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Fine-mapping of the gene responsible for a new variant of multiple endocrine neoplasia in the rat

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To my father

1 Introduction

A new variant of multiple endocrine neoplasia (MEN) has been identified in a subline of the Sprague Dawley laboratory rat. The affected animals develop inborn bilateral cataracts (*Sprague Dawley white eye*, *SD*^{we}) with a clear cornea and a well-differentiated retina. The typical alterations include: a swollen lens epithelium, rupture of the posterior lens capsule, formation of deep clefts and vacuoles in the lens cortex, the presence of a yellow, amorphous structure in the lens core.

The new rat phenotype, initially identified by the presence of cataracts, includes also neoplasia of multiple endocrine tissues. The spectrum of affected organs in *SD*^{we} rats, as well as the mode of inheritance differ from MEN1 and MEN2 syndromes known in human. To emphasize these differences the new MEN syndrome was designated MENX. So far, in the reports on cataract in the rat (Yokoyama et al., 2001; Yamashita et al., 2002; Takamura et al., 2002) no associated endocrine malignancies were described. Sporadic endocrine tumors commonly observed in rats do not appear in young animals (Lee et al., 1982), whereas endocrine malignancies characteristic for MENX rats are developed within the first year of life.

1.1 Cancer of neuroendocrine cells

The neuroendocrine tissues of mammals are found in a number of morphologically distinct secretory organs, including the pituitary, thyroid, parathyroid, and adrenal. In addition, the neuroendocrine cells contribute to the diffuse endocrine tissues, such as the gastric secreting cells of the gastrointestinal tract (Waldum et al., 1993). The neuroendocrine cells derive from neural crest and produce hormones such as ACTH, calcitonin, vasoactive intestinal peptide (VIP), growth hormone-releasing hormone (GHRH), pancreatic polypeptide, corticotropin-releasing hormone (CRH), neurotensin, and somatostatin (SRIH).

It has been postulated that the progression of the neuroendocrine tumors might be stimulated by overexpression of growth factors involved in cell proliferation

(Nilsson O. et al., 1995). Each tissue produces hormone secretions in response to the biological signals that modulate not only the secretory activity but also the cellularity. Thus, in situations of chronic demand the number of secretory cells can be increased. In abnormal physiological circumstances (including excess secretion of trophic hormones) this can lead to hyperplasia, e.g. chronic hypocalcaemia/ hyperphosphatemia provokes expansion of the parathyroid cell mass, leading to secondary parathyroidism. Long term stimulation by positive growth signals can lead to a transition of the hyperplastic tissues into neoplasia. Neoplasms of endocrine organs include pituitary adenoma, bilateral medullary thyroid cell neoplasia, bilateral parathyroid hyperplasia, bilateral adrenal pheochromocytoma, and multiple extra-adrenal pheochromocytoma.

Pituitary adenomas are the most common type of pituitary tumors. They are usually benign and cause a variety of disorders (Table 1).

Syndrome	**OMIM**	**Gene**	**Proposed function/defect**
MEN1	131100	*MEN1*	Nuclear, tumor suppressor interaction with junD
Familial acromegaly	102200	*GNAS1?*	--
McCune-Albright syndrome	174800	*GNAS1*	Signal transduction/ constitutive cAMP elevation
Carney complex, type1	160980	*CNC1*	PKA signalling defect for activating GH

Table 1. Genetic syndromes involving pituitary adenomas (modified from Melmed, 2003).

A candidate gene for pituitary oncogenesis, the pituitary tumor-derived transforming gene (*PTTG*), was identified in rat pituitary tumor cells (Pei and Melmed, 1997). *PTTG* has been suggested to play a role as a transcriptional activator (Chien and Pei, 2000; Pei, 2001). Hunter et al. (2003) confirmed the presence of *PTTG* in pituitary adenomas and demonstrated a significant correlation with growth hormone secretion. FGF receptors (FGFRs) also play a role of in pituitary tumorigenesis. Tumor-derived N-terminally truncated isoform of FGFR4 (ptd-FGFR4) has been shown to cause pituitary tumorigenesis in transgenic mice (Ezzat et al., 2002). ptd-FGFR4 involvement in human pituitary adenomas was shown by Qian et al. (2004). Also, the increased level of peripheral serum sEGFR (the soluble epidermal growth factor receptor) was

shown to be associated with the development of pituitary adenoma (Kong et al., 2004).

Medullary thyroid carcinoma (MTC: OMIM 155240) is a malignant tumor derived from calcitonin-secreting thyroid C cells which are sparsely distributed in the thyroid gland. MTC may occur sporadically or as a component of the familial cancer syndrome, multiple endocrine neoplasia type 2. In hereditary forms, MTC and/or its precursor lesion, C cell hyperplasia, is the single component in familial MTC (FMTC) or is part of the multiple endocrine neoplasia type 2 syndromes (MEN2A and 2B). Familial MTC occurs from mutation in the *RET* gene. The specific *RET* codon mutation correlates with the MEN2 syndromic variant, the age of onset of MTC, and the aggressiveness of MTC (Mulligan et al., 1994; Brandi et al., 2001), it can also be caused by mutations in the *NTRK1* gene (Gimm et al., 1999), an NGF receptor which forms chimeric fusion oncogenic proteins in papillary thyroid carcinomas (Russel et al., 2000). Marsh et al. (2003) analyzed a series of sporadic and familial MTCs characterized for mutations in *RET* for chromosomal imbalances. Chromosomal imbalances in primary MTC tumors were largely identical with those observed in the MTC cell line (Marsh et al., 2003). Activation of MEK/ERK pathway was shown to be induced in MTC cells by IL-1β (Park et al., 2005). The affected chromosomal regions harbor tumor suppressor genes neurofibromin 2 (*NF2*) and the checkpoint kinase 2 gene (*hCHK2*) as well as genes encoding glial cell line-derived neurotrophic factor family receptors alpha-2-4 and their ligands that facilitate RET dimerization and downstream signaling.

Parathyroid tumors may cause increased levels of parathyroid hormones secreted by the parathyroid glands, leading to hyperparathyroidism. Most parathyroid tumors are benign adenomas. Primary hyperparathyroidism is usually the result of a parathyroid adenoma manifesting with hypercalcemia, kidney stones and decalcification of bone. Secondary hyperparathyroidism develops due to excessive secretion of parathyroid hormone and results in decalcification of bone. The overexpression of the *cyclin D1/PRAD1* gene was reported to be involved in parathyroid tumors (Hemmer et al., 2001), whereas the *retinoblastoma* tumor suppressor gene was completely inactivated in most specimens from nine patients with parathyroid carcinoma but not in those with

parathyroid adenoma (Cetani et al., 2004). Parathyroid carcinoma can also be caused by mutation in the *HRPT2* gene encoding a protein called parafibromin (Carpten et al., 2002) which has been postulated to function as a tumor suppressor (Woodard et al., 2004). Inactivating germ-line mutations in *HRPT2* were identified in patients with hereditary hyperparathyroidism–jaw tumor (HPT-JT) syndrome, or hyperparathyroidism 2 (OMIM 145001) (Carpten et al., 2002). Germ-line *HRPT2* mutations were also reported in tumor samples from patients with apparently sporadic parathyroid carcinoma (Shattuck et al., 2003).

The adrenal tumors are usually pheochromocytomas, whereas an extra-adrenal tumor is termed paraganglioma (usually a nonfunctional, i.e. non-catecholamine secreting) neoplasm. Pheochromocytoma and paraganglioma are neoplasms of chromaffin cells which can be found in the adrenal medulla. The chromaffin tissue is a part of the diffuse neuroendocrine (APUD) system derived from the embryonic neuroectodermal crest. The pheochromocytoma is one of the characteristics of von Hippel Lindau syndrome (VHL, OMIM 193300), an autosomal dominant disease also manifesting with multiple cysts in kidney, liver, and pancreas, as well as with retinal and cerebellar hemangioblastoma. The *VHL* gene involved in the disease, codes for a classical tumor suppressor (Kim and Kaelin, 2004). The *VHL* gene was shown to be involved in the regulation of transcriptional elongation: its product, pVHL, binds to and is stabilized by two transcription factors, elongin B and C. Schoenfeld et al. (2000) showed that elongins B and C are stabilized through their interactions with each other and VHL which protects the VHL-elongin complex against proteasomal degradation. Because the VHL-elongin binding site is frequently mutated in cancers, Schoenfeld et al. suggested that loss of elongin binding causes lack of VHL protein stability and/or potential VHL ubiquitination functions. The ubiquitination activity of VHL protein was shown (Okuda et al., 2001; Kuznetsova et al., 2003) and subsequently the C-terminal alpha-helical domain of VHL was identified to play a role in its ubiquitin ligase activity (Lewis and Roberts, 2004). Zatyka et al. (2002) identified cyclin D1 as one of the new targets of *VHL* gene and suggested that genetic modifiers influence the phenotypic expression of VHL disease.

Pheochromocytoma and paraganglioma also occur in families without other associated clinical features, with susceptibility transmitted in an autosomal dominant fashion which suggests that other genes are probably involved in isolated familial pheochromocytoma and paraganglioma. Genes involved in hereditary pheochromocytomas and paragangliomas (Gimm et al., 2000; Astuti et al., 2001; Yip et al., 2004) which are characteristic also for MENX have been isolated, however they account for only a small fraction of the sporadic tumor cases (Maher and Eng, 2002; Parren et al., 2002).

Yang et al. (2003) performed gene expression profiling of sporadic pheochromocytoma and found 341 genes expressed differently in pheochromocytoma and normal adrenal gland, among them genes participating in cell division and apoptosis. Familial pheochromocytoma with or without susceptibility to paraganglioma as well as sporadic pheochromocytoma occurs also with mutations in the succinate dehydrogenase complex, subunit B, D, and C (Maher and Eng, 2002). Paragangliomas are rarely associated with familial and hereditary pheochromocytoma, except in Carney's syndrome (OMIM 160980) which is associated with a functioning extra-adrenal paraganglioma.

1.1.1 Multiple endocrine neoplasia type 1 and type 2

Multiple endocrine neoplasia (MEN) is a group of autosomal dominant disorders characterized by endocrine tumors. There are two major multiple endocrine neoplasia syndromes: MEN1 and MEN2 recognized by different organ associations.

Multiple endocrine neoplasia type 1 (MEN1, OMIM 131100) was first described as the autosomal dominant transmission of parathyroid adenoma, pancreatic islet cell adenoma or carcinoma, and pituitary adenoma in 1954 (Wermer, 1954). Tissues affected in MEN1 include parathyroid, pituitary, and pancreatic islet cells. Tumors are mostly benign and manifest by oversecretion of hormones: prolactin or somatomedin C (pituitary adenomas), insulin and proinsulin (insulinomas), gastrin, vasoactive intestinal polypeptide, or glucagon (patients with MEN1 pancreatic disease). The *MEN1* gene responsible for the multiple endocrine neoplasia type 1 was identified in 1997 and codes for a 610 amino acid protein: menin (Chandrasekharappa et al., 1997). Stewart et al. (1998) investigated *MEN1* gene expression pattern in the mouse and showed

that *MEN1* expression was not restricted to organs affected in MEN1, suggesting that menin has a significant function in many different cell types including the CNS and testis. The protein product of the *MEN1* gene is targeted to the nucleus (Guru et al., 1998) and has been reported to interact with a variety of proteins that regulate transcription, including JunD (Agarwal et al., 1999), Smad3 (Kaji et al., 2001), and NF-κB (Heppner et al., 2001). A comparative genomic approach was used to determine important functional domains of *MEN1* gene via the identification of evolutionary conserved regions (Khodaei et al.,1999). Amino acids affected by inactivating missense mutations in MEN1 patients in this region are completely conserved between human and zebrafish. Guru et al. (2001) identified a *MEN1* ortholog in *Drosophila melanogaster*, *MEN1*, that encodes a 763 amino acid protein sharing 46% identity with the human *MEN1*. Moreover, the majority of the missense mutations and in-frame deletions observed in patients with MEN1 appear in amino acid residues conserved between the *Drosophila* and human protein, suggesting the importance of the conserved regions (Guru et al., 2001). Wautot et al. (2002) stated that most missense and in-frame MEN1 genomic alterations affect one or all domains of menin interacting with JunD, Smad3, and NfkappaB. Crabtree et al. (2001) suggested that menin might play a role in osteoblast formation and differentiation. Sowa et al. (2004) found that menin interacts with Runx2 transcription factor physically and functionally, promoting osteoblast differentiation. In 2003 menin was found to interact with the 32-kDa subunit (RPA2) of replication protein A (Sukhodolets et al., 2003).

MEN1 mutations identified so far are loss of function mutations, and the tumors of MEN1 patients show loss of the wild-type allele (Poisson et al., 2003). Germline mutations in *MEN1* have been found in the majority of MEN1 kindreds and the likelihood of finding an *MEN1* mutation correlates with the number of MEN1-realted tumors and family history (Ellard et al., 2005). The germ line *MEN1* mutations, mostly missense mutations or in-frame deletions-insertions, are also found in familial isolated hyperparathyroidism (FIHP) (Pannett et al., 2003). The somatic mutations in the *MEN1* gene have been identified in sporadic parathyroid adenomas, gastrinomas, insulinomas, and bronchial carcinoids (Carling et al., 1998; Goebel et al., 2000; Wang et al.,

1998; Gortz et al., 1999). Mutations that were reported more than three times in patients with no apparent relationship are listed in table 2.

Mutation	Exon	Clinical presentation
Arg98ter	2	MEN1-F, nonfunctional PET
357del4	2	MEN1-F, Neuroendocrine tumor
359del4	2	MEN1-F, MEN1-S
416delC	2	MEN1-F, MEN1-S
465delK	2	MEN1-F, nonfunctional PET, Parathyroid adenoma
512delC	2	MEN1-F
738del4	3	MEN1-F, MEN1-S
739del4	3	MEN1-F
G893+1A	Intron4	MEN1-F, MEN1-S
G894-9A	Intron4	MEN1-F, MEN1-S
Trp341ter	7	MEN1-F
Arg415ter	9	MEN1-F, nonfunctional PET, Parathyroid adenoma
Asp418Asn	9	MEN1-F, MEN1-S, parathyroid adenoma
Arg460ter	10	MEN1-F, MEN1-S, parathyroid adenoma
1650insC	10	MEN1-F, lung carcinoid
1657insC	10	MEN1-F
Arg527ter	10	MEN1-F

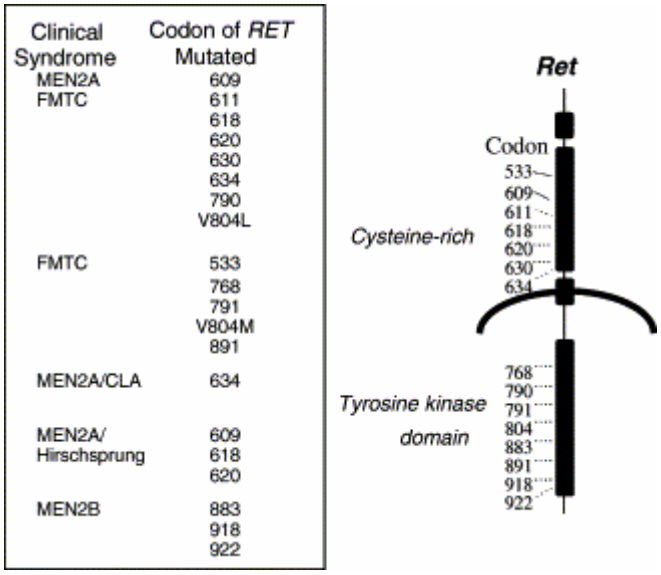
Table 2. Most frequent *MEN1* gene mutations observed in unrelated patients. MEN1-F, MEN1-familial; MEN1-S, MEN1-sporadic; PET, pancreatic endocrine tumor (from Guo and Sawicki, 2001).

Multiple endocrine neoplasia type 2 (MEN2) is a dominantly inherited cancer syndrome that comprises two clinical subtypes: MEN type 2A (MEN2A) and MEN type 2B (MEN2B). Both syndromes have autosomal transmission patterns. The MEN2 families are predisposed to develop medullary thyroid carcinoma, pheochromocytoma, and parathyroid hyperplasia with hyperparathyroidism. MEN2A (OMIM 171400) is characterized by medullary thyroid carcinoma, pheochromocytoma and parathyroid adenoma (parathyroid hyperplasia). No developmental abnormalities are known to be associated with MEN2A. MEN2B (OMIM 162300) resembles MEN2A, in that both include medullary carcinoma of the thyroid, pheochromocytoma, and autosomal dominant inheritance (Table 3).

Symptom	MEN2A	MEN2B
Medullary thyroid carcinoma	100%	100%
Pheochromocytoma	50%	50%
Hyperparathyroidism	10-20%	0%
Intestinal ganglioneuromatosis and mucosal neuromas	0%	>90%
Marfanoid attributes	0%	>90%

Table 3. Tissue involvement in the MEN2 syndromes (Jimenez and Gagel, 2004).

