region q11–q12 there were very large *Not*I fragments that did not resolve under the electrophoresis conditions that we used and are therefore given as running in the nonresolution zone (sizes over 1 Mb).

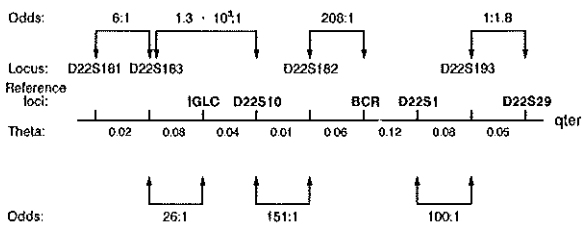

FIG. 1. Most likely order and sex-averaged recombination rates between gene markers on chromosome 22. Reference loci were used as anchor points for placing four new markers on the map. The odds against inversion of the marker order are shown above or below the brackets and are based on multipoint linkage analyses. The localization of D22S193 is based on physical mapping data.

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Appendix

A new polymorphic probe on chromosome 22: NB17 (D22S181)

Nucl. Acids Res. 19:686, 1991

A new polymorphic probe on chromosome 22: NB35 (D22S182)

Nucl. Acids Res. 19:686, 1991

A new polymorphic probe on chromosome 22: NB84 (D22S183)

Nucl. Acids Res. 19:687, 1991

A new polymorphic probe on chromosome 22q: NB129 (D22S193)

Nucl. Acids Res. 19:687, 1991

A new polymorphic probe on chromosome 22: NB5 (D22S201)

Nucl. Acids Res. 19:1963, 1991

A new polymorphic probe on chromosome 22: NB17 (D22S181)

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Source/Description: NB17 is a 0.7 kb HindIII-EcoRI fragment isolated from two pooled chromosome 22 specific libraries (ATCC # 57733 and ATCC # 57714) and was subcloned into pUC9.

Polymorphism: BglII or TaqI digestion of genomic DNA and hybridization with the probe detects a two allele polymorphism: BglII (A1: 12.5 kb, A2: 4.0 kb) with a constant band at 3.0 kb; TaqI (B1: 2.9 kb, B2: 2.2 kb). The polymorphisms detected with these two enzymes are almost in complete linkage disequilibrium.

Frequency: Estimated from 92 (BglII) and 95 (TaqI) unrelated Caucasians.

A1: 0.69 B1: 0.65
A2: 0.31 B2: 0.35

Not Polymorphic For: BglI, DraI, MspI and PstI.

Chromosomal Localization: Regional localization was established by hybridization to a panel of somatic cell hybrids: PgMe-25Nu, containing only human chromosome 22; PgMo-22 and 1CB-17ANu, respectively containing both products of the Philadelphia translocation at 22q11; A3EW2-3B, containing the t(11;22) from Ewing's sarcoma (at 22q12) and 1/22 AM27 containing a t(1;22) at 22q13 (1, 2). The probe was assigned to chromosome 22 between 22pter and 22q11.

Mendelian Inheritance: Mendelian inheritance has been demonstrated in extended pedigrees of Gilles de la Tourette syndrome families (n = 380).

Probe Availability: Available for collaboration.

References: 1) Goyns,M.H., Young,B.D., Geurts van Kessel,A., de Klein,A., Grosveld,G., Bartram,C.R. and Bootsma,D. (1984) *Leukemia Res.* **8**, 547–553. 2) Geurts van Kessel,A., Turc-Carel,C., de Klein,A., Grosveld,G., Lenoir,G. and Bootsma,D. (1985) *Mol. Cell Biol.* **5**, 427–429.

A new polymorphic probe on chromosome 22: NB35 (D22S182)

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Source/Description: NB35 is a 0.8 kb HindIII-EcoRI fragment isolated from two pooled chromosome 22 specific libraries (ATCC # 57733 and ATCC # 57714) and was subcloned into pUC9.

Polymorphism: BglII digestion of genomic DNA and hybridization with the probe detects a two allele polymorphism: 8.7 kb (A1) and 8.0 kb (A2). No constant bands were present.

Frequency: Estimated from 96 unrelated Caucasians.
A1: 0.76 A2: 0.24.

Not Polymorphic For: BglI, DraI, MspI, PstI and TaqI.

Chromosomal Localization: Regional localization was established by hybridization to a panel of somatic cell hybrids: PgMe-25Nu, containing only human chromosome 22; PgMo-22 and 1CB-17ANu, respectively containing both products of the Philadelphia translocation at 22q11; A3EW2-3B, containing the t(11;22) from Ewing's sarcoma (at 22q12) and 1/22 AM27 containing a t(1;22) at 22q13 (1, 2). The probe was assigned to chromosome 22 between 22pter and 22q11.

Mendelian Inheritance: Mendelian inheritance has been demonstrated in extended pedigrees of Gilles de la Tourette syndrome families (n = 380).

Probe Availability: Available for collaboration.

References: 1) Goyns,M.H., Young,B.D., Geurts van Kessel,A., de Klein,A., Grosveld,G., Bartram,C.R. and Bootsma,D. (1984) *Leukemia Res.* **8**, 547–553. 2) Geurts van Kessel,A., Turc-Carel,C., de Klein,A., Grosveld,G., Lenoir,G. and Bootsma,D. (1985) *Mol. Cell Genet.* **5**, 427–429.

A new polymorphic probe on chromosome 22: NB84 (D22S183)

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Source/Description: NB84 is a 1.0 kb HindIII-EcoRI fragment isolated from two pooled chromosome 22 specific libraries (ATCC # 57733 and ATCC # 57714) and is subcloned into pUC9.

Polymorphism: PstI digestion of genomic DNA and hybridization with the probe detects a two allele polymorphism: 4.6 kb (A1) and 4.0 kb (A2). No constant bands were present.

Frequency: Estimated from 91 unrelated Caucasians.
A1: 0.19 A2: 0.81.

Not Polymorphic For: BglI, BglII, DraI, MspI and TaqI.

Chromosomal Localization: Regional localization was established by hybridization to a panel of somatic cell hybrids: PgMe-25Nu, containing only human chromosome 22; PgMo-22 and 1CB-17ANu, respectively containing both products of the Philadelphia translocation at 22q11; A3EW2−3B, containing the t(11;22) from Ewing's sarcoma (at 22q12) and 1/22 AM27 containing a t(1;22) at 22q13 (1, 2). The probe was assigned to chromosome 22 between 22pter and 22q11.

Mendelian Inheritance: Mendelian inheritance has been demonstrated in extended pedigrees of Gilles de la Tourette syndrome families (n = 380).

Probe Availability: Available for collaboration.

References: 1) Goyns,M.H., Young,B.D., Geurts van Kessel,A., de Klein,A., Grosveld,G., Bartram,C.R. and Bootsma,D. (1984) *Leukemia Res.* 8, 547−553. 2) Geurts van Kessel,A., Turc-Carel,C., de Klein,A., Grosveld,G., Lenoir,G. and Bootsma,D. (1985) *Mol. Cell Biol.* 5, 427−429.

A new polymorphic probe on chromosome 22q: NB129 (D22S193)

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Source/Description: NB129 is a 0.6 kb HindIII-EcoRI fragment isolated from two pooled chromosome 22 specific libraries (ATCC # 57733 and ATCC # 57714) and is subcloned into pUC9.

Polymorphism: PstI digestion of genomic DNA and hybridization with the probe detects a two allele polymorphism: 1.6 kb (A1) and 1.3 kb (A2). No constant bands were present.

Frequency: Estimated from 98 unrelated Caucasians
A1: 0.45 A2: 0.55.

Not Polymorphic For: BglI, BglII, DraI, MspI and TaqI.

Chromosomal Localization: Regional localization was established by hybridization to a panel of somatic cell hybrids: PgMe-25Nu, containing only human chromosome 22; PgMo-22 and 1CB-17ANu, respectively containing both products of the Philadelphia translocation at 22q11; A3EW2−3B, containing the t(11;22) from Ewing's sarcoma (at 22q12) and 1/22 AM27 containing a t(1;22) at 22q13 (1, 2). The probe was assigned to the long arm of chromosome 22 between 22q11 and 22q12.

Mendelian Inheritance: Mendelian inheritance has been demonstrated in extended pedigrees of Gilles de la Tourette syndrome families (n = 380).

Probe Availability: Available for collaboration.

References: 1) Goyns,M.H., Young,B.D., Geurts van Kessel,A., de Klein,A., Grosveld,G., Bartram,C.R. and Bootsma,D. (1984) *Leukemia Res.* 8, 547−553. 2) Geurts van Kessel,A., Turc-Carel,C., de Klein,A., Grosveld,G., Lenoir,G. and Bootsma,D. (1985) *Mol. Cell Biol.* 5, 427−429.

A new polymorphic probe on chromosome 22: NB5 (D22S201)

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Source/Description: NB5 is a 0.5 kb HindIII-EcoRI fragment isolated from two pooled chromosome 22 specific libraries (ATCC # 57733 and ATCC # 57714) and was subcloned into pUC9.

Polymorphism: TaqI digestion of genomic DNA and hybridization with the probe detects a four allele polymorphism: 1.75 kb (A1), 1.6 kb (A2), 1.4 kb (A3) and 1.3 kb (A4). No constant bands were present.

Frequency: Estimated from 97 unrelated Caucasians.
A1 : 0.072
A2 : 0.86
A3 : 0.052
A4 : 0.015

Not Polymorphic For: BglI, BglII, DraI, MspI and PstI.

Chromosomal Localization: Regional localization was established by hybridization to a panel of somatic cell hybrids: PgMe-25Nu, containing only human chromosome 22; PgMo-22 and 1CB-17ANu, respectively containing both products of the Philadelphia translocation at 22q11; A3EW2-3B, containing the t(11;22) from Ewing's sarcoma (at 22q12) and 1/22 AM27 containing a t(1;22) at 22q13 (1, 2). The probe was assigned to chromosome 22 between 22q13 and 22qter.

Mendelian Inheritance: Mendelian inheritance has been demonstrated in extended pedigrees of Gilles de la Tourette syndrome families (n = 380).

Probe Availability: Available for collaboration.

References: 1)Goyns,M.H., Young,B.D., Geurts van Kessel,A., de Klein,A., Grosveld,G., Bartram,C.R. and Bootsma,D. (1984) *Leukemia Res.* **8**, 547–553. 2)Geurts van Kessel,A., Turc-Carel,C., de Klein,A., Grosveld,G., Lenoir,G. and Bootsma,D. (1985) *Mol. Cell Biol.* **5**, 427–429.

Chapter III

Cytogenetic, molecular genetic and pathological analyses in 126 meningiomas

submitted for publication

Cytogenetic, Molecular Genetic and Pathological Analyses in 126 Meningiomas

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Abstract

In a series of 126 meningiomas, tumor and patient characteristics were investigated and statistically analyzed. A combined cytogenetic and molecular genetic approach was used to study chromosomal abnormalities and loss of markers on chromosome 22q. This approach was successfully applied to 93 meningiomas. In 66 cases complete or partial loss of chromosome 22 was observed and in at least 12 of them this chromosome was involved in structural aberrations. In addition to chromosome 22 changes, chromosomes 1, 6, 11, 13, 14, 18, 19, X, and Y were also frequently involved in structural and numerical aberrations. Statistical analysis revealed a significant association between the number of chromosomal abnormalities and tumor grade. Complex karyotypes predominated in the group of grade II/III meningiomas. Furthermore, other variables showed statistically (or marginally statistically) significant differences. Meningiomas from the convexity were more often grade II/III, displayed predominantly (partial) loss of chromosome 22 and had complex karyotypes more often. These features were frequently found in meningiomas from males. Base meningiomas on the other hand occurred more often in females, they were usually grade I, showed loss of (parts of) chromosome 22 less often and displayed fewer additional chromososmal abnormalities.

Introduction

Meningiomas are considered to be histologically benign neoplasms that account for 13-19% of all primary brain tumors treated by surgery (Russell and Rubinstein 1989). However, all meningiomas may display, to a certain extent, atypical or anaplastic features (Jääskeläinen et al., 1985).

Cytogenetically, meningiomas are the most extensively studied human solid tumors and they display a consistent chromosome aberration (Zang and Singer, 1967; Zang, 1982). These cytogenetic studies and molecular genetic analyses strongly suggest that loss of (parts of) chromosome 22 is a primary event in the development of these tumors and also indicate that several chromosome 22 loci might be involved in the process (Seizinger et al., 1987; Rey et al., 1988; Dumanski et al., 1990; Lekanne Deprez et al., 1991a; Rey et al., 1993; Lekanne Deprez et al., 1994). In addition, other less common non random chromosomal changes occur in some tumors, which might play a role in tumor progression (Katsuyama et al., 1986; Al Saadi et al., 1987; Casalone et al., 1987; Maltby et al., 1988; Rey et al., 1988; Vagner-Capodano et al., 1993; Biegel et al., 1994). One of these changes could also be the primary cause in a subset of meningiomas without chromosome 22 involvement, as a meningioma/ependymoma family did not show linkage to DNA markers flanking the NF2 locus on chromosome 22 (Pulst et al., 1993). More complex chromosome abnormalities appear to be associated with more atypical features (Vagner-Capodano et al., 1993).

Most meningiomas occur sporadically, although they are also found in patients with the autosomal dominantly inherited predisposition for Neurofibromatosis type 2 (NF2) or in multiple meningioma patients or very rarely as sole tumor type clustered in some families (Butti et al., 1989; Domenicicci et al., 1989; Russell and Rubinstein, 1989; McDowell, 1990; Sieb et al., 1992). Positional cloning approaches have resulted in the isolation of the *NF2* gene on chromosome 22q12 and mutations were observed in both the germ-line of NF2 patients and in sporadic tumors (Rouleau et al., 1993; Trofatter et al., 1993). Recently, mutation analyses of the *NF2* gene have revealed mutations in about 30% of the sporadic meningiomas (Lekanne Deprez et al., 1994; Ruttledge et al., 1994). This indicates that the *NF2* gene is important in the development of a large number of sporadic meningiomas, although it is still very well possible that other loci on chromosome 22 and/or other chromosomes are involved (see above). Studies on the association between chromosomal

abnormalities, histopathological and other tumor and patient characteristics of meningiomas are scarce (Zang, 1982; Al Saadi et al., 1987; Maltby et al., 1988; Casalone et al., 1990; Sanson et al., 1992; Vagner-Capodano et al., 1993) and in only two of these studies statistical analyses on some of these characteristics (Casalone et al., 1990; Vagner-Capodano et al., 1993) were performed. Investigation of the different characteristics of meningiomas could help in identifying different subclasses, which might explain the genetic alterations, not affecting the *NF2* gene, found in a subset of tumors. This study describes cytogenetic and molecular genetic findings in a large number of meningiomas. A significant association was found between characteristics like sex, site of tumor origin, histological subtype, grade, chromosome 22 loss and number of chromosomal abnormalities.

Materials and methods:

Tumor collection and tissue culture:

From November 1988 to September 1993 we collected 126 meningiomas (Table 1). If possible, tumor material obtained at surgery was divided in three or more parts. These parts were used for: 1) histopathology, 2) DNA isolation and 3) cytogenetic analysis. Tumor parts were frozen and stored in liquid nitrogen prior to DNA isolation and pieces were used directly for interphase in situ hybridization and tissue culture. Both preparation for tissue culture and tissue culture conditions were as described elsewhere (Koper et al., 1990).

Histological examination and localization:

Histopathological assessment was performed on formalin fixed 5 μm paraffin embedded tumor sections. The tumors were classified according to the WHO histological typing of tumors of the central nervous system (Zülch 1979) and Kepes et al. (1982). The following subtypes or mixtures of them were identified in our series: transitional (T), fibroblastic (F), meningotheliomatous (M), psammomatous (Ps), angiomatous (A), papillomatous (P), anaplastic (Ana), xanthomatous (X), lipoblastic (L), pleiomorphic (Pl), oncocytic (O), and haemangiopericytic (H).

The grading system used in this study was as described by Jääskeläinen et al. (1985). In this system a higher grade corresponds to an increased degree of anaplasia, which is

defined by evaluating 6 histological parameters (loss of architecture, increased cellularity, nuclear pleomorphism, mitotic figures, focal necrosis and brain infiltration). This resulted in three different grades: grade I (benign), grade II (atypical), and grade III (anaplastic). The grading of the meningiomas was carried out by one of us (SZS).

The anatomic sites of tumor origin were subdivided in convexity (C), base (B), falx (F), and intraventricular (IV).

Cytogenetic analysis and in situ hybridization:

Cytogenetic analysis was carried out on cultured samples, from passage 0 (p0) corresponding to the first outgrowth of cells to passage 3 (p3). Cytogenetic results were only taken into account when the cells were cultured for less than 50 days. Cells were harvested and fixed according to standard methods. Chromosomes were identified using RFA, QFQ and GTG banding techniques and described according to ISCN (1991).

Fluorescent in situ hybridization (FISH) was performed on interphase nuclei directly obtained from the fresh tumor sample or after short term culture. Competitive in situ hybridization was applied using at least two chromosome 22 specific cosmid or centromere probes following the technique as reported by Arnoldus et al. (1991). For all probes used, the number of signals per nucleus after hybridization was counted in at least 100 nuclei. As a control, 34 lymphocyte cultures of the patients (when available) and 23 lymphocyte cultures of normal donors were used. In both control groups we observed similar hybridization results: one spot (apparent monosomy) in about 5% of nuclei and two spots (disomy) in more than 90% of the nuclei. This allowed us to conclude the absence of constitutional loss of chromosome 22 in patients lymphocytes. Control data were used to determine the sensitivity of the technique used and to define the cut off rate (mean % of nuclei with one spot + two times the standard deviation) of 13.5% and 12%, respectively above which a cell population with (partial) monosomy could be safely identified.

The following probes cited from centromere to telomere of chromosome 22 were used: p22/1:2.1 (centromere, McDermid et al. 1986); 2 cosmids in the lambda immunoglobulin light chain gene region: lambda 2.2, lambda 3.1 (Goyns et al., 1984); 4 cosmid probes 40D2, 58B12, 50B11, 76A4 ordered from centromere to telomere within 100 kb from D22S193 (Chapter VI); 29B5 (D22S72); 103D12 (D22S56); 4 cosmid probes in the NF2 region: 115D7 (NEFH), 121G10, 96C10, 74B1. The cosmid probes in the vicinity of

D22S193, D22S72, D22S56 and NF2 were obtained by screening two chromosome 22-specific cosmid libraries (LL22NCO1 and LL22NC03: de Jong et al., 1989; Zucman et al., 1992).

LOH analysis of chromosome 22:

High-molecular-weight DNA was isolated from stored tumor tissue according to standard procedures. Before DNA isolation the percentage of tumor cells was determined by microscopic examination of a cryostat section of the tumor specimen. Only tumor samples with more than 80% tumor cells were used for DNA analysis. Most specimens contained more than 90% tumor cells. When available, constitutional DNA was isolated from peripheral blood leucocytes. Restriction endonuclease digestion, agarose gel electrophoresis, Southern transfer, hybridization to ^{32}P labeled probes and autoradiography were performed as reported (Lekanne Deprez et al., 1991a). The following DNA markers for loci on chromosome 22 were used: D22S181, D22S183 (Lekanne Deprez et al., 1991b), D22S10 (Hofker et al., 1985), D22S182 (Lekanne Deprez et al., 1991b), D22S1 (Barker et al., 1984), D22S193 (Lekanne Deprez et al., 1991b), 17.1 3'(3 prime part of the MN1 cDNA 17.1, which detects a Pvu II polymorphism, unpublished result), D22S29 (Rouleau et al., 1989), D22S45 (Budarf et al., 1991), D22S201 (Lekanne Deprez et al., 1991c) and D22S205 (van Biezen et al., 1993). D22S201 and D22S205 are located distal to D22S193, but their orientation relative to each other or D22S45 is not known. The other probes are ordered from centromere to telomere. For most of the tumor DNA samples analyzed in this study constitutional DNA was not available, therefore loss of heterozygosity (LOH) for a specific marker was assumed when a heterozygous DNA sample showed clear reduction of intensity of one of the 2 alleles.

Statistics:

The statistical analyses were performed with the Statistical Package for the Social Sciences (SPSS) and with the Epidemiological Graphics, Estimation, and Testing package (EGRET). We used the X^2 test, the Exact Homogeneity test, and Fisher's exact test. A significant association was concluded when $P \leq 0.05$. The following variables were mutually analyzed: the sex of the patients, the age at surgery (over and under 50 years of age), the site of tumor origin, the histological subtype, the grade, the loss of (parts of) chromosome 22, and the

number of clonal chromosomal abnormalities (chr abn). To analyze the latter variable the number of clonal chromosomal abnormalities was divided in two groups: $\leq$ 2 chr abn or $>$ 2 chr abn as seen by chromosome banding analysis.

Results

The 126 meningiomas that were studied are listed in table 1. This table shows the sex and age at surgery of the patients, the site of tumor origin, the histological subtype, grade, and the chromosome 22 status of the tumors. This series contains 13 tumors derived from 9 patients with two or more meningiomas, which were located at different sites, and 2 meningiomas from 2 sisters with NF2 (table 1). Of the 110 single meningioma patients without evidence of NF2, 82 were from females (average age 55.3) and 28 were from males (average age 57.5). In the complete group of meningiomas the site of tumor origin was subdivided in convexity 52.9% of the cases, base 37.2%, falx 7.4% and intraventricular 2.5%. Histopathologically 52.3% of the meningiomas were transitional, 16.7% fibroblastic, 11.9% meningotheliomatous and 19.1% were mixed or had a different subtype. Grading of the meningiomas resulted in 73% grade I (benign), 19.8% grade II (atypical) and 7.2% grade III (anaplastic) tumors. The chromosome 22 status (last 3 columns in table 1) is the result of karyotyping, FISH and LOH analysis. These analyses were carried out depending on the quantity of material available. FISH analysis of freshly obtained tumor cells was applied only in the second half of this series and proved to be a very sensitive tool for the detection of (partial) loss of chromosome 22 (table 1 and 2). In total 97 tumors could be examined with at least one of these techniques. For the interpretation of (partial) loss of chromosome 22 and number of clonal chromosomal abnormalities in the tumors (below and table 4), the tumors 10, 133 and 177 were excluded because discrepancies between the methods were found. This was probably due to inhomogeneity in these tumors with regard to the percentage tumor vs normal cells. Tumor 16 was excluded because only one cell could be analyzed. This cell, however, showed a very abnormal karyotype, including loss of chromosome 22. Tumor 176 was excluded with regard to the interpretation of number of clonal chromosomal abnormalities because incomplete cytogenetic data were available. FISH analysis in this tumor clearly showed loss of chromosome 22 (table 1). Therefore, we established that in 66

(71%) of the 93 meningiomas complete or partial loss of chromosome 22 was found.