The gene involved in the development of MEN2 is *RET* (rearranged during transfection) (Donis-Keller et al., 1993; Hofstra et al., 1994) which was found to be an oncogene activated by DNA rearrangement (Takahashi et al., 1985). *RET* codes for a tyrosine kinase receptor which is expressed predominantly in tissues of neuroendocrine origin. RET is a transmembrane protein containing four extracellular cadherin-like domains participating in calcium binding; a cysteine-rich region, located close to the cell membrane, promoting dimerization after ligand-stimulation; tyrosine-kinase domain mediating the activation of intracellular signal transduction. The signaling through RET is essential for the maintenance and regeneration of various neurons of the enteric nervous system, as well as for kidney development (Putzer and Drosten, 2004). The point mutations in the *RET* proto-oncogene result in the MEN2A and MEN2B syndromes. The mutations in exons 10 and 11 account for 98% all mutations associated with the MEN2A and the most commmon among them (80% of all MEN2A mutations) is cystein substitution C634R which leads to constitutive receptor activation. The 95% cases of MEN2B are caused by M918T mutation in the *RET* intracellular kinase catalytic domain and the possible result is the shift of RET autophosphorylation sites. The M918T mutation is frequently a *de novo* mutation located on the allele inherited from the patient's father (Santoro et al., 2002; Alberti et al, 2003). The phenotypes associated with specific mutations in *RET* are shown in Fig. 1.



Clinical Syndrome	Codon of *RET* Mutated
MEN2A	609
FMTC	611
	618
	620
	630
	634
	790
	V804L
FMTC	533
	768
	791
	V804M
	891
MEN2A/CLA	634
MEN2A/ Hirschsprung	609
	618
	620
MEN2B	883
	918
	922

Fig. 1. Mutations of the *RET* proto-oncogene and the clinical phenotypes associated with specific mutations. MEN2A, multiple endocrine neoplasia type 2A; FMTC, familial medullary thyroid carcinoma; MEN2A/CLA, MEN2A associated with cutaneous lichen amyloidosis; MEN2A/Hirschsprung, MEN2A associated with Hirschsprung disease; MEN2B, multiple endocrine neoplasia type 2B (Jimenez and Gagel, 2004).

The catalytic activity of RET is initiated by the binding of the ligand to the extracellular domain of the receptor. As a result of this activation conformational rearrangements within the receptor take place which enables the transmission of a growth signal into the cell (Salvatore et al., 2000). The ligands for RET are members of the GDNF (glial cell line-derived neutrophic factor) family related to the transforming growth factor-ß family: GDNF, neurturin, artemin and persephin. Unlike other tyrosine kinase receptors, RET does not interact directly with the GDNF ligands but through another protein, GDNFR-a. GDNF-family ligands require glycosylphosphatidylinositol (GPI)-linked glycoprotein receptors (GFRα-1 to -4) to enable the RET dimerization and activation of RET (Fig. 2).

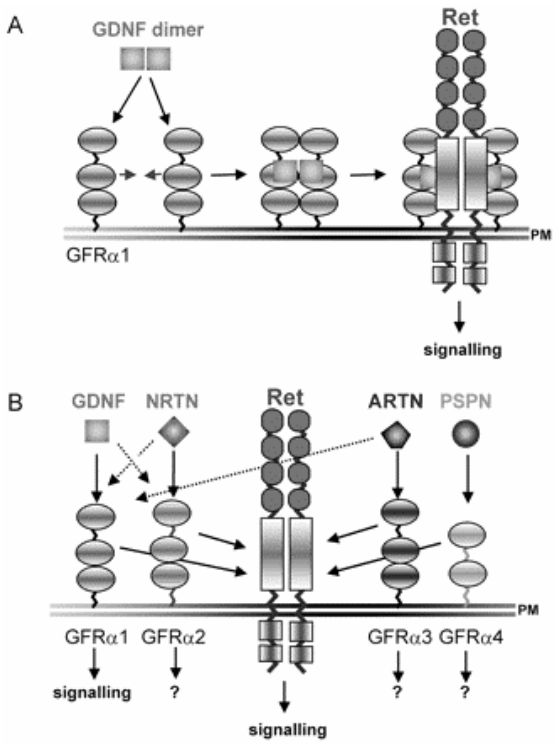


Fig. 2. The RET signaling. (A) glial-cell-line-derived neurotrophic factor (GDNF) dimer brings together two molecules of GDNF family receptor alpha1 (GFRalpha1). This leads to dimerization of two molecules of RET and transphophorylation of RET tyrosine kinase domains. (B) GDNF family ligands activate RET via different GFR receptors. Dotted arrows indicate possible ligand-receptor interactions (from Sariola and Saarma, 2003).

GDNF preferentially binds to GFRα1, neurturin to GFRα2, artemin to GFRα3 and persephin to GFRα4 (Putzer and Drosten, 2004).

Following the interaction with its ligand coreceptor, RET undergoes autophosphorylation and then interacts with multiple intracellular effectors such as Grb-2 Ras, Src, and Shc (Hennige et al., 2000; Califano et al., 2000; Kato et al., 2002; Knauf et al., 2003). The GDNF signal mediated through RET and GFRα-1 is essential for both the development of the enteric nervous system and the kidney, as demonstrated in the studies on mice with null mutations in *GDNF*, *RET* or *GFRα-1* genes (Schuchardt et al., 1994; Sanchez et al., 1996; Cacalano et al., 1998).

A variety of mutations in the *RET* gene (deletion, insertion, frameshift, missense, nonsense) are responsible for the development of Hirschsprung disease (HSCR, OMIM 142623) which manifests through an intestinal malformation absence of ganglia in some or all of the large intestine or colon

(Parisi and Kapur, 2000). Bordeaux et al. (2000) suggested that Hirschsprung disease may result from apoptosis of RET-expressing enteric neuroblasts, since five different HSCR-associated *RET* mutants induce cell death independent of the presence of RET ligand, possibly as a result of the loss of cell survival signal. Iwashita et al. (2001) found that the molecular mechanisms involved in HSCR include partial impairment of RET kinase activity, complete impairment of RET kinase activity and severe impairment of RET expression in neuronal cells. Carasquillo et al. (2002) showed that genetic interaction between *RET* and *EDNRB*, encoding a G protein-coupled receptor, is responsible for the development of Hirschsprung disease.

Although the genes and molecular mechanisms of the inherited MEN syndromes are already well studied, only little is known about the responsible genes and their function in tumorigenesis for many sporadic forms of neuroendocrine tumors. It may be assumed that target genes for the *MEN1*-mediated transcriptional activation and target proteins for the *RET*-mediated phosphorylation can be targets for mutations leading to tumorigenesis. The fact that in many sporadic neuroendocrine tumors, mixed neuroendocrine syndromes or the new MENX syndrome described in this thesis neither *MEN1* nor *RET* were found to be mutated (Fritz et al., 2002) does not exclude the possibility that the diseases are caused by defects in other elements of the *RET* or *MEN1* signaling pathway.

1.1.2 Multiple endocrine neoplasia- like phenotypes

In 1982 Lee et al. described the tumor spectrum of the Long-Evans rat strain, which they proposed as an animal model for a mixed multiple endocrine neoplasia syndrome (Lee et al., 1982). In a colony of aging male Long-Evans rats, 50% of the animals developed pituitary nodules. The nodules were in most cases associated with diffuse hyperplasia of prolactin cells adjacent to the nodules. The animals also demonstrated a high incidence of C cell hyperplasia and/or carcinoma, adrenal medullary nodules, and parathyroid hyperplasia. Like the MENX, the syndrome described by Lee et al. is characterized by a mixture of symptoms characteristic of both the MEN1 and MEN2 (Table 4).

Disorder	Phenotype
Mixed multiple endocrine neoplasia in Long-Evans rats	pituitary nodules C cell hyperplasia and/or carcinoma, adrenal medullary nodules, and parathyroid hyperplasia
MEN1	pituitary adenoma, insulinoma, parathyroid adenoma
MEN2	pheochromocytoma, medullary thyroid hyperplasia, neuroma, paraganglioma, parathyroid adenoma
MENX	pituitary adenoma, parathyroid adenoma, pheochromocytoma, medullary thyroid hyperplasia, paraganglioma

Table 4. Phenotype comparison between the mixed multiple endocrine neoplasia observed in Long-Evans rats, MEN syndromes, and MENX (Lee et al., 1982; Fritz et al., 2002).

Tumors of the neuroendicrine system can also arise following targeted genetic alteration in the mouse: Schulz et al. (1992) examined transgenic mice over-expressing the *c-mos* protooncogene driven by the Moloney murine sarcoma virus long terminal repeat. *c-mos* expression was predominantly limited to the brain of the affected animals, with low levels found in the adrenal and thyroid tissues. The mice in the three of four of *c-mos* transgenic lines developed a high frequency of multicentric pheochromocytomas and/or medullary thyroid neoplasms in tissues where *c-mos* was active. Moreover, the cataract formation in mice described by Schulz et al., like the one observed in SD^{we} rats (Fritz et al., 2002), was caused by aberrant lens-fiber cell differentiation which allows the assumption that there exists a specific cataract form associated closely with neuroendocrine malignancies.

Mixed MEN syndromes in humans have been described in the past, however, the genes involved in those mixed-type MEN syndromes are not known. Khairi et al. (1975) described a disorder they termed the MEN3, inherited in a dominant manner. Patients were diagnosed with mucosal neuroma, medullary thyroid carcinoma and pheochromocytoma, as well as hypertrophied corneal nerves, skeletal defects and gastrointestinal tract abnormalities. The MEN of mixed type (designated as multiple endocrine adenomatosis, MEA) was diagnosed by Hansen et al. (1976) in a patient with multiple neurofibroadenomatosis, medullary thyroid carcinoma, multiple adenomata of

the parathyroids, adrenal cortical adenoma and small cell anaplastic bronchogenic carcinoma. Nathan et al. (1980) suggested the possibility of a mixed MEN syndrome upon diagnosing a patient with malignant gastrinoma and pheochromocytoma. In 1987 Bertrand et al. reported on a case of mixed MEN characterized by acute congestive heart failure and hypertension, as well as phaeochromocytoma, bilateral medullary thyroid carcinoma, parathyroid adenoma and macro-prolactinoma. Tamasawa et al. (1994) described another case of mixed MEN in a patient with benign pheochromocytoma associated with malignant multiple islet cell carcinoma. In 1997 Hoppner and Ritter reported on a distinct MEN2A phenotype resulting from a duplication in the cysteine rich domain of *RET*. The high incidence of parathyroid disease was observed in the affected members of the family, however, none of them developed pheochromocytoma. Luo et al. (2003) investigated clinical features of the MEN associated with pheochromocytoma and found in three patients mixed type of MEN manifesting with either left or right adrenal pheochromocytoma, insulinoma and parathyroid hyperplasia. However, no genetic evidence, or molecular study, of these cases has been conducted. The MEN1 variant showing more frequent prolactinoma and less frequent gastrinoma than typical MEN1 was described by Hao et al. (2004). The variant is reproducible among kindreds but the mechanisms underlying its development are not known.

The MEN-like phenotypes have also been described in other cancer syndromes. Farndon et al. (1986) reported on a non-MEN medullary thyroid carcinoma (MTC) where patients had no extra-thyroidal manifestations of the MEN2A or 2B (hyperparathyroidism, phaeochromocytomas or mucosal neuromas). In the McCune-Albright syndrome (OMIM 174800) besides changes in the skeleton and skin, endocrine organs are effected (thyroid and pituitary tumors). The pathogenesis in McCune-Albright syndrome involves an activating mutation of the cAMP-regulating *GNAS1* gene product Gsα resulting in constitutive activation of adenylate cyclase and subsequent cAMP formation as a second messenger (Bhansali et al., 2003).

1.1.3 Multiple endocrine neoplasia type X

A new variant of the MEN (MENX), which is described in this work, developed spontaneously in the Sprague-Dawley rat. In order of frequency the most commonly observed neoplasms in MENX-affected animals were pheochromocytoma (tumors of the adrenal medulla), multiple paragangliomas (extra-adrenal pheochromocytomas), thyroid medullary C-cell hyperplasia/ neoplasia, pituitary adenoma, and hyperplasia of the parathyroid gland. The neoplasia affecting adrenal, parathyroid, and medullary thyroid tissues are almost exclusively bilateral (Fritz et al., 2002). Tumors affecting the neuroendocrine system develop within the first year of life. Pituitary and adrenal tumors associated with the MENX in the rat have been shown to be multifocal (Palme, Pellegata, and Atkinson unpublished). The combination of tissues affected in MENX, differ from MEN1 and MEN2 (Table 5).
Another important feature of the MENX is the mode of inheritance. In contrast to known MEN syndromes MENX is transmitted as a recessive trait. Moreover no mutations have been found in either *MEN1* or *RET* genes (Fritz et al., 2002).

Phenotype	**MEN1**	**MEN2A**	**MEN2B**	**MENX**
Pituitary adenoma	Yes	No	No	Yes
Insulinoma	Yes	No	No	No
Parathyroid adenoma	Yes	Yes	No	Yes
Phaeochromocytoma	No	Yes	Yes	Yes
Medullary thyroid hyperplasia	No	Yes	Yes	Yes
Paraganglioma	No	Yes	No	Yes
Neuroma	No	No	Yes	No
Cataract	No	No	No	Yes

Table 5. Comparison of human MEN phenotypes with rat MENX phenotype (modified from Fritz et al., 2002).

The characteristics of MENX suggests that the development of the syndrome results in a mutation of an unknown gene; *Sprague-Dawley white eye* rats represent an animal model suitable for mapping of the *MENX* locus.

1.2 Gene mapping in the rat

1.2.1 Genetic studies of the rat as a model organism of human cancer

The rat as a model organism is important for the study of human health and disease, particularly in the fields of physiology, behavior, pharmacology, and toxicology (Szechtman et al., 2001; Hosseini-Yeganeh et al., 2002). The usefulness of rat for the laboratory research is based on the ease of breeding and the short time needed to reach sexual maturity. Genetic and molecular resources available for the rat, such as DNA markers, genetic maps, molecular probes, libraries and finally the whole genome sequence make the labroratory rat a suitable tool to identify disease genes. The large number of rat models that mimic the human diseases used in biomedical research contributes to the generation of the vast amount of experimental data. The exploration of such data demands rat-specific genetics tools and resources which enable genotype or phenotype analyses. The identification of the genes involved in the pathophysiological mechanisms in rats can lead to the identification of its human counterparts.

1.2.2 Rat genome

Rat genome is 2.75 Gb large and it has been recently published by Gibbs et al. (2004). The reported sequence of *Rattus norvegicus* covers over 90% of the genome. About 28% of the rat genome aligns only with the mouse, about 39% align in all three species: rat, mouse and human. Approximately 2.9% of the rat DNA is in segmental duplications (duplicated regions of the genome that are repeated over at least 5 kb of length and with >90% identity), whereas the human genome has 5–6% (Bailey et al., 2002) and the mouse genome 1.0–2.0% duplicated regions (Cheung et la., 2003). It is estimated that 90% of rat genes possess strict orthologues in both mouse and human genomes.
Through the sequencing of the rat genome great improvement in the resources for physical and genetic mapping has been made.

1.2.3 Linkage analysis

A genetic linkage map of rat genome is based on recombination frequencies for different loci. The two genes or genetic markers that are close together on a chromosome will be separated by cross-overs less frequently than the two genes or markers that are more distant from one another. Loci that lie very close together on a chromosome are rarely separated, because only a cross-over located precisely in the small space between them can create recombinants. Therefore sets of alleles on the same small chromosomal segment can be transmitted as a block (haplotype) through a pedigree. Based on recombination frequencies for different pairs of genes/ markers the maps of their relative positions are constructed. Genetic markers required for performing linkage analysis can be provided by microsatellite markers (Dib et al., 1996) which are repetitive sequences about 100 bp in size disperred through the genome. These short repetitive sequences show a high variability during evolution and thus accumulate considerable allelic differences between different rat strains.

In this study the result of linkage analysis was expressed in LOD scores. The LOD (logarithm of odds) score indicated the likelihood of linkage (Ott, 1985). For a mendelian character, a LOD score greater than +3 is an evidence of linkage, and the one that is less than -2 is an evidence against linkage.

1.2.4 Radiation hybrid mapping strategy

Radiation mapping approach involves artificially breaking human chromosomes and transferring their fragments into rodent cells (Goss and Harris, 1975; Walter et al., 1994). Upon exposure of donor cells to a lethal dose of radiation (3,000-8,000 rads) chromosomes break up randomly into fragments and the larger the X-ray dose, the smaller are the generated fragments. After irradiation the donor cells are fused with recipient cells of a different species. In 1998 Deloukas et al. created a physical map of over 30,000 human genes and in 2001 the physical map of the mouse genome has been obtained largely by radiation hybrid mapping. To map as many markers as possible, a high throughput PCR screening method was developed. The resulting map spanned the whole mouse genome and showed the positions of over 11,000 genes (Hudson et al., 2001).

1.3 Aim of this work

The characteristics of the new variant of multiple endocrine neoplasia MENX and the absence of mutations in MEN1- and MEN2 in the MENX-affected rats suggest that a yet unknown gene is involved in the development of the newly discovered hereditary cancer syndrome.

In order to gain insight into the molecular aspects of MENX pathogenesis, the study presented here aimed at mapping the gene responsible for MENX. A genetic linkage analysis was performed in a set of both MENX-affected and non-affected rats using microsatellite markers and the radiation hybrid panel. Following the mapping of the MENX gene to a single chromosome, the critical region was restricted using new informative microsatellites, and finally the candidate genes were identified based on synteny maps between rat mouse and human, as well as analysis of the genetic information provided by public data bases.

Sprague-Dawley white eye rats used in this work represent a suitable animal model for human MEN-like, or -mixed diseases. The study of the MENX model will contribute to our understanding of a new genetic mechanism of neuroendocrine tissue tumorigenesis.

2 Materials

2.1 Chemicals

Acrylamide (40%)	Appligene, Heidelberg
Agarose	USB/ Pharmacia Biotech, Freiburg
Aluminum Potassium Sulfate	Roth, Karlsruhe
Ampuwa distilled water	Fresenius Kabi, Bad Homburg
APS (ammoniumperoxodisulfate)	Sigma, Deisenhofen
Big Dye Terminator	Applied Biosystems, Weiterstadt
Boric acid	Roth, Karlsruhe
Bromophenol blue	Merck, Darmstadt
Chloral Hydrate	Roth, Karlsruhe
Chlorophorm	Merck, Darmstadt
Citric acid	Roth, Karlsruhe
Cresol Red	Sigma, Deisenhofen
Dimethyl Sulfoxide (DMSO)	Sigma, Deisenhofen
DNeasy Tissue Kit	Qiagen, Hilden
dNTP set	Pharmacia Biotech, Freiburg
EDTA (ethylenediamine tetra-acetic acid)	Roth, Karlsruhe
Eosin	Sigma, Deisenhofen
Ethanol	Merck, Darmstadt
Ethidium Bromide	Sigma, Deisenhofen
Formamide	Sigma, Deisenhofen
Gel Extraction Kit QIAquick	Qiagen, Hilden
Hematoxylin	Sigma, Deisenhofen
Hydrochloric acid	Merck, Darmstadt
Isopropanol	Merck, Darmstadt
Loading buffer	Applied Biosystems
Molecular Weight Marker VIII	Promega, Mannheim
Oligonucleotides	GSF Neuherberg MWG Biotech, Ebersberg
PAGE- PLUS Concentrate (40%)	Amresco, Solon/USA

PCR buffer 10x	Pharmacia Biotech, Freiburg
Proteinase K	Roche, Mannheim
Radiation hybrid panel	Research Genetics, Huntsville/USA
RNA extraction kit	Qiagen, Hilden
RNase-free water	Gibco, Karlsruhe
Sodium Acetate	Sigma, Deisenhofen
Sodium Dodecylsulfate (SDS)	Merck, Darmstadt
Sodium Hydroxide	Roth, Karlsruhe
Sodium Iodate	Merck, Darmstadt
Sucrose	Merck, Darmstadt
Taq DNA polymerase	USB/ Pharmacia Biotech, Freiburg
TEMED (N,N,N´N´-tetramethyl-ethylendiamine)	Sigma, Deisenhofen
Tris	USB/ Pharmacia Biotech, Freiburg
TRIZOL Reagent	Invitrogen, Karlsruhe
Urea	Amresco, Solon/USA
Xylene	Merck, Darmstadt

2.2 Solutions and buffers

2.2.1 Histology

Hematoxylin	Hematoxylin	1g
	Sodium Iodate	0.2g
	Aluminum potassium sulfate	91.8g
	Chloral Hydrate	50g
	Citric acid	1g
	Filled up with H_2O to the final volume o	
	1000ml	
Eosin (stock)	Eosin	10g
	H_2O	1000ml
(working solution)	Eosin (stock)	20ml
	H_2O	180ml

2.2.2 Agarose electrophoresis

Agarose gel 3% (w/v)	agarose	3g
	1x TBE buffer	100ml
	ethidium bromide solution (10 mg/ml)	3µl
5x TBE buffer	Tris	
	boric acid	0.4M
	EDTA	0.4M
	pH 8.0	0.01M

2.2.3 Polyacrylamide gel electrophoresis

Gel 10%	40% Acrylamide 19:1	6.67ml
	5x TBE buffer	4ml
	10% APS	140µl
	TEMED	7µl
	H_2O	4ml

2.2.4 Polymerase Chain Reaction

Reaction mix	forward primer (5pmol)	1µl
	reverse primer (5pmol)	1µl
	dNTPs (2mM)	1µl
	PCR buffer 10x	1µl
	sample buffer	13µl
	Taq DNA polymerase (5000U/ml)	0.2µl
Sample buffer	cresol red	4mg
	sucrose	6g
	H_2O	10ml

2.3 Devices and accessories

Adhesive sealing foils (autoclavable)	ABgene
Agarose electrophoresis chamber B1A/B2	OWL Scientific
Balances Basic BA 4100	Sartorius
F1 310	Fischer
Camera RA2	Haiser
Centrifuges Biofuge pico	Heraeus Instruments
Cover glass	Roth
Cover glass glue Pertex	Medite
Digital camera HV-D225	Hitachi
DNA Sequencer ABI377	ABI Prism
Glassware	Shott
Homogenizer Type T.801 Ultra- Turrax	IKA Labortechnik
Laboratory film Parafilm “M”	American National Can
Microscope Axioplan Imaging 2	Carl Zeiss
Microtome HM355	Microtom
Microtome blade A35	Feather
Microwave Cooktronic M716	Philips
PCR cycler Gene Amp 9700	Perkin Elmer
PCR plates (96 well)	Peqlab
Pipettes 5-100 µl Research Pro	Eppendorf
P 1000, 200, 20, 2 Pipetman	Gilson
5ml,10ml, 25ml	Gilson
Plastic tubes 15ml, 50ml	Falcon
Polyacrylamide electrophoresis chamber	Attro
Power supply (electrophoresis) Pac 300	Bio Rad
Reaction tubes: 0.5, 1.5, 2.0 ml,	Eppendorf
Gene Amp	Perkin Elmer
Slides for histology	Roth
Spectrophotometer printer LX-850	Epson
Thermomixer Compact	Eppendorf
UV- Spectrophotometer DU-62	Beckman
UV- Transilluminator	Bachofer

Vacuum infiltration processor Tissue-Tek VIP	Miles Scientific
Video copy processor P66E	Mitsubishi
Vortex Reax top	Heidolp

2.4 Software

Acrobat Reader 4.0 (Mac)
Adobe Photoshop 3.0.5 (Mac)
Endnote 4.0.1 (Mac)
Fisher Exact Test (http://www.physics.csbsju.edu/stats/fisher.form.html)
Gene Scan 3.1 (Mac)
Internet Explorer (MacOS9: Internet Explorer 5)
LSM5 Image Browser version 2.80.1123 (PC)
Map Manager QT version 3.0b29 (Mac)
Matrox Intellicam version 2.07 (PC)
Microsoft Excel (MacOS9: Microsoft Office 2001)
Microsoft PowerPoint (MacOS9: Microsoft Office 2001)
Microsoft Word (MacOS9: Microsoft Office 2001)
Primer 3 Input:
(http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi)
Rat Genome Blast:
http://www.ncbi.nlm.nih.gov/genome/seq/RnBlast.html
Sequencher 4.1 (Mac)

3 Methods

3.1 Histology

Histological examination was performed for MENX- affected animals in order to determine the spectrum of affected tissues and the level of their malignancy. Paraffin- embedded tissues were stained with hematoxylin- eosin, which is a common stain combination used in histological evaluation of the tissues with the use of light microscope. The basic radical of hematoxylin is attracted by acidic cell nuclei, which upon staining appear dark purple or blue, whereas eosin stains acidophilic cytoplasmic structures pink (Papanicolaou staining).

3.1.1 Paraffin embedding of the tissue

After rat autopsy tissue fragments were placed in 4% formalin phosphate for one day (the volume of the solution was 6 times the size of the tissue fragment). Fixed tissue was then subjected to the mechanical embedding process in the vacuum infiltrations processor (VIP) for 12 hours. Ready paraffin bocks were subsequently left to cool down in the freezer to enable easy processing and cut with the use of microtome into 1μm thin slices. Finally, tissue slices were placed in a water bath (50°C) to flatten, transferred onto glass slides, and dried (60°C, overnight).

3.1.2 Hematoxylin - Eosin staining

Slides with paraffin- embedded tissue were placed in Xylene for 10 min to remove the paraffin. Next, the slides were submerged in the following solutions:

1. Ethanol 100% - 96% - 80% - 70% - 50% - 30% (10 sec each)
2. Distilled water (10 sec)
3. Hematoxylin (4 min)

Slides were then washed with running water (10min), placed in eosin solution for 2 min and submerged in:

1. Ethanol 70% - 96%- 100% (10 sec each)
2. Xylene (5 min)

Finally, cover glass was glued to the slides, which were then left to dry (60°C, overnight).

3.2 DNA extraction

Extraction of rat genomic DNA for genetic linkage analysis was performed with the use of DNeasy Tissue Kit (Qiagen) according to manufacturer's protocol. First, rat tails were cut in 0.4 cm fragments and left to lyse with Proteinase K (overnight at 55°C). Further on the lysate was loaded onto the column and spinned down to let the DNA bind to the membrane of the column. The contaminants were then removed in two washing steps followed by DNA elution. Finally, DNA concentration was estimated by UV spectroscopy measurement.

3.2.1 UV/VIS spectroscopy

DNA yield was determined by measuring the concentration of DNA in solution by its absorbance at 260 nm:
DNA concentration = 50 μg/ml x A_{260} x dilution factor
A_{260} / A_{280} (where A_{280} is protein absorbance) ratio of 1.8 or higher was considered as representing pure DNA sample.

3.3 Polymerase Chain Reaction

Polymerase chain reaction (PCR) was used to amplify microsatellite markers (short repetitive DNA sequences) and in result estimate their inheritance pattern (homozygous versus heterozygous) among the affected and unaffected animals. To permit the amplification the heat-stable DNA polymerase, deoxynucleoside triphosphates (dNTPs) and two oligonucleotide primers (usually 15-25 nucleotides long) complementary to the target DNA are required.

3.3.1 Oligonucleotides

The oligonucleotide sequences for amplification of most of the microsatellite markers were obtained from the following databases:

- Jackson Laboratory (www.informatics.jax.org/rat)
- Otsuka GEN Research Institute (http://ratmap.ims.u-tokyo.ac.jp)
- Wellcome Trust Centre for Human Genetics (www.well.ox.ac.uk/pub/genetics/ratmap/)

All other remaining primers for both microsatellite markers and genes were designed manually or through the use of Primer 3 Input computer program. The design of primers was based on the published rat genome sequence (http://www.ncbi.nlm.nih.gov/genome/guide/rat/).

Self-designed primer sequences for chromosome 4 are presented in Table 6.

Name	Forward primer sequence	Reverse primer sequence	Annealing temp./ Number of cycles	Product length (bp)
D4GSF1	ACCAAGGTGGATGAGTCCTG	TGCTGTGAAATCTCTCTCTCCTC	58/37	376
D4GSF2	CCCTCAGTCCAATGAGGATG	GGACACAGGAGCAAAGATCC	55/35	193
D4GSF3	CATCAAAATGCTGGGTCCTC	TCTTCTTGCAACAATACCAAGAG	58/37	233
D4GSF4	ACGGCCATGCAGAACCTAC	TTCCTGTCAGCTAGTTTTGGTG	56/35	186
D4GSF5	AAGGCACTGGAAACAACTGG	AAAAGCCCAAGAAATGCAAC	56/35	175
D4GSF6	GGGTGGAAAGTGACAGAGGA	TCTGCTAACCGAATGCATGA	55/35	234
D4GSF7	GCAAACCCCTTCAGCTCATA	TGCTTCATGTGGGGTGTTTA	55/35	217
D4GSF8	GAAGGAAAACTCCAACCCAAG	GCCTTTTCTTTGGGGAGTTG	55/35	230
D4GSF9	TACCAGATGTCCTTCCCTGG	TCTCTGGCCAAAGTACTCTCC	55/35	327
D4GSF10	CATGTGCTACATAGACATACATGC	AAAGAGGAAGAAAACACAGTGC	55/35	240
D4GSF11	ACATGGGAGAGCCCACAG	CGAGGGACACACACACTCAC	55/35	276
D4GSF12	CTTACCTTCCCAGGGCTCAC	CCAGTCAGCATTCTGGTTCC	55/35	198
D4GSF13	CCAAGCTAGGCTCTGGAAAC	TAAGGGGCAAGTTCCAGTTG	55/35	243
D4GSF14	TTCCAGGCAGACTACAGTGC	CGGGCAACTGAAGTTAAAGG	55/35	237
D4GSF15	ATCCCGAGGTCTCTTCTGTG	GCCCATACACCAAGTAACTCAG	55/35	243
D4GSF16	GGGAGGCAGTCTCATGTAGC	AGAGGTAGAGGCTGGCTGTG	55/35	241
D4GSF17	GGAGGAAGATTGCCACACAT	AGGAAATCCTGGTCCAGACA	55/35	185
D4GSF18	TGCTGTTCATTGTTGTGTGG	TCATATGCATTGCCGTCTTC	55/35	176
D4GSF19	ATCCCAAGCAATGGAACAAG	AATCCGAGGCAACACATACA	55/35	166
D4GSF20	CAGGCTCCTGTCAGCATGTA	CATTCAGTGCCTGCTCCATA	55/35	151
D4GSF21	CCTGCATATGAGCATTTTGG	TCCTCTTCCACTCAGTCCAGA	55/35	172
D4GSF22	TTTTGTGACCGGTATTGTGG	CATGCAGAGAGGATCGTCAA	55/35	248
D4GSF23	TCCAAGAGGAAAGAGTTTTGGA	GCATGCAGAGAAGATGGTCA	55/35	189
D4GSF24	CACAGAAGAGGTTTTGTGACCA	ATCCTGCAGAGGTTGCAGTT	55/35	180
D4GSF25	TCCAGTCGCATTCAGCATAC	CATTCAGAGCCTGCTCCAC	55/35	151

D4GSF26	TGCTTTGTCCTGCAAACACT	TGGTGAAAGTTTGCATCCTG	55/35	184
D4GSF27	CCCAAACCTTGAGGCTACTG	TCCAAGCTTCATGACAGAGAAA	55/35	184
D4GSF28	AAGAGTGACCACACCCATCC	TCTCTGGAGCCATAGGCGTAAA	55/35	190
D4GSF29	TTGCTTTTGGTGTTGGTTGA	CCATAAGCATGTCTTTAATCTTAGCA	55/35	202
D4GSF30	CAGGCCTCTTCTCTGACACC	GCTTGGAATTTCTTTCCATCC	55/35	159
D4GSF31	CCATTATTCCTTGGTGGTTTG	AATCAATCACGCGATCAAAA	55/35	171
D4GSF32	AGGCAGGAGGAGACCACATA	TCTTGGTTTTGTTGCTTGGAA	55/35	179
D4GSF33	TCTCTGTTCAAGGGAGGATTG	TTTCCAGCCATCATACGTGA	58/35	164
D4GSF34	CAGTGGGCCTCTTCTCTGAC	TGCTTGGAATCTCTTTCTGTCC	55/35	152
D4GSF35	GGCAGAGGCAAATGAATCTC	TTGTTTGCTTGCTTGTTTGG	55/35	218
D4GSF36	TGGTCAACAGAAAACAAACTCC	AACATTTCCCCTCCCCAAA	55/35	150
D4GSF37	CCCAACATGCTGTCTTGATG	TCCATTTGAAACACTTAACTGTAAAC	55/35	235
D4GSF38	TTCTCCTTTCAGCATGCACTT	TTGGTTATTCCCGAGACAGC	52/35	214
D4GSF39	TTGTCATGTTCTGGCTGAGTC	TTGGAGTGGAGACTGAAGGAA	55/35	219
D4GSF40	TGACATCTGCTGAATTAAAGTTTC	GCTGGTCTAGAAGTAAGTGAATCTG	55/35	498
D4GSF41	TGCTTCTATGAGAGTGCTCACC	AGATGCCACACCTGGCTATG	55/35	247
D4GSF42	TGATTGGATTGTTTGTTTTTCAC	GCCAAAAGAATGGGAGAAATC	55/35	249
D4GSF43	AGGTGGTTGGTTGGGACTG	CTCCCAATCCACCTTACACAG	55/35	399
D4GSF44	TACACGAGGCTCTCCTCCAC	GAGCCCAGCATCAGTCTCAG	55/35	291
D4GSF45	GGATGTATTTAAAGTCATACCCACAG	TAACCAGCTGAAAGGGAAGC	55/35	298
D4GSF46	TCATCAGGCAGCTCACATTT	GCCTCCCATTAATTCCAGTGT	58/35	845
D4GSF47	CTTTACGCTCGGCCTTCAC	TGGAGCCATATCCTACCAGA	58/35	150
D4GSF48	ATTCCCGAGTGACCTTTTCC	CCCTTCCAAATTCACATGCT	58/35	293
D4GSF49	TGCCTGTTAATCTGGCTCCT	TAGGGAATGCTGGGTCTTTG	58/35	200
D4GSF50NN	GCCAGAGTTAGCCTTCCACTT	TTATACCGCCCACCAGAGTC	58/35	542
D4GSF51	CCAGGAATGGGGGTAAGAAG	GCAACTTGATTGGATTGAGGA	58/35	246
D4GSF52	CGTTCTGCTTTAGGCTGACA	AAGAGCCAAAAGACCAGGAT	58/35	775