Table 1. Characteristics of patients with meningiomas

No.	Sex/Age (yr)	Site of Tumor Origin[1]	Histological Subtype (grade)[1]	#22 status		
				Karyotype[2]	FISH(% loss)[3]	LOH[4]
1	M/66	C	T (I)			nc
3	F/60	C	M (II)			-22*
4	F/59	B	T (I)	-22		
5	F/55	B	T (I)			
6	F/63	F	T (II)			
7@	F/62	C	F (I)	-22,C		-22
8	M/65		F (I)			
9	F/47	C	T (II)	der(22),C		der(22)
10	F/67	F	T (I)	-22,C		2n
11	M/43	C	M/L/A/Ps (II)			
12	F/43	C	M (II)			nc
13	F/41	B	T (I)			
14	F/22	IV	T (II)	-22,C		
15	F/53	B	T (I)	2n		
16	M/68	IV	M/H (III)	-22,C?		
17	M/49	B	T (I)			
18	M/73	C	T (I)			-22
19	F/64	B	T (I)			

No.	Sex/Age (yr)	Site of Tumor Origin[1]	Histological Subtype (grade)[1]	#22 status		
				Karyotype[2]	FISH(% loss)[3]	LOH[4]
20	F/66	C	T (I)			
21	F/75	B	M (I)			
22@	M/39	C	T/Ps (I)			der(22)
23	M/49	C	T (I)			
24	F/31	B	T (I)	2n		
25	M/66	C	F (I)			-22
26	M/55	C	M/L (II)			
27	F/47	C	T/Ps (I)			-22*
28	F/64	B	Ps (I)			-22
29	F/84	C	T (I)	-22,C		-22
30	F/68	IV	M (III)	-22,C		-22
31	F/71	C	T (II)			-22
32@	M/64	C	T (II)	der(22),C		der(22)
33	F/41	C	T (I)			
34	F/57	B	T (I)			
35	F/40	B	T (I)	-22		
36	F/21	C	T (I)			
37	F/38	C	T (I)	2n		2n
38	F/66	C	T (I)			-22
39	F/63	C	T (II)	der(22),C		der(22)
40	M/59	C	T (II)			

No.	Sex/Age (yr)	Site of Tumor Origin[1]	Histological Subtype (grade)[1]	#22 status		
				Karyotype[2]	FISH(% loss)[3]	LOH[4]
41	F/71	C	T (II)			der(22)
43	M/65	B	T (I)			
44	M/42	C	M (III)			-22
46[@]	F/74	C	M/Ps (I)	-22		-22
48	F/51	C	T (I)			nc
50	F/59	B	Ps (I)	-22		-22
51	M/70	C	F (I)			der(22)
53	F/75	C	T/Ps (I)	-22		-22
54	M/55	C	T (I)	-22,C		
55A[5@]	F/30	C	F (I)			-22
55B	F/32	C	F (I)	-22		-22
55C	F/33	B	F (I)	-22		-22
55D	F/33	B	F (I)	-22		-22
59	F/72	B	M (II)	2n		2n
60[6]	F/47	C	M (II)	-22		-22
63[@]	F/78	B	T (I)			2n
64	F/36	B	T (I)	2n		2n
69	F/54	B	T (II)	-22,C		
70	F/56	B	T/Ps (I)			
71	F/59	C	M/A (I)			-22
72	F/62	B	T/Ps (I)	2n		

No.	Sex/Age (yr)	Site of Tumor Origin[1]	Histological Subtype (grade)[1]	#22 status		
				Karyotype[2]	FISH(% loss)[3]	LOH[4]
73	F/63	B	T (I)	2n		2n
74	M/73	C	T (I)			
76	F/48	B	T (I)	2n		
77	M/67	B	T (I)	2n		
78[7]	M/71	C	T (III)	der(22),C		
81	F/66	B	M (II)	+22,C		+22
82	M/33	C	T (II)	der(22),C		-22
85	F/46	C	T/Ps (I)	2n		2n
86	F/62	B	T (I)	2n		
87	F/57	C	T (I)			
88	F/34	C	M (I)	2n		2n
89[@]	F/55	B	T (I)	2n		
90	M/64	B	T (I)	2n		2n
92	F/43	C	T (II)			-22
93	F/74	C	M (III)		87%(dir)	-22
94[7]	M/72	C	Ana (III)			-22
95	F/70	C	Pl/A (II)			
99	F/69	C	T (I)		69%(P3)	-22
102	F/59	C	F (I)			-22
103	F/61	F	F (I)			-22
108	F/49	C	F (I)	-22	75%(P1)	-22

No.	Sex/Age (yr)	Site of Tumor Origin[1]	Histological Subtype (grade)[1]	#22 status		
				Karyotype[2]	FISH(% loss)[3]	LOH[4]
109	M/69	B	M (II)		73%(dir)	-22
111	F/64	C	Ps (I)	-22	88%(dir)	-22
116	M/41	C	M/A/O/Pl/L (III)			-22
118	F/20	C	T (I)			2n
119	F/71	B	T (I)		60%(dir)	-22
120	F/70	B	T (I)		4%(dir) ns	
121[6]	F/55	B	F (I)	der(22)	76%(P0)	der(22)
125	F/45	C	A/P (III)		74%(P0)	-22
126	F/74	F	T (III)	-22,C	74%(P1)	ni
127	F/34		T (I)	-22	90%(P1)	-22
128	M/50	C	T (II)	2n		
129	F/67	B	F (I)	-22	62%(dir)	-22
130	F/67	B	A (I)			
132	F/4	B	Ps (I)			
133	F/55	B	T (I)	2n	17%(dir)	2n
135	F/75	F	T/Ps (I)	-22,C	71%(P1)	-22
136	F/72	C	T (I)			2n
137	F/53	B	T (I)	2n	3%(P1) ns	
138	F/64	B	T (I)			2n
140	M/61	C	T/A (II)			der(22)
141	F/50	C	F (I)			nc

No.	Sex/Age (yr)	Site of Tumor Origin[1]	Histological Subtype (grade)[1]	#22 status		
				Karyotype[2]	FISH(% loss)[3]	LOH[4]
143	F/46	C	X (I)		63%(dir)	
144	M/62	C	T (II)		71%(dir)	der(22)
145	F/78	C	T (I)		54%(dir)	-22
147	M/21	F	M (I)	-22,C	74%(P1)	-22
148	F/58	C	F (I)	-22	53%(dir)	-22*
149	F/30	B	M (I)	-22	67%(P1)	
150	F/48	C	F (I)			-22
153	F/61	B	T/Ps (I)			2n
154[@]	M/?		F (I)			2n
158	F/45	B	T (I)	2n	5%(P0) ns	
161	F/64	C	F (I)			-22
163	M/72	F	F (I)	-22,C	54%(dir)	
164[&@]	F/61	C	F (I)		70%(dir)	
165	F/55	B	M (I)		20%(dir)	
166	F/45	B	T (I)	2n	8%(P0) ns	
170	F/39	F	T (I)	-22	50%(dir)	
172[&@]	F/61	C	F (I)	der(22),C	87%(dir)	
174	F/69	C	T (I)			
176	M/45	F	M (I)	-22?	77%(dir)	
177	F/61		T (I)	2n	28%(dir)	
179	F/30	B	M (II)			

No.	Sex/Age (yr)	Site of Tumor Origin[1]	Histological Subtype (grade)[1]	#22 status		
				Karyotype[2]	FISH(% loss)[3]	LOH[4]
180	F/76	B	T (I)	2n	9%(dir) ns	
181	F/42	C	T (I)			
183	M/56	C	T (II)	-22,C	49%(dir)	

[1] The abbreviations are given in the methodology section.

[2] For more details see table 3. -22 = clonal loss of one #22 in all or part of the metaphases; 2n = two normal #22; der(22) = partial deletion of #22 due to structural rearrangement; C = complex karyotype (> 2 chromosomal abnormalities); ? = cytogenetic analysis failed to draw a reliable conclusion.

[3] % of nuclei showing one spot for the #22 specific markers (see methods). Dir = fresh tumor; P0 = primairy outgrowth of the tumor; P1 = first passage in culture; P2 = second passage etc.; ns = no significant loss of #22 markers.

[4] LOH = Loss of heterozygosity for #22 specific polymorphic markers (see methods). -22 = loss of at least two #22 specific polymorphic markers; 2n = no loss of #22 observed; der(22) = partial deletion of #22 (see figure 1); ni = not informative; * = polymorphic for only one marker, which shows loss; nc = not conclusive, for technical reasons.

[5] No.55 A,B,C and D are meningiomas obtained from a patient with multiple meningiomas (Lekanne Deprez et al., 1994).

[6] No.60 and No.121 are 2 meningiomas found in 2 sisters from an NF2 family (Delleman et al., 1978).

[7] No.78 and No.94. Tumor 94 is a recurrence of tumor 78.

[8] No.164 and No.172. Two meningiomas located at different sites from one patient.

[@] Patient with at least two meningiomas located at different sites.

Table 2. **Comparison of FISH, cytogenetic and LOH techniques for detection of (partial) loss of chromosome 22 in meningioma**

	Number of tumors (%)			
Technique	Total*	Disomy 22	(partial) loss of 22	not evaluable
FISH	30	5 (16.7%)	25 (83.3%)	0
Cytogenetics	54	20 (37%) (13X: normal) (7X: other abn.)	34 (63%) (10X: -22 only) (24X: -22 and other abn.)	0
LOH	69	13 (18.8%)	50 (72.5%)	6 (8.7%)

* The following tumors were excluded for reasons described in the result section, FISH: 133, 177; Cytogenetics: 10, 16, 133, 176, 177; LOH: 10, 133.

The results of cytogenetic analysis of 59 meningiomas cultured for less than 50 days are shown in table 3. Longer culture times usually resulted in normal karyotypes, which was probably due to outgrowth of normal cells initially present in low numbers in the cultures. This was seen also when repeated cytogenetic analysis of meningiomas with an altered karyotype resulted in normal karyotypes in the course of time (data not shown). Thirteen meningiomas showed a normal or non-clonal karyotype and in 41 cases clonal chromosomal abnormalities were found. Twenty three of the cases with chromosomal abnormalities showed $\leq$ 2 changes, including chromosome 22 aberrations in 17 cases. In eighteen meningiomas more than 2 chromosomal abnormalities, incuding chromosome 22, were identified. In at least 12 meningiomas chromosome 22 was involved in structural abnormalities. These are further discussed below. Partial or complete loss of chromosome 22 was the most frequently observed abnormality (63%) in this series of meningiomas. In addition to this other chromosomes also showed non random abnormalities in all or sublines of the cells. Of these, chromosomes 1 (27.7%), 6 (13%), 11 (13%), 13 (14.8%), 14 (18.5%), 18 (13%), 19 (24.1%), X (20.5%), and Y (20%) were most often involved (table 3). Complete or partial loss of these chromosomes was the predominant abnormality.

Table 3. Summary of chromosomal findings in 59 meningiomas

No.	Days in culture	Karyotypes and/or clonal rearrangements [no. of cells]
4	P2	44,X,-X,-22 [16]
7@	15	47,XX,+7,+21,-22 [18]/ idem, 9q+ [3]
9	35	45,XX,-19,-22,+dic(22pter->q11?::19p12?->qter) [6]/ 42-44,idem 1p-,6q-,11p+,-21,+variable additional aberrations [7]
10	4	43,XX,-14,-19,-22 [14]/ 42,XX,tas(7;19)(pter;qter),-14,-19,-22 [2]
14	33	44,XX,dic(1qter->q10::19pter->qter),add(2)(p21),der(2)t(2;8)(q14?;q21), add(4)(q21),der(8)t(2;8)(p21;q21),del(12)(p12),t(14;16)(q11;q11-12), der(18)t(2;18)(q14;p11),-22 [10]/ 46,XX [3]
15	19	46,XX [5]/ 44-45, random losses [3]
16	17	85,XX,1p-[2x],1q-[2x],-10,-11,-12,-16[2x],-20[2x],-22[2x],+6 markers [1]
24	38	47,XX,+mar(?del(13)(q14q22)) [16]
29	27	45,XX,dic(1;19)(1qter->p13::19q11->pter),del(13)(q13q21),+20,-22 [5]/ 46,idem,+20 [11]/ 45,idem,+20,-21 [8]. Additional structural aberrations were observed in 10 of the cells in the different clones.
30	19	41,XX,add(1)(p12),dic(1;2)(p10;q10),-3,-4,der(6)t(1;6)(q12;q14), der(8)t(8;13?)(q24;q14?),del(11)(q11),-13,der(16)t(4;16)(q21;p13), der(18)t(11;18)(q12;q12),der(19)t(3;19)(q11;q12),-22 [7]/ 42,idem,+ring [6]/ 84,XXXX,idem tetraploid,+2 ring [3]
32@	15	45,XY,-1,t(4;22)(p16?;q11),der(22)t(1;22)(p11;q11) [8]/ 44,idem,13p+,-14 [2]/44,idem,-10,+12,13p+,-14 [6]/ 45,idem,-10,+12,13p+,-14,+16 [12]/90,idem,tetraploid [2]

No.	Days in culture	Karyotypes and/or clonal rearrangements [no. of cells]
35	6	45,XX,-22 [14]/ 90,XXXX,-22,-22 [2]
37	9	46,XX,add(2)(q22) [20]
39	49	45,XX,1q-,3q+,-10 [3]/ 44,XX,2q-,-6,-7,10p+,11p+,13q+,19q+,-22,22q+,+fragment [10]/ 45,XX,2q-,-6,-7,10p+,11p+,-13,19q+,-22,22q+,+fragment,+ring [3]/ 46,XX [5]
46[a]	14	45,XX,-22 [16]
50	14	45,XX,-22 [12]/ 45,XX,r(17),-22 [3]/ 44,XX,-22,tas(9;17)(qter;pter)[1]
53	28	45,XX,-22 [13]/ very aberrant tetraploid including tas [2]
54	35	43,XY,-14,-19,-22 [3]/ 42,X,-Y,-14,-19,-22 [6]/ 46,XY [6]
55B[b,a]	14	45,XX,-22 [1]/ 44,X,-X,-22 [8]/ 40-43,idem,with random losses [5]
55C	10	45,XX,-22 [24]/ 89,hypotetraploid,-22[2x],with additional losses and rearrangements [1]
55D	39	45,XX,-22 [3]/ 46,XX [2]
59	18	46,XX [16]
60[b]	35	45,XX,-22 [4]/ 46,XX [12]
64	49	46,XX [16]
69	32	43,XX,der(1)t(1;16)(p11;q11),dic(6qter->p12::16q11->pter or 22q11-qter),-14, -16,-22 [16]

No.	Days in culture	Karyotypes and/or clonal rearrangements [no. of cells]
72	21	46,XX [16]
73	14	46,XX [16]
76	21	46,XX [23]
77	18	46,XY [20)]
78[7]	5	Very aberrant hypodiploid to hypotetraploid karyotype. Clonal aberrations: t(1;2)(q41?;q22?),-19,19q+,22q+ [13]. Also many tas and HSR
81	25	56(54-59),X,-X,1p-,+1p-,+3,+5,6q+,+13,+17,+18,(+19),+20,(+20),(+22), +various markers [9]/ 46,XX [1]
82	3	44,der(X),Y,1p-,t(1;6)(p32;p21),2q-,-4,-5q-,+6q-,6p+,der(7),10q+,12q-, der(12)t(12;17)(q10;q10),-15,-19,22q-,der(22)t(2;22)(q11;?;q11) [7]/ idem,+additional aberrations:9q+ or -9,11p+ or -11,-21 [10]
85	14	46,XX [16]
86	35	46,XX,t(7;14)(q35;q11-12) [9]/ 46,XX [3]
88	32	46,XX [18]/ tetraploid [2]
89[@]	21	45,XX,-11 [3]/ 47,XX,+X [4]/ 46,XX [42]
90	38	46,XY [14]/ tetraploid [4]
108	10	45,XX,-22 [16]/ 44,XX,dic(13;22)(p11;p11),-22 [4]/ 44-45,XX,-22,and variable tas and whole arm translocations [6]
111	28	45,XX,-22 [12]/ 44,XX,-22,dic or tas involving chromosome 19 [4]
121[6]	49	46,XX,22q- [7]

No.	Days in culture	Karyotypes and/or clonal rearrangements [no. of cells]
126	32	46,XX,1p-,-6,11p+[2x],-14,-18,-21,-22 [1]/ 40,X,idem,-X,8q-,9p+,-13 [1]/ 39,X,idem,-X,-7,8q-,9p+,-13,20p+ [4]/ 39,X,idem,-X,-8q-,9p+,-13 [10]
127	32	45,XX,-22 [33]
128	28	45,X,-Y [3]/ 46,XY [13]
129	5	45,XX,-22 [31]/ idem,+tas [7]
133	49	46,XX [13]/ 92,XXXX [2]
135	28	42,X,-X,-1,-12[3],-14,-17[3],-18[3],-19[6],-20[3],-22,+r[10] [cp17]
137	21	46,XX [20]
147	46	42,XY,i(1q),-4,-14,-18,-22 [18]/ tetraploid,idem [1]
148	11	44,XX,-18,-22 [10]/ 46,XX [2]
149	28	45,XX,-22 [16]
158	7	46,XX,t(3;7)(p12?;q35) [20]
163	8	42,X,-X,-14,-19,-22 [6]/ 41,idem,-21 [6]/ 41,idem,-13 [4]. In 10 cells various tas were observed involving 19[9x],3[7x],14[6x] etc.
166	10	46,XX [17]
170	9	45,XX,-22 [9]/ 43-44,idem,+random losses [9]

No.	Days in culture	Karyotypes and/or clonal rearrangements [no. of cells]
172[&@]	6	44,X,del(X)(q13),trc(1;22;19)(1qter->p12::22cen->q11::19cen->qter), inv(7)(p14-15q34-35),der(15),der(16)t(16;22)(q24;q?),i(18p), and various additional rearrangements in some cells [30]
176	17	46,XY [5]/ 30-34,-22,incomplete [3]
177	10	46,XX [16]
180	5	46,XX [16]
183	6	42-43,XY,dic(1;19)(p12;p13)[23], del(11)(p12)[2],dic(11;21)(p11;p12)[3], tas(11;21)(pter;pter)[5],-14[23],tas(19;21)(qter;pter)[3],-21[5],-22[23] [cp23]

For abbreviations see legends table 1.

Structural abnormalities of chromosome 22 were identified in 12 meningiomas. The breakpoints observed after cytogenetical analysis were all in band 22q11 (table 3). In 10 tumors we were able to map the breakpoints using a combined LOH and FISH study (figures 1,2). Five tumors (9, 22, 39, 41 and 140) showed retention of proximal chromosome 22 markers and loss of distal markers with breakpoints scattered between D22S181 and 17.1 3'. Tumor 121, a meningioma in an NF2 patient, revealed an interstitial deletion with the proximal breakpoint between D22S181 and D22S183 and the distal breakpoint between D22S29 and D22S45. In the tumors 51 and 144 loss of proximal chromosome 22 markers and retention of distal markers were observed. In these latter meningiomas chromosome 22 could either be involved in an interstitial deletion or a translocation resulting in a deletion of the proximal part of this chromosome. Two tumors (32, 82) showed structural aberrations of both chromosome 22 copies (table 3). In meningioma 32 one copy of chromosome 22 was involved in a reciprocal translocation t(4;22) from which the 22q- and the 4p+ derivative chromosomes were the products and the other copy (22q+) was involved in a translocation with chromosome 1 resulting in der(22)t(1;22) as previously reported (fig.2; Lekanne Deprez et al., 1991a). FISH and RFLP analysis on hybrid cell lines segregating the reciprocal translocation products and on cultured cells derived directly from meningioma 32 enabled us

to map both breakpoints (data not shown). The t(4;22) breakpoint was located about 80 kb telomeric from D22S193 and the breakpoint on the 22q+ marker mapped distal to cos 76A4 (fig. 2). In meningioma 82 one der(22) contained a part of chromosome 2 and another unidentified chromosomal fragment, the other copy of chromosome 22 was present as a 22q- marker (table 3). LOH study in this tumor revealed only 2 informative markers (17.1 3' and D22S45), which both showed LOH (data not shown). This indicated that the breakpoint, on one of the 2 markers, was located proximal to D22S193.

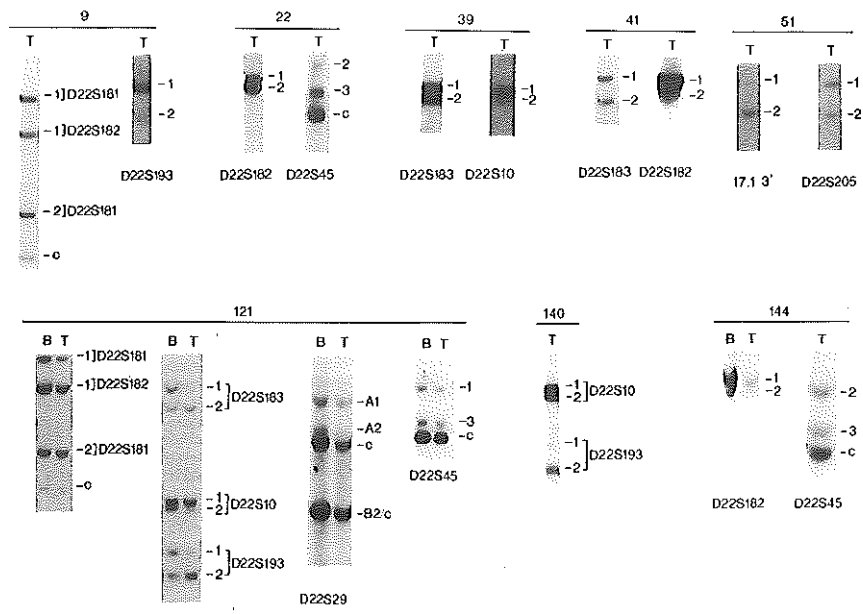

Figure 1. Southern blot analysis of meningiomas displaying partial deletions for 22q. T, tumor tissue DNA; B, blood leucocyte DNA. For each tumor both chromosome 22 markers showing loss and markers showing retention of heterozygosity are depicted respectively. The polymorphic alleles are indicated with 1, 2 and 3. C represents a constant band. D22S29 detects 2 polymorphisms: A1/A2 and B2. The weak remaining signal is probably caused by contamination with normal cells of the tumor sample. The following marker/enzyme combinations were used: D22S181 and D22S182/BglII; D22S183, D22S193 and D22S10/PstI; 17.1 3' and D22S45/PvuII; D22S29 and D22S205/TaqI.

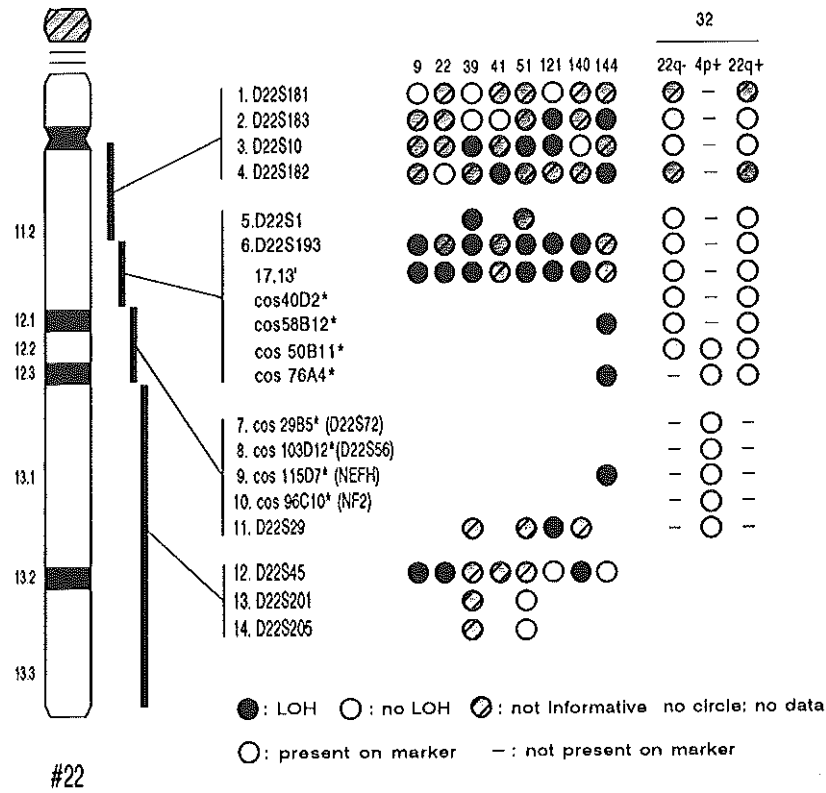

Figure 2. Schematic representation of deletions of chromosome 22q. The localization of the different markers on 22q with their most likely order are indicated (see methods). Markers with asterisks (*) were used for FISH and all the others for Southern blot analysis (figure 1). For meningioma 32 we investigated both hybrid cell lines segregating the 22q- and the 4p+ derivative chromosomes respectively and cultured tumor 32 cells using FISH and Southern blotting.

Statistical analyses concerning age and sex were performed only on the sporadic meningioma cases, omitting the multiple meningioma and NF2 patients, to avoid any kind of bias. All the other analyses were carried out on the complete group minus the tumors described above in the case of loss of chromosome 22 or number of chromosomal abnormalities. When we looked at the age distribution, after correcting for population distribution (Damhuis et al., 1993), we observed a biphasic curve in both male and female patients with a decline in incidence between the age of 50 to 54 years. Two other studies revealed a similar decline in number of female patients (Dumanski et al., 1990; Vagner-Capodano et al., 1993), but one other study by Casalone et al. (1990) did not. A subdivision of our patients in two groups, over and under 50 years of age at surgery, did not result in any significant association with one of the in the methods section mentioned variables. Therefore, larger studies are necessary to further investigate the significance of this incidence profile, if any. Other patient and tumor characteristics however revealed some interesting associations (table 4). Three of them were marginally significant: 1) We found that only 18.5% of the male meningiomas were located at the base, whereas 43.8% of the female tumors were observed at that site (P=0.058). 2) Female patients predominated in the group of meningiomas with $\leq$ 2 chromosomal abnormalities (74.3% versus 33.3%, P=0.06). 3) Loss of (parts of) chromosome 22 was found in 88.5% of the grade II/III tumors but in only 64.2% of the grade I tumors (P=0.079). Statistically significant associations were observed in all the following instances: 1) Grades II/III meningiomas were more often found in male than in female patients (46.4% versus 22%). 2) Base meningiomas were mostly grade I (88.9%) and grade II/III meningiomas were more frequently derived from the convexity (70.6%). 3) In 87.8% of the convexity meningiomas partial or complete loss of chromosome 22 was found, but in only 40.6% of the base tumors this was the case. All intraventricular and falx meningiomas showed (partial) loss of chromosome 22. 4) Almost all (92%) base meningiomas had $\leq$ 2 chromosomal abnormalities, whereas 52.4% of the convexity tumors and only 14.3% of the intraventricular and falx meningiomas had $\leq$ 2 chromosomal abnormalities. 5) All fibroblastic meningiomas were grade I, while 60% of the meningothelial meningiomas were graded II/III and grade II/III comprised only 27.4% of all tumors analyzed. 6) Most (94.7%) of the fibroblastic meningiomas showed (partial) loss of chromosome 22. Tumors without loss of chromosome 22 were predominantly of the transitional type (74.1%). 7) In 78.6% of the grade II/III meningiomas $>$ 2 chromosomal

abnormalities were found, whereas in only 17.5% of the grade I tumors this was the case. 8) Loss of (parts of) chromosome 22 was observed in 94.4% of the tumors with > 2 chromosomal abnormalities but in only 47.2% of the meningiomas with ≤ 2 chromosomal abnormalities.