D4GSF53	TGAGGAGTTCCTACAAGACAAGC	AAACCCTGGAGAGGGAGAGA	58/37	233
D4GSF54	CTGATGCTGTGCTTTAAGGTG	TCACCACTCCATCTTCTGTTT	58/37	697
D4GSF55	CTCTTGGAAGAGAGTCTCCTTGT	ATTTGTCCCCACCACTGTGT	58/37	597
D4GSF56	GGTGCCCCTCTTTTGTGTAA	GGGGACAAAACATGTCCAAC	58/37	551
D4GSF57	TCAGAAACGAATGCTTGTGC	CATGGGTCAGCCTCGATAAT	58/37	398
D4GSF58	TGCTGTTTGAACATTCCATTG	TCAGAAACGAATTGCTTGTGC	58/37	348
D4GSF59	GTCACTGGGGGACAAAACAT	GGTGCCCCTCTTTTGTGTAA	58/37	556
D4GSF60	ACCCCAGAGTGGTGCATTT	TGGACTTAGAATGTTAAGTGGTTTT	58/37	249
D4GSF61	GCTCCTGTCAGCATGCAC	AACCTGCCCCACATGGAT	58/35	222
D4GSF62	CCCAGCCATTGAACTGATTAAG	TTGCTCAAATTGGCCTTTAG	58/35	227
D4GSF63	AGGGTAGAAGCATGCCAGTC	TTTCCCTTCTTTGTTGTGTTTC	58/35	248
D4GSF64	TGATGTTGAGAATTATTAATGACCAG	GAAACTCCATACCTTCAAAAAGAAG	58/35	235
D4GSF65	AGTTCCCTGGCTTTCTCC	TGGGAAGGGGAGTTGAGAAG	55/35	206
D4GSF66	AGACAAATGGGTCGAACTGG	TCAGTGAGTGCATACCTTGTTTG	55/35	196
D4GSF67	AGGCCTGGAGTCACTTCTCA	CACCATGGACTAAGGCTGGT	55/35	229
D4GSF68	AAGAAATTAGGTATTTTGGGACCT	CCCAGGGTCAGTCCATAACA	55/35	180
D4GSF69	GCTTGCTCCATAGCTCAAGTG	TGAGGAGGAGGTATGGGATG	58/35	399
D4GSF70	AGTCTCCTTTCAGCATGCAC	TATTCCCGAGACAGCCTCAC	55/37	218
D4GSF71	TTGACCTCTATGTGCAAGCTG	CCTGTAGCACTTAATCACCATTG	57/37	241
D4GSF72	CCTGTAGCACTTAATCACCATTG	TTGACCTCTATGTGCAAGCTG	57/37	241
D4GSF73	CTGGGCCACATAGTGAGTTC	TTCCTTTCAAGTAACTACACTTTAACC	57/37	279
D4GSF74	AGCCATCTCCAACACTTTCC	GATGGGAGCAGAAATGATGC	57/37	187
D4GSF75	ACACACAAATGCCTGCACAC	TGGTTCATCAACTCGAAGACTC	57/37	365
D4GSF76	GGGTAAACCCACAAAACTTTACTC	AAAGGTGGAGATTGATTGAGC	57/37	286
D4GSF77	TGAAGAGCTCGTTTTAAAG	GATTTGGCTCCCTCTTCTGG	57/37	239
D4GSF78	CCATATTAGCTGAAGCTATACAGAG	TCATAGAAGGTGACCCCATAATATC	57/37	234
D4GSF79	TTCCACATGCACTTGTAGGC	AGTCTCACCAGGGGAACTAGC	57/37	273

D4GSF80	AAAAGTGAGAAGTGAAAACATGAA	AAAGGTGTGGACAGTTGTGG	57/37	248
D4GSF81	TTGCACTCAGACTGTTTTCTCC	TGCAACTGTCTAATTGCCAAA	55/37	150
D4GSF82	TGTATCTCATGTAGCCCAGGA	CCAAGAGGAAGTTATCCTTGGAA	55/37	250
D4GSF83	TCGCTTTGAAAAATTGAGAGG	CACATTCACTTTCACACTCTCTTACA	55/37	155
D4GSF84	AAAGTTCGCTTTGAAAAATTGA	TGCACTCTCTCTTTCACACACA	55/37	250
D4GSF85	AAAGGTGGAGATTGATTGAGC	CAAACTTAGGGTAAACCCACAAA	55/37	294
D4GSF86	AGGGCATATACCTCGCATGT	TTGGCAGTGTGTGTTTTAAATATC	55/37	398
D4GSF87	ATCCTAAAGGTTGGGCGAAT	GGCACATACTCCCACACACA	55/37	565
D4GSF88	CCAGAGGAATGTCTCTAACTTCCA	AGTCCTGGCACAGTACTTAAAAA	55/37	399
D4GSF89	CTGGCCTCCACACACAAGTA	TTTGACATTGGAGAGTCCTAAA	55/37	207
rDUSP161	CACAGCACCATTACATCATCG	ACAGAGGCCAGGGAAACAAC	55/37	474
rDUSP162	TCCCAAGATGTTGGTTCTCTG	TGGCCCATAAAATTAAAGTTCG	55/37	596
rDUSP163	CCACCTTAGTCCTGGGATTG	AGCATCCTCTGACGAGAAGC	55/37	589
rDUSP164	TTCGTATTCAGCCAGTGTGG	GCCGCCTACGAACAGAATAG	55/37	581
rDUSP165	CAGCCATCTATGGAGGCAAC	CAAACAAACAAAACCCTTGC	55/37	580
D4Ntf3	TTCAGATATGCCTGTGCTGG	TGCAGGTAAAACATCCGTACAC	55/35	293
rWNT5-1	CACAGCCACAGAGGACAATG	CAGCACCCTCAGCTTTCTTG	55/35	397
rCCND2-1	TTTGCATGTGCTAATCGCTC	TCCCTTTCTGCTGGACTTTG	55/35	437
D4CD9	CCGGAAACCAAAAGAATCAA	ATGCATGGTTTTGACTGGGT	55/35	296
rCdkn1b-1	TCTGTCAGCCATTGTTCTCG	CCAGCTAGGGTGTCAGTTTTG	55/35	247
D4Ptpro	CCAATGAGACAGGCAATCTG	TCCAACACACAATCATCGAG	55/35	272
rMKP7-1	GAAGGAGGTGGGAAAAGAGG	CACATCCATTGGAGGCTTTT	55/35	681
rMKP7-2	AGAAAATTTTGCCGTGGTTG	AGGACTGGTTTCGGGAGTCT	55/35	611
rMKP7-3	ATGGGACCAACAAGCTATGC	CACCATAGCTCCATTTTCCA	55/35	672
D4Siat8a	GCAAGCTTCCTTGTGTAGCC	TGGAATACGTGCAATGTTGG	55/35	248
D4Ret2	GCCGACAGTGTGATGTCTTC	ATAGCCTGGAATGGTTGTGC	52/35	207
D4Ret3	TCCTGCTCATACACACACTCG	CACTGTGACAGCAGGACAGC	60/35	244

D4Ret7	TGCCTCGTCATAGGAGTTGC	CCATGATAGGATTGGCTCTTTC	58/37	236
D4Ret8	CGTCATTCCGAGGGTACG	AGTCCTGTGCTCAGTTCAAGC	55/35	239
D4Ret9	TTTGGGACACTTGTGTGTTTTC	TTGTTGGTTATCCCCACAAC	55/35	248
D4Ret10	TGGAAATAGAGGACCCTTGC	CAAGGCAAACTCGATCCTTC	55/35	385
D4Ret11	TGGAAAGGTGCTTTGCCTAC	CAGATCATCTTTGCCAGTATCC	55/35	299
D4Ret12	TGCTGGAGCGTGTTAGTTTG	CCTACATCCAGCCAGAAATAGC	55/35	228
D4Ret13	CCCTCTGAGCTCCTACATCC	ACCGTCCCAGAAGAAAATTG	55/35	225
D4Ret14	AGATGCAAAGAGGGAACAGG	TGGGGTCTCAGACTATATTGACC	55/35	580
D4Ret15	CCACCTCTCTACCACAGCAAG	TGGGAACCTTAAACTTATCAGGAG	55/37	235
D4Ret16	CTGCCACTAGATGGACCAAAC	GCATGGATACCCACAAACAC	55/37	232
D4Ret17	AGCTGGCAGGATTCTTGATG	TCCAATTAATCCCCCATGAC	55/37	173
D4Ret18	TGGTTCCATTTCCTCAATCAC	TTGCCAATTGTTTTCTTATCACTC	55/37	226
D4Ret19	ATATGCAGAGTGGGGTGAGG	TCCCTGGTTTCTTCAGTTGC	55/37	386
D4Ret20	CACCGAAGCATGCCATATC	AGCCTGAGGACTCTGCACTC	55/37	228
D4Ret21	GCACTGTGCAGTTCACTTCC	GTAGCCGTCTGCTTGTCTCC	55/37	217
D4Ret22	AACACAGAAGGCACGACTCC	TTCCAAAGACTTGCCTGTCC	55/37	211
D4Ret23	CGGATTTGTCTTTGGAAAATG	CGCCATGACTTACCTTCCAC	55/37	189
D4Ret24	CCAGGTCTAAAGAAAGCGATG	GTGACAGAGAACACGCATGG	55/37	192
D4Ret25	AGCCCTTTAACCTCCATTGC	GCAAGCTCCCTGTGTTTCTC	55/37	179
Ret(RH)	CGAGAGCCGATGGCAC	CAGTATGGTGTGCACAAAGTGG	58/35	203
Hrh1(RH))	TGGCCTCAGTTCATCACTTCT	AGGGACAAATGTGCTTTTCTCT	58/35	134
Kcna5(RH)	GCCGATCCATTCTTCATCGT	GAAGATGGCCACGACAT	58/35	137
Alox5(RH)	CAGCTTAACTGTGAGTACGG	AGCAGTCCATCATCAC	58/35	143
Tnfr(RH)	TTCCTTGGTGCTACCGACTT	GCCCTGAGAAGCTTTGTTTG	58/35	188
Rho(RH)	CCAGAGACTGTGGCTGACTG	GGTCTTGGTGGATGGATGTC	58/35	201

Table 6. Self-designed primer sequences for microsatellite markers and genes.

Primers were synthesized by U. Linzner (GSF- Institute of Pathology, AG-BIODV). Lyophylisates that were dissolved in distilled water (Ampuwa) to a final concentration of 10 pmol. All primer stock solutions were stored at -20°C.

3.3.2 PCR conditions

PCR amplification of microsatellite markers was performed in a 96-well plate format. After pipetting 20µg of the template (which was genomic DNA extracted from affected and unaffected rats) reaction mix (15µl) was added to each well to a final volume of 22µl per reaction. Plates were then sealed with a non-transparent adhesive foil and placed in a thermocycler. The following standard PCR conditions were used for all microsatellites, unless specifically stated otherwise (Table 7). The DNA samples were amplified in 30-37 cycles.

Step	Temperature	Time
Denaturation	94°C	30 sec
Annealing	52-60°C	40 sec
Elongation	72°C	45-60 sec
Additional cycle Annealing Elongation	 52-60°C 72°C	 45 sec 5 sec

Table 7. PCR conditions for amplification of microsatellite markers and gene fragments.

3.4 Agarose electrophoresis

Agarose gel electrophoresis was used to determine both the yield and purity of DNA isolation and for analysis of PCR amplification of the microsatellites. Different concentrations of agarose gels were used (2% or 3%), depending on the expected size of the fragments. Agarose was dissolved in TBE buffer by heating, ethidium bromide (10mg/ ml) was added for UV detection of the DNA fragments. Warm gel solution was poured into a tray with a well-forming comb is put into it. The solified gel was placed into a horizontal electrophoresis apparatus and covered with 1x TBE buffer. Molecular weight marker (MWM) VIII

of the resolution 50-1000bp was used to determine the size of the amplified fragments. Marker solution was prepared as follows:

MWM stock	1µl
Bromophenol blue	1µl
H_2O (Ampuwa)	5µl

DNA samples (7µl) were loaded into the sample wells and electrophoresed at 90-150mA for 0.5- 1 hour at room temperature. Molecular weight marker solution was run in an adjacent lane to allow size determination of the DNA fragments. After electrophoresis the gel was visualized under UV light and the image digitalized for analysis.

3.5 Polyacrylamide gel electrophoresis

Polyacrylamide gels were used when greater resolving power was needed to distinguish the differences in microsatellite size that were not detected on agarose gels. For this purpose 7-12% acrylamide gels were used.
Gel solutions were prepared as described in 2.2.2, pipetted into casting apparatus and left for one hour to fix. 10µl of PCR product per well were loaded. Additionally Molecular weight marker VIII solution (see 3.3) was loaded in an adjacent lane to enable the sample size determination after visualization. Gel was run at 150-200mA for 3-4 hours.

3.5.1 Gel staining

After completed electrophoresis the gel was placed in 1x TBE solution (500ml) containing 10µl ethidium bromide (10mg/ ml) for 5 min to allow DNA staining. Stained gels were visualized under UV light and photo documented.

3.6 Genetic mapping

3.6.1 Animal breeding

Animals used in this study were obtained in the back crossings as follows: (Wis/Nhg x SD^{WE}) x SD^{WE} ,

where: Wis stands for Wistar/Neuherberg (wild type)

SD^{WE} stands for Sprague-Dawley white eye (MENX- affected).

Wild-type Sprague-Dawley and Wistar rats for breeding were obtained from a comercial source (Charles River Germany, Sulzfeld, Germany) (Fritz et al., 2002).

3.6.2 Determination of rat genotype

First, a whole-genome screen for the *MENX* locus in affected and unaffected rats was performed using PCR amplified microsatellite markers. Marker length (allelotype) was determined by either agarose or polyacrylamide electrophoresis (see above). The inheritance of each microsatellite marker was determined and correlated with phenotype: a single band on the gel was interpreted as homozygosity for a given marker (allele inherited from the affected SD/SD parent), whereas double band was interpreted as heterozygosity (one SD 'affected' allele and one Wistar 'unaffected' allele). Markers, which were inherited either in a homozygous or heterozygous state in both parents, were considered non-informative. Finally, a statistical test (Fisher Exact Test) was performed to obtain p values for each microsatellite marker (p values were calculated online:

http://www.physics.csbsju.edu/stats/fisher.form.html).

Markers for which $p \geq 0.01$were considered not linked and thus excluded from further studies.

3.6.3 Haplotype analysis

Homozygosity/ heterozygosity of inherited microsatellite markers showing linkage was represented graphically and the percentage of recombination between neighboring markers was calculated (Fig. 3) based on the definition of one centimorgan (cM): one cM equals the distance between two genes (markers) that will recombine with a frequency of exactly one per cent (a per cent of cross-over was proposed as a genetic map unit by A. Sturtevant in 1913; This unit is now by convention called 'centimorgan' after T. H. Morgan).

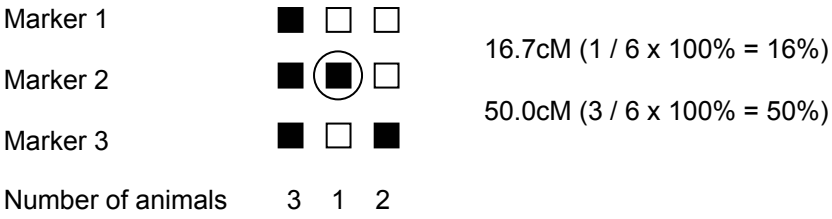


Fig. 3. Principle of the haplotype analysis. Black squares represent homozygosity, whereas white squares represent heterozygosity for a single microsatellite marker. The numbers at the bottom show the number of animals with a particular haplotype pattern. On the left, estimated genetic distances between two markers are shown. Circled square represents a double recombination event.

Standard error was calculated according to the following formula:

$$SE= \sqrt{R(1-R)/n}$$

Where: R is the number of recombinants and n is the total number of the animals.

Finally, markers were ordered along the chromosome in such a way that the double recombination events were reduced to minimum.

3.6.4 Radiation Hybrid

To map the markers and genes, which were not informative and therefore not useful for haplotype analysis, radiation hybrid mapping was performed. Microsatellite repeats and gene fragments were amplified on a rat-hamster radiation hybrid panel (Research Genetics) under PCR conditions described in 3.3.2. After agarose electrophoresis and visualization under UV light samples which contained a specific DNA fragment, and hence produced the specific PCR product were annotated as "1", those which did not give the product as "0", and those which were not clear as "2". After introducing “0”, “1” or “2” for each of the 108 amplified panel templates into Map Manager (Manly and Olson, 1999; http://mapmgr.roswellpark.org/mmQTX.html) computer program PCR results were automatically analyzed and markers ordered to minimize double cross-over events.

3.7 Synteny groups

Generation of groups of conserved synteny for gene mapping is based upon the similarities in chromosomal structure (gene order) between different species. A synteny group represents a set of genes arranged in a conserved order between the different species. Synteny groups are useful in studies aiming at identifying new genes in organisms which genomes are poorly studied. For the purpose of this project a synteny group between rat, mouse and human was generated in order to find genes that are unknown in the rat but are already localized in the latter species. Comparing the order of syntenic groups in mouse and human could further lead to map those genes in the rat.

3.8 Automated chain termination DNA sequencing

3.8.1 Sample preparation

PCR products were cut out of the agarose gel under UV light and purified using Gel Extraction kit (Qiagen). Gel slices were weighed, solubilized and placed in a column. In the presence of high salt DNA adsorbed to the silica- membrane and (after washing away the impurities) eluted in water.

3.8.2 Sequencing reaction

During the sequencing reaction (denaturation, annealing and extension) a single sequencing 5'- primer (unlabeled) is used. Four fluorescently- labeled dideoxytriphosphonucleotides (ddATP, ddTTP, ddCTP and ddGTP) are incorporated into the DNA strand being synthesized by DNA polymerase. Each fluorescent dye emits light at a different wavelength upon excitation. The synthesis of nascent DNA chains is terminated by the incorporation of the dye - labeled nucleotide (protocoll modified from Sanger et al., 1977 by Applied Biosystems: http://docs.appliedbiosystems.com/pebiodocs/04339923.pdf).
Sequencing reaction mix (total volume of 10µl) consisted of:

Big Dye Terminator mixture	2µl
Primer (10µM)	1µl
DMSO	0.3µl
Template	2µl
H_2O	4.7µl

DNA samples were amplified (35 cycles) under the following conditions:

1. Denaturation at 94°C for 10 sec
2. Annealing at 50°C for 5 sec
3. Elongation at 60°C for 4 min

3.8.3 Precipitation

After the completed amplification samples were precipitated with 3M Sodium Acetate (1µl), and 96% Ethanol (25µl) at room temperature. After washing with 70% Ethanol, the DNA pellets were vacuum- dried. Dried samples were dissolved in 1.5µl of a mix of Formamide and Loading Buffer, denatured at 86° and stored on ice for 5 min.

3.8.4 Sequencing gel

The components of the polyacrylamide denaturing gel were as follows:

Urea	18g
H_2O	18ml
TBE (5x)	10ml
PAGE- PLUS Polyacrylamide (40%)	6ml
APS (10%)	300µl
TEMED	28µl

Prior to pouring the gel solution was degassed and filtered. Poured gels were left for one hour to polymerize.

The fluorescently- labeled samples (3µl) were loaded on the gel for separation (ABI Prism Sequencer). Sequence was read by digital laser- induced capture of fluorescence in each sample lane.

Sequences were finally analyzed with Sequencher computer program in order to determine any possible changes between samples derived from wild type and mutated animals.

4. Results

4.1 Phenotype of MENX rats

4.1.1 Histological appearance of tumors

4.1.1.1 Pituitary

In the affected pituitary the division into *pars neuralis*, *pars intermedia* and *pars distalis* (Fig. 4A) did not exist. Pituitary tumors (classified as adenomas) formed large nodules with hemorrhagic areas, the normal tissue was prominently compressed by tumor mass (Fig. 4B). Round cells characteristically arranged in cord-like clumps present in a normal organ (Fig. 4C) changed into large polygonal tumor cells. Under higher magnification it was possible to observe isolated mitotic cells indicating increased proliferation activity within the malignant tissue (Fig. 4D). Pituitary tumors were multifocal (B. Palme, GSF; personal communication).

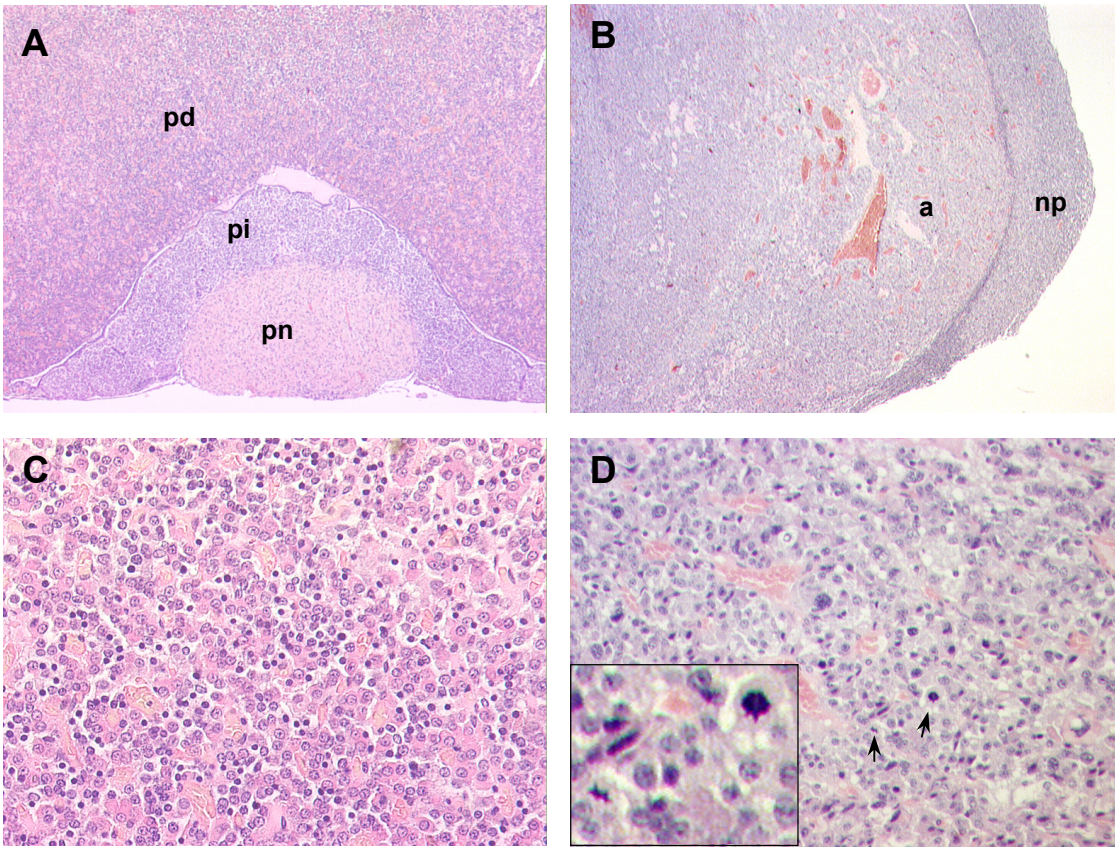


Fig. 4. Cross-sections of the rat pituitary gland stained with hematoxylin- eosin. A- normal organ (unaffected animal), 25x; B- pituitary adenoma (affected animal), 25x; C- normal pituitary (*pars distalis*), 100x; D- pituitary adenoma, 100x: arrows point at mitotic cells, which are shown in higher magnification in the box (magnification 200x). a- adenoma with hemorrhagic lesions (red); np- normal pituitary pressed to the edge of the organ by tumor mass; pd- *pars distalis*, pi- *pars intermedia*, pn- *pars neuralis*.

4.1.1.2 Adrenal gland

In the healthy adrenal the division into cortex and medulla is clearly seen (Fig. 5A). The affected adrenals developed pheochromocytomas in the medullary part, arranged in nest- like structures clearly seen under the higher magnification as rosettes (Fig. 5B). The three parts of the healthy cortex: *zona glomerulosa*, *zona fasciculata*, and *zona reticularis* (Fig. 5C) are not present in the affected organ. Individual tumor cells were often polygonal with hyperchromatic nuclei (Fig. 5D). Tumors were bilateral.

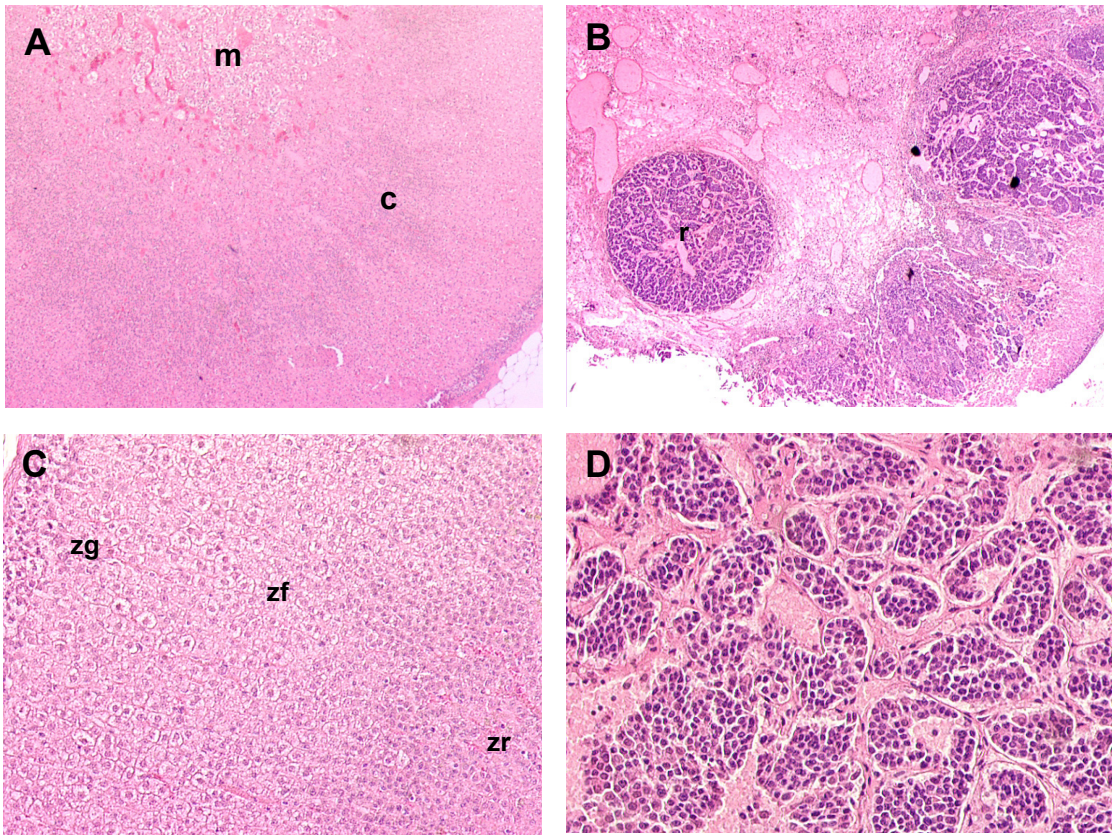


Fig. 5. Cross-sections of the rat adrenal gland stained with hematoxylin-eosin. A- normal organ (unaffected animal), 25x; B- multifocal pheochromocytoma (affected animal), 25x: in the medullary part of the adrenal gland cells form nest- like structures (rosettes); C- normal adrenal gland (cortex), 100x; D- pheochromocytoma rosette, 100x. c- cortex; m- medulla; r- rosettes; zg- *zona glomerulosa*, zf- *zona fasciculata*, zr- *zona reticularis*.

MENX- affected animals develop malignancies before reaching one year of life, whereas unaffected animals do not show any symptoms at this stage.

4.2 Inheritance of MENX syndrome

Animals used for the purpose of this project belonged to two families generated by the interstrain backcross (Wis/Nhg x SD^{WE}) x SD^{WE}. The recessive mode of inheritance (according to mendelian genetics) was confirmed in the F2 crossings (the appearance of cataract was used as the early marker of the MENX cancer syndrome). As expected, F1 generation remained unaffected (they showed neither cataract, nor endocrine tumors), whereas half of the F2 animals became affected (Table 8).

Generation	Percentage of affected animals (Expected)	Percentage of affected animals (Observed)
F1	0%	0%
F2	50%	48.8% (9 male; 11 female)

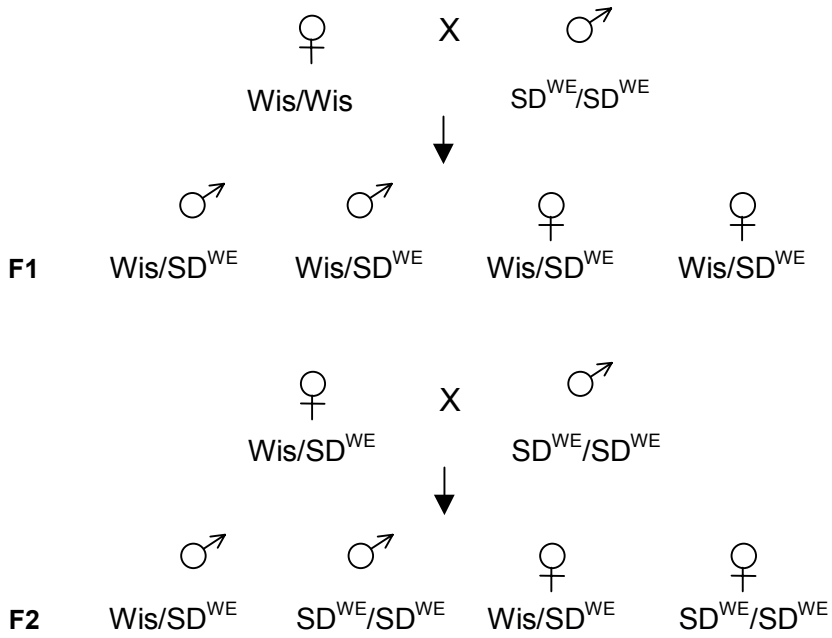
Table 8. Number of affected and unaffected backcross offspring: theoretical (Expected) and observed among the animals used in this study (Observed). Total number of animals: 41.

On the basis of the data shown in Table 8 it was possible to conclude that MENX syndrome affecting animals used in this study is a genetic disease of a recessive trait.

4.3 Genetic analysis

4.3.1 Mapping of MENX gene locus to a single chromosome

For the purpose of this project two kinds of genetic mapping were performed: haplotype analysis and radiation hybrid panel analysis. The cohort of 41 rats used in this study was obtained in the crossing: (Wis/Nhg x SD^{WE}) x SD^{WE}:



The animals belong to two families (1 and 2) listed in Table 9a and 9b.

Animal Number	Phenotype	Sex
307	Affected	Male
308	Affected	Male
309	Unaffected	Female
373	Affected	Male
374	Affected	Male
375	Unaffected	Male
376	Affected	Female
377	Unaffected	Female
378	Unaffected	Female

Table 9a. Characteristics of the rats used in the genotyping study: F2 generation, backcross family 1 (A. Fritz, previously GSF- Institute of Pathology, unpublished).

Animal Number	Phenotype	Sex
310	Unaffected	Male
311	Unaffected	Male
63/1a	Unaffected	Male
312	Affected	Female
313	Affected	Female
314	Affected	Female
315	Affected	Female
316	Affected	Female
317	Unaffected	Female
318	Unaffected	Female
319	Unaffected	Female
379	Affected	Male
63/4	Affected	Male
63/5	Unaffected	Male
63/6	Unaffected	Male
63/7	Unaffected	Male
63/8	Affected	Female
63/8	Affected	Female
63/10	Unaffected	Female
63/11	Unaffected	Female

Table 9b. Characteristics of the rats used in the genotyping study: F2 generation, backcross family 2 (A. Fritz, previously GSF- Institute of Pathology, unpublished).

Animals listed above in the table belong to two backcross families. The offspring presented in table 9a counts as few as nine animals- a rather low number compared to breeding among healthy rats. MENX- affected animals did not breed very well in general. The higher incidence of a poor breeding efficiency was previously associated with the MENX syndrome itself: the maturation of the ovaries in the affected females is disturbed (A. Fritz, previously GSF- Institute of Pathology; personal communication). Mating an affected female with an

unaffected male gave in result a very low number of F1 animals, or no animals at all, whereas mating an unaffected female with an affected male gave bigger chances for obtaining enough F1 rats to further generate F2 animals, which could be used for the genotyping.

4.3.2 Haplotype analysis

The haplotype analysis was performed after the genome-wide screen of the rats. The screening was based on the amplification of the microsatellite markers using oligonucleotide primers which sequences were available in public databases. Microsatellites were amplified with 41 DNA samples derived from both affected (20) and non-affected (21) animals. Informative markers showing both homozygous (SD/SD) and heterozygous (SD/Wis) pattern on the gel (Fig. 6) were selected for the genotypic analysis. Fig. 6 shows the partial correlation between the phenotype and the genotype of tested animals in terms of the inheritance of the marker *D4Rat82*. In Fig. 6A the genotype of both affected and unaffected animals exactly corresponds to their phenotype. However, among 17 offspring rats shown in Fig. 6B the genotype correlates with phenotype only in 10 cases.

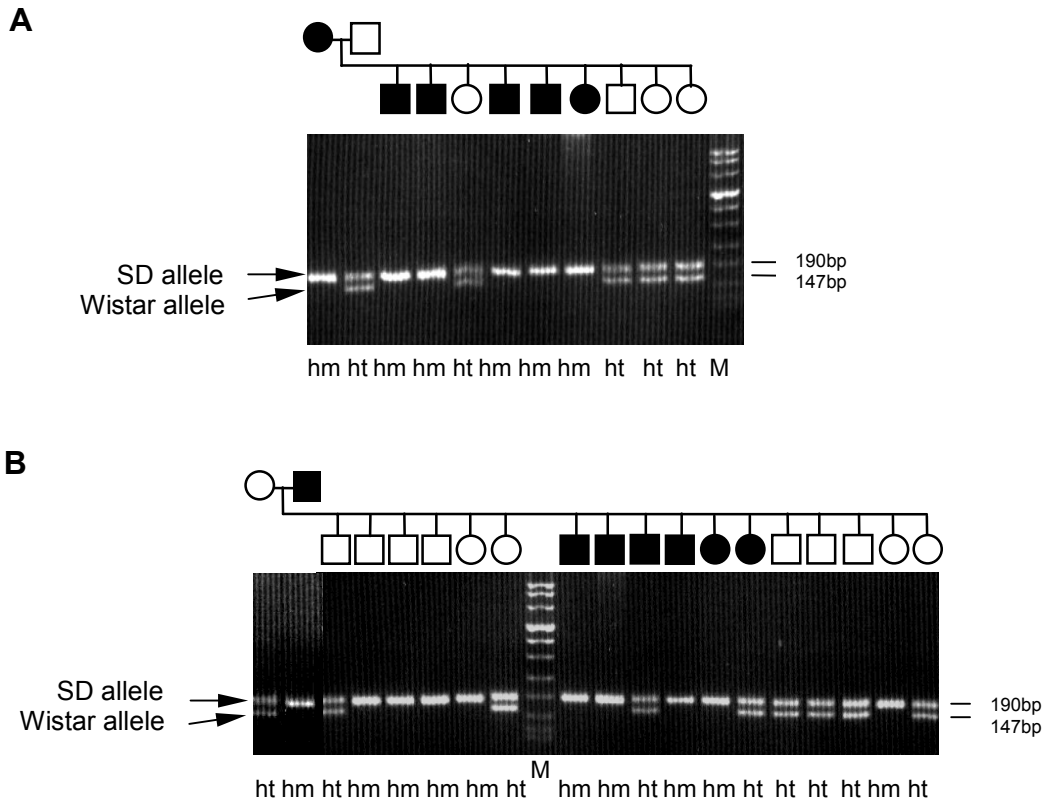


Fig. 6. Homozygosity (single band) and heterozygosity (two bands) of the animals in terms of microsatellite marker inheritance (*D4Rat82*), agarose electrophoresis. A- genotyping of the animals, which belong to backcross family 1; B- genotyping of the animals, which belong to Backcross family 2 (fragment); Phenotypes of the given animals are shown above the gel images: filled squares- affected, empty squares- non-affected; Genotypes are given below the images: hm- homozygosity, ht- heterozygosity; M- molecular weight marker VIII spanning the size of the PCR fragments from about 1100bp to 30bp.