Table 4. Frequency tables between different tumor and patient characteristics showing statis significant (or marginally significant) associations

Sex and Site.

	M	F	Tot
C	18	37	55
B	5	35	40
F and IV	4	8	12
Tot	27	80	107

Exact homogeneity test (2 sided); P=0.058

Sex and Grade.

	M	F	Tot
I	15	64	79
II/III	13	18	31
Tot	28	82	110

$X^2(1) = 5.029$; P=0.025

Sex and number of Chr abn.

	M	F	Tot
≤2 Chr abn	3	26	29
>2 Chr abn	6	9	15
Tot	9	35	44

Fisher's exact test (2 sided); P=0.06

Grade and Site.

	I	II/III	Tot
C	41	24	65
B	40	5	45
F and IV	7	5	12
Tot	88	34	122

$X^2(2) = 10.074$; P=0.006

Chromosome 22 loss and Site.

	Loss #22	No Loss	Tot
C	43	6	49
B	13	19	32
F and IV	10	0	10
Tot	66	25	91

Exact homogeneity test (2 sided); P<0.001

Number of Chr abn and Site.

	≤2 Chr abn	>2 Chr abn	Tot
C	11	10	21
B	23	2	25
F and IV	1	6	7
Tot	35	18	53

Exact homogeneity test; P<0.001

Grade and Subtype.

	I	II/III	Tot
F	21	0	21
T	49	17	66
M	6	9	15
Mixed and Others	16	8	24
Tot	92	34	124

$X^2(3) = 16.601$; P=0.001

Chromosome 22 loss and Subtype.

	Loss #22	No Loss	Tot
F	18	1	19
T	25	20	45
M	9	3	12
Mixed and Others	14	3	17
Tot	66	27	93

Exact homogeneity test; P=0.008

Chromosome 22 loss and Grade.

	Loss #22	No Loss	Tot
I	43	24	67
II/III	23	3	26
Tot	66	27	93

$X^2(1) = 6.028$; P=0.079

Number of Chr abn and Grade.

	≤2 Chr abn	>2 Chr abn	Tot
I	33	7	40
II/III	3	11	14
Tot	36	18	54

$X^2(1) = 16.183$; P<0.001

Chromosome 22 loss and number of Chr abn

	Loss #22	No Loss	Tot
≤2 Chr abn	17	19	36
>2 Chr abn	17	1	18
Tot	34	20	54

$X^2(1) = 12.434$; P=0.002

The following abbreviations, else than described in the methodology section, were: M = male; F = female; Tot = total number.

Discussion

This series of meningiomas contained 13 tumors from 9 patients with more than one meningioma, in whom there was no evidence of NF2. Therefore, the frequency of multiple meningiomas and/or meningiomatosis in our study is 7.6% (9/119). This is in the same range, between 4.5% and 10.5%, with other published series since the introduction of CT scanning (Domenicucci et al., 1989 and cited references). No differences could be observed between these multiple meningioma cases and the sporadic cases in all factors investigated in this study, in accordance with the findings of Domenicucci et al. (1989).

In agreement with previous reports all cytogenetically abnormal meningiomas, except tumor 81, showed a hypodiploid karyotype, with loss of (parts of) chromosome 22 as the most common aberration. FISH analysis performed in the second half of this study suggested that the % of meningioma with loss of chromosome 22 was higher than detected by cytogenetic and/or LOH studies. This is in support with a postulated role of chromosome 22 as the primary event in the development of (the majority of) meningiomas. In spite of this, other changes involving chromosomes 1, 6, 11, 13, 14, 18, 19, X, and Y were also frequently found. These observations corroborate and extend previous findings (Katsuyama et al., 1986; Al Saadi et al., 1987; Casalone et al., 1987; Maltby et al., 1988; Rey et al., 1988; Logan et al., 1990; Vagner-Capodano et al., 1993; Biegel et al., 1994). The most consistent aberrations described in many papers are those of chromosome 1 with changes of both the p- and q-arm and of chromosome 14. When the non-22 chromosomal aberrations found in our series were independently compared with the histological grade of the tumors, all these changes were predominantly observed in grade II and III tumors. Therefore, it is difficult to unmask specific changes that underlie progression or a more anaplastic histology. The association between abnormalities of chromosomes 1 and 14 and a more malignant histology was also found by Doney et al. (1993). In addition, they observed that loss of 14q24-32 is the smallest region of overlap on chromosome 14. Interestingly we found reciprocal translocations with chromosome 14 both in band 14q11-12 in two meningiomas (tumors 14 and 86). In tumor 86 it is the only cytogenetic abnormality found and this case showed a benign histopathology. Changes concerning chromosome 6 were recently reported to be the most common secondary alteration in pediatric meningiomas (Biegel et al., 1994). The frequent involvement of chromosome 6 was also found by us but our tumors were all

from adult patients, indicating that aberrations of chromosome 6 are not restricted to childhood meningiomas. It remains to be determined whether some of these changes are important in the pathogenesis of meningiomas or are a result of genetic instability. A gene on a chromosome other than 22 might even be the primary cause of meningioma development in (some of) the cases without chromosome 22 involvement as the studies of Ruttledge et al. (1994) and Pulst et al. (1993) suggest that in a subclass of meningiomas chromosome 22 is not involved.

Structural chromosomal alterations are an important tool in defining the localization of genes involved in cancer development and progression. This approach has resulted in the designation of three different loci on chromosome 22 that might be involved in the development of meningiomas. The most proximal chromosome 22 locus was defined because a reciprocal t(4;22) in a meningioma disrupts a gene, located in band 22q11 (Lekanne Deprez et al., 1991a; Chapter VI). The second locus represents the recently isolated *NF2* gene at 22q12 (Rouleau et al., 1993; Trofatter et al., 1993). The meningiomas described in this paper were used also for a mutation study of the *NF2* gene (Lekanne Deprez et al., 1994). The most distal locus has been defined because 2 meningiomas showed terminal deletions of chromosome 22 with the most distal breakpoint telomeric to the *MB* gene (Dumanski et al. 1990; Rey et al., 1993). Therefore, this locus might be located on 22q12.3-qter. In our study we found 12 tumors with structural aberrations of chromosome 22. The common region that is lost in these tumors includes all three of the above mentioned loci.

The statistical analyses suggest that different meningioma subclasses do exist. In our series of meningiomas we found 8 statistically significant associations and 3 interesting trends. These associations corroborate and extend previous findings, as the other studies did not investigate as many variables and statistical analyses was only performed in two of these studies. The statistically significant associations observed were that grade II/III meningiomas predominated in males and that base meningiomas were mainly grade I, whereas grade II/III meningiomas were most often found in the convexity (this study, Vagner-Capodano et al., 1993). Furthermore, we found that base meningiomas compared with tumors derived from the convexity had less chromosomal abnormalities, which was also observed by Casalone et al. (1990). The following observations were described as trend or mentioned by others but were statistically significant in our series of patients. The observation that meningothelial meningiomas showed more malignant features was described by Zang (1982) as well. The

finding that the majority of grade II/III meningiomas had complex karyotypes was in agreement with findings of Vagner-Capodano et al. (1993). They also found that telomeric associations were restricted to grade II/III tumors an observation which we did not confirm. The difference between the grading of the tumor and loss of chromosome 22 was shown to be a trend in our study but statistically significant in the study of Sanson et al. (1992). In addition, we and others found that female patients predominated in the group of meningiomas with less complex karyotypes (Zang, 1982; Maltby et al., 1988; Casalone et al., 1990). However, this association was marginally significant (this study, Casalone et al., 1990). Statistically significant associations that were not described in other papers were the observation that base meningiomas were hardly observed in men (trend), and that loss of chromosome 22 was mainly observed in convexity, falx or intraventricular meningiomas and found in only 40% of the base tumors. Furthermore, most fibroblastic meningiomas revealed (partial) loss of chromosome 22 and loss of (part of) this chromosome was found in almost all meningiomas with complex karyotypes, whereas only half of the meningiomas with less complex karyotypes revealed loss. Therefore, one might speculate that in meningiomas with chromosome 22 involvement genetic instability is induced.

Recent studies might suggest that a subclass of meningiomas exists in which genes on chromosome 22 are not involved (Pulst et al., 1993; Ruttledge et al., 1994). Furthermore, studies in meningiomas have suggested that several chromosome 22 loci might play a role (see above). One or more of the statistically significant differences observed in this study could be the result of the distinct genetic alterations found in meningiomas. Longer follow-up of these patients will enable us to find out whether these differences are of predictive value concerning the clinical behaviour of the meningiomas. This might eventually lead to a classification system that predicts the clinical consequence of a certain tumor for the patient. Interestingly in this regard is the study of de la Monte et al (1986). They found a statistically significant association between meningioma recurrences and different histopathological features.

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Chapter IV

Familial anaplastic ependymoma: evidence of loss of chromosome 22 in tumor cells

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Familial anaplastic ependymoma: evidence of loss of chromosome 22 in tumor cells

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Abstract

A family is presented with anaplastic ependymomas, histologically verified in 3 cases, and neuroradiologically suggested in a 4th case. Two healthy brothers both had 2 affected sons. All 4 male patients were younger than 5 years old at the time of diagnosis. Two boys died before the age of 3 years. Genotype analysis (using polymorphic DNA markers for chromosome 22 and interphase cytogenetic analysis) of one of the tumors, showed a subpopulation of tumor cells with monosomy of (part of) chromosome 22. Nonneoplastic cells of this patient showed a normal karyotype. These findings give further evidence for the role of a tumor suppressor gene on chromosome 22 in the pathogenesis of familial ependymal tumors.

Introduction

Familial occurrence of brain tumors is infrequent, and has been reported especially for medulloblastomas, glioblastomas and astrocytomas. Concurrence of ependymal tumors in 2 sisters made up the first report on familial brain tumors, but subsequent familial ependymal tumors have been reported rarely (Tijssen et al., 1982).

Cytogenetic and DNA studies on ependymal tumors are relatively scarce (Ranson et al., 1992). Reviewing the literature, deletions or translocations of chromosome 22 were

observed in 12 out of 30 analysed ependymomas. Monosomy 22 was found in one tumor of a family with 2 sisters and a maternal male cousin with ependymomas (Savard and Gilchrist, 1989). We present a family with 4 male cousins whose fathers are brothers, with verified ependymal brain tumors in 3, and neuroradiological evidence in the 4th.

Family study

The pedigree of the family is shown in figure 1. No signs of neurofibromatosis were present in patients A to D, nor in their relatives. The mothers of the patients were not related neither consanguinous to their partners, and did not use medication during or around pregnancy. Spontaneous abortion occurred once in the mother of patient A and B, and four times in the mother of patients C and D. A sister of the (affected childrens') paternal grandfather died at 2 years of age, after an operation for a brain tumor, but no medical or pathology records were available. Tracing of the family pedigree according to paternal links, was possible back to 1689. This showed several childhood deaths in each generation, possibly because of higher childhood mortality at that time. Cerebral MRI of the father of patients C and D was normal.

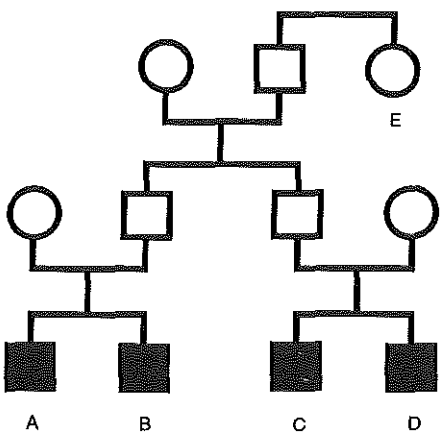

Figure 1. Family pedigree. Patients A to D had proved ependymal brain tumors, patient E died at 2 years after operation for a brain tumor (anecdotal).

PATIENT A, born 1977, presented at the age of 5 with headache, vomiting, somnolence, and right hemiataxia. CT-scan showed a tumor in the fourth ventricle and right cerebellar hemisphere (fig. 2A). Treatment consisted of tumor extirpation, postoperative radiotherapy and chemotherapy (methotrexate, vincristine and prednisolone). Hydrocephalus developed, but the patient improved after drainage. The patient is now 15 years old and is quite well, except for moderate cognitive disturbances and slight ataxia.

PATIENT B, born 1981, a brother of patient A, presented at the age of 3 days old with irregular respiration, and slight icterus. A low-quality CT-scan did not allow a specific diagnosis. Six months later, he was readmitted for drowsiness, vomiting and weight loss. CT-scanning showed a large tumor in the fourth ventricle, with contrast enhancement and hydrocephalus (fig. 2B). Despite ventricular shunting he died several days later. Autopsy was not permitted, and there is no histology of the tumor.

PATIENT C, born 1989, a first cousin of patients A and B, was admitted at the age of 21 months, with vomiting, lethargy, increasing skull circumference and delayed development of motor skills. CT-scanning and MRI revealed a large intraventricular supratentorial tumor with contrast enhancement (fig. 2C). Angiography showed a large tumor blush with vascularisation mainly from choroid arteries. Craniotomy revealed a highly vascular subependymal tumor. He died 4 months after the biopsy.

PATIENT D, born 1990, a brother of patient C, was followed regularly because of the family history. At the age of 2 months a cerebral CT-scan was normal. At the age of 8 months he started vomiting,and showed delayed motor skills. CT scanning and MRI showed an infratentorial tumor with contrast enhancement (fig. 2D). The tumor was surgically removed, and used for genetic analysis. Postoperative treatment consisted of chemotherapy with vincristine, procarbazine and methotrexate. At 2.5 years old a routine MRI showed a small tumor recurrence at the operation site, which has been extirpated recently.

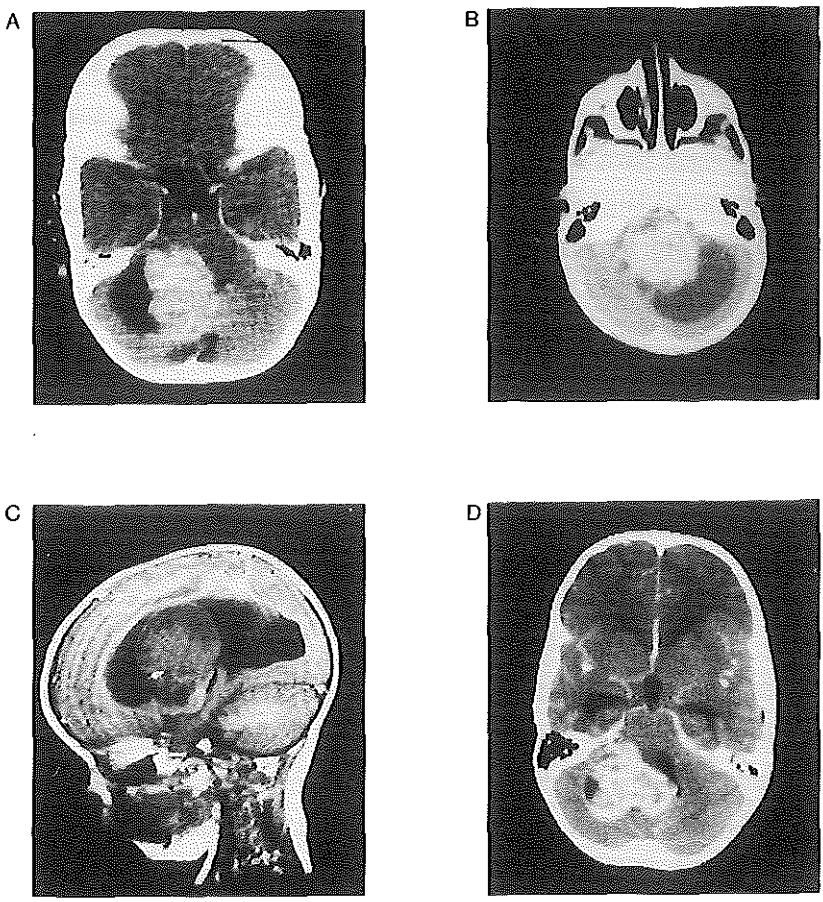

Figure 2. Cerebral scans. A, CT of patient A, showing a infratentorial tumor with contrast enhancement. B, CT of patient B. C, MRI of patient C, showing a large supratentorial intraventricular tumor. D, CT of patient D.

Microscopic examination (patients A,C &D,fig. 3). All tumors were well vascularized and highly cellular, with nuclear polymorphism, several mitoses and irregular amounts of cytoplasm. Immunohistochemistry was positive for GFAP, EMA, NSE, S-100, LEU-7, LCA, vimentin and $\alpha_1$antitrypsin, and negative for several other labels . In patient C some cells were arranged in ribbons, and rosettes and microcystic spaces were found. In patient D typical tubuli and some necrosis were present. Electronmicroscopy of this tumor showed a uniform population of cells, with well developed rough endoplasmatic reticulum and Golgi-complex. Desmosomal structures and interdigitating villi were seen frequently.

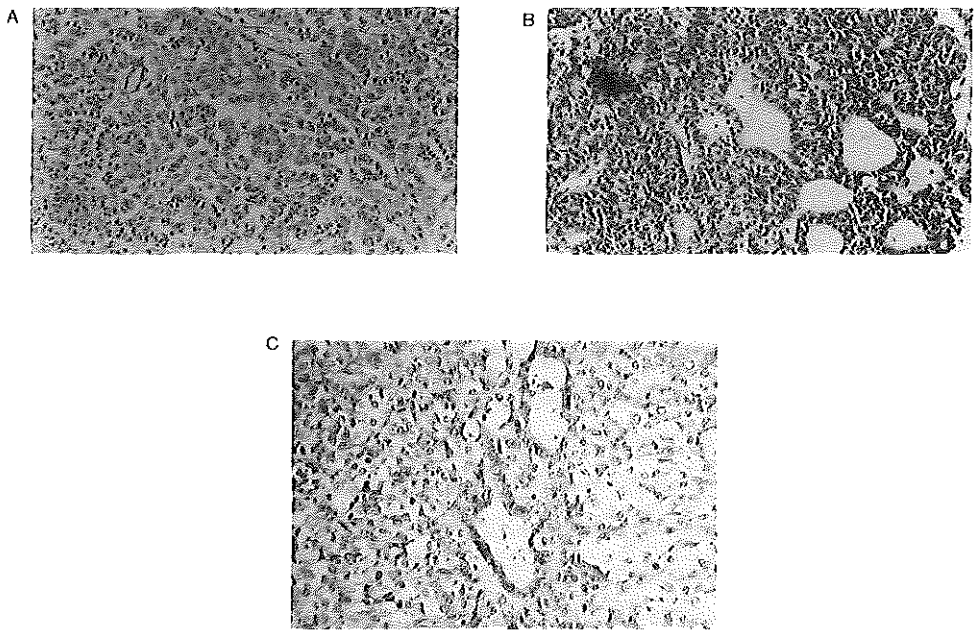

Figure 3. Histopathology, hematoxylin and eosin stain. A, patient A (obj 25X). B, patient B (obj 25X). C, patient D (obj 40X).

Genetic analysis

Fresh tumor tissue obtained from patient D at neurosurgery was used for DNA extraction and interphase cytogenetic analysis. DNA extraction, Southern blotting and hybridization were performed according to standard procedures. The following polymorphic DNA markers for loci on chromosome 22 were used: D22S181, D22S183, D22S10, D22S182, D22S1, D22S193, cos 50B11. The probes are ordered from centromere to telomere (van Biezen et al., 1993).

INTERPHASE CYTOGENETICS. Nuclei isolated from freshly removed tumor cells, were investigated for aberrations of chromosomes #1, 6, 7, 10, 11, 17, 18, 22, X and Y by fluorescent in situ hybridisation (FISH), according to Arnoldus et al (1990). In addition, we used probes specific for the centromeric regions of chromosome 22 (p22/1:2.1), and two cosmid probes (cos 58B12 & cos 50B11) that are located close to D22S193 on band 22q11. One hundred cells were counted for each probe. Cultured lymphocytes from blood of an unrelated subject were used as control.

Results

Cytogenetic analysis of respectively lymphocytes and fibroblasts in patients C and D showed a normal male karyotype. The tumor of patient D was investigated in more detail. Interphase cytogenetic analysis performed on freshly removed tumor tissue with probes for chromosome #1,6,7,10,11,15,17,18,X and Y revealed results in the normal range. This indicates a diploid tumor, without gross chromosomal aberrations of these chromosomes.

Additional techniques were applied to investigate chromosome 22. Interphase cytogenetics on freshly removed tumor tissue showed only one fluorescent spot in about 76% of nuclei, using 3 probes for chromosome 22 (table 1). Moreover, 7 polymorphic probes for chromosome 22 were used to study possible loss of heterozygosity (LOH) in tumor DNA. D22S193 was the only informative probe. This probe clearly showed LOH when DNA derived from freshly removed tumor tissue was compared with DNA from fibroblasts of the same patient. In addition, we looked at mutations in the recently cloned *NF2* gene, using RNA SSCP analysis. No evidence for mutations was observed in RNA from this tumor. However, the same method showed mutations in 36% of 53 meningiomas and schwannomas

(Lekanne Deprez et al., 1994).

Table 1. **Interphase cytogenetic analysis of tumor of patient D**

		Number of spots per nucleus		
Tissue	Probes	0	1	2
fresh tumor	p22/1:2.1	6	73	21
	cos 58B12	8	76	16
	cos 50B11	6	79	15
blood control*	p22/1:2.1	7	10	83
	cos 58B12	8	13	79

*Cultured lymphocytes from blood of an unrelated subject.

Discussion

The histology and immunohistochemistry, intraventricular location, age of onset, and identical neuroradiological patterns indicate that the tumors in all four patients are similar, and of ependymal origin. While they are not typical ependymomas, they lack the major criteria for ependymoblastomas. Considering age of onset, location, morphology and immunohistochemistry, we designate these tumors as anaplastic ependymomas. Familial ependymal tumors are very rare, and to our knowledge 9 families have been reported. These include three families with 2 or more ependymal tumors (Tijssen et al., 1982; Savard and Gilchrist, 1989), and 5 ependymal tumors associated with glioma, plexus papilloma and medulloblastoma (Tijssen et al., 1982; Sato et al., 1984). While coincidence may account for part of these concurrences, the occurrence of 4 affected sons in a single generation, born from 2 healthy brothers, strongly suggests the involvement of a genetic predisposing mutation.

DNA and interphase cytogenetics of the tumor of patient D showed loss of (part) of

chromosome 22 in a major percentage of tumor cells, indicating the involvement of a tumor suppressor gene. Although loss of chromosome 22 in tumor tissue might be a secondary effect of tumorigenesis, the consistent observation of loss of chromosome 22 in our and other reported cases, suggest a causal relationship. Because ependymomas are sometimes found in neurofibromatosis type 2, we searched for mutations in the *NF2* gene, in RNA from the tumor of patient D. No mutation was detected, thus we have no evidence that the *NF2* gene is involved in this family. However, there are indications for additional loci on chromosome 22 involved in the development of NF2 related tumors.

The likely transmission of the disorder by 2 healthy brothers, their exclusively male offspring, the occurrence of spontaneous abortions and the history of a brain tumor in a paternal aunt, raise questions on the possible mode of inheritance. It cannot be explained by simple classic mendelian genetics, and variation in expression of an autosomal dominant gene is a proposed explanation, but lacks precision. Whether sex-related effects of the transmitting parent (imprinting), or a gene with a variable insert size may be involved remains open to speculation, and awaits the isolation and characterization of the gene involved.

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Chapter V

A t(4;22) in a meningioma points to the localization of a putative tumor suppressor gene

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A t(4;22) in a Meningioma Points to the Localization of a Putative Tumor-suppressor Gene

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Summary

Cytogenetic analysis of meningioma cells from one particular patient (MN32) displayed the stem-line karyotype 45, XY, −1, 4p+, 22q−, 22q+, which thus had rearrangements of both chromosomes 22. The 22q+ marker appeared as a dicentric: 22 pter→q11::1p11→qter. The reciprocal product of this translocation has presumably been lost because it lacked a centromere. The 22q− chromosome also appeared to have lost sequences distal to band q11. We assumed that this marker could have been the result of a reciprocal translocation between chromosomes 4 and 22. To investigate the 4p+ and 22q− chromosomes in more detail, human-hamster somatic cell hybrids were constructed that segregated the 22q− and 4p+ chromosomes. Southern blot analysis with DNA from these hybrids showed that sequences from 22q were indeed translocated to 4p+ and that reciprocally sequences from 4p were translocated to 22q−, demonstrating a balanced t(4;22)(p16;q11). On the basis of these results we presume that in this tumor a tumor-suppressor gene is deleted in the case of the 22q+ marker and that the t(4;22) disrupts the second allele of this gene. The latter translocation was mapped between D22S1 and D22S15, a distance of 1 cM on the linkage map of this chromosome. The area in which we have located the translocation is within the region where the gene predisposing to neurofibromatosis 2 has been mapped.

Introduction

The involvement of a tumor-suppressor gene in the etiology or pathology of a particular type of cancer is often indicated by loss of a specific chromosome or part of a chromosome (Cavence et al. 1986). Until now five tumor–suppressor genes have been isolated; these include the retinoblastoma gene (Weinberg 1989), the p53 gene (Eliyahu et al. 1989; Finlay et al. 1989), a gene called "DCC" which is involved in the pathology of colorectal carcinoma (Fearon et al. 1990), a gene involved in Wilms tumor associated with the WAGR syndrome (Call et al. 1990; Gessler et al. 1990; Rose et al. 1990), and the gene which is involved in the predisposition to neurofibromatosis 1 (NF1; Cawthon et al. 1990; Viskochil et al. 1990; Wallace et al. 1990).

Meningioma is a common benign mesenchymal intracranial or intraspinal tumor arising from arachnoidal cells surrounding the brain and spinal cord. Cytogenetic analysis and RFLP analysis of these tumors reveal a specific chromosomal aberration. In about 50% of the tumors one copy of chromosome 22 is lost (Zang 1982). In a few percent of the cases other aberrations of chromosome 22, such as translocations (breakpoints are mapped in region 22q11–22q12) or deletions of the larger part of the long arm, are found (Katsuyama et al. 1986; Al Saadi et al. 1987; Casalone et al. 1987; Dumanski et al. 1987; Meese et al. 1987; Seizinger et al. 1987*a*; Maltby et al. 1988; Rey et al. 1988; Casartelli et al. 1989; Poulsgård et al. 1989). These findings suggest that inactivation of both alleles of a tumor-suppressor gene on chromosome 22 is a causal event in the development of meningioma.

The autosomal dominant disorder neurofibro-

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matosis 2 (NF2) predisposes to acoustic neurinomas, meningiomas, and other tumors of the central nervous system. Loss of sequences on chromosome 22 has also been observed in tumors associated with NF2 (Seizinger et al. 1986; Seizinger et al. 1987*b*; Rouleau et al. 1990). The gene predisposing to NF2 has been located between D22S1 and D22S28 by using linkage analysis (Rouleau et al. 1990). At present it is not clear whether the gene predisposing to NF2 is the same as the gene involved in sporadic meningioma.

In the course of our search for the exact position of the meningioma locus on chromosome 22, we analyzed tumor cells from a patient in whom both copies of chromosome 22 were involved in translocations. One of the marker chromosomes was the result of a reciprocal translocation between chromosomes 4 and 22. We presume that this translocation has inactivated a tumor-suppressor gene. Using hybrid cell lines we mapped the translocation breakpoint to a position between D22S1 and D22S15.

Material and Methods

Patient

The patient, born in 1925, had a history of head injuries: in 1931 he had a car accident resulting in brain damage, and in 1947 he had a concussion of the brain, after which his sense of smell was impaired. In 1968 an olfactorial meningioma was removed surgically. In 1989 a second meningioma (MN32), located on the right temporal side of the brain, was removed. This tumor showed a combination of three histological types: syncytial, transitional, and fibroblastic. These are characteristic of all meningiomas. The localization of the tumor was completely outside the previous operation position, and it was not considered to be a local recurrence.

Tissue Culture, Hybrid Cell Lines, and Cytogenetics

The meningioma specimen was obtained within 30 min after surgery. Both preparation for tissue culture and tissue culture conditions were as described elsewhere (Koper et al. 1990). Chromosome analysis was carried out on cultured cells after one passage using G- (trypsin-Giemsa), R- (acridine orange), and Q- (quinacrine) banding.

The somatic cell hybrids were constructed according to a method described elsewhere (Geurts van Kessel et al. 1981). Interspecies hybrid cell lines were obtained by fusion of thymidine kinase–deficient (TK^-) A3 Chinese hamster cells with cultured meningioma cells. Inactivated Sendai virus was used as the fusogen. Independent hybrid clones were selected in F10 medium supplemented with HAT (hypoxanthine, aminopterin, and thymidine), 2 μM Ouabain, and 15% FCS (Biological Industries). The hybrid cell lines were analyzed by R-banding; at least 10 metaphases/hybrid cell line were studied. This analysis was repeated several times during culture of the hybrid cell lines. Possible rearrangements between hamster and human chromosomes were verified using chromosome painting. This was done by biotinylation of human DNA and by in situ hybridization on metaphase spreads of the hybrid cell lines. The eventual presence of the marker 22q+, which resembles hamster chromosome 6, was also analyzed by using this technique.

DNA Extraction, Southern Blotting, Hybridization, and Densitometric Analysis

High-molecular-weight DNA was isolated from cultured tumor cells (first passage) and from hybrid cell lines according to standard procedures. DNA samples were digested to completion with restriction enzymes (Boehringer Mannheim and Promega) and were separated by electrophoresis, transferred to nylon membranes (Hybond N^+; Amersham), and hybridized to radiolabeled probes according to a method described by Feinberg and Vogelstein (1983). Conditions for hybridization are slightly modified from those described by Amasino et al. (1986). Blots were hybridized at 65°C in 0.25 M sodium phosphate pH 7.2, 0.25 M NaCl, 7% SDS (biochemical 44244; British Drug Houses), 1 mM EDTA, and 10% polyethylene glycol (M_r 6 × 10^3, biochemical 44271; British Drug Houses). After hybridization, blots were washed once in 3 × SSC, 0.1% SDS for 20 min at 65°C; once in 1 × SSC, 0.1% SDS for 20 min at 65°C; and once for 20 min at 65°C in 0.3 × SSC, 0.5% SDS. Membranes were exposed to Kodak XAR-5 film at −70°C with an intensifying screen. The following loci were examined: c-abl (0.6-kb *Eco*RI/*Bam*HI fragment; Heisterkamp et al. 1983), bcr (a third-exon *Pst*I c-DNA probe of 393 bp; Heisterkamp et al. 1985), D22S1 (Barker et al. 1984; Julier et al. 1988), D22S15 (Rouleau et al. 1988), myoglobin (MB, a third-exon PCR probe; Weller et al. 1984), D22S201 (this probe has been localized on 22q13-qter; N. A. van Biezen, unpublished data), D4S62 (Thayer et al. 1987), F5.53 (Bakker et al. 1987), and D4S125 (Nakamura et al. 1988). The sequence of these probes on the long arm of chromosome 22 is centromere–bcr–D22S1–D22S15–MB–c-sis–telomere. The location of these probes on the

short arm of chromosome 4 is centromere–D4S62–F5.53/D4S125–telomere. Quantitative densitometric scanning of the X-ray films was performed with a model 620 video scanning densitometer (Bio-Rad Laboratories).

Results

Cytogenetic analysis of meningiomas after surgery and short-term tissue culture was carried out routinely. In one case (MN32) we observed that both copies of chromosome 22 were altered. Table 1 shows the results of the cytogenetic analysis, indicating that all meningioma cells show an abnormal karyotype. Besides the basic chromosomal aberrations, additional aberrations were found in some of the cells. These additional aberrations probably arose after the tumor had been formed. The basic stem line 45, XY, −1, 4p+, 22q−, 22q+ is shown in figure 1. One marker chromosome (22q+) is involved in a translocation with chromosome 1, creating a dicentric chromosome: 22 pter→q11::1p11→qter. The 22q− marker has also lost sequences distal to band q11. The aberrations of chromosome 22 are not constitutional, because control cells of the patient displayed a normal karyogram.

To investigate the presence of sequences on the long arm of chromosome 22 in the tumor cells, we prepared a Southern blot carrying tumor DNA versus human placental DNA and hybridized it with probes located on chromosome 22. As a control a probe for the c-abl gene, which is located on chromosome 9, was used. The results are illustrated in figure 2, together with the densitometric analysis of the hybridization signals. When the hybridization signals of the meningioma lane and those of the placental control lane are compared, it appears that in the meningioma cells probably two copies of c-abl, bcr, and D22S1 are present and that presumably only one copy each of D22S15 and D22S201 is present. Also, densitometric analysis of the autoradiographs is in agreement with this hypothesis (fig. 2). The placental lane contains about 1.8 times more DNA than does the meningioma lane. Thus, these findings suggest that the chromosome 22 probes located at 22q11 (bcr and D22S1) are still present in two copies and that sequences distal to D22S1 (e.g., D22S15 and D22S201) are definitely present in MN32 but probably in only one copy.

The karyotype of tumor MN32 indicates that both marker chromosomes (22q− and 22q+) have lost sequences distal to band q11. However, hybridization results (fig. 2) showed that these sequences are retained in the tumor cells. Furthermore, the karyotype showed a 4p+ marker suggesting a possible t(4;22). This possibility was investigated using somatic cell hybrids. MN32 tumor cells were fused with a hamster cell line (A3). This resulted in (*a*) five independent human-hamster hybrid cell lines that segregated the aberrant chromosome 22q− and (*b*) three independent hybrid cell lines that segregated the 4p+ marker chromosome. Representatives of this group of hybrid cell lines are hybrids 6A and 14G-10. In 80% of the cells from cell line 6A the 22q− chromosome was found; additional human chromosomes in this line were 3, 6, 9, 11–13, 15–17, 20, and X. Cell line 14G-10 contained the 4p+ marker in 90% of the cells, next to human chromosomes 3, 5, 8, 9, 14, 16, 17, 19–21, and X. No hybrid cell lines which segregated the 22q+ chromosome were obtained.

DNA isolated from the hybrid cell lines and from human and hamster control cells was used for Southern blot analysis with probes from the relevant chromosomal regions. The results obtained with hybrids 6A (22q−) and 14G-10 (4p+) are illustrated in figure 3. DNA derived from hybrid 6A (22q−) hybridized with D22S1 (fig. 3*A*) but not with D22S15 (fig. 3*B*). DNA from hybrid 14G-10 (4p+) hybridized only with D22S15 (fig. 3*B*). Other probes distal to D22S15, e.g., MB, behaved in a manner analogous to that of

Table 1

Cytogenetic Findings in Meningioma 32 (MN32)

No. of Chromosomes	Sex Chromosomes	Missing or Abnormal Chromosomes	No. of Cells
45	XY	−1, 4p+, 22q−, 22q+	8
44	XY	−1, 4p+, 13p+, −14, 22q−, 22q+	2
44	XY	−1, 4p+, −10, +12, 13p+, −14, 22q−, 22q+	6
45	XY	−1, 4p+, −10, +12, 13p+, −14, +16, 22q−, 22q+	12
90	XY	Same, tetraploid	2

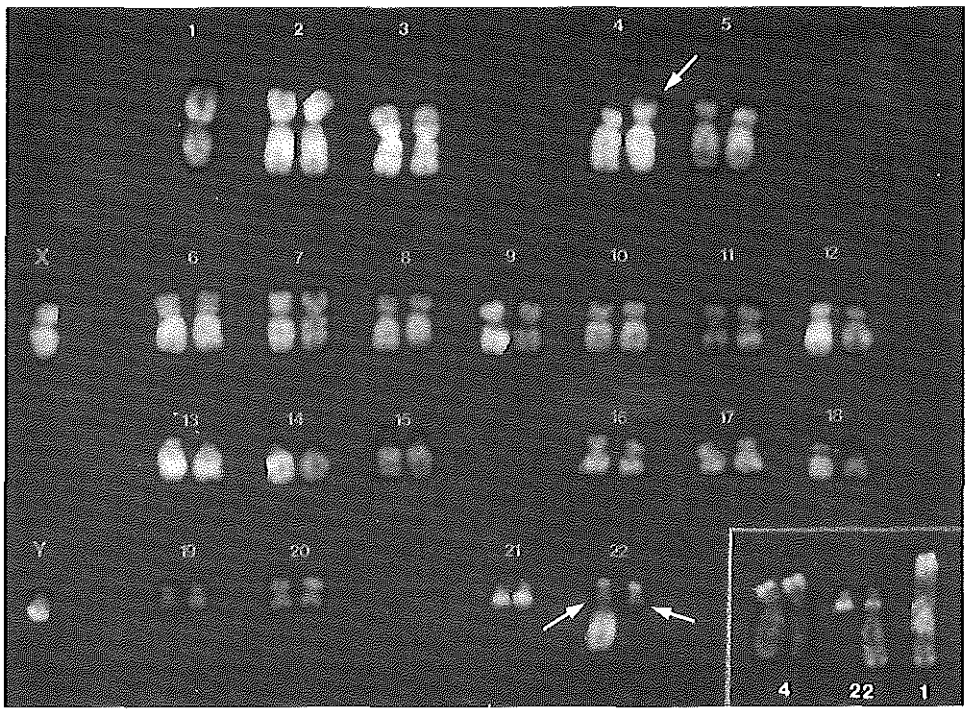

Figure 1 Basic stem-line karyotype of meningioma 32 (MN32): 45, XY, −1, 4p+, 22q−, 22q+. Arrows indicate structural abnormalities (Q-banding). Insert shows the normal and aberrant chromosomes from pairs 4, 22, and 1 (R-banding).

D22S15 (results not shown). This indicates that sequences on 22q distal to D22S1 are indeed present on the 4p+ marker. To investigate the possibility of a balanced translocation t(4;22), we hybridized the same blots with probes located on 4p16. Probe D4S62, which has been located at position 4p16.2-16.1, recognizes a 2.4-kb fragment in both placental and 14G-10 lanes (fig. 3C). This suggests that sequences up to position 4p16.2 are still retained on the 4p+ marker chromosome. Probe F5.53 (fig. 3*D*) recognizes in placental DNA the cognate fragment of 6.5 kb and a cross-hybridizing band at 3.4 kb. Both bands were mapped on chromosome 4, at positions 4p16.3 (6.5 kb) and 4p16.1-15.1 (3.4 kb) (G. J. van Ommen, personal communication). The 3.4-kb band is also present in lane 14G-10. Thus, sequences from 4p16.1-15.1 are retained on the 4p+ chromosome, a finding which confirms the results obtained with probe D4S62. The cognate 6.5-kb band is found in hybrid 6A, suggesting that this part of chromosome 4 has been translocated to the 22q− marker chromosome. This conclusion was confirmed by the finding that probe D4S125 (located at 4p16.3) also hybridizes to DNA from hybrid 6A but not to that from 14G-10 (result not shown). The same reciprocal marker segregation pattern was observed with the other hybrid cell lines.

Thus, taken together the hybridization results strongly suggest that the 4p+ and 22q− marker chromosomes are indeed the products of a reciprocal translocation t(4;22) (p16;q11). Figure 4*A* shows an outline of the position of the probes on chromosomes 4 and 22, and in figure 4*B* their position on the translocation products is indicated. Hybridizations with 25 other single-copy probes for chromosome 22 (N. A. van Biezen, unpublished data) showed that all probes

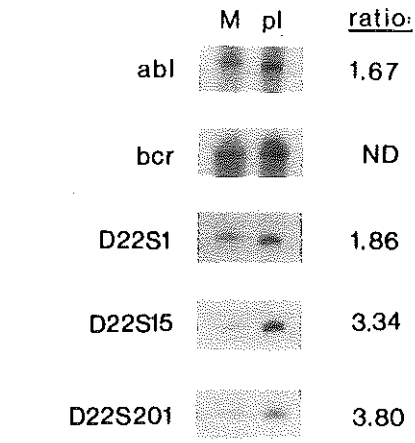

Figure 2 Analysis of copy number of different probes in MN32. Lane M, DNA isolated from MN32. Lane pl, DNA isolated from a human placental control DNA. Also indicated is the ratio of the area under the curve of placental and MN32 signals after densitometric scanning. ND = not done.

could be located either on the 4p+ or on the 22q− chromosomes (results not shown). This finding underlines the conclusion that the t(4;22) is indeed reciprocal and that no substantial parts of chromosome 22 appear to be missing.

Discussion

To localize the putative tumor-suppressor gene involved in meningioma, we selected a tumor (MN32) in which all cells contained the basic chromosomal aberrations −1, 4p+, 22q−, and 22q+, with additional aberrations appearing in some of the cells (table 1). The basic aberrations are probably involved in the etiology of the tumor and included two structurally abnormal chromosomes 22. This is in agreement with the indicated role of chromosome 22 in the development of meningioma (Zang 1982). The meningioma described in our study was the second meningioma in this patient. The second tumor was found far away from the position of the first, which argues against recurrence of the first tumor. To explain the occur-

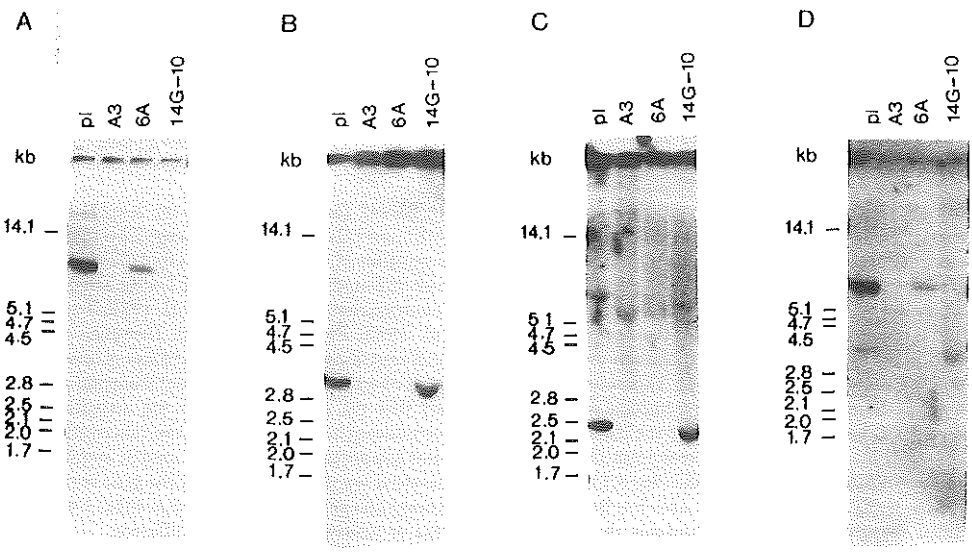

Figure 3 Southern blots of DNA from (hybrid) cell lines. Lane pl, Human placental control DNA. Lane A3, Chinese hamster (A3) DNA. Lane 6A, Hybrid MN32/A3 6A (22q−). Lane 14G-10, Hybrid MN32/A3 14G-10 (4p+). *A*, *Hin*dIII-digested DNA probed with D22S1. *B*, *Eco*RI-digested DNA probed with D22S15. *C*, *Eco*RI-digested DNA probed with D4S62. *D*, *Hin*dIII-digested DNA probed with F5.53. Besides the cognate hybridization signal at 6.5 kb, there is also a cross-hybridizing band (3.4 kb), which is located at 4p15.1-16.1 (G. J. van Ommen, personal communication).

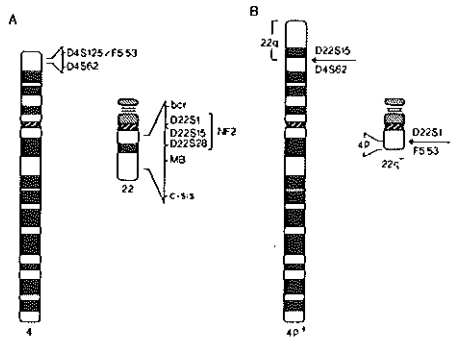

Figure 4 *A*, Regional localization of probes for loci on normal chromosomes 4 and 22. The area in which the NF2 gene has been mapped is indicated. *B*, Schematic representation of reciprocal t(4;22), creating 4p+ and 22q− marker chromosomes in meningioma MN32. The breakpoints (indicated with arrows) are shown according to DNA probes for chromosomes 4 and 22.

rence of two meningiomas one could argue that the patient is in fact suffering from NF2. However, this is not very likely, considering the finding that the tumor cells contained two aberrant chromosomes 22 whereas control cells of the patient displayed a normal karyotype. It is known that meningiomas may occur following trauma (Preston-Martin et al. 1980, 1983). This patient suffered from several head injuries, which may explain the occurrence of two meningiomas in this case.

In MN32 both chromosomes 22 are involved in translocations. One copy of chromosome 22 (22q−) is involved in a balanced translocation with chromosome 4: t(4;22)(p16;q11). Both products of the reciprocal translocation are still present in the tumor. In this case, inactivation of a meningioma tumor–suppressor gene at or near the translocation breakpoint represents a very likely hypothesis. If this mechanism of inactivation is operative, we would expect that the exact localization of the breakpoint corresponds to the localization of the gene. Hybridization experiments using hybrid cell lines segregating the reciprocal products of the t(4;22) were performed to map the breakpoints on chromosome 4 and 22. The breakpoint on chromosomes 4 is located between D4S62 (4p16.1) and F5.53 (4p16.3) (fig. 3*C* and *D*). This area has been investigated intensively because it contains the gene presumed to be responsible for Huntington chorea (Bucan et al. 1990). The distance between D4S62 and F5.53 is estimated to be 3 cM, on the basis of multilocus linkage analysis (Cheng et al. 1989). On chromosome 22 the breakpoint was mapped between D22S1 and D22S15 (fig. 3*A* and *B*). The distance between D22S1 and D22S15 is at most 1 cM, and the cumulative lod score between these loci is 5.35 at a recombination fraction of zero (Roulcau et al. 1989; Zhang et al. 1990). This localization agrees with the localization of translocation breakpoints in six other meningiomas, which were all mapped at 22q11 (Casalone et al. 1987; Maltby et al. 1988; Rey et al. 1988). A schematic representation of the reciprocal translocation t(4;22) is indicated in figure 4*B*.

The other copy of chromosome 22 (marker 22q+) is also involved in a translocation, leading to a dicentric chromosome: 22 pter→q11::1p11→qter. The reciprocal product of this dicentric chromosome was not found, probably because it lacks a centromere. As D22S1 is probably still present in two copies in the tumor DNA (fig. 2), it seems that the breakpoint in this marker is distal to D22S1. Sequences distal to D22S1 are presumably present in only one copy in MN32 (fig. 2), and we showed that these sequences were present on marker chromosome 4p+ as a result of the reciprocal t(4;22). Therefore it seems that the translocation breakpoint in the dicentric 22q+ chromosome is also located between D22S1 and D22S15. This rearranged chromosome could have lost the tumor-suppressor gene together with the reciprocal product of the translocation. If this is the case, we would expect that the translocation in the 22q+ marker is closer to the centromere than is the breakpoint in the 22q− chromosome. It is also possible that this translocation, too, disrupts the tumor-suppressor gene.

In MN32 the localization of the t(4;22) between D22S1 and D22S15 on chromosome 22 is identical to the position of the translocation t(11;22)(q24;q12), which is found in most cases of Ewing sarcoma and of neuroepithelioma (McKeon et al. 1988; Turc-Carel et al. 1988; Zhang et al. 1990). However, the balanced translocation in Ewing sarcoma is reminiscent of that observed in chronic myeloid leukemia and suggests that the t(11;22) leads to the activation of a proto-oncogene rather than to the inactivation of a tumor-suppressor gene as is the case in meningioma. Therefore we would expect that the gene involved in meningioma and the one involved in either Ewing sarcoma or neuroepithelioma are different.

So far, two earlier reports have suggested a localization of the meningioma tumor–suppressor gene. Both are in conflict with the localization suggested by our experiments. Dumanski et al. (1987) describe a men-

ingioma in which cytogenetic analysis shows a partial deletion of the q arm of one copy of chromosome 22. RFLP analysis of DNA derived from this tumor shows loss of one copy of the c-sis gene. Although the MB probe in this patient was not informative, the authors claim that densitometric analysis of the autoradiographs shows that the MB gene is still present in two copies. This suggests that localization of the tumor-suppressor gene should be distal to MB. MB is approximately 20 cM distal to D22S1/D22S15 (Julier et al. 1988). On the basis of these data and the mapping of the NF2 gene, it has been suggested that the meningioma tumor-suppressor gene and the gene predisposing to NF2 are different genes. The second report (Zhang et al. 1990) describes a meningioma also with a cytogenetically observed 22q – chromosome. In situ hybridization with probe D22S15 suggests that this fragment is still present on the 22q – chromosome and that, consequently, the suppressor gene is expected to be distal to D22S15. Thus, as of yet there is no consensus on the position of the meningioma tumor-suppressor gene. It could be that there are indeed two genes, with the more distal one being responsible only for sporadic meningioma and with the proximal one being involved in both sporadic meningioma and NF2. However, it is also possible that the t(4;22) induces a position effect, which could alter the expression of a distally located gene.

The present report is the first detailed localization of a reciprocal translocation breakpoint in meningioma. We presume that this translocation interferes with either the structure or the expression of a tumor-suppressor gene. Recently, two translocations that have been described in families with NF1 have led to the successful isolation of the gene predisposing to this disease (Cawthon et al. 1990); Viskochil et al. 1990; Wallace et al. 1990). The area in which we have located the translocation is within the region where the gene predisposing to NF2 has been mapped (Wertelecki et al. 1988; Rouleau et al. 1990). Therefore, on the basis of our results it is possible that the putative meningioma tumor-suppressor gene located at the t(4;22) and the gene predisposing to NF2 are one and the same.