None of the markers for rat chromosome 1 (where *Men1* gene responsible for multiple endocrine neoplasia type 1 is placed) showed statistical significance. This resulted from the poor correlation between phenotype and genotype of the tested animals (Fig. 7).

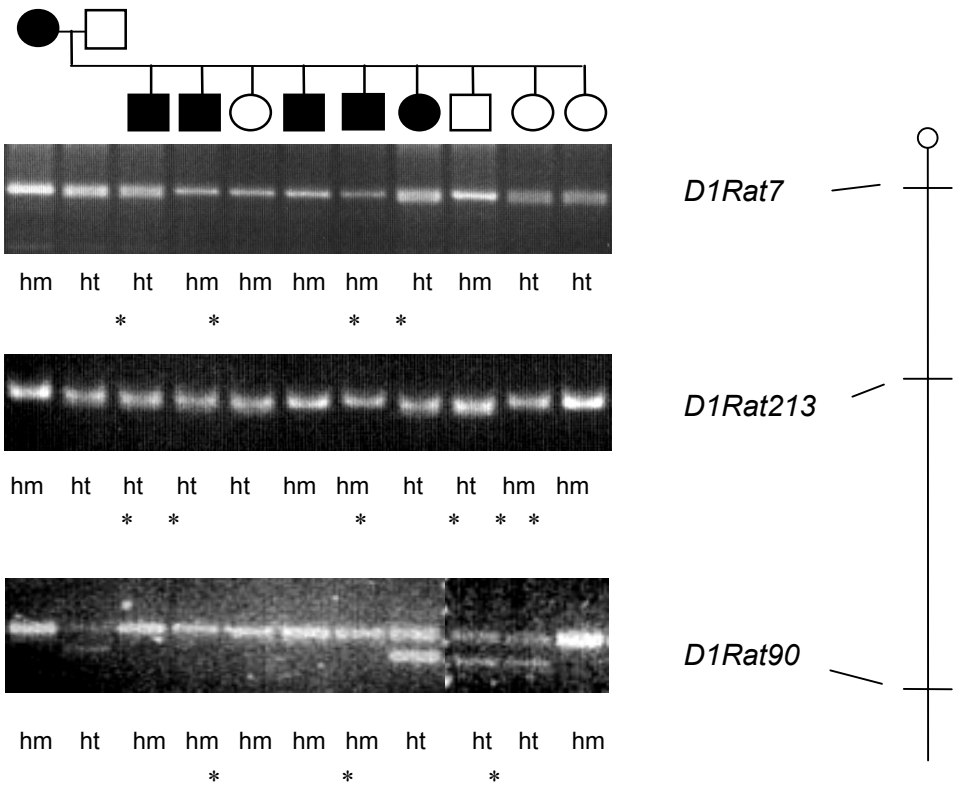


Fig. 7. Genotype analysis of the animals from backcross family 1 (chromosome 1). Phenotypes of the given animals are shown above the gel images: filled squares- affected, empty squares- non-affected; Genotypes are given below the images: hm- homozygosity, ht- heterozygosity. Genotypes which are not in agreement with the animals' phenotype are indicated with the asterisk. On the right- schematic representation of rat chromosome 1 with the position of tested microsatellite markers indicated.

As shown in Fig. 7 genotypes of 4, 5, and 3 out of 9 animals (44%, 55%, and 33%, respectively) were in disagreement with the phenotype for markers: *D1Rat7*, *D1Rat213*, and *D1Rat90*. The association with the phenotype was identified for markers on chromosome 4 (Table 10) (for marker *D4Rat61* see Fig. 8).

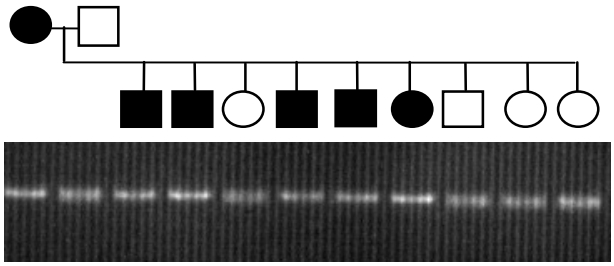


Fig. 8. Genotype analysis of the animals from backcross family 1 in terms of marker *D4Rat61* inheritance (chromosome 4). Single band mean homozygosity, double band- heterozygosity.

Results presented in Fig. 8 indicate marker *D4Rat61* is strongly linked to MENX locus.

4.3.3 Statistical significance of marker inheritance

Among 79 markers tested in the genome-wide screen (with at least two markers per chromosome selected) 60 were informative. After the pattern of inheritance for each of the informative markers was estimated and correlated with animals' phenotype statistical analysis was performed to determine the statistical significance (potential linkage) for every marker (Table 10).

Chromosome	Marker name	Backcross 1 (9 animals)	Backcross 2 (32 animals)	Backcross 1+2 (41 animals)
1	*D1Rat213*	p=0.642	p=0.615	p=0.552
1	*D1Rat7*	p=0.642	p=0.369	p=0.321
1	*D1Rat90*	p=0.404	p=0.148	p=0.078
1	*D1Ra132*	p=0.642	p=0.039	p=0.042
2	*D2Mgh19*	p=0.595	p=0.077	p=0.133
2	*D2Rat116*	p=0.119	p=0.383	p=0.557
2	*D2Rat38*	p=0.595	p=0.369	p=0.436
3	*D3Rat75*	p=0.642	p=0.398	p=0.328
3	*D3Rat56*	p=0.642	p=0.517	p=0.442
3	*D3Rat116*	p=0.642	p=0.335	p=0.308
3	*D3Rat5*	p=0.642	p=0.369	p=0.321

4	*D4Rat25*	p=0.166	p=0.636	p=0.280
4	***D4Rat57***	**p=0.007**	**p=0.039**	**p=0.001**
4	***D4Rat82***	**p=0.007**	**p=0.016**	**p=0.0004**
4	***D4Rat206***	**p=0.166**	**$p=2.8x10^{-8}$**	**$p=1.6x10^{-8}$**
4	***D4Rat61***	**p=0.007**	**p=0.00046**	**$p=4.8x10^{-6}$**
5	*D5Rat126*	p=0.166	p=0.535	p=0.222
5	*D5Rat85*	p=0.595	p=0.084	p=0.078
5	*D5Rat83*	p=0.642	p=0.015	p=0.042
5	*D5Rat50*	p=0.357	p=0.224	p=0.430
5	*D5Rat93*	p=0.642	p=0.082	p=0.139
6	*D6Rat105*	p=0.277	p=0.005	p=0.037
6	*D6Rat68*	p=0.277	p=0.182	p=0.404
6	*D6Rat124*	p=0.642	p=0.069	p=0.133
7	*D7Rat27*	p=0.357	p=0.630	p=0.442
7	*D7Rat44*	p=0.357	p=0.384	p=0.569
8	*D8Rat52*	p=0.595	p=0.158	p=0.222
8	*D8Rat43*	p=0.444	p=0.335	p=0.563
8	*D8Rat44*	p=0.444	p=0.335	p=0.563
8	*D8Rat16*	p=1	p=0.615	p=0.557
9	*D9Rat10*	p=0.119	p=0.517	p=0.436
9	*D9Rat103*	p=0.357	p=0.411	p=0.585
9	*D9Rat79*	p=0.642	p=0.5	p=0.563
10	*D10Rat126*	p=0.642	p=0.384	p=0.447
10	*D10Rat165*	p=0.642	p=0.384	p=0.447
10	*D10Rat56*	p=0.119	p=0.251	p=0.563
11	*D11Rat37*	p=0.119	p=0.535	p=0.430
11	*D11Rat43*	p=0.278	p=0.267	p=0.448
12	*D12Rat23*	p=0.642	p=0.369	p=0.321
12	*D12Rat27*	p=0.642	p=0.517	p=0.442
13	*D13Rat8*	p=0.642	p=0.205	p=0.308

13	*D13Rat72*	p=0.166	p=0.125	p=0.037
14	*D14Rat1*	p=0.357	p=0.253	p=0.103
14	*D14Rat98*	p=0.119	p=0.138	p=0.430
15	*D15Rat45*	p=0.642	p=0.588	p=0.585
15	*D15Rat19*	p=0.357	p=0.444	p=0.585
15	*D15Mgh4*	p=0.642	p=0.482	p=0.557
16	*D16Rat32*	p=0.642	p=0.778	p=0.078
16	*D16Rat53*	p=0.404	p=0.304	p=0.5
17	*D17Rat2*	p=0.555	p=0.155	p=0.216
17	*D17Rat10*	p=0.166	p=0.267	p=0.080
17	*D17Rat64*	p=0.166	p=0.578	p=0.263
18	*D18Rat115*	p=0.722	p=0.555	p=0.595
18	*D18Rat14*	p=0.722	p=0.535	p=0.585
19	*D19Rat14*	p=1	p=0.5	p=0.423
19	*D19Rat25*	p=1	p=0.630	p=0.547
19	*D19Rat91*	p=0.357	p=0.353	p=0.216
20	*D20Rat47*	p=0.642	p=0.353	p=0.436
20	*D20Mit4*	p=1	p=0.155	p=0.133
20	*D20Rat29*	p=0.119	p=0.335	p=0.133
X	*DXRat5*	p=0.047	p=0.155	p=0.020
X	*DXRat25*	p=0.166	p=0.517	p=0.220

Table 10. Statistical analysis of microsatellite markers used in the first screening determined using Fisher Exact Test. In bold- markers showing statistical significance (linkage): $p < 0.01$.

p values calculated for the microsatellites listed in Table 10 show the statistical significance only for chromosome 4, which indicates *MENX* locus is linked to this particular chromosome, specifically to the region between markers: *D4Rat61* and *D4Rat206*. These data excluded remaining chromosomes from further genotypic analysis and also pointed out to *Ret* as a possible gene involved in MENX (*Ret* was known to be associated with marker *D4Rat61*).

4.3.4 Mapping of MENX to rat chromosome 4

For this purpose the percentage of recombination between two subsequent markers was estimated based on homozygous/ heterozygous pattern of inheritance of a given marker. The optimal (i.e. the most probable) order of the markers was obtained by placing them in such a way that the number of double recombination events was minimized (Fig. 9).

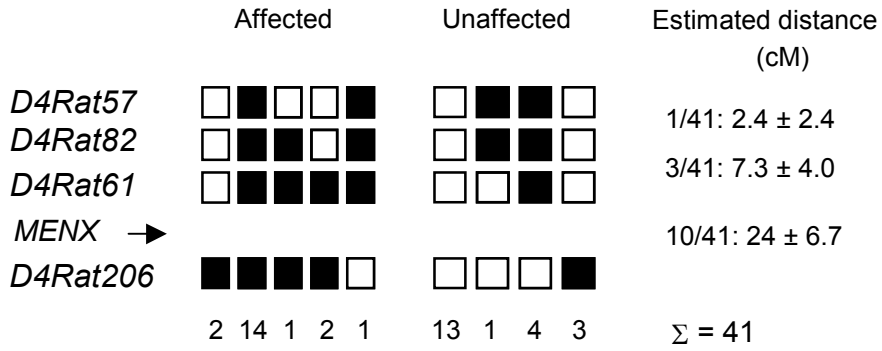


Fig. 9. Haplotype analysis of affected and unaffected animals; filled squares mean homozygosity of the individual animal for a given marker, whereas empty squares mean heterozygosity. On the right estimated genetic distances between two neighboring markers are presented together with the standard error values. The arrow shows the most probable location of the gene responsible for MENX. Numbers below indicate the number of animals with a particular haplotype pattern.

In the group of affected rats 14 out of 20 animals showed homozygous pattern of the marker inheritance for the whole region. However, the haplotype pattern of three affected animals suggests the gene responsible for *MENX* is most probably located within a 24 cM region between markers *D4Rat61* and *D4Rat206* (one animal is heterozygous for *D4Rat206*, which suggests the gene cannot be found in the close distance to this marker, at the same time two other animals are heterozygous upstream from *D4Rat61*, which suggests *MENX* locus cannot be found in the close distance *D4Rat61* or above it).

Since marker *D4Rat61* was associated with *Ret* (below *Ret* gene), gene responsible for multiple endocrine neoplasia type 2A and 2B (Donis-Keller et al., 1993; Hofstra et al., 1994), based on the above genotyping data it was possible to exclude *Ret* as the gene responsible for MENX.

To further investigate this region it was necessary to find more markers within it. For that purpose a publicly available radiation hybrid map of microsatellite markers (Fig. 10), as well as rat data recourses deposited on the server of The Jackson Laboratory, USA was used.

Only markers mapped in the interval between *D4Rat61* and *D4Rat206* were chosen for the further analysis. Since marker *D4Rat206* was not positioned in the available release of the radiation hybrid map shown in Fig. 10 it was not possible to define the lower border of the region of interest and therefore all informative markers below *D4Rat61* (indicated by the red arrow in Fig. 10) had to be tested. Altogether 94 microsatellites from the publicly available data recourses were tested for allele- specific polymorphisms.

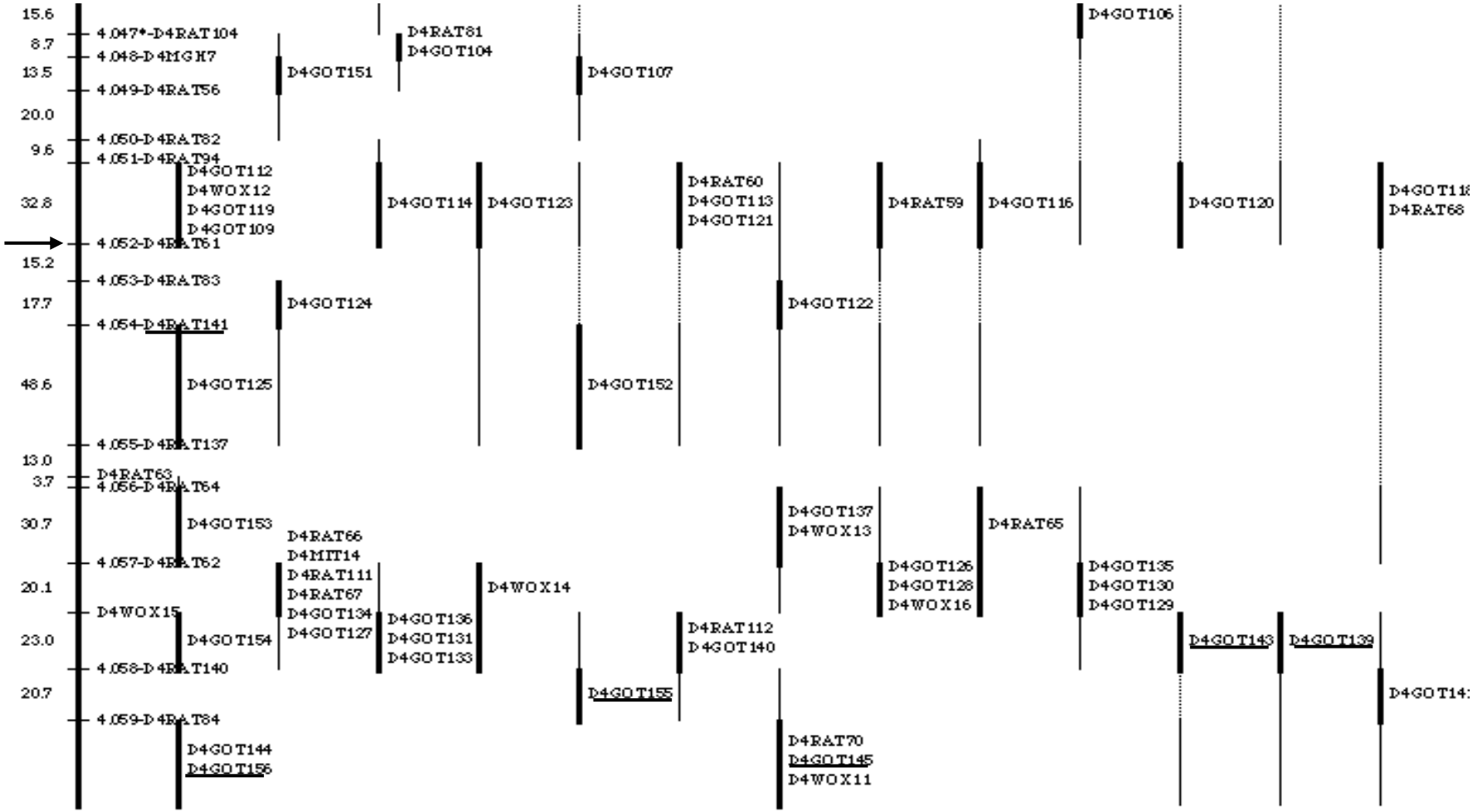


Fig. 10. Fragment of the radiation hybrid map available at: www.well.ox.ac.uk/rat_mapping_resources/Comprehensive_maps/comp_map_chr04.html. Numbers on the left show the distances in centirays (cR) between two neighboring markers. Framework markers are placed on the first bar from left. Remaining bars represent the most probable position (thick line) and less probable position (thin line) of a given marker. The arrow indicates the position of *D4Rat61* defining the upper border of the critical region determined in the first genome screen. Underlined markers are the only informative markers mapping to the interval. These were used later in the second screening.