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Chapter VI

Molecular cloning of a gene disrupted by a balanced translocation in a meningioma

to be submitted

Molecular Cloning of a Gene Disrupted by a Balanced Translocation in a Meningioma

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Abstract

We have previously mapped a putative meningioma suppressor gene at the chromosome 22 breakpoint of a t(4;22)(p16;q11), which was observed in meningioma 32. We now report the isolation of a gene (*MN1*) from chromosome 22, which is disrupted by the translocation breakpoint. The *MN1* gene spans about 70 kb and consists of at least two large exons of approximately 4.7 kb and 2.8 kb. The 4.7 kb exon is disrupted by the t(4;22). There is no obvious homology in the nucleotide and putative amino acid sequences with other known genes. The gene is highly conserved in evolution and northern blots show a ubiquitously expressed mRNA of 8 kb with an alternative 4.5 kb transcript in skeletal muscle. In meningiomas the expression pattern is very variable. Some, including meningioma 32, show no expression suggesting that the gene could function as a tumor suppressor gene for meningeal cells. Paradoxically, however, a very high expression is sometimes also observed in meningiomas.

Introduction

Meningiomas are primary tumors of the arachnoidal layer of the meninges and they usually occur as benign and slow-growing tumors, that account for 13%-19% of all intracranial and 25% of all intraspinal tumors (Russell and Rubinstein, 1989). The majority of the meningiomas are sporadic. However, they are also frequently observed in patients with the hereditary disorder neurofibromatosis type 2 (NF2), in patients with multiple meningiomas or (rarely) as familial meningiomas (Butti et al., 1989; Domenicucci et al., 1989; Russell and Rubinstein, 1989; McDowell, 1990; Sieb et al., 1992). This could suggest that there might be a genetic predisposition for the development of meningiomas distinct from that for NF2. Another indication for genetic heterogeneity is that recently linkage analysis in a meningioma/ependymoma family excluded the NF2 region (Pulst et al., 1993). Both cytogenetic and molecular genetic studies have shown that complete or partial loss of chromosome 22 is a very frequent event in the development of meningiomas (Zang, 1982; Seizinger et al., 1987; Dumanski et al., 1990) suggesting that a tumor suppressor gene(s) located on the q-arm of chromosome 22 plays a crucial role in the pathogenesis of these tumors. Similar results were obtained in both sporadic and NF2 associated (vestibular) schwannomas (Seizinger et al., 1986; Bijlsma et al., 1992). The *NF2* gene has been isolated recently (Rouleau et al., 1993; Trofatter et al., 1993). Mutations in the gene, that were observed in both sporadic and NF2 associated tumors, suggest that this gene is indeed frequently involved in the tumorigenesis of meningiomas and vestibular schwannomas (Rouleau et al., 1993; Trofatter et al., 1993; Bianchi et al., 1994; Bourn et al., 1994; Irving et al., 1994; Jacoby et al., 1994; Lekanne Deprez et al., 1994b; Pykett et al., 1994; Ruttledge et al., 1994; Sainz et al., 1994; Twist et al., 1994). Apart from the putative existence of a meningioma locus which was not linked to the NF2 region (Pulst et al., 1993) other evidence exists for aberrations on chromosome 22 which are not in the vicinity of the *NF2* gene. These include the telomeric region which shows LOH in some meningiomas (Dumanski et al., 1990; Rey et al., 1993), a more proximal region in which we observed a germline deletion in a patient with multiple meningiomas and a tumor associated balanced t(4;22) (Lekanne Deprez et al., 1991; Lekanne Deprez et al., 1994a). In this paper we describe the molecular analysis of the latter breakpoint and the identification and isolation of a gene, *MN1*, which is disrupted by the translocation breakpoint and which is presumed

to play a role in the development of meningiomas.

Methodology

PFGE, Southern and northern blots. Agarose plugs were prepared by imbedding $0.5x10^6$ T24 and $3x10^6$ hybrid (6A, 14G-10, 9B) cells in low-melting temperature agarose (FMC). Following deproteinization steps, DNA in the blocks was digested to completion with the appropriate restriction enzymes. After digestion the blocks were rinsed with TE (10mM Tris pH 7.5, 1mM EDTA) and one third was applied to a 1% agarose gel. Electrophoresis was carried out in 0.5xTBE for 20 hours at 12.5 °C, 160 V, 165 mA and a switch time of 45 sec using a Contour-clamped Homogeneous Electric Field (CHEF) system (BioRad). After electrophoresis gels were stained with ethidium bromide and the DNA was transferred to Hybond N^+ filters (Amersham) by electroblotting in 0.5xTBE at 120 V, for 2 h in a cooled BioRad transblot cell. DNA extraction, southern blotting, radiolabeling of probes, hybridization and autoradiography were performed as reported (Lekanne Deprez et al., 1991). Northern blot analysis was carried out using northern filters containing 2 μg of poly A^+ RNA from different human tissues (Human MTN, Clonetech, CA) and filters containing glyoxal denatured total RNA isolated from primary meningioma specimens using the guanidinium thiocyanate method (Chirgwin et al., 1979). Total RNA (20 μg/lane) was separated by electrophoresis on a 1% agarose gel and transferred to a nylon membrane (GeneScreen, NEN). Hybridization was in the presence of 50% formamide at 42°C. After washing twice with 2xSSC at 20°C, twice with 2xSSC, 0.1% SDS for 20 min at 65 °C and once with 1xSSC at 20°C the membranes were either exposed to Kodak XAR films at -70°C using intensifying screens or the hybridization signals were evaluated using a phosphorimager (Molecular Dynamics).

Screening for conserved sequences. Ten μg of DNA from each species was digested with EcoRI and electrophoresed, blotted, hybridized and washed as previously described (Lekanne Deprez et al., 1991). Hybridization and washing was at 60°C. The final wash was performed in 0.3xSSC, 0.1%SDS for 20 minutes.

Hybrid cell lines. The somatic cell hybrids 6A, 14G-10 and 9B were derived by fusing A3 Chinese hamster cells with cultured meningioma 32 cells (MN32), harbouring the reciprocal t(4;22)(p16;q11). Hybrids 6A segregates the 22q- derivative chromosome and hybrid 14G-10 the 4p+ marker chromosome of this translocation as reported previously (Lekanne Deprez et al., 1991). Hybrid 9B harbours normal chromosome 22 with additional human chromosomes 8, 9, 13, 17, 21 and Y, derived from normal cells of patient 32. PgMe25NU is a mouse-human hybrid cell line with human chromosome 22 as its only human component (Goyns et al., 1984). Cells were grown in DMEM medium supplemented with 10% FCS, penicillin and streptomycin.

Construction of a cosmid contig. D22S193 was used to start the isolation of a cosmid contig. To generate the contig, two chromosome 22 specific cosmid libraries (LL22NC01 and LL22NC03), gridded in microtiter plates and containing approximately four genome equivalents of chromosome 22, were used (de Jong et al., 1989; Zuckman et al., 1992). Isolated single copy fragments and cosmid DNA from previous screens were used for the isolation of overlapping clones. Cosmid probes or other repeat containing probes were treated as follows: before hybridization 100 nanograms of the cosmid DNA was digested with a restriction enzyme, labeled by random priming using alpha ^{32}P-dATP and prehybridized for 2hr in 5xSSC at 65°C in a total volume of 500 μl containing 100 μg of total sheared human genomic DNA and 100 μg of predigested cosmid DNA from the previous walk. Hybridization, washing and autoradiography were as reported previously (Lekanne Deprez et al., 1991). The EcoRI map from this contig was made by hybridizing cosmid blots and blots containing human genomic DNA with the isolated cosmids and single copy restriction enzyme fragments from this region.

Screening of cDNA libraries. Two different humane fetal brain cDNA libraries were used. About 1x10^6 clones of an oligo(dT) primed λgt11 cDNA library (Clonetech, HL1065) or the same amount of an oligo(dT) and random primed λZAP II cDNA library (Stratagene, 936206) were plated on E.coli LE392 or PC2495 respectively. Filters were prepared according to standard techniques, and the library was initially screened with isolated genomic restriction fragments from the cosmid contig and subsequently with end fragments of the isolated cDNAs. Fragments containing repetitive sequences were first prehybridized with

total sheared human genomic DNA before hybridization as described in the previous section. Labeling, hybridization and washing conditions were as described elsewhere (Lekanne Deprez et al., 1991). Positive plaques were purified and subcloned into pTZ plasmid or rescued as pBluescript plasmid from the λZAP II phages.

cDNA sequencing. Sequencing was performed on both strands using double-stranded and/or single stranded pTZ, pBluescript and M13 subcloned fragments. The isolated cDNAs and subcloned fragments were sequenced using the universal and reverse primers, as well as primers derived from the obtained sequence. The dideoxy chain termination and deaza G/A sequencing kits from Pharmacia were used. The nucleotide sequence was compared with the EMBL and NCBI databases to search for homologous sequences and the GRAIL (Gene Recognition and Analysis Internet Link) program to define putative ORFs (Uberbacher and Mural, 1991).

Results

Pulsed-field analysis of t(4;22)(p16;q11)

For the characterization of the reciprocal t(4;22)(p16;q11) on chromosome 22 in meningioma 32 we used different probes from the 22q11 region for analyses of membranes from pulsed field gels. Figure 1 shows the result of NruI digested DNA probed with D22S193. Lanes T24 and 9B contain DNA from cell lines with normal chromosome 22 and serve as controls; T24 is a human bladder carcinoma cell line and 9B a hamster/human hybrid cell line with normal chromosome 22 from patient 32. In these lanes a fragment of approximately 120 kb is recognized. In DNA isolated from hybrid 6A, which harbours the 22q- marker of the reciprocal t(4;22), an aberrant 250 kb NruI band is observed (Fig.1, lane 6A). DNA from hybrid 14G-10, containing the 4p+ derivative chromosome, shows no specific hybridization, indicating that D22S193 is not located on that part of chromosome 22 that was translocated to chromosome 4p (Fig.1, lane 14G10). The other signals observed on this blot are probably the result of aspecific hybridization with excess DNA in the non-resolution zone of the gel (Fig.1, all lanes) and/or methylation differences of the NruI sites (Fig.1, lane T24). In addition, DNA digested with MluI, NotI and SalI showed altered bands in DNA from hybrid

6A (data not shown). These data suggest that D22S193 recognizes the breakpoint on the 22q- marker chromosome.

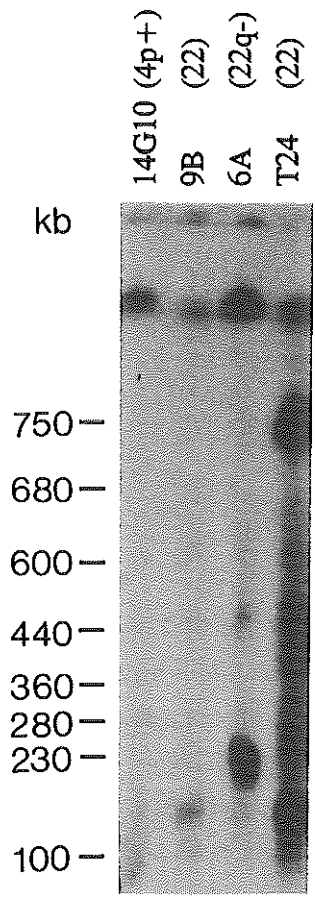

Figure 1. Pulsed-Field Gel Analysis of DNA from hybrids containing the products of the t(4;22) of meningioma 32. Lane T24: control DNA from a bladder carcinoma cell line containing normal chromosome 22. Lane 6A: DNA from hybrid 6A segregating the 22q- marker chromosome of t(4;22). Lane 9B: DNA from a control hybrid cell line containing normal chromosome 22 from patient 32. Lane 14G10: DNA from hybrid 14G10 containing the 4p+ marker chromosome from this translocation. DNA was digested with NruI and hybridized to D22S193. The length markers are indicated on the left.

Genomic cosmid contig spanning the translocation breakpoint

Two chromosome 22 specific cosmid libraries (LL22NC01 and LL22NC03) were used to isolate a contig starting from D22S193. This resulted in the isolation of four overlapping cosmids, which were used to construct a genomic EcoRI restriction map encompassing 120 kb (Fig.2). Further details concerning the isolation and construction of the map are given in the methodology section. To investigate the localization of the newly isolated cosmids relative to the breakpoint we used these cosmids and isolated restriction fragments for fluorescent in situ hybridization (FISH) on metaphase chromosomes and southern hybridization on DNA derived from the hybrid cell lines segregating the translocation products. Both approaches confirmed that the position of the breakpoint was confined in the contig (results not shown). Further mapping revealed that the breakpoint was located on a 8 kb genomic EcoRI fragment (Fig.2, see below).

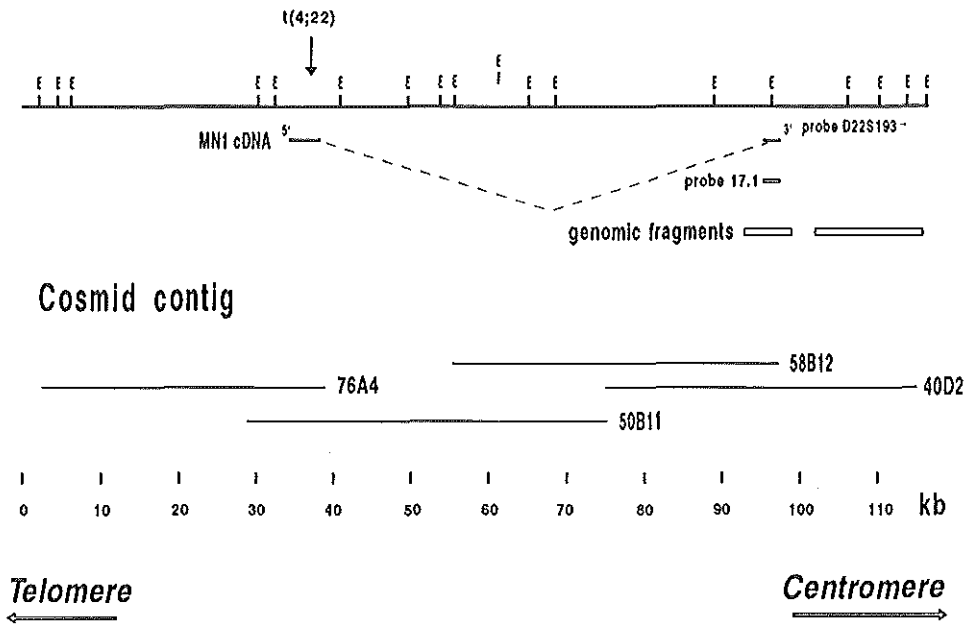

Figure 2. Physical map around the t(4;22) breakpoint and the *MN1* gene on chromosome 22q11. The deduced EcoRI (E) restiction map with the localization of the t(4;22) breakpoint, the complete *MN1* cDNA and cDNA clone 17.1 (probe 17.1) are shown. One EcoRI site is lifted from the map because its exact localization within the map is unknown. The overlapping cosmids spanning this region are shown at the bottom from telomere to centromere. The genomic fragments used to screen the cDNA library are indicated by open boxes.

Isolation and characterization of the cDNAs

To search for transcripts in the region covered by the contig, genomic fragments (Fig.2), were used to screen a fetal brain cDNA library (Clontech, HL1065). Sequence analysis and restriction mapping of the isolated cDNA clones proved that all clones were derived from the same gene and one of them (cDNA 17.1) contained a poly (A) tail with a polyadenylation signal 22 bp upstream. In total about 3 kb of the cDNA was isolated. The insert isolated from one of these clones, cDNA probe 17.1, was used on a northern blot and recognized a messenger of approximately 8 kb (Fig. 4). Therefore, the previous and another cDNA library (Stratagene 936206) were screened again with probes located at the most 5' end from former screens. Some clones derived from the second library contained composite inserts and therefore were discarded unless carefully checked. In total overlapping cDNAs were isolated which together span approximately 7.5 kb of the messenger. We do not know yet if we isolated a full-length cDNA. We have called the gene from which these cDNAs were derived *MN1*.

Restriction mapping of cDNA clones and subcloned genomic fragments from this region showed that the isolated 7.5 kb transcript consists of two large exons of 4.7 kb at the 5' end and 2.8 kb at the 3' end (data not shown). The 5' exon-intron boundary was sequenced and revealed a consensus splice acceptor site (data not shown). The localization of the gene on the isolated chromosome 22 contig is depicted in figure 2 and shows that the gene spans approximately 70 kb and is interrupted by one 60 kb intron.

Mapping of the translocation breakpoint

Fine mapping of the breakpoint was done on mapping panels with 2 genomic probes and a cDNA probe located in the 8 kb genomic EcoRI fragment in which the breakpoint was found (Fig. 3). EcoRI restriction patterns obtained with these probes on DNA extracted from cultured meningioma 32 tumor cells (MN32), hybrids harbouring the 22q- (6A) or 4p+ (14G-10) marker chromosomes of meningioma 32 and controls (human, 9B, A3, PgMe25Nu and mouse) are shown in figure 3. Genomic probe A, located at the telomeric site of this 8 kb genomic fragment, detects in addition to the normal 8 kb band an aberrant band of 7 kb in meningioma 32 DNA (Fig. 3A, lane MN32). The 8 kb band corresponds to hybridization with the other copy of chromosome 22 present in the tumor. The aberrant band is the result of hybridization with the breakpoint containing fragment on the 4p+ marker. Furthermore,

probe A is conserved in hamster DNA and detects a fragment of about 7 kb (Fig. 3A, lane A3). Therefore, DNA from 14G10 shows hybridization with two fragments of about 7 kb, the 4p+ breakpoint fragment and the hamster homologue (Fig. 3A, lane 14G10). The 7 kb fragments observed in lanes 6A and 9B and the 20 kb fragment in PgMe25NU are also the result of hybridization with conserved hamster and mouse sequences, respectively (Fig. 3A). Genomic probe B, located at the centromeric site of the 8 kb fragment, detects in meningioma 32 DNA the normal 8 kb band and a 2.5 kb band (Fig. 3B, lane MN32). The 2.5 kb band is also found in DNA from hybrid 6A indicating that this band is produced by the translocation breakpoint (Fig. 3B, lane 6A). These results prove that the translocation breakpoint is located between probe A and B (Fig. 3, physical map). The localization of the *MN1* gene relative to the t(4;22) in meningioma 32 was investigated with cDNA probe C, which is part of the 5' exon and is located between the genomic probes A and B. Figure 3C shows the EcoRI restriction fragments which hybridize with cDNA probe C. This probe recognizes both products of the reciprocal t(4;22), as the 2.5 kb fragment is derived from the 22q- marker (Fig. 3C, compare lanes MN32 and 6A) and the 7.0 kb fragment is the result of the hybridization with the 4p+ marker (Fig. 3C, compare lanes MN32 and 14G-10). These results show that the 5' exon of the *MN1* gene is disrupted by the translocation in meningioma 32. Figure 3C also shows weakly hybridizing fragments of approximately 6 and 7.5 kb (lanes human, MN32). This is the result of hybridization with homologous human sequences that are not located on chromosome 22. In PgMe25NU, a hybrid cell line with only human chromosome 22 in a mouse background, these fragments were not observed (Fig 3C, lane PgMe25NU). cDNA probe C detects in both hamster and mouse DNA an EcoRI fragment of 8.5 kb, suggesting that this DNA fragment is highly conserved (Fig 3C, lanes A3, mouse). In the hybridizations shown in figure 3 it is obvious that the normal 8 kb EcoRI band is underrepresented in tumor DNA (lane MN32). The normal copy of the *MN1* gene in tumor 32 is located on a der(22)t(1;22)(p11;q11) with the breakpoint on chromosome 22 between cos 76A4 and the *NF2* gene. The underrepresentation of this chromosome in MN32 DNA preparations was also observed when we used RFLP probes for the more proximal region of chromosome 22. However, in in situ hybridizations with cosmids from the *MN1* gene region on MN32 metaphase spreads, no deviation from the contribution of both marker chromosomes 22 was observed (results not shown). Therefore, we must conclude that the apparent underrepresentation of the der(22)t(1;22) in DNA preparations is an artefact and is

probably caused by the less efficient release of this chromosome during cell lysis.

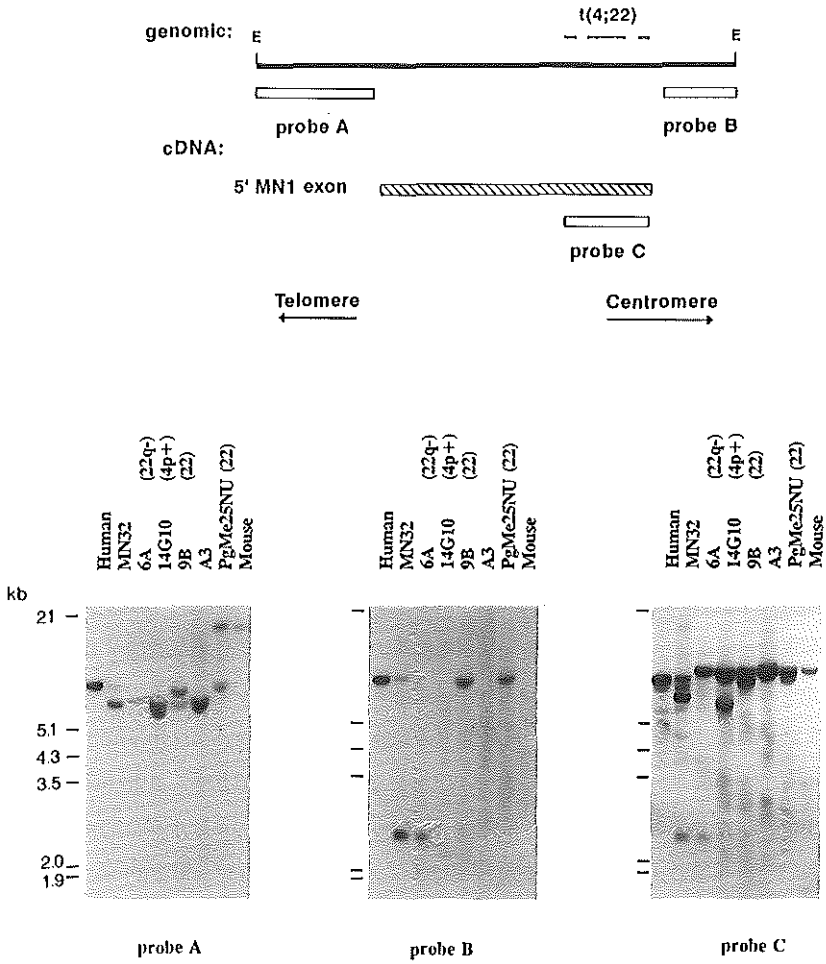

Figure 3. Fine mapping of the t(4;22) breakpoint in meningioma 32. On top the physical map of the 8 kb genomic fragment with the localization of the 5' *MN1* exon, the translocation breakpoint and the probes (genomic probes A and B, cDNA probe C) used for the mapping are shown. Below the hybridizations of EcoRI digested DNA with probes A, B and C are shown. Lane Human: control DNA from leucocytes of an unrelated individual. Lane MN32: DNA from cultured meningioma 32 cells. Lanes 6A, 14G10 and 9B: see figure 1 legend for a description. Lane A3: Chinese hamster (A3) control DNA. Lane PgMe25NU: control DNA from (mouse) hybrid PgMe25NU containing only human chromosome 22. Lane Mouse: control mouse DNA. The sizes of the markers are indicated on the left.

Southern, northern blots and evolutionary conservation.

DNA isolated from 71 meningiomas was investigated for rearranged fragments using probes from the region spanning the *MN1* gene. Probes, which recognize all different EcoRI fragments from this region, were used to hybridize southern blots containing BglII, EcoRI, HindIII and PvuII digested meningioma DNA. Using this method we detected alternative fragments in DNA isolated from meningioma 55. This is a meningioma derived from a patient with multiple meningiomas. The rearranged fragments are the result of an interstitial deletion and a point mutation in the germline of patient 55, which disrupt two EcoRI sites located approximately 10 kb downstream of the *MN1* gene as was reported previously (Lekanne Deprez et al., 1994a). No other meningiomas showed aberrations using this analysis.

A northern blot, containing 2 μg of poly A^+ RNA extracted from 8 different human tissues, was hybridized with cDNA probe 17.1 (Fig. 2). All tissues showed hybridization of a transcript of about 8 kb, with the highest expression level and an additional 4.5 kb transcript found in skeletal muscle (Fig. 4A). At present it is not known if this transcript is the result of alternative splicing or hybridization with a homologous gene. An example of northern blots made from total RNA isolated from meningiomas is shown in figure 4B. In meningiomas only the 8 kb messenger is observed and different meningiomas show a variable expression level of the *MN1* gene. Some meningiomas show a high level, others a low or undetectable level (Fig. 4, lanes 1-7). The ß-actin cDNA probe was used to quantitate the relative levels of the transcript. RNA isolated from cells cultured from meningioma 32, containing only tumor cells, as was established by cytogenetic analysis, showed no expression of the *MN1* gene (data not shown).

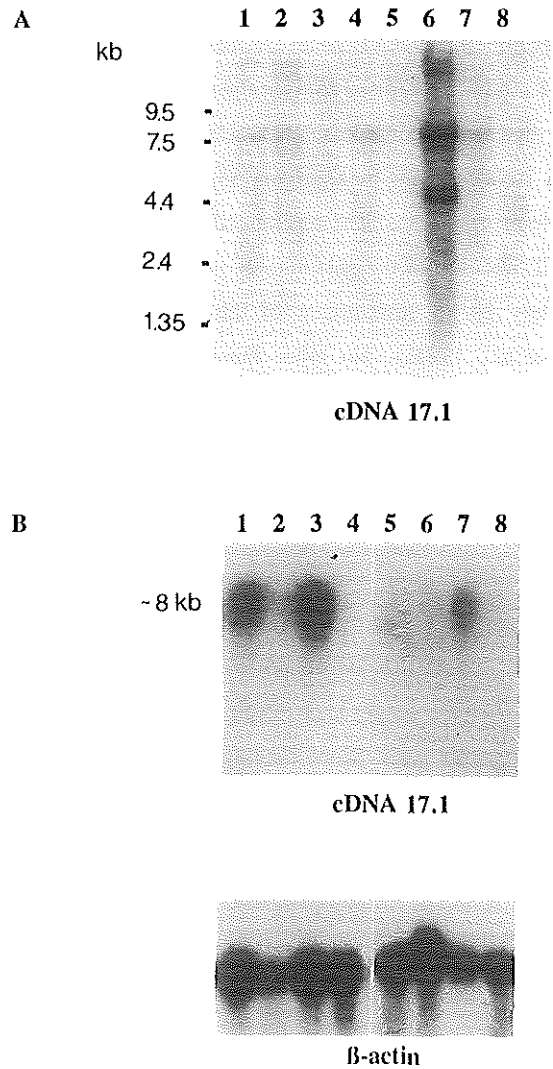

Figure 4. Northern blot hybridization of *MN1* cDNA 17.1. A: Autoradiogram of a Northern blot (Human MTN, Clonetech, CA), containing 2 μg of Poly A^+ RNA from 8 different human tissues, hybridized with cDNA probe 17.1. Numbers 1-8 correspond to: heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas tissue, respectively. B: Autoradiogram of a Northern blot, containing 20 μg total RNA isolated from 7 independent meningiomas (lanes 1-7) and total brain (8), hybridized with cDNA probe 17.1 (upper part) or with ß-actin (lower part).

The evolutionary conservation of the *MN1* gene was investigated by hybridization of cDNA probe C (Fig. 3) to genomic DNA, extracted from a number of different species, after digestion with EcoRI. Hybridization signals were observed in very different species, including species as evolutionarily distant as Xenopus Laevis and Drosophila Melanogaster (Fig.5). This result provides evidence that the *MN1* gene has been highly conserved during evolution.

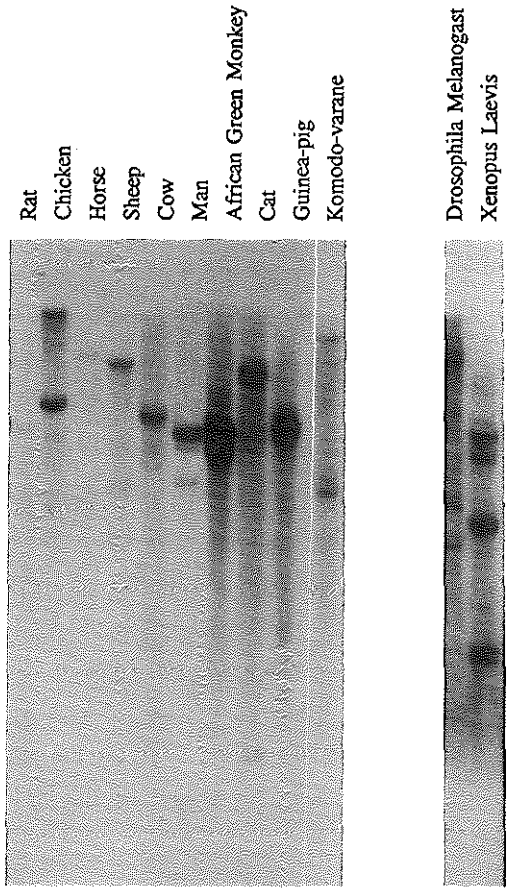

Figure 5. Zoo blot analysis of EcoRI digested DNA isolated from several species hybridized with *MN1* cDNA C. The first 10 lanes were exposed for 16 hours and the last 2 lanes for 7 days.

cDNA sequence analysis

Sequence analysis was performed in both orientations on different selected overlapping *MN1* cDNA clones. Sequencing this *MN1* transcript was not an easy task, as the first exon (4713 nucleotides) has a high overall GC content (69%). The numerous sequence artifacts encountered were dissolved by sequencing other independent cDNA clones, subcloned fragments and/or by using shifted internal primers with and without the interchange of dATP and dGTP for 7-deaza dATP and 7-deaza dGTP in the sequencing reactions. The nucleotide sequence which we have analyzed at the moment this chapter was written did not predict a single contiguous ORF. Therefore, we used the GRAIL program to deduce the potential protein coding regions from the nucleotide sequence. The program predicts two putative ORFs with a reasonable length with the quality score excellent protein coding region. The amino acid sequences of both ORFs between the stop codons are depicted in figure 6. The most 5' ORF (Fig. 6A) contains 6 methionine codons, of these the ones at positions 53 and 88 show the closest match with the Kozak consensus sequence for translation initiation sites (Kozak, 1987). Upstream of this ORF 3 additional ATGs are situated, all followed by stop codons (data not shown). The 3' ORF (Fig. 6B) contains 8 methionine codons, of these the ones at positions 88, 275, 548 and 651 fit best with the Kozak consensus sequence.

The MN1 nucleotide sequence revealed two trinucleotide (CAG) repeat sequences located between both ORFs. These repeats were investigated for tumor associated length variation in our series of meningioma DNAs and control DNAs using radioactive PCR with primers flanking the repeats and separation on sequence gels (Verkerk et al., 1991). The most 5' CAG repeat was not polymorphic in any of the 114 DNA samples analyzed, including 89 meningiomas. However, the other CAG repeat was polymorphic and detected three alleles differing six or nine nucleotides from the smallest allele respectively. The frequencies of the alleles, as estimated from 92 unrelated DNAs of Caucasian origin, were 5% for the smallest, 73% for the middle one and 22% for the largest fragment. In 63 meningioma DNA samples all three alleles were recognized, but no increase in length was observed (data not shown).

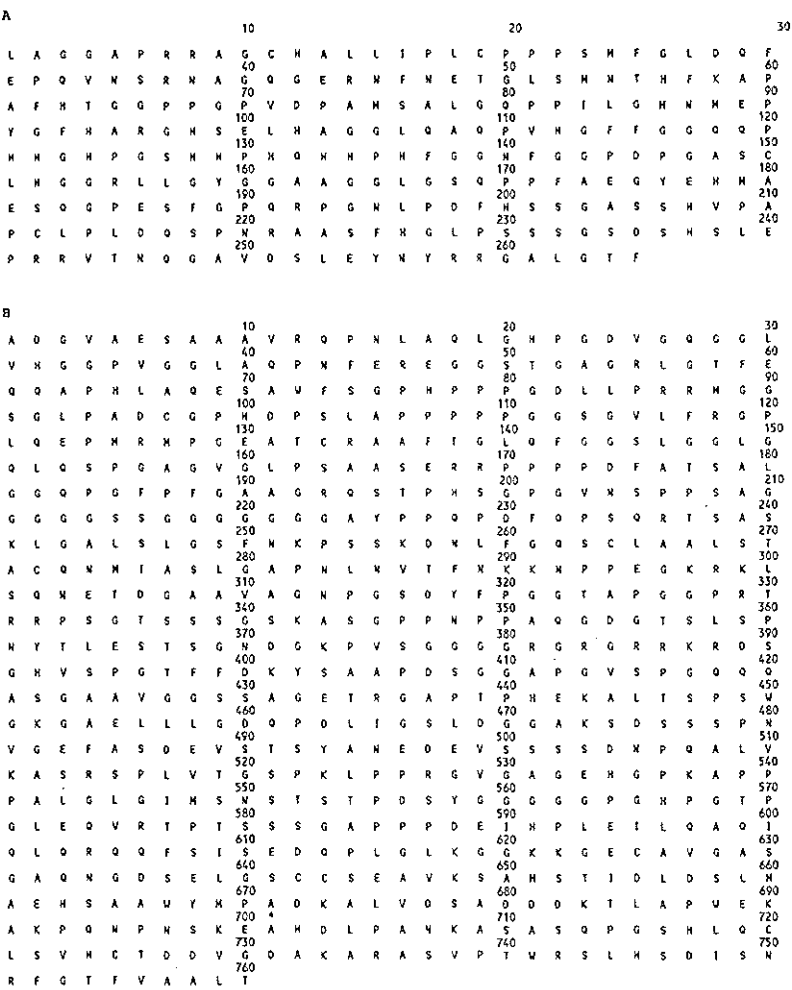

Figure 6. The predicted amino acid sequence between the stop codons from the 2 most likely ORFs as we have deduced from the nucleotide sequence derived from the *MNI* transcript. A: The amino acid sequence of the 5' ORF. B: The amino acid sequence of the 3' ORF. The location of the intron is shown with an asterisk (*).

Discussion

Characterization of the reciprocal t(4;22)(p16;q11) in meningioma 32 has led to the identification of a new gene, consisting of at least two exons of about 4.7 kb (5' end) and 2.8 kb (3' end). The entire 5' exon and genomic sequences upstream (results not shown) are extremely GC rich. This area apparently is a very large GC island. The translocation breakpoint is located in the 5' exon, which strongly suggests that the gene is directly involved in the tumorigenesis of meningioma 32. Therefore, we have called this gene *MN1*, for candidate meningioma gene 1. Many reports describe the use of reciprocal translocations for the isolations of cancer genes. Specific chromosomal translocations are involved in the activation of proto-oncogenes as is the case in many leukemias (Solomon et al., 1991). In addition, they can also inactivate a tumor suppressor gene. For instance, in neurofibromatosis type 1 (NF1) two reciprocal translocations involving band 17q11.2 resulted in the successful isolation of the *NF1* tumor suppressor gene (Cawthon et al., 1990; Viskochil et al., 1990; Wallage et al., 1990). In agreement with the observed loss of chromosome 22 in meningiomas this might suggest that the *MN1* gene is a tumor suppressor gene and that the translocation in meningioma 32 inactivates the gene. Northern blot analysis of meningioma 32 RNA supports this. Southern blot analysis of 71 meningiomas with probes from the MN1 region revealed a point mutation and a 1.5 kb homozygous deletion in one tumor (55). This tumor was from a patient with multiple meningiomas. Further analysis showed that the alterations were found also in the germline of this patient and were located about 10 kb downsteam of the *MN1* gene (Lekanne Deprez et al., 1994a). One might speculate that these alterations interfere with *MN1* gene function. However, this gene was highly expressed in meningioma 55. Northern analysis of the *MN1* gene in other meningiomas also showed high expression in some and low or no expression at all in others. This might indicate that the expression of the *MN1* gene is deregulated in meningiomas. Further study at the protein level is however needed to investigate this in more detail.

Although the nucleotide sequence as we have obtained so far would suggest 2 possible ORFs, in vitro transcription/translation experiments suggest that only one large protein of approximately 3.8 kb is formed (Molyn et al., personal communication). Therefore, we expect that both ORFs are part of this large protein and that some sequence artifacts are still present in the 1 kb that separate them. The scanning model for the initiation of translation

postulates that the first AUG codon in the mRNA located in the right context defines the beginning of the protein (Kozak, 1991). Therefore, we expect that the methionine at position 53 (Fig.6B), which is the first AUG with a reasonable Kozak sequence, is the start point for translation. Because in the upstream leader sequence 4 other AUG codons are found and this sequence is very GC-rich (highly structured) we presume that the transcript is poorly translated. These features are observed in many proto-oncogenes, transcription factors, receptors, signal transduction components and proteins involved in the immune response (Kozak, 1991). All these proteins play a critical regulatory role and one might speculate that the unfavourable translation context of the *MN1* gene is a way of tightly regulating its expression. Amino acid sequences deduced from both the predicted ORFs (Fig.6A,B) and the nucleotide sequences of the transcript were analyzed for homology with other genes using the EMBL and NCBI databases. No significant homology was obtained which could predict a putative function for the gene. That the gene product must have an important function is, however, suggested by the finding that the gene is highly conserved during evolution. An almost complete alignment was found with two identical expressed sequence tags (gene bank accession numbers Z28469 and HSXT02462). Furthermore, two CAG repeats, probably located in the putative ORF, were observed. Instability (expansion) of polymorphic trinucleotide repeats has been found to be responsible for a number of (neurological) diseases (Warren and Nelson, 1993). Expansion of the CAG repeats in the *MN1* gene, however, was not found in meningiomas. Further analysis of these repeats in patients with neurodegenerative disorders is under way.

The neurofibromatosis type 2 gene (*NF2*) was recently isolated and is located about 1.7 Mbp distal to the *MN1* gene on the q-arm of chromosome 22 (McDermid et al., 1993; Rouleau et al., 1993; Trofatter et al., 1993). Mutation analysis of the *NF2* gene transcript in sporadic meningiomas revealed mutations, which probably inactivate the gene, in about 30% of the tumors (Ruttledge et al., 1994; Lekanne Deprez et al., 1994b). However, in both meningiomas (32, 55) with aberrations in or close to the *MN1* gene, no mutations in the *NF2* gene were found, neither by using RT-PCR-SSCP (Lekanne Deprez et al., 1994) or by SSCP analysis of exons 1-12 on the DNA level (Zwarthoff et al., unpublished results). This corroborates the putative role of the *MN1* gene in the development of (a subset of) meningiomas. Interestingly, both these meningiomas were from multiple meningioma patients. Moreover, most partial chromosome 22 deletions found in meningiomas so far

involve loss of both the *MN1* and the *NF2* gene. Reciprocal translocations in meningiomas are very rare and the three reported cases are located in region 22q11 (Casalone et al., 1990; Lekanne Deprez et al., 1991) and 22q12 (Maltby et al., 1988), but none except ours was precisely mapped to investigate *MN1* or *NF2* gene involvement. To further adress the question about the extent of the contribution of the *MN1* gene to meningioma development a detailed mutation study of the *MN1* gene is necessary. This gene might also be involved in other tumors with known chromosome 22 deletions, such as breast cancer (Larsson et al., 1990), colon cancer (Okamoto et al., 1988), ependymomas (James et al., 1990), gliomas (Rey et al., 1993), pheochromocytomas (Khosla et al., 1991; Tanaka et al., 1992) and rhabdoid tumors (Biegel et al., 1990; Newsham et al., 1994).

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Chapter VII

Constitutional DNA-level alterations in chromosome 22 in a patient with multiple meningiomas

Genes Chrom. Cancer 9:124-128, 1994

Constitutional DNA-Level Aberrations in Chromosome 22 in a Patient With Multiple Meningiomas

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We describe a patient who developed multiple meningiomas but had no clear evidence of neurofibromatosis type 2. Four of the tumors, derived from three different sites, were analyzed cytogenetically and/or at the DNA level using chromosome 22 specific probes. All four tumors showed loss of the same copy of chromosome 22. On the chromosome that was retained in the tumors, we found two constitutional aberrations, a 1.5 kb deletion and a point mutation. The patient had inherited both alterations from her father. The father has not developed any meningiomas so far but he has been treated for a well-differentiated adenocarcinoma of the lung and a brain metastasis from this tumor. The mother and 75 unrelated individuals did not show any of the chromosome 22 alterations. The multiple tumors found in the patient suggest that she has a predisposing gene for the development of meningiomas. The finding that all investigated tumors lost the same, constitutionally normal copy of chromosome 22 could indicate that the predisposing gene resides on chromosome 22 and was affected by the constitutional mutations. *Genes Chrom Cancer 9:124–128 (1994).*

INTRODUCTION

Meningioma is a common benign intracranial or intraspinal tumor of the arachnoidal layer of the meninges. The highest incidence is during the fifth and sixth decades of life. On average, twice as many females are affected as males (Zang, 1982), and an accelerated growth has been found during pregnancy (Roelvink et al., 1987). There are three patterns of meningioma occurrence: 1) sporadic cases with solitary tumors, 2) meningiomas as part of the hereditary syndrome neurofibromatosis type 2 (NF2), and 3) multiple or familial aggregations of meningiomas (Butti et al., 1989; Domenicucci et al., 1989; Russell and Rubinstein, 1989; McDowell, 1990; Sieb et al., 1992). Some of the latter cases could indicate the existence of a genetic predisposition for the development of meningiomas distinct from that for NF2.

Loss of (parts of) chromosome 22 is a very frequent event in both sporadic and NF2 associated meningiomas, suggesting the involvement of a tumor suppressor gene or genes located on chromosome 22 (Zang, 1982; Seizinger et al., 1987a,b; Wullich et al., 1989; Dumanski et al., 1990). Recently, the NF2 gene was cloned; it is located between *NEFH* and D22S360 (Rouleau et al., 1993; Trofatter et al., 1993). As yet it is not clear whether one or more than one gene on chromosome 22 can lead to these diseases. Evidence for aberrations on chromosome 22 which are not in the vicinity of the NF2 gene have been described by several authors. These include a reciprocal translocation t(4;22) (Lekanne Deprez et al., 1991a), and loss of sequences distal to D22S28 on chromosome 22 (Dumanski et al., 1990) in meningiomas, and a reciprocal translocation in an acoustic neuroma in which band q13 is involved (R. Wolff, personal communication). To further investigate the importance of other putative loci on chromosome 22, the study of genetic changes in cases with multiple or familial meningiomas without evidence of NF2 may be relevant.

We describe a patient with multiple meningiomas. In the four tumors that were analyzed, loss of the same copy of chromosome 22 was observed. The retained copy of chromosome 22 showed constitutional genetic aberrations that we cloned in an effort to identify the postulated predisposing gene for meningioma.

MATERIALS AND METHODS

Case Report

The female patient (patient 55) was born in December 1956. In 1986, she came to the Department of Neurology with neurological complaints. Clinical examination and imaging (CT scan, angiography, MRI) suggested multiple meningiomas infra- and supratentorially at both sides of the cer-

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ebellopontine angle and intraspinally. In 1987, several parasagittal meningiomas were removed surgically. The tumors were histologically diagnosed as fibrous meningiomas (designated MN55A). In December 1989, a recurrence of a parasagittal meningioma on the left side was removed and histologically diagnosed as a fibrous meningioma (MN55B). Multiple small nodules of hyperplasia were observed in this region. In October 1990, two meningiomas from the right (MN55C) and the left (MN55D) cerebellopontine angle were excised at an interval of 2 weeks; both were of the fibrous type. The patient's parents have no history of meningiomas. However, her father (born in 1929) developed a well-differentiated adenocarcinoma of the lung in 1984 and 2 years later a brain metastasis of this tumor. A CT scan performed in 1990 did not reveal any intracranial tumors. When this paper was written, he was alive and without complaints.

Tissue Culture and Cytogenetic Analysis

The meningioma specimens (MN55B,C,D) were obtained within 30 min after surgery. The preparation for tissue culture and tissue culture conditions were as described (Koper et al., 1990). Chromosome analysis was carried out on cultured cells after one to three passages using R-(acridine orange) and Q-(quinacrine) banding. The karyotypes were described according to ISCN (1991).

DNA Extraction, Southern Blotting, and Hybridization

High molecular weight DNA was isolated from cultured and fresh tumor tissue and peripheral blood leucocytes from the patient, her parents, and unrelated individuals according to standard procedures. The percentage of tumor cells in the fresh tumor sample was established by microscopic examination of a frozen tissue section. DNA analyses were performed as reported by Lekanne Deprez et al. (1991a). The following DNA markers for loci on chromosome 22 were used: D22S181, D22S183 (Lekanne Deprez et al., 1991b), D22S10 (Hofker et al., 1985), D22S182 (Lekanne Deprez et al., 1991b), D22S1 (Barker et al., 1984), D22S193 (Lekanne Deprez et al., 1991b), D22S45 (Budarf et al., 1991), D22S201 (Lekanne Deprez et al., 1991c), and D22S205 (van Biezen et al., 1993). The probes are ordered from centromere to telomere when possible (van Biezen et al., 1993).

Molecular Cloning and Characterization of Cloned Fragments

A λEMBL4 library containing size-fractionated, *Eco*RI digested meningioma DNA from patient 55 (MN55B) was constructed according to Frischauf et al. (1983) using λEMBL4 predigested *Eco*RI arms (Stratagene). The library was probed with D22S193, and four phage clones were isolated and their restriction maps were established. This map was compared with the map constructed from control DNA isolated from the CMLO λEMBL3 library (de Klein et al., 1986). When relevant, double-stranded sequencing of pUC19 subcloned fragments was performed using a sequencing kit (Pharmacia).

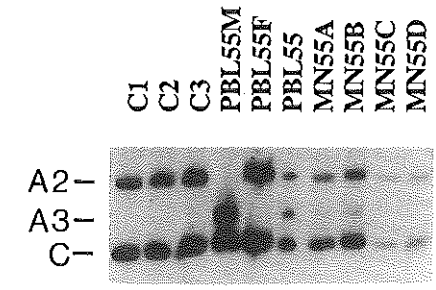

Figure 1. Loss of heterozygosity for D22S45 in DNA from leucocytes and meningiomas of patient 55. C1, C2, and C3: Control DNAs from leucocytes of three unrelated individuals. PBL55M and F: DNA from leucocytes of the mother (M) and the father (F) of patient 55. PBL55: DNA from leucocytes of patient 55. MN55A-D: DNA from meningioma MN55A, B, C, and D of patient 55. *Pvu*II digested DNA was probed with D22S45. On the left are the observed fragment sizes as described by Budarf et al. (1991). A2: allele 2 (2.6 kb), A3: allele 3 (2.1 kb), and C: a constant band of 1.8 kb.

RESULTS

Four intracranial meningiomas from patient 55 were analyzed cytogenetically and/or at the DNA level. Cytogenetic analyses performed on short-term cultures of three of them showed loss of chromosome 22 in all the cases (Table 1; MN55B,C,D). Tumor MN55B also showed loss of one of the X chromosomes in most of the cells. Nine chromosome 22 specific polymorphic probes were used to analyze loss of heterozygosity (LOH) in DNA isolated from the tumors. D22S10 and D22S45 were found to be polymorphic. The results with D22S45 are shown in Figure 1, showing loss of maternal allele A3. Identical results were obtained with D22S10 (results not shown). This suggests that the same (paternal) copy of chromosome 22 is preserved in all four tumors. The presence of residual signal of the lost allele (Fig. 1) and cells with a normal karyotype (Table 1) were probably due to an admixture of normal cells in the tumor.

In our search for a gene involved in the devel-

TABLE 1. Cytogenetic Findings in Meningioma Tumors from Patient 55

Tumor #	Passage in culture	No. of cells karyotyped	Karyotype
MN55A	Not done		
MN55B	p0 (primary outgrowth)	15	45,XX,−22[1]/44,X,−X,−22[8]/40–43, idem, with random losses [5]
MN55C	p1	25	45,XX,−22[24]/89, hypotetraploid, −22, −22, with additional losses and rearrangements [1]
MN55D	p3	5	45,XX,−22[3]/46,XX[2]

opment of meningioma, we used different chromosome 22 specific probes. One of the probes, D22S193, which has been mapped to band 22q11 (van Biezen et al., 1993), detected an aberrant *Eco*RI fragment in patient 55. Figure 2 shows the hybridization of D22S193 with *Eco*RI digested DNA. This probe detects, in control DNA and DNA isolated from leucocytes of the mother of patient 55, a 2.6 kb *Eco*RI fragment (lanes C1, C2, C3, and PBL55M). In DNA isolated from leucocytes of patient 55 and her father, D22S193 recognizes two alleles, the 2.6 kb fragment and an abnormal sized band of 15 kb (lanes PBL55F and PBL55). In addition, this probe showed loss of the normal (2.6 kb) allele and retention of the abnormal allele in all four meningiomas from the patient (lanes MN55A, B, C, and D). The relatively weak appearance of the 15 kb allele compared with the 2.6 kb allele is due to less efficient transfer of larger fragments. Additional investigation of 75 unrelated individuals showed only the 2.6 kb *Eco*RI fragment (data not shown). These results show that this patient has a constitutional aberration on chromosome 22, which she inherited from her father, and that this copy of chromosome 22 is preserved in all meningiomas investigated.