Among the markers showed in Fig. 10 only six were informative (indicated with red underline), as they showed no polymorphism, whereas only one additional marker from The Jackson Laboratory database (*D4Rat201*) turned out to be informative. For these markers statistical significance of linkage was determined (Table 11).

Chromosome	**Marker name**	**Backcross 1 (9 animals)**	**Backcross 2 (32 animals)**	**Backcross 1+2 (41 animals)**
4	*D4Rat141*	p=0.039	p=0.0004	$p=2.6x10^{-5}$
4	*D4Got143*	p=0.166	$p=1.4x10^{-6}$	$p=6.0x10^{-7}$
4	*D4Got139*	p=0.166	$p=2.4x10^{-7}$	$p=1.1x10^{-7}$
4	*D4Got155*	p=0.167	$p=2.4x10^{-7}$	$p=1.1x10^{-7}$
4	*D4Got156*	p=0.722	$p=1.4x10^{-6}$	$p=1.1x10^{-5}$
4	*D4Got145*	p=0.404	$p=1.4x10^{-6}$	$p=2.7x10^{-6}$
4	*D4Rat201*	p=0.007	$p=7.7x10^{-5}$	$p=6.0x10^{-5}$

Table 11. List of markers used in the second screen, covering the critical region on chromosome 4, and the calculated p values indicating statistical significance ($p< 0.01$). The order of the markers was determined from the radiation hybrid map.

p values listed in Table 11. are of high statistical significance ($p< 0.01$) indicating strong linkage of the listed markers to the *MENX* locus, which suggests it is located on chromosome 4.

Analogously to the first screen the percentage of recombination and subsequently the genetic distances between two neighboring markers were calculated and their most probable position was chosen based on the exclusion of double recombination events, as in case of the first haplotype analysis. 17 of 20 affected rats were homozygous for all listed markers), which suggests that the *MENX* locus is to be found within the region covered by the analyzed microsatellites. On the basis of the haplotype pattern of two affected animals (one being heterozygous downstream from marker *D4Rat206*, and the other being heterozygous upstream from marker *D4Rat201*), it was concluded that the gene responsible for MENX must be located between *D4Rat206* and *D4Rat201* in a region about 22cM in size (Fig. 11).

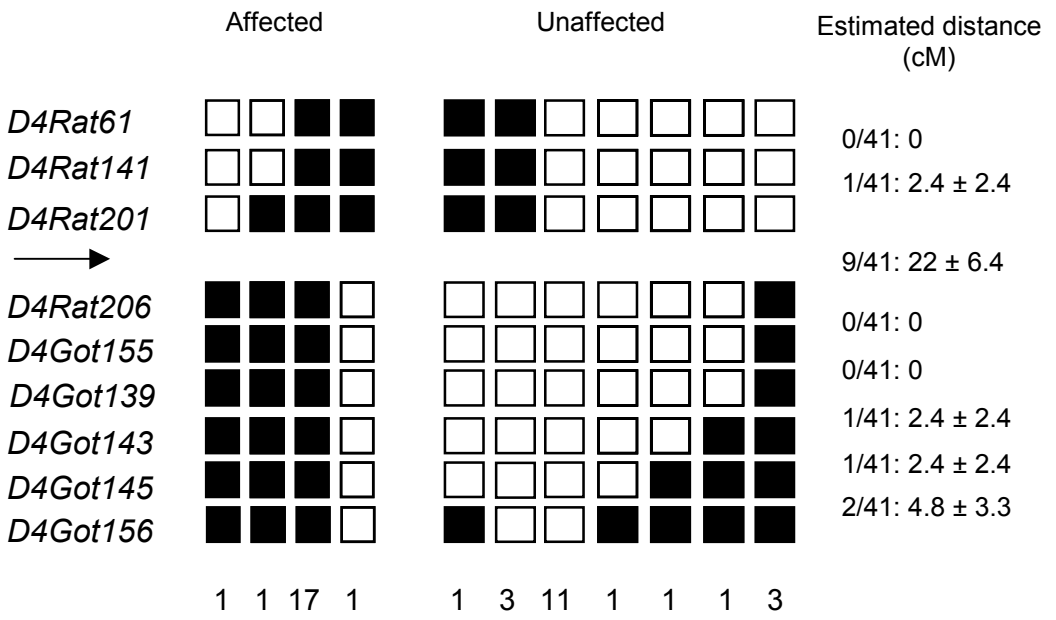


Fig. 11. Secondary screening of affected and unaffected animals. Filled squares indicate homozygosity (SD/SD) of the individual animal for a given marker, whereas empty squares mean heterozygosity (Wis/SD). On the right estimated genetic distances between two neighboring markers are presented together with the standard error values. The arrow shows the most probable location of the MENX gene. Numbers below indicate the number of animals with a particular haplotype pattern.

Two affected animals shown in Fig. 11 point out to the region between the markers: *D4Rat61* and *D4Rat201* as the site of *MENX* gene. Together with the information provided by the group of 17 animals (*MENX* locus can be found anywhere in the shown region), as well as the remaining animal, which haplotype suggests the gene is located distal to marker *D4Rat141* it can be concluded that the region of interest on chromosome 4 previously defined by markers *D4Rat61* and *D4Rat206* was reduced by 2cM, with its upper border shifted down and defined by marker *D4Rat201*. This marker, however, proved not to be very close to the *MENX* gene (Fig. 12).

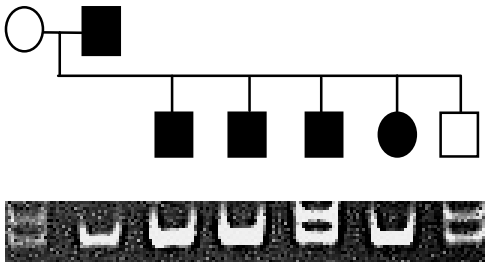


Fig. 12. Inheritance of the marker *D4Rat201* among some of the members of a backcross rat family (F2 generation). In one affected animal (indicated with an asterisk) the marker is inherited in a heterozygous state, which indicates *D4Rat201* is not highly linked to the MENX locus.

As shown in Fig. 12 one affected offspring animal is a recombinant in terms of *D4Rat201* inheritance, which proves this marker is not highly linked to the *MENX* locus. Unfortunately, no other markers allowing the further narrowing down the 22cM interval containing *MENX* locus.

4.3.5 Interval mapping on rat chromosome 4

The results of the second haplotype analysis were further verified by the analysis performed using Map Manager computer program. The microsatellite marker inheritance patterns for each marker among all 41 animals were introduced to the Map Manager program in order to perform the interval mapping, i.e. the generation of a curve showing the region most probably harboring MENX locus (Fig. 13).

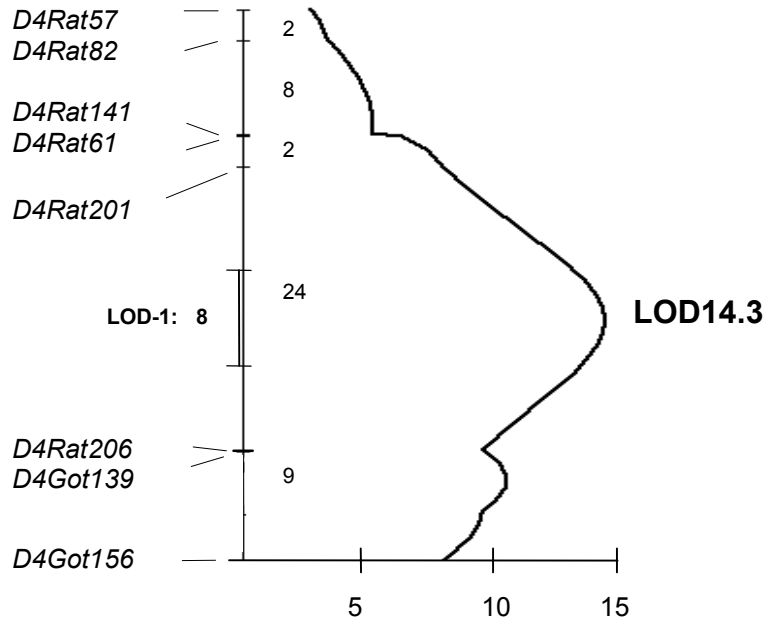


Fig. 13. Interval mapping on the distal part of rat chromosome 4 created by Map Manager program. Distances between two neighboring microsatellite markers are indicated in cM. The peak (LOD14.3) indicates the site of the *MENX* locus. The 8cM distance defined by LOD-1 indicates the most probable location of the *MENX* gene.

Fig. 13 shows that *MENX* locus is to be found with the highest probability between markers: *D4Rat201* and *D4Rat206* (LOD14.3), which is in agreement with the results obtained in the haplotype analysis (Fig. 11).

The lack of informative markers in the region of interest (Table 12) prevented further restriction of the critical region on chromosome 4.

Name of the marker	Database
D4Rat63	http://www.well.ox.ac.uk/rat_mapping_resources/
D4Rat64	Comprehensive_maps/comp_map_chr04.html
D4Rat68	
D4Rat111	
D4Rat112	
D4Rat137	
D4Got122	
D4Got124	
D4Got126	
D4Got128	
D4Got129	
D4Got130	
D4Got131	
D4Got133	
D4Got135	
D4Got136	
D4Got137	
D4Got140	
D4Got141	
D4Got142	
D4Got143	
D4Got144	
D4Got152	
D4Got153	
D4Wox16	
D4Mit14	http://www.well.ox.ac.uk/~bihoreau/Chr4frame.html
D4Mit19	
D4Mit20	

Table 12. List of the tested microsatellite markers mapped to the critical region on rat chromosome 4, which were not informative.

4.3 Synteny groups

To refine mapping genes known to map to chromosome 4 were studied to find those present within the 22cM region containing the MENX locus. In order to do that a synteny map of rat, mouse and human was obtained (Fig. 14).

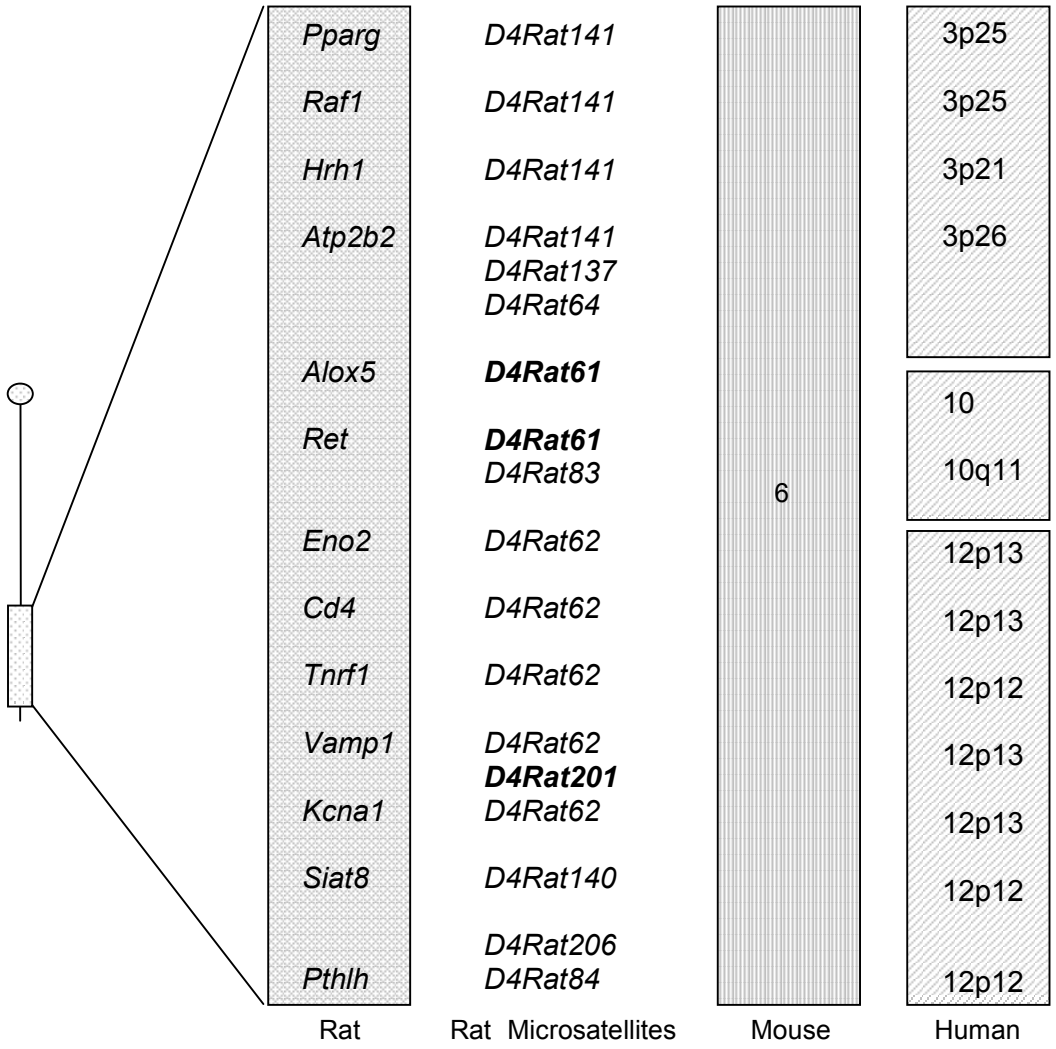


Fig.14. Synteny map of rat and human. The critical region on the chromosome 4 in the rat (left column) corresponds to a fragment of chromosome 6 in the mouse (middle column) and to three fragments of human genome on chromosomes: 3p, 10 and 12p and human (chromosomal position shown inside the three columns on the right). In the middle rat microsatellite markers linked to the genes within the synteny region are shown. *Alox5*- arachidonate 5-lipoxygenase; *Atp2b2*- ATPase isoform 2, Na^+K^+ transporting, beta polypeptide 2; *Cd4*- CD4 antigen; *Eno2*- enolase 2, gamma; *Hrh1*- Histamine receptor H1; *Kcna1*- potassium (K^+) channel protein, voltage dependent; *Pparg*- peroxisome proliferator activated receptor, gamma; *Pthlh*- parathyroid hormone-like peptide; *Raf1*- proto-oncogene serine/threonine-protein kinase; *Ret*- ret proto-oncogene; *Siat8*- Sialyltransferase 8a; *Tnfr1*- Tumor necrosis factor receptor; *Vamp1*- Vesicle-associated membrane protein 1. Source: http://ratmap.ims.u-tokyo.ac.jp/cgi-bin/Mapview_rat.pl?RNO04

As shown in Fig. 14 the distal part of rat chromosome 4 is equivalent to the fragment of mouse chromosome 6, as well as parts of the human chromosomes: 3p, 10, and 12p. This enabled finding genes and microsatellite markers around those genes, as well as determination if they can provide any information on the position of the *MENX* locus.

According to the results obtained in the haplotype analysis *MENX* locus is located below the marker *D4Rat61*. Since microsatellite markers placed above this marker on the synteny map (Fig. 14) refer to human chromosome 3 it was possible to exclude genes mapped on this chromosome from the further analysis.

Since the rat sequence was not known at this time it was not possible to find microsatellite repeats inside the genes. The only way to check if they are relevant to this study was to use the new markers known to be close to them, shown in the middle column of Fig. 14. Unfortunately none of the tested markers (both shown in Fig. 14 and obtained from other databases) were informative (Table 12).

4.4 Radiation hybrid mapping

Radiation hybrid mapping was used to find the physical position and the relationship of the genes and markers that were tested in the haplotype analysis, and further to determine if ret was present within the critical region on the chromosome 4. The genes to be mapped using the radiation hybrid panel, were chosen from the relevant synteny region, i.e. fragments of the human chromosomes: 10 and 12p (Table 13).

Acc#	Symbol	Description	Human chr	Mouse chr
34361	*Ret*	Ret proto-oncogene (MEN2A, MEN2B and medullary thyroid carcinoma 1, Hirschsprung disease)	10	6
34425	*Sdf1*	Stromal cell-derived factor 1	10	6
41174	***Alox5***	5 - Lipoxygenase	10	6
5	*A2m*	Alpha-2-macroglobulin	12	6
127	***Cacna1c***	Ca channel, voltage-dependent, L type, alpha 1c subunit	12	6
33908	***Eno2***	Enolase 2, gamma, neuronal	12	6
33995	*Grin2b*	Glutamate receptor, ionotropic, N-methyl D-aspartate 2B	12	6
34081	*Iapp*	Islet amyloid polypeptide	12	6
34129	***Kcna1***	Potassium (K+) channel protein, voltage dependent	12	6
34135	*Kras2*	Kirsten rat sarcoma viral oncogene homologue 2	12	6
34145	*Ldhb*	Lactate dehydrogenease B	12	6
34340	***Pthlh***	Parathyroid-like peptide	12	6
34507	*Tpi1*	Triosephosphate isomerase 1	12	6
35122	***Cd4***	CD4 antigen (p55)	12	6
35124	*Nkg2-d*	Natural killer lectin like receptor, orthologue of human NKG2D	12	6
35126	*Cd9*	CD9 antigen (p24)	12	6
39180	*Klrd1*	CD94 antigen (located within the rat natural killer gene complex)	12	6
39292	*Scnn1a*	Sodium channel, nonvoltage-gated 1, alpha (epithelial)	12	6
41164	***Siat8a***	Sialyltransferase 8 A	12	6
41218	*Mgp*	Matrix Gla protein	12	6
44360	*Gys2*	Glycogen synthase 2 (liver)	12	6
44361	***Vamp1***	Vesicle-associated membrane protein 1 (synaptobrevin 1)	12	6
44362	***Tnfrsf1a***	Tumor necrosis factor receptor superfamily, member 1a	12	6
44448	*Gucy2c*	Guanylate cyclase 2C (heat stable enterotoxin receptor)	12	6
45108	*Cdkn1b*	Cyclin-dependent kinase inhibitor 1B	12	6

Table 13. List of the genes present in the rat-mouse-human synteny region containing MENX locus. Acc #- NCBI accession number. In bold- genes analyzed on the radiation hybrid panel. Source: NCBI.