To further characterize the genetic alteration of chromosome 22, we cloned the aberrant 15 kb *Eco*RI fragment and compared it with control DNA. Figure 3 shows the restriction map of this region. Restriction mapping of subcloned fragments and hybridization with the appropriate probes established that one *Eco*RI site in patient 55 was lost because of an internal deletion of 1.5 kb (Fig. 3), and sequence analysis showed that the other *Eco*RI site was lost due to a point mutation (data not shown). These results explain the appearance of the aberrant 15 kb *Eco*RI fragment. In order to determine whether these germline mutations were within a gene, we searched for transcribed sequences in this part of chromosome 22. Using genomic subcloned fragments of approximately 10 kb from this region (Fig. 3), we screened three different human cDNA libraries (fetal brain, temporal cortex, and T47D). No cDNA clones could be isolated from this area of

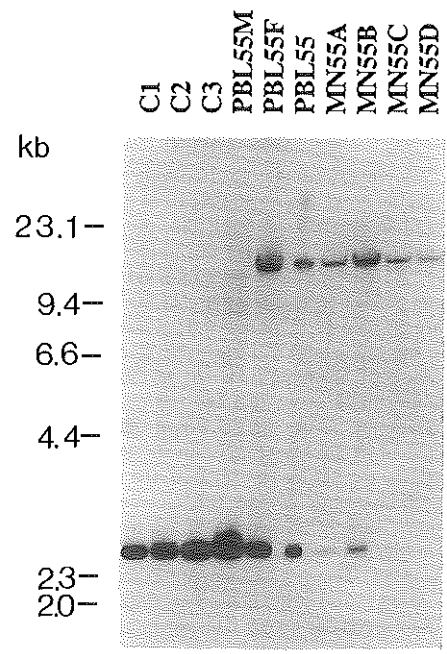

Figure 2. Analysis of the constitutional aberrations in patient 55 and her parents. See Figure 1 legend for a description of the DNA samples used. EcoRI digested DNA was probed with D22S193. The sizes of the markers are indicated on the left.

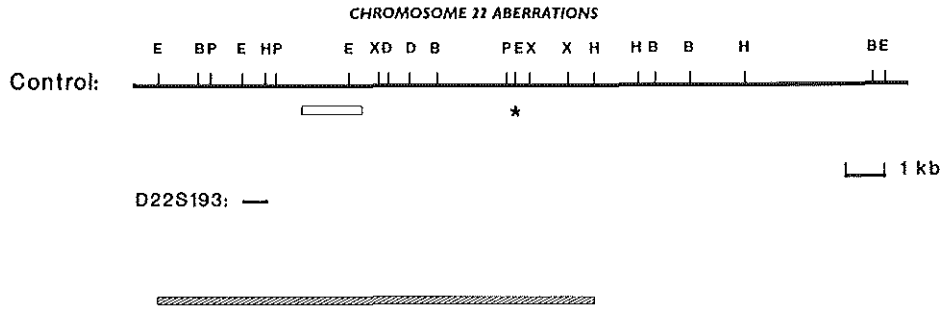

Figure 3. Physical map from the area containing the constitutional alterations of patient 55. Control: Physical map for control DNA. The aberrations observed in patient 55 are indicated by an open box for the 1.5 kb deletion and an asterisk (*) for the point mutation. Probe D22S193 is indicated by a black box. The fragment used to screen cDNA libraries is indicated by the hatched box. Abbreviations: B, *Bgl*II; D, *Dra*I; E, *Eco*RI; H, *Hind*III; P, *Pst*I; X, *Xba*I. Not all *Dra*I, *Pst*I, and *Xba*I sites are shown.

chromosome 22. Also, zoo blots, containing hamster, mouse, and rat DNA, showed no clear conserved sequences, except for a weak conservation of D22S193 (data not shown).

DISCUSSION

We postulated that the multiple meningiomas probably arose as a consequence of a predisposing germline mutation distinct from the gene predisposing to NF2. LOH study with D22S10 and D22S45 performed in four meningiomas derived from patient 55 suggested loss of the same copy of chromosome 22. This result was further supported by the study of D22S193. The finding that the same copy of chromosome 22 was lost in all four tumors could mean that the postulated germline mutation is on the copy of chromosome 22 which is retained in the tumor cells. In the multiple meningioma patient we investigated, the retained copy of chromosome 22 had two constitutional alterations, namely, a deletion of 1.5 kb and a point mutation, both of which were detected with probe D22S193. The same abnormalities were not found in 75 unrelated individuals. This suggests that they either represent a very rare polymorphism or that they might be involved in the pathogenesis of the multiple meningioma syndrome of patient 55. We investigated the latter possibility by searching for transcribed sequences in the region surrounding the deletion and point mutation but found no evidence for a gene in the immediate vicinity of the aberrations. However, we recently cloned a gene which is located approximately 10 kb from the germline aberrations in patient 55. This gene was cloned because it is disrupted by the reciprocal translocation t(4;22) found in a meningioma (Lekanne Deprez et al., 1991a; Lekanne Deprez, unpublished results). Thus it is possible that the alterations found in patient 55 interact with the expression of this gene and that this plays a role in meningioma development. Further investigations are necessary to explore this hypothesis.

An alternative explanation for the retention of the same copy of chromosome 22 in the four meningiomas could be that this copy has been subject to genomic imprinting. The postulated germline mutation could be in the imprinting gene, which does not have to reside on chromosome 22, a situation analogous to that proposed for some familial cases of Wilms' tumor (Haber and Housman, 1992). At present, we consider this explanation less likely in view of the fact that no evidence for genomic imprinting of chromosome 22 has been found in sporadic meningiomas and NF2 (Fontaine et al., 1990, 1991; Sanson et al., 1990).

Only two reports have described constitutional chromosome abnormalities and the occurrence of meningioma. Once a Robertsonian t(14;22) was found in familial meningioma (Bolger et al., 1985). In another patient with multiple meningiomas, a ring chromosome 22 was found, resulting in deletion of sequences between 22q13.3 and 22qter (Arinami et al., 1986). It is conceivable that these abnormalities lead to somatic instability of chromosome 22. In neither case was cytogenetic analysis performed on the tumors to investigate this possibility. We therefore conclude that the findings presented in this paper give a better indication for the proposed existence of a predisposing gene on chromosome 22 in multiple/familial meningiomas.

The father of patient 55 is also a carrier of the alterations but has not developed any meningiomas. He is now 63 years old. The age of onset in seven families with multiple meningiomas was reported to vary between 8 and 72 years. Thus he could still be at risk. An alternative explanation

could be that he is a case of mosaicism and that at least the meninges are not affected by the mutations. Interestingly, he developed lung cancer at age 55 and a brain metastasis of this tumor 2 years later. Both were diagnosed as well-differentiated adenocarcinomas. As far as we are aware, the combination of meningiomas and lung cancer has not been described before.

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Chapter VIII

Frequent NF2 gene transcript mutations in sporadic meningiomas and vestibular schwannomas

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Frequent *NF2* Gene Transcript Mutations in Sporadic Meningiomas and Vestibular Schwannomas

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Summary

The gene for the hereditary disorder neurofibromatosis type 2 (NF2), which predisposes for benign CNS tumors such as vestibular schwannomas and meningiomas, has been assigned to chromosome 22 and recently has been isolated. Mutations in the NF2 gene were found in both sporadic meningiomas and vestibular schwannomas. However, so far only 6 of the 16 exons of the gene have been analyzed. In order to extend the analysis of an involvement of the NF2 gene in the sporadic counterparts of these NF2-related tumors, we have used reverse transcriptase–PCR amplification followed by SSCP and DNA sequence analysis to screen for mutations in the coding region of the NF2 gene. Analysis of the NF2 gene transcript in 53 unrelated patients with meningiomas and vestibular schwannomas revealed mutations in 32% of the sporadic meningiomas (n = 44), in 50% of the sporadic vestibular schwannomas (n = 4), in 100% of the tumors found in NF2 patients (n = 2), and in one of three tumors from multiple-meningioma patients. Of the 18 tumors in which a mutation in the NF2 gene transcript was observed and the copy number of chromosome 22 could be established, 14 also showed loss of (parts of) chromosome 22. This suggests that in sporadic meningiomas and NF2-associated tumors the NF2 gene functions as a recessive tumor-suppressor gene. The mutations detected resulted mostly in frameshifts, predicting truncations starting within the N-terminal half of the putative protein.

Introduction

Neurofibromatosis type 2 (NF2) is a dominant hereditary disorder that predisposes patients to the development of a number of benign CNS tumors. These are typically (bilateral) vestibular schwannomas (tumors of the eighth cranial nerve), (multiple) meningiomas, schwannomas, and ependymomas. The incidence of NF2 is ~1:40,000, with >95% penetrance (Kanter et al. 1980; Evans et al. 1992). The tumors that occur in NF2 patients also occur sporadically. For instance, intracranial meningiomas represent ~13%–19% of all primary brain tumors that are treated by surgery (Russell and Rubinstein 1989). The actual incidence of meningiomas is probably higher, since they are observed in 33% of intracranial neoplasms found incidentally at necropsy (Wood et al. 1957).

The NF2 gene has been assigned to chromosome 22 by tumor deletion studies and linkage mapping (Seizinger et al. 1986, 1987*a*, 1987*b*; Rouleau et al. 1990) and recently has been isolated (Rouleau et al. 1993; Trofatter et al. 1993). The gene presumably encodes a 595-amino-acid protein called "merlin," or "schwannomin," of which the N-terminal 340 residues display a high homology to moesin, ezrin, and radixin. These proteins have been postulated to play a role in mediating interactions between the cell membrane and the cytoskeleton. In some NF2-related tumors the inacti-

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vating germ-line mutation was accompanied by loss of heterozygosity (LOH) of chromosome 22 (Rouleau et al. 1993; Trofatter et al. 1993), suggesting a classical tumor-suppressor–gene model analogous to that originally proposed by Knudson (1971).

In sporadic meningiomas and schwannomas, LOH of chromosome 22 is a frequent event, suggesting loss or inactivation of a tumor-suppressor gene(s) on chromosome 22 as the underlying mechanism for the development of these tumors (Zang 1982; Seizinger et al 1986, 1987*a*; Dumanski et al. 1990; Bijlsma et al. 1992). In a study by Rouleau et al. (1993), 30 meningiomas and 30 vestibular schwannomas from either NF2 or sporadic patients were analyzed for mutations by denaturing gradient gel electrophoresis analysis of 6 of the 16 exons of the gene. In total, six mutations were observed. Even when the limited region of the gene that so far has been investigated is considered, this raises the question about the extent in which the NF2 gene is involved—in particular, because there are indications of additional loci on chromosome 22 that may play a role in the development of these tumors (Dumanski et al. 1990; Lekanne Deprez et al. 1991; D. R. Cox, personal communication).

In the present paper we describe the analysis of mutations in the NF2 gene in 44 sporadic meningiomas, in 4 sporadic vestibular schwannomas, in 2 tumors from NF2 patients, and in 3 tumors from patients with multiple meningiomas. Mutations were detected in one-third (19/53) of the cases, and these were often (14/18) associated with loss of chromosome 22. This suggests that in a significant number of sporadic NF2-related tumors, the NF2 gene plays an important role in tumor development.

Material and Methods

Tumor Samples and RNA Isolation

Fifty-three tumor samples were obtained from patients during surgery. The tumors were classified according to the World Health Organization histological typing of tumors of the CNS (Zülch 1979). The tumors were called "sporadic" when there was no family history for NF2 and when they were solitary cases. Patients were diagnosed as having NF2 when they fulfilled the criteria for NF2 according to the National Institute of Health Consensus Conference Statement on Neurofibromatosis (1987). When more than two meningiomas that did not fulfill the criteria for NF2 were found in one patient, this patient was considered as having multiple meningiomas. The tissue samples were kept frozen in liquid nitrogen until needed. The percentage of tumor cells in the tumor specimen was established by microscopic examination of a frozen tissue section. Most specimens contain >90% tumor cells. These samples and one cultured meningioma were used for total RNA isolation using the guanidinium thiocyanate method (Chirgwin et al. 1979).

RNA PCR Amplification

First-strand cDNA synthesis was carried out by denaturing 1.5 μg of total RNA at 65°C for 10 min in 12 μl of diethylpyrocarbonate–treated water with 30 pmol of each of the primers A3/3′ and B3/3′. The denatured RNA was chilled on ice for 2 min and incubated for 10 min at room temperature and then for 60 min at 42°C in a final volume of 20 μl containing 4 μl 5 × reverse-transcriptase buffer (Promega), 10 mM DTT, 0.5 mM each dNTP, and 200 units of reverse transcriptase (catalog M530; Promega). The reaction was terminated by heating to 95°C for 5 min and cooling on ice. A first amplification by PCR, resulting in the amplification products A and B, was performed by combining the following reagents in a 50-μl reaction: 2 μl of the reverse-transcribed product, 5 μl 10 × PCR buffer (Boehringer-Mannheim), 200 μM each dNTP, 1.5 units of *Taq* DNA polymerase (Perkin Elmer/Roche), 20 pmol of each primer (product A, A1/5′ and A3/3′; and product B, B1/5′ and B3/3′). Nested PCR amplifications A1 (A1/5′ and A1/3′), A2 (A2/5′ and A2/3′), and A3 (A3/5′ and A3/3′) were performed using 1 μl of amplification product A. Nested PCR amplifications B1 (B1/5′ and B1/3′), B2 (B2/5′ and B2/3′), and B3 (B3/5′ and B3/3′) were performed with 1 μl of amplification product B. The amplified products were separated on a 3% agarose gel (NuSieve 3:1; FMC). The conditions for nested PCR were identical to those for the amplification of fragments A and B. Amplification was carried out in a GeneAmp 9600 machine (Perkin Elmer), with the following parameters: initial denaturation for 5 min at 94°C, 32 three-step cycles (denaturation at 94°C for 15 s, annealing at 58°C for 15 s, and elongation at 72°C for 1 min 15 s), and 3 min at 72°C. The oligonucleotide primers used in reverse transcription and PCR amplification were as depicted in table 1.

SSCP Analysis

For SSCP analysis, identical nested PCR reactions were performed as described above, except that 20 μM dCTP and 0.1 μl of [α-^{32}P] dCTP (3,000 Ci/mmol; Du Pont–New England Nuclear) were added. After amplification, PCR products were diluted (1:6) with 0.1%

Table 1

Oligonucleotides for PCR and Sequence Analysis

Name	Sequence (5'→3')	Position[a]	Expected Size (bp)
A1/5'.....	CATGGCCGGGCCATCGCTTCC	−1/21	335
A1/3'.....	CCTGAACCAGCTCCTCTTCAGC	313/334	
A2/5'.....	TCAAAGGAAGAACCAGTCACC	259/279	353
A2/3'.....	TCAGCTTCATCCCTGGCTCG	592/611	
A3/5'.....	GGAGAGAATTACTGCTTGGTAC	555/576	370
A3/3'.....	CATAAATAGATCATGGTTCCCGAT	901/924	
B1/5'.....	CCTCAAAGCTTCGTGTTAATAAGC	860/883	365
B1/3'.....	TTCCTGCTCAGCCTCTGCGGC	1204/1224	
B2/5'.....	GGAGGCAAAACTTCTGGCCCAG	1179/1200	339
B2/3'.....	GACAGGCTGTCACCAATGAGG	1497/1517	
B3/5'.....	CAATTCCAGCACCGTTGCCTCC	1457/1478	350
B3/3'.....	GGGTGGCTGGGTCACCTGCT	1787/1806	

[a] Relative to the initiation codon (Rouleau et al. 1993).

SDS and 10 mM EDTA. A 5-μl sample of the diluted reaction was then mixed with 5 μl of stop solution (U.S. Biochemical Corporation). After denaturing at 94°C for 2 min and chilling on ice, 3 μl of the sample was loaded onto a 0.5 × Mutation Detection Enhancement gel (J. T. Baker) and electrophoresed at 8 W constant power for 14–16 h at room temperature, with 0.6 × TBE (Tris-borate EDTA) buffer. Gels were transferred to 3 MM Whatman paper, dried, and exposed to Kodak XAR-5 film.

Subcloning and Sequencing

Individual bands were carefully excised from dried SSCP gels and were placed in 100 μl of deionized water for 3 h at 37°C, with gentle shaking to elute the DNA from the gel. Five microliters of eluted DNA was reamplified using the appropriate primers as described above. Amplified products were subcloned into the plasmid vector pCM™II (Invitrogen). A mixture of four independent plasmids from one PCR amplification were sequenced with the appropriate NF2 primers from both orientations by using double-stranded recombinant plasmids as template for the dideoxy chain-termination method (U.S. Biochemical).

In Situ Hybridization, Cytogenetic and RFLP Analysis of Chromosome 22

The copy number of chromosome 22 was determined using three different techniques, depending on the material available. Karyotyping was performed after 3–50 d of culture. Cosmids from the q arm of chromosome 22 and a chromosome 22–specific centromere probe were used for in situ hybridization on both metaphase spreads and interphase nuclei of cultured and fresh tumor tissue. Chromosome 22–specific cosmid and centromere probes were used along the centromere and the long arm of chromosome 22. RFLP analysis was done by using 10 polymorphic probes for loci on chromosome 22. Only tumor samples with >80% tumor cells were used for this analysis. No constitutional DNA was available for most of the tumor DNA samples. Therefore, LOH for a specific marker was only scored when a heterozygous DNA sample showed considerable reduction of intensity of one of the two alleles. Further details of this study and information about the probes will be published elsewhere.

Results

RNA samples isolated from a total of 48 meningiomas (1 NF2 associated, 3 from patients with multiple meningiomas, and 44 sporadic tumors) and 5 vestibular schwannomas (1 NF2 associated and 4 sporadic tumors) were investigated for mutations in the *NF2* gene transcript. Reverse-transcriptase PCR was used to amplify the whole coding region of the NF2 mRNA in six different overlapping fragments of ~350 bp. All PCR-amplified products were first analyzed by agarose gel electrophoresis to search for large deletions and insertions in *NF2* gene transcripts. Some tumors revealed fragments of altered size. After this preliminary study, SSCP analysis was carried out. All cases showing alterations on agarose gels also detected aberrant fragments by SSCP techniques. Aberrantly migrating fragments

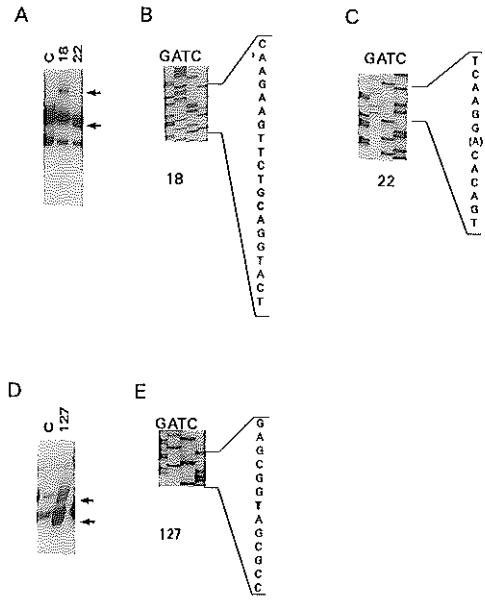

Figure 1 *NF2* gene–transcript mutation analysis. *A* and *D*, SSCP PAGE of reverse-transcribed and PCR-amplified RNA from tumors 18, 22, and 127. The aberrantly migrating SSCP fragments are indicated by arrows. Lanes C, Control RNA samples. *B*, *C*, and *E*, DNA sequence analysis of the area surrounding the mutations in tumors 18, 22, and 127. In *B* the 8-bp insertion in tumor 18 is indicated by unblackened letters; in *C* the 1-bp deletion in tumor 22 is indicated by parentheses; and in *E* the C→T transition in tumor 127 is indicated by the unblackened letter. The 5'→3' orientation is from top to bottom.

were isolated from the SSCP gels. These fragments were amplified once more and were subcloned into a plasmid. In each case, four separate clones were combined for double-stranded sequence analysis in both orientations. Sequence analysis demonstrated the presence of deletions, insertions, and point mutations in all but one sample, which displayed an altered migration on agarose and/or SSCP gels. In figure 1, examples of all three types of alterations are depicted. Figure 1*A* and *D* shows the SSCP analysis of RNAs from tumors 18, 22, and 127, and figure 1*B*, *C*, and *E* shows the corresponding sequences that identify an 8-bp insertion in tumor 18, a 1-bp deletion in tumor 22, and a point mutation in tumor 127.

Table 2 shows a compilation of the results of the *NF2* gene–transcript mutation analysis in the 44 sporadic meningiomas, 1 meningioma from an NF2 patient, and 3 meningiomas from patients with multiple meningiomas. A summary of the chromosome 22 status of these tumors is also included. The copy number of chromosome 22 in the meningiomas and vestibular schwannomas was studied by cytogenetic analysis and by FISH and RFLP analysis, with a variety of chromosome 22–specific probes. Thirteen meningiomas revealed both mutations in the *NF2* gene and loss of (parts of) chromosome 22, one of which represented meningioma from a multiple-meningioma patient (tumor 22) and one of which represented meningioma from a NF2 patient (tumor 121). In 14 (32%) of the 44 sporadic meningiomas, mutations in *NF2* gene transcripts were observed. In tumor 128 we detected two different mutations. It remains to be established whether these represent different mutations in each of the two alleles of the *NF2* gene. Two meningiomas showed SSCP variations due to point mutations not resulting in amino acid substitutions (table 2, tumors 1 and 94). In the meningiomas analyzed here, we found no correlation between the presence of *NF2* mutations and age or sex of the patients (data not shown). A slight overrepresentation of mutations was found in meningiomas with a (partial) fibroblastic histology. However, this was not statistically significant (two-tail Fisher's exact test; $P = .075$).

Table 3 summarizes the results obtained with RNA samples from vestibular schwannomas. Two of four sporadic vestibular schwannomas showed mutations in the *NF2* gene transcript; none showed loss of (parts of) chromosome 22. We also performed mutation analysis on RNA from a meningioma (tumor 121) and a vestibular schwannoma (tumor 106), both of which were derived from NF2 patients. Both cases showed mutations in the *NF2* gene: in tumor 121, an in-frame insertion of 105 bp was detected (table 2), and in tumor 106 a 21-bp deletion was observed, creating a stop codon at the junction (table 3). Both tumors also showed loss of (parts of) chromosome 22.

Discussion

In this report we have shown that mutations in *NF2* gene transcripts are a frequent event associated with sporadic meningiomas. RNA SSCP analysis of the coding region of the *NF2* gene in 53 unrelated patients with meningiomas and vestibular schwannomas revealed mutations in 32% of the sporadic meningiomas ($n = 44$), in 50% of the sporadic vestibular schwannomas

Table 2

***NF2* Gene–Transcript Mutations in Meningiomas**

Tumor	Loss of (parts of) Chromosome 22[a]	Mutation	Position[b]	Result
1	?	GAA→GAG	387	Polymorphism
3	−22			
7	−22			
9	−22	TAT→TAA	303	Stop
10	−22			
12	?			
15	Diploid	96-bp deletion	329–424	In frame
18	−22	8-bp insertion	241	Frameshift
22[c]	−22	1-bp deletion	209	Frameshift
25	−22	128-bp insertion and 3-bp deletion	448 and 449–451	Frameshift
32	−22			
35	−22			
41	−22			
48	?			
54	−22			
55[c]	−22			
82	−22			
85	Diploid			
88	Diploid			
90	Diploid			
92	−22			
93	−22	1-bp deletion	1107	Frameshift
94	−22	GCC→GCT	219	Polymorphism
99	−22	20-bp deletion	448–467	Frameshift
108	−22	CAA→CT	53–54	Frameshift
109	−22			
111	−22	1-bp deletion	76	Frameshift
116	−22			
118	Diploid			
119	−22			
121[d]	−22	105-bp insertion	517	In frame
125	−22			
127	−22	CAG→TAG	1009	Stop
128	Diploid	2-bp deletion	36–37	Frameshift
		82-bp deletion and 18-bp insertion	496–577	Frameshift
130	Not done			
133	Diploid			
135	−22			
136	Diploid			
140	−22	14-bp deletion	1223–1236	Frameshift
141	?	2-bp deletion	68–69	Frameshift
143	−22			
144	−22			
145	−22	119-bp insertion	364	Frameshift
147	−22			
149	−22			
150	−22	13-bp deletion	153–165	Frameshift
153	Diploid			
154[c]	Diploid			

[a] −22 = Complete or partial loss of chromosome 22; Diploid = no loss of chromosome 22; and ? = not conclusive.
[b] Relative to the initiation codon (Rouleau et al. 1993).
[c] Patient with multiple meningiomas.
[d] Meningioma in an NF2 patient.

Table 3

***NF2* Gene–Transcript Mutations in Vestibular Schwannomas**

Tumor	Loss of (parts of) Chromosome 22[a]	Mutation	Position[b]	Result
68	Not done			
106[c]	−22	21-bp deletion	38–58	Stop
110	Diploid	53-bp deletion	440–492	Frameshift
117	Diploid	22-bp deletion	1023–1044	Frameshift
151	Diploid			

[a] −22 = Complete or partial loss of chromosome 22; and Diploid = no loss of chromosome 22.
[b] Relative to the initiation codon (Rouleau et al. 1993).
[c] Vestibular schwannoma in an NF2 patient.

($n = 4$), in 100% of the tumors found in NF2 patients ($n = 2$), and in one of three tumors from multiple-meningioma patients. The mutations found can be divided into three categories: point mutations, deletions, and insertions. Three tumors carried mutations creating a stop codon at the mutated site (table 2; tumors 9, 106, and 127). Most insertions and deletions introduced frameshifts leading to premature termination of the reading frame within 14–200 bp of the site of mutation. The insertions found in RNA from tumors 18, 25, and 121 occurred at the border of two exons, indicating that they could be the result of splice-junction mutations. The 20-bp deletion in RNA from tumor 99 is at the beginning of exon 5 and is probably caused by a mutation in the splice acceptor site of exon 5. In our series of samples, no deletions of complete exons were observed. In two cases, an in-frame deletion of 96 bp and a 105-bp in-frame insertion were observed in RNA from tumors 15 and 121, respectively (table 2). The mutations observed predict major alteration in the structure of the merlin protein, suggesting that these represent real loss-of-function mutations, leading to inactivation of the *NF2* tumor-suppressor gene.

Most of the NF2 mutations described in this paper map to the N-terminal region of the predicted merlin protein (fig. 2), leading predominantly to deletions in the C-terminal part of the protein. However, it is as yet unclear whether the N-terminal moesin-ezrin-radixin-homology region of the merlin protein is more frequently associated with mutations in meningiomas. Mutations in sporadic vestibular schwannomas also appear to cluster somewhat in the N-terminal region of the merlin protein. However, mutations also have been found scattered throughout the *NF2* gene coding region in multiple tumor types (Bianchi et al. 1994). More extensive studies will be required to satisfactorily address the question about the pattern of mutations in human tumors.

The alterations observed are in agreement with a recessive tumor-suppressor gene model, because, in 13 of 15 meningiomas and in 1 of 3 vestibular schwannomas, mutations in *NF2* gene transcripts were observed together with loss of (parts of) chromosome 22, which is in accordance with the two-hit hypothesis first described by Knudson (1971). In two tumors derived from NF2 patients, both mutations in the *NF2* gene and loss of (parts of) chromosome 22 were observed. Thus, these mutations most likely represent germ-line alteration responsible for the disease in these patients. Further analysis of somatic tissue should prove this. Patient 121 belongs to a family in which the patients suffer mostly from multiple meningiomas (e.g., see patient FIII,2 in Delleman et al. 1978). However, bilateral vestibular schwannomas, the hallmark for NF2, were

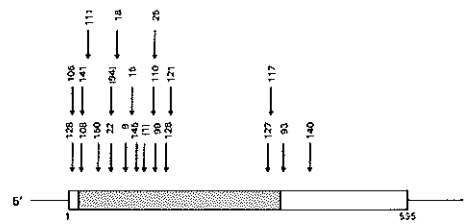

Figure 2 Outline of the position of the different mutations, with respect to the open reading frame of the NF2 protein. The region that displays homology to moesin, ezrin, and radixin is hatched. The two tumor samples showing silent mutations are in parentheses.

observed at autopsy in one member of the family in 1939. This, together with the presence of an *NF2* gene mutation in one of the tumors, suggests that this is indeed an NF2 family.

In most cases where we discovered aberrant fragments by agarose gel electrophoresis and/or SSCP, the intensity of the aberrant fragment was less than that of the wild-type fragment; this was also so in cases where, in addition to the mutation, loss of chromosome 22 was observed (fig. 1*A* and *D*). A possible explanation for the differences in intensity between wild-type and mutant RNA could be that a considerable number of normal endothelial cells may be present in the tumor. This is also obvious from RFLP studies, where complete loss of one allele is hardly ever observed (authors' unpublished results). An additional explanation is that the mutant RNA molecules, which in most cases can lead only to very short, truncated proteins, are less stable than their normal counterparts (Sachs 1993).

The frequent occurrence of *NF2* gene mutations in meningiomas and vestibular schwannomas suggests that this gene is important for the development of these types of tumors in both their sporadic form and their hereditary form. The actual frequency of *NF2* gene mutations may be even considerably higher, in light of the limits of techniques such as SSCP analysis, which depends on factors such as fragment size and position of the mutation (Sheffield et al. 1993). In the paper by Sheffield et al. (1993), the detection frequency of fragments of 300–400 bp is ~60%. Thus, when the presumed efficiency of the SSCP method is corrected for, the mutation frequency would increase to 50%. In addition, mutations in the promoter of the gene and very large deletions and insertions will not be detected by SSCP analysis. However, it is very much the question whether, when all these limitations might be solved, the percentage of mutations would reach 100. This is especially interesting because evidence exists for putative additional loci on chromosome 22. Aberrations of chromosome 22 that are not in the vicinity of the *NF2* gene have been described by Dumanski et al. (1990) and D. R. Cox (personal communication). In addition, we have recently cloned a gene proximal to the *NF2* gene, which is disrupted by a reciprocal translocation t(4;22) in a meningioma (table 2, tumor 32; Lekanne Deprez et al. 1991, 1994). Close to this gene we have found a germ-line deletion in a patient with multiple meningiomas (table 2, tumor 55; Lekanne Deprez et al. 1994). Neither case revealed mutations in NF2 transcripts by SSCP analysis of the coding region (present paper). This suggests that, besides the *NF2* gene, other genes on chromosome 22 may exist that play a role in the pathogenesis of meningiomas and vestibular schwannomas.

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Summary and Discussion

Meningioma is a common benign central nervous system tumor (CNS) arising from arachnoidal cells surrounding the brain and spinal cord. Cytogenetic studies as early as 1967 and molecular genetic analyses revealed that loss of chromosome 22 is the most frequent aberration observed in these tumors. In addition, 50% of the patients with the autosomal dominant inherited disorder NF2 have meningiomas. Linkage analysis in affected families has assigned the predisposing gene to chromosome 22. This indicates that both NF2 and sporadic meningioma presumably are the result of mutations in one or more genes on chromosome 22. Consistent and specific chromosome deletions suggest the involvement of tumor suppressor genes. A tumor suppressor gene plays a role in tumorigenesis when both alleles of the gene are inactivated. Loss of such a gene is one of the mechanisms involved in functional inactivation. Therefore, a tumor suppressor gene(s) on chromosome 22 is (are) probably involved in the development of meningiomas. The goal of the work described in this thesis was to localize and isolate this (these) gene(s) on chromosome 22.

In order to identify the region(s) on chromosome 22 responsible for meningioma development we first collected meningiomas for cytogenetic and DNA analysis. At that time only a limited number of markers from this chromosome were available. Therefore, a chromosome 22 specific phage library was used for the isolation of additional probes. The 25 isolated single-copy probes were further characterized by testing whether they recognized restriction fragment length polymorphisms (RFLPs) on human genomic DNA and they were regionally localized on chromosome 22 using a panel of somatic cell hybrids. The polymorphic probes were used to construct a linkage map (Chapter II).

These probes and markers obtained from other laboratories were subsequently used to investigate our collection of meningiomas. Chapter III describes the results of the combined cytogenetic and molecular genetic study in our series of meningiomas. Furthermore, the observed chromosomal changes were compared with other patient and tumor characteristics and statistically analyzed. In accordance with other reports loss of chromosome 22 was most frequently observed. We found complete or partial loss of this chromosome in 71% of the tumors. Statistical analysis revealed a significant association between the number of

chromosomal abnormalities and tumor grade. Complex karyotypes predominated in the group of grade II/III meningiomas. This suggests that in the progression of meningiomas multiple genetic hits are required. In addition, other variables showed statistically significant differences indicating that meningioma subclasses exist. For instance, meningiomas from the convexity were more often grade II/III, displayed predominantly (partial) loss of chromosome 22 and had complex karyotypes more often. These features were frequently found in meningiomas from males. Base meningiomas on the other hand occurred more often in females, they were usually grade I, showed loss of (parts of) chromosome 22 less often and displayed fewer additional chromosomal abnormalities. Future studies of patient survival, recurrence rate and invasive growth of the tumors should establish whether these observations are of clinical importance.

The combined cytogenetic and molecular genetic approach was also applied to an anaplastic ependymoma, which revealed loss of markers on the long arm of chromosome 22 (Chapter IV). This tumor was obtained from an 8 month old boy born from a family in whom two healthy brothers each had two sons with anaplastic ependymomas. Normal cells from this boy showed a normal karyotype. This finding provides evidence for the role of a tumor suppressor gene on chromosome 22 in the pathogenesis of ependymal tumors. The clustering of ependymal tumors in four nephews from one family suggests that a predisposing germ-line mutation is involved in the pathogenesis. However, because neither the fathers nor the grandparents developed ependymomas there is no evidence for a simple dominant mode of inheritance of the disease in this family. A possible explanation could be that there is mendelian inheritance of the disease but that the inheritance involves a gene which is inactivated by paternal imprinting rather than by an inactivating mutation. An alternative model is based on, for instance the fragile X syndrome, in which the expansion of a trinucleotide repeat in the *FMR-1* gene can continue over several generations untill a final step suddenly reveals the disease in members of the same generation.

Cytogenetic analysis of our meningiomas revealed one tumor, meningioma 32, with a reciprocal t(4;22)(p16;q11). We hypothesized that this translocation disrupts a potential tumor suppressor gene. To further investigate this translocation we fused the meningioma cells with a hamster cell line. This resulted in hybrids segregating one of the two products of the

translocation (Chapter V). These hybrid cell lines were subsequently used to map the breakpoint with probes from the relevant region of chromosome 22. A combination of pulsed field analysis and chromosome walking resulted in both the recognition and crossing of this breakpoint on chromosome 22 respectively. From this region a gene, designated *MN1*, was isolated that was disrupted by the translocation (Chapter VI). Northern blot analysis of this gene in meningiomas showed a very irregular expression pattern. Some, including meningioma 32, showed no expression and others a relatively high expression level. This might suggest that the *MN1* gene acts as a tumor suppressor gene at least in the meningiomas without expression. However, on the other copy of chromosome 22 in meningioma 32 the *MN1* gene was intact as judged from Southern blotting and because the breakpoint of the der(22)t(1;22)(p11;q11) was mapped just distal to this gene. Southern blot analysis of DNA isolated from 71 meningiomas with probes from the region spanning the *MN1* gene revealed alternative fragments in one case that will be discussed below. The *MN1* gene is highly conserved in evolution, though sequence analysis and data base searches have not yet provided clues to its function. The nucleotide sequence of the 8 kb MN1 mRNA would suggest 2 distinct and physically separated open reading frames (ORF). Computer analysis of these sequences (GRAIL program) suggests that they are excellent exon candidates. Preliminary in vitro transcription/translation experiments suggest that only one large protein is formed. We speculate that both identified ORFs are part of this large protein and that the sequence in between still contains some sequence artifacts. The 5' leader sequence of the *MN1* transcript is very GC-rich and contains 4 AUG codons upstream of the putative translational start site, both features suggest that this gene is poorly translated. These characteristics are also found in mRNAs for proto-oncogenes, transcription factors, receptors and signal transduction components. This suggests that such leader sequences are important in the regulation of expression at the level of translation.

In another patient (patient 55) who suffered from multiple meningiomas, which suggests a genetic predisposition, we observed a germ-line deletion and point mutation close to the *MN1* gene on chromosome 22. The other copy of chromosome 22 was lost in all four investigated tumors (Chapter VII). This might suggest that the constitutional mutations and the loss of chromosome 22 inactivate the postulated predisposing gene, which could be the *MN1* gene. Although the position of the germ-line alterations are very suggestive we found a high

expression of the *MN1* gene in one of the tumors. Furthermore, the father of this patient revealed the same germ-line alterations without signs of meningiomas. Thus so far, the involvement of the *MN1* gene in meningioma development in this patient remains an open question.

The recent cloning of the *NF2* gene, which is located about 1.7 Mb distal to the *MN1* gene, suggests that different genes on chromosome 22 might play a role in meningioma development. To elucidate the extent in which the *NF2* gene is involved in meningioma development we performed RT-PCR SSCP mutation analysis in 44 sporadic meningiomas (Chapter VIII). In 14 tumors mutations, predominantly resulting in premature termination of the putative protein, were observed and this often was accompanied by loss of (parts of) chromosome 22. These results suggest that inactivating mutations of the *NF2* gene are frequently involved in sporadic meningioma development and that this gene functions as a recessive tumor suppressor gene. However, in both meningioma 32 and patient 55, with aberrations in or close to the *MN1* gene we were not able to identify mutations in the *NF2* gene using the RT-PCR SSCP approach.

In addition to the isolated *NF2* and *MN1* genes, other studies suggest even more loci involved in meningioma development. Molecular genetic analyses in two meningiomas revealed aberrations distal to the *NF2* gene on chromosome 22. Furthermore, recently linkage analysis in a meningioma/ependymoma family excluded 15 Mb of chromosome 22, including the *NF2* gene, as the region harbouring the predisposing mutation. This indicates that another menigioma locus exists probably on another chromosome. This hypothesis assumes that mutations in different genes can lead to the development of the same tumor type. Such a situation resembles the development of Wilms' tumors in which three loci, two on chromosome 11p and a third one still unknown, have been identified. One obvious explanation for this observation might be that all these putative genes participate in the same signal transduction pathway and that abrogation of separate steps in the pathway can lead to the same type of tumor. Another possibility might be that these different genetic alterations are responsible for the development of the same tumor type with specific for instance histological characteristics or that a combination of both takes place. Interesting in this respect is the fact that both meningiomas showing alterations in or close to the *MN1* gene

were derived from patients with more than two meningiomas located at different sites in the brain. Moreover, one or more of the mentioned subclasses of meningiomas (Chapter III) might be the result of mutations in different genes.

The search for genes that might play a role in menigioma development have resulted in the isolation of two genes and the suspected involvement of at least two others that are still unknown. Therefore, much more research is required to completely understand the pathogenesis and progression of meningiomas. Concerning the *MN1* gene, mutation analysis in meningiomas must be performed to investigate the importance of this gene in meningioma development in general. This mutation analysis should be extended to other tumor types with aberrations of chromosome 22 in order to determine the role of MN1 in cancer development. At present it is not clear whether *MN1* gene mutations are the primary or secondary cause of tumor development in (some) meningiomas. Interesting in this respect is the observation that meningioma 32 cells could be cultured for a very long time, whereas other meningioma cultures stopped dividing after a few weaks or became overgrown by normal cells initially present in low numbers in the cell cultures. Therefore, alterations of *MN1* might contribute to immortalisation. Functional studies such as transfection experiments of mutated or wild-type MN1, the generation of (knock-out) transgenic mice and protein studies should provide insight into the normal function of the *MN1* gene and its contribution to neoplastic disease.

Recent work by others and our mutation study have clearly indicated that the NF2 gene is involved in meningioma development. Sequence comparison of the NF2 gene with moesin, ezrin and radixin suggests that this gene plays a role in mediating interactions between the cell membrane and the cytoskeleton. These findings should facilitate future studies about the exact role of this gene in tumorigenesis. The generation of antibodies and transgenic animals might provide useful tools for this analysis.

Cloning of the as yet unkown genes and the continuation of mutation analysis of all identified putative meningioma genes might result in a better understanding of the involvement of the individual genes and might eventually lead to a model for meningioma development.

Samenvatting

Meningeomen zijn goedaardige tumoren van het centrale zenuwstelsel (CZS) en ontstaan uit cellen afkomstig van het vlies, dat de hersenen en het ruggemerg omgeeft. Chromosoomstudies, reeds uitgevoerd vanaf 1967, en moleculair-genetische analyses hebben laten zien, dat verlies van chromosoom 22 de meest frequente afwijking in deze tumoren is. Daarnaast komen meningeomen frequent voor bij patiënten met de dominant overervende aandoening NF2. Koppelings-onderzoek in NF2 families heeft aangetoond, dat het verantwoordelijke gen op chromosoom 22 gelocaliseerd moet zijn. Dit wijst erop dat zowel NF2 als sporadische meningeomen waarschijnlijk veroorzaakt wordt door één of meerdere genen op chromosoom 22. Het consequent aanwezig zijn van een specifieke chromosomale afwijking, in dit geval verlies van chromosoom 22, doet vermoeden dat we hier te maken hebben met een tumorsuppressorgen (genen). Een tumorsuppressorgen draagt bij tot het ontstaan van een tumor, wanneer beide copieën van het gen geïnactiveerd zijn. Verlies van een dergelijk gen is één van de mechanismen om het gen te inactiveren. Deze bevindingen veronderstellen dat ten minste één tumorsuppressorgen op chromosoom 22 betrokken is bij het ontstaan van meningeomen. Het doel van het werk, beschreven in dit proefschrift, was om zulke genen op chromosoom 22 te isoleren en te bestuderen.

Het onderzoek werd opgestart met de isolatie van DNA merkers, die specifiek voor chromosoom 22 zijn. Bij deze merkers hebben we onderzocht of ze in staat waren polymorfismen in restrictie enzym knipplaatsen te detecteren in normaal menselijk DNA. Voor de localisatie van de merkers op chromosoom 22 hebben we gebruik gemaakt van DNA, geïsoleerd uit een aantal hybride cellijnen, die stukken van chromosoom 22 bevatten. De polymorfe merkers zijn ook gebruikt om een genetische koppelingskaart van een deel van chromosoom 22 te maken.

Onze polymorfe merkers en merkers, die van andere laboratoria afkomstig waren, hebben we gebruikt om DNA, geïsoleerd uit onze serie meningeomen en een aantal andere tumoren van het CZS, te bestuderen op afwijkingen van chromosoom 22. De tumoren zijn vervolgens op chromosomaal niveau nader gekarakteriseerd door gebruik te maken van cytogenetische analysetechnieken. Deze methoden stelden ons in staat om (delen van) chromosomen aan te

wijzen, die mogelijk voor meningeoom ontwikkeling van belang zouden kunnen zijn. De resultaten van deze gecombineerde studie zijn vergeleken met een aantal kenmerken van de patiënten en hun tumoren. In overeenstemming met de bevindingen van andere onderzoeksgroepen was verlies van chromosoom 22 de meest voorkomende chromosomale afwijking. Ook het anaplastisch ependymoom vertoonde verlies van chromosoom 22. In 71% van de meningeomen werd volledig of gedeeltelijk verlies van dit chromosoom geobserveerd. Statistische analyse toonde een significant verschil tussen het aantal chromosomale afwijkingen en de gradering van de tumor. Complexe karyogrammen werden vaker gevonden in tumoren van graad II en III. Dit doet vermoeden dat bij de progressie van meningeomen meerdere genetische gebeurtenissen een rol spelen. De statistisch significante verschillen, die tussen de variabelen gevonden waren, suggereren dat meningeomen in verschillende subgroepen onder te verdelen zijn. Convexiteit meningeomen toonden meer verlies van (delen van) chromosoom 22, meerdere chromosomale afwijkingen, waren in hoofdzaak van graad II en III en kwamen vaak bij mannen voor. Meningeomen gelocaliseerd in de basis daarentegen vertoonden weinig verlies van (delen van) chromosoom 22, weinig andere chromosomale afwijkingen, waren meestal van graad I en werden vaker bij vrouwen gevonden.

Chromosoom-analyse resulteerde in de identificatie van een tumor, meningeoom 32, met een gebalanceerde t(4;22)(p16;q11). Onze werkhypothese was dat deze translocatie een tumorsuppressorgen kapot zou maken en dat het verder in kaart brengen van het translocatie breukpunt ons zou kunnen helpen bij het isoleren van dit gen. Hiervoor hebben we de tumorcellen gefuseerd met een hamster-cellijn, waardoor twee hybride cellijnen verkregen werden, die elk één translocatieproduct bevatten. Dit maakte het mogelijk om met chromosoom 22 specifieke merkers de ligging van het breukpunt te bepalen. Eén van deze merkers bleek het breukpunt te herkennen. Met behulp van genomische en cDNA banken werd het gebied van chromosoom 22, waarin het breukpunt gelegen is, geïsoleerd en een gen in dit gebied geïdentificeerd. Nadere plaatsbepaling van dit gen, door ons *MN1* genoemd, toonde aan dat het door de translocatie kapot gemaakt werd. Verdere karakterisatie van het gen door middel van sequentie-analyse en vergelijking met genen-banken hebben geen aanwijzingen voor zijn mogelijke functie opgeleverd. Het gen is wel sterk geconserveerd gedurende de evolutie. Southern-blot technieken zijn vervolgens gebruikt om afwijkingen in

het gebied rondom het *MN1* gen te bestuderen in 71 andere meningeomen. In één van deze tumoren, afkomstig van een patiënt met meerdere individuele meningeomen (patiënt 55), detecteerden we afwijkingen in dit gebied. Deze afwijkingen bleken ook aanwezig te zijn in DNA geïsoleerd uit bloed van patiënt 55. De afwijkingen, een intersitiële deletie en een punt mutatie, bevinden zich op ongeveer 10 kb afstand van het *MN1* gen. Op dit moment is niet bekend of deze afwijkingen voor de meningeomen in deze patiënt predisponeren en de functie van het *MN1* gen beïnvloeden. Nader onderzoek moet uitwijzen hoe belangrijk dit gen is voor de ontwikkeling van meningeomen.

Op grond van literatuurgegevens zijn er aanwijzingen dat meerdere genen een rol zouden kunnen spelen bij meningeoom ontwikkeling. Recentelijk is het op chromosoom 22 gelegen *NF2* gen gecloneerd. Bovendien doen afwijkingen distaal van het *NF2* gen in een tweetal meningeomen vermoeden dat een derde, nog niet geïsoleerd, gen een rol zou kunnen spelen. Verder laat koppelingsonderzoek in een ependymoma/meningeoma familie zien, dat een groot deel van chromosoom 22, waaronder het *NF2* gen, niet verantwoordelijk is voor het ontstaan van de ziekte in deze familie. Waarschijnlijk is het predisponerende gen op een ander chromosoom gelegen. Mutatie-studie van het *NF2* gen hebben we uitgevoerd om de rol van dit gen bij meningeoom ontwikkeling te onderzoeken. In 30% van de tumoren was het mogelijk mutaties op te sporen. Bijna alle mutaties zouden leiden tot een sterk verkort NF2 eiwit, waardoor het eiwit waarschijnlijk geïnactiveerd wordt. In de meeste tumoren gingen de mutaties samen met verlies van (delen van) chromosoom 22. Deze bevindingen doen sterk vermoeden dat we hier te maken hebben met een recessief tumorsuppressorgen en dat dit gen een belangrijke rol vervult bij het ontstaan van meningeomen. Echter dit sluit de betrokkenheid van andere genen niet uit. Verder onderzoek zal moeten bepalen wat de (onderlinge) functie van deze genen is bij het proces, dat aan de ontwikkeling van meningeomen ten grondslag ligt.

Curriculum vitae

30 juni 1965	geboren te Oegstgeest
1984	eindexamen VWO aan het Rijnlands Lyceum te Oegstgeest
1984 - 1988	studie Gezondheidswetenschappen aan de Faculteit der Geneeskunde Rijksuniversiteit van Leiden (cum laude) *Bijvakstages:* 1 Afdeling Klinische Oncologie, Academisch Ziekenhuis Leiden begeleider: Dr. P.J. Schrier 2 Afdeling Klinische Oncologie, Academisch Ziekenhuis Leiden begeleider: Prof. Dr. F.J. Cleton 3 Afdeling Nierziekten, Academisch Ziekenhuis Leiden begeleider: Prof. Dr. M.R. Daha 4 Afdeling Medische Biochemie, Sylvius Laboratorium Leiden begeleider: Dr. J.A. Maassen *Hoofdvakstage:* Afdeling Medische Biochemie, Sylvius Laboratorium Leiden begeleider: Dr. J.A. Maassen
1989 - 1994	Promotieonderzoek, afdeling Pathologie, Erasmus Universiteit Rotterdam promotor: Prof. Dr. D. Bootsma (afd. Genetica) co-promotor: Dr. E.C. Zwarthoff
1994 -	Wetenschappelijk onderzoeker, afdeling Anatomie & Embryologie, Academisch Medisch Centrum, Amsterdam

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Nawoord

Dit boekje is tot stand gekomen na vijf jaar onderzoek op de afdeling Pathologie. Terugkijkend kom ik tot de conclusie dat deze periode voor mij alle ingrediënten bevatte, die voor de toekomst van een (ex-)promovendus zeer waardevol kunnen zijn. Het was een tijd van hard werken, enerverende ontdekkingen ('ups en downs'), leerzame ontwikkelingen, en stimulerende samenwerkingen binnen de Erasmus Universiteit en daarbuiten. Dat ik altijd met veel plezier op het lab gewerkt heb, dank ik aan mijn direkte collega's. Zonder de indruk te wekken volledig te zijn, wil ik op deze plaats diegenen, die voor mijn promotie van groot belang zijn geweest, met name noemen.

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