106 DNA templates of the radiation hybrid panel were amplified with the primers for selected microsatellites and gene fragments. The PCR results were then coded as follows: ‘0’ (no product), ‘1’ (presence of the product), and ‘2’ (not determined) (Table 16). The results of the amplification of the radiation hybrid panel samples were submitted online to the Rat RH Map Server (http://rgd.mcw.edu/RHMAPSERVER/) in the number- coded form (Fig. 15).

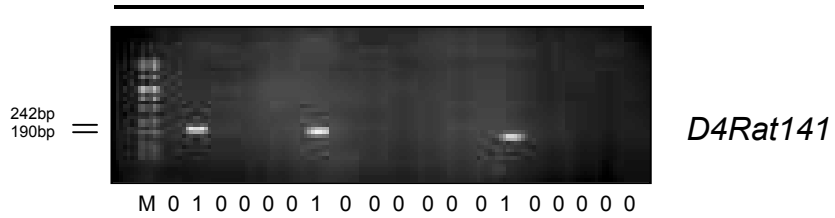


Fig. 15. Radiation hybrid mapping. Fragment of the RH panel (agarose electrophoresis): PCR product assigned as ‘1’, whereas no PCR product is described as ‘0’. M- molecular weight marker.

The server performed the mapping in such a way that microsatellites/ genes were placed against the framework maps of the server’s database. LOD cutoff was set on 15.0 to minimize multiple linkages of the genes/ markers within the rat genome. Finally, genes and microsatellite markers were mapped on the radiation hybrid panel.

Position (cR) of the markers/ genes that were linked to a single chromosome (no multiple linkage) was estimated in relation to the framework markers deposited in the Map Server database. The mapping data were summarized in a report (Table 15) and markers linked exclusively to chromosome 4 were presented as a graph (Fig. 16).

Summary Report (LOD: 15.0)
Markers in data set: 36
Markers in data set that are placed: 26
Markers in data set that are not placed: 10
Markers in data set that are multilinked: 0

Table 15. Fragment of the report on the radiation hybrid mapping performed by RHMAPSERVER.

Alox5	00000000000000000000**1**0**1**000**1**0000000**1**00**1**0000**1**0000000000000**1**0**1**0000000000000**1**00**1**0000**1**000000000000000000**1**10**11**0**1**000
c-raf	00000000000000000000**11**000**1**0000000**1**00**1**0000**1**0000**1**00**1**00**1**0**1**0000000000000**1**00**1**0000**1**0000000**1**0000000**1**0**1111**000
Pparg	00000000000000000000**11**000**1**0000000**1**00**1**0000**1**0000**1**00**1**00**1**0000000000**1**000**1**00**1**0000**1**0000000**1**000000**1**0**1**0**11**00**1**000
Hrh1	00000000000000000**1**00**11**000**1**0000000000**1**0000**1**0000**11**0**1**0000**1**0**1**0000000**1**000**1**00**1**0000**1**000000000000000000**1**0**11**0**11**000
Atp2b2	00000000000000000000**11**000**1**0000000**1**00**1**00**1**000000**11**0**1**0000**1**0**1**0000000**1**000**1**00**1**0000**1**000000000000000000**1**0**11**0**11**000
Vhl	00000000000000000000**1**000**1**0000000**1**00**1**00**1**000000**1**00**1**0000**1**0**1**0000000000000000**1**0000**1**000000000000000000**1**0**11**00**1**000
Rho	00**1**0**1**000000000000000**11**000**1**0000000**1**00**1**000000000**1**00**1**0000**1**000000000000000**1**00**1**0000**1**0000000**1**0000000**1**0**1111**00
Siat8	00000000000000000000000**1**00**11**0**1**000000**1**00**1**0000**1**00**1**0**11**0**1**00**1**0**1**0000000000000000000**1**0000**1**000000000000000000000**1111**000
Pthlh	00000000**1**0000000**11**0000**1**000**1**0**1**000000**1**00**1**0000**1**00**1**0**1**00**1**00**1**0**1**0000000000000000000**11**000**1**0**1**0**1**00**1**0000000**1**00**1**0**11**000
Vamp	00000000**1**0000000000000**1**000**1**0**1**000000**1**00**1**000000000**1**00**1**0000**1**000000000000000000**1**0000000000000000000**111111101**000
Cd4	00000000**1**0000000000000**1**000**1**0**1**000000**1**00**1**000000000**1**00**1**0000**1**000000000000000000**1**0000000000000000000**111111101**000
Kcna5	00000000**1**0000000000000**1**000**1**0**1**000000**1**00**1**000000000**1**00**1**0000**1**000000000000000000**11**000000000000000000**110111**00**1**000
Tnfr	00000000000000000000000000**1**0**1**000000**1**0**1**000000000000000000**1**0000000000000000000**1**0000000000000000000**1110**0**1**00**1**000
D4Rat201	00000000**1**0000000000**11**0000**1**0000000**1**00**1**000000**2**0**1**0000000**1**0**1**000000000000**1**00**1**0000**1**0000000000**1**00**1**0**111**00**1**0000
D4Got139	00000000**1**0000000000000**1**000**1**0**1**000**1**00**1**00**1**0000**1**00**1**0**11**0**1**00**1**0**1**0000000000000000000**1**0000**1**000000000000000**1**00**1111**000
D4Rat141	00000000**1**0000000000**1**00**11**000**1**0000000**1**00**1**0000**1**0000**11**0**1**00**1**0**1**0000000**1**00**1**000**1**00**1**0000**1**0000000**1**0000000**1**0**1**0**11**000**1**00
D4Rat61	00000000000**1**0000000000000**1**0000000**1**00**1**0000**1**0000**11**0**1**0000**1**0**1**0000000000000**1**00**1**0000**1**000000000000000**1111111**000
Ret	00000000000**1**0000000**111**000**1**0**1**000000**1**00**1**000000000**1**00**1**0000**1**0**1**0000000000000**1**00**1**0000**1**0**1**000000000000**1**0**1111111**000
D4Got134	00000000000000000000000**1**000**1**0000000**1**00**1**000000000**11**0**1**00**1**0**1**0000000000000000000**1**0000000000000**1**000**1**00000**11111**000
D4Wox1	00000000000000000000000000**110**1000000000**1**0000000000**11**0000**1**0**1**0000000000000000000**1**0000000000000**1**000**1**0000**111110**000
D4Rat94	000**1**0000000000000000000**1**0000000000**1**00**1**000000000**11**0**1**0000**1**000000000000000000000**1**100**111**0000000000000000000**11**0**11**000
D4Rat83	00000000000000**11**0000**1**0**111**000**1**0000000**1**00**1**0000**1**0000**11**0**1**00**1**0**1**0000000000000**1**00**1**0000**1**0000000**1**0000000**1111111**000
D4Rat104	00000000000000000000000**1**000**1**0000000**1**00**1**000000000**11**0**1**00**1**0**1**0000000000000000000**1**0000000000000**1**000**1**00000**11111**000
D4Rat206	00000000**1**0000000000000**1**00**11**0**1**000000**1**00**1**0000**1**00**1**0**11**0**1**00**1**0**1**0000000000000000000**1**0000**1**000000000000000000**11111**000
D4Got136	00000000**1**0000000000000**1**000**1**0000000**1**00**1**000000000**1**00**1**0000**1**0000000000000000000**1**0000000000000000000**1**00**111111**00**1**000
D4Got126	00000000000000000000000**1**000**1**0**1**000000**1**00**1**000000000**1**00**1**0000**1**0000000000000000000**1**0000000000000000000**111111**0**11**000
D4Wox14	00000000**1**0000000000000**1**000**1**0**1**000000**1**00**1**000000000**1**00**1**0000**1**0000000000000000000**1**0000000000000000000**11111111**000
D4Rat67	00000000**1**0000000000000**1**000**1**0**1**000000**1**00**1**000000000**1**00**1**0000**1**0000000000000000000**1**0000000000000000000**11111111**000
D4Got127	00000000**1**0000000000000**1**000**1**0**1**000000**1**00**1**000000000**1**00**1**0000**1**0000000000000000000**1**0000000000000000000**11111111**000
D4Got155	00**1**000001000000000000001000**1**0**1**000**1**00**1**00**1**0000**1**00**1**0**11**0**1**00**1**0**1**0000000000000000000**1**0000000**1**000**1**0000000**1**00**1**0000000
D4Got143	00000000**1**0000000000000**1**00**11**0**1**000000**1**00**1**0000**1**00**1**0**11**0**1**00**1**0**1**0000000000000000000**1**0000**1**000000**1**0000000000**11111**000
D4Got145	00000000000000**1**0000000**1**000**1**0**1**000**122**1**00**1**0000**1**00**1**0**1**00**1**00**1**0000000000000000000**1**0000**111**0**1**000**1**000**1**000**1**00**1**0001000
D4Rat65	00000000**1**0000000000000**1**000**1**00000**1**00**1**00**1**0000000**1**0**1**00**1**0000**1**0000000000000000000**1**0000**1**0000000000**1**00**1111**00**11**000
D4Rat65	00000000**1**0000000000000**1**000**1**00000**1**00**1**00**1**0000000**1**0**1**00**1**0000**1**0000000000000000000**1**0000**1**0000000000**1**00**1111**00**11**000
D4Rat65	00000000**1**0000000000000**1**000**1**00000**1**00**1**00**1**0000000**1**0**1**00**1**000**1**0000**1**000**1**0000000**1**0**1**0000**1**000**1**0000000**1**00**1111**00**11**00***2*
D4Rat65	00000000**1**0000000000000**1**000**1**00000**1**00**1**00**1**0000000**1**0**1**00**1**000**1**0000**1**000**1**0000000**1**0**1**0000**1**000**1**0000000**1**00**1111**00**11**00***2*

Table 16. Data used for radiation hybrid mapping. The numbers represent the PCR results: 0- no product, 1- presence of the product, 2- not determined.

Two additional genes were included in the radiation hybrid mapping analysis: Von Hippel-Lindau syndrome homolog (*Vhl*) gene and rhodopsin (*Rho*). *Vhl* is involved in the development of vascular tumors of the central nervous system, renal clear cell carcinomas, and pheochromocytomas (Bauer et al., 2002), and *Rho* is a transmembrane receptor expressed in retina, involved in an autosomal dominant disease retinitis pigmentosa (Berson et al., 1990). Both genes mapped to chromosome 4 were analyzed (despite the lack of evidence for their presence in the synteny region) for their potential relevance to this study: *Vhl* because of the disease phenotype it causes (malignant and benign neoplasms), and *Rho* because of its involvement in an ocular disease and its structure (transmembrane receptor).

Fig. 16 shows *Ret* gene (underlined) placed below marker *D4Rat61* and above marker *D4Rat201*. Since the latter specifies the upper border of the region on chromosome 4 containing the *MENX* locus it was concluded that ret **is not** present in the critical region, and therefore **is not** the gene responsible for the development of the new variant of multiple endocrine neoplasia.

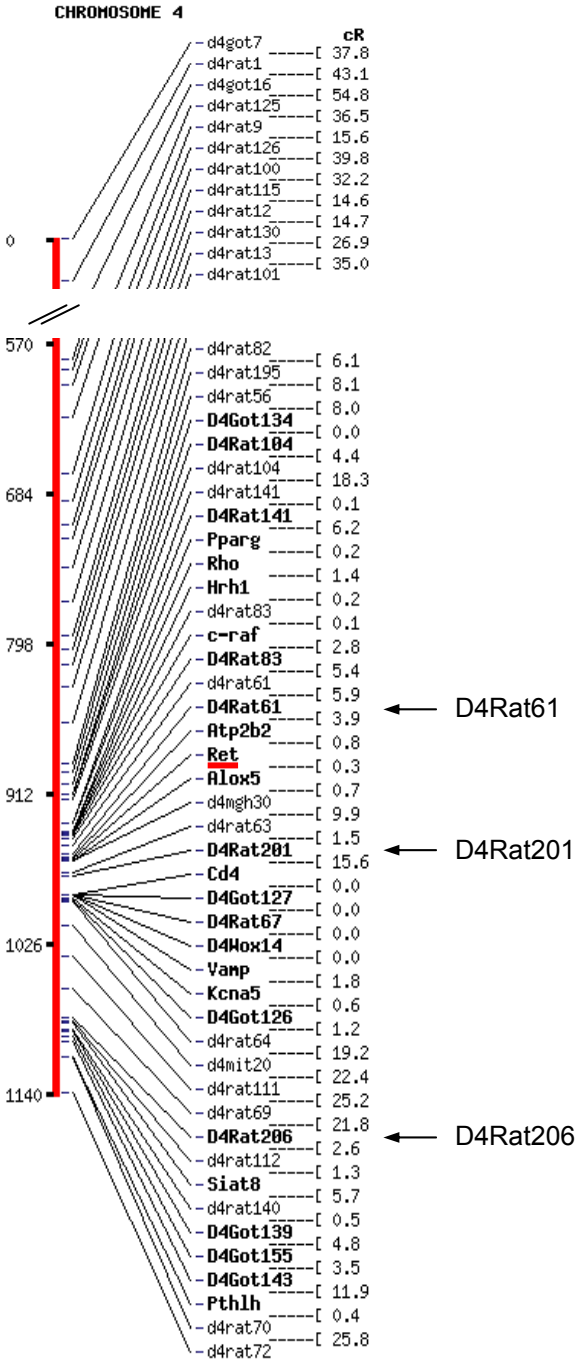


Fig. 16. Radiation hybrid mapping. Data generated by the RH Map Server of the Rat Genome Database. In bold- markers/ genes linked exclusively to chromosome 4 shown with respect to the position of framework markers. cR distances between two neighboring markers/ genes are shown. *Alox5*- arachidonate 5-lipoxygenase; *Atp2b2*- ATPase isoform 2, Na^+K^+ transporting, beta polypeptide 2; *Cd4*- CD4 antigen; *Eno2*- enolase 2, gamma; *Hrh1*- Histamine receptor H1; *Kcna1*- potassium (K^+) channel protein, voltage dependent; *Pparg*- peroxisome proliferator activated receptor, gamma; *Pthlh*- parathyroid hormone-like peptide; *Raf1*- proto-oncogene serine/threonine-protein kinase; *Ret*- ret proto-oncogene; *Rho*- Rhodopsin; *Siat8*- Sialyltransferase 8a; *Tnfr1*- Tumor necrosis factor receptor; *Vamp1*- Vesicle-associated membrane protein 1.

Rho maps far above the interesting region (Fig. 16), which excludes it from the further study. Rat map server could not perform the placement of *Vhl* at LOD cutoff of 15.0 (Fig. 16) however, by setting the cutoff at 10.0 the placement was performed (Fig. 17):

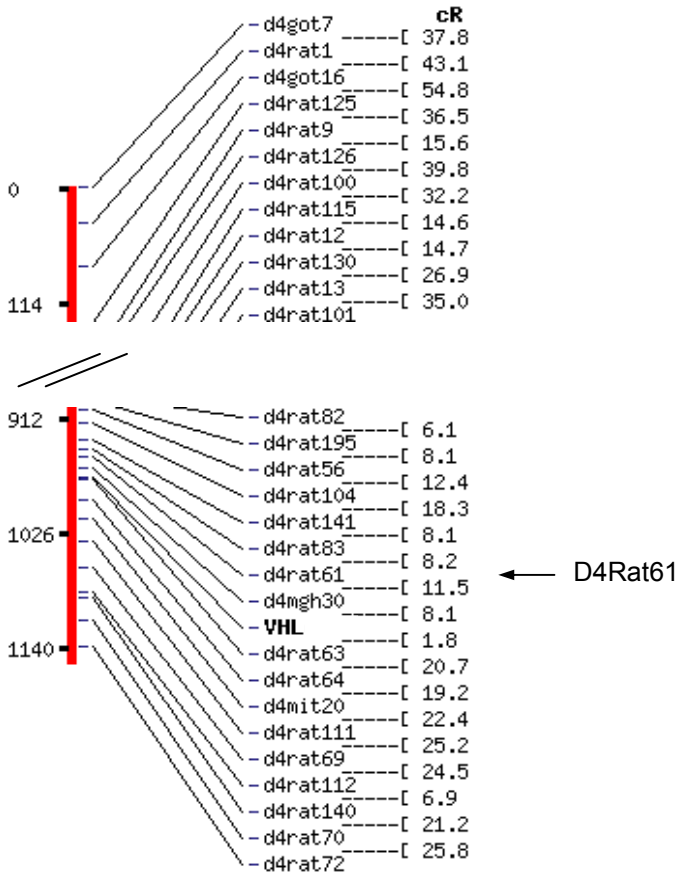


Fig. 17. Radiation hybrid mapping. Data generated by the RH Map Server of the Rat Genome Database. In bold- von Hippel-Lindau syndrome homolog (*Vhl*) gene. cR distances between two neighboring markers/ genes are shown.

By comparing Fig. 16 and Fig. 17 it was possible to conclude that *Vhl* gene maps to the region above marker *D4Rat201*: it is placed proximal to the framework marker *D4Rat63* (Fig. 16), whereas *D4Rat201* maps distal to it (Fig. 17). This excludes *Vhl* from the further analysis.

The only genes, which could be mapped on the radiation hybrid panel, present within the region of interest on chromosome 4 (Fig. 16), that is between markers: *D4Rat201* and *D4Rat206*, were: *Vamp1*, *Kcna5*, and *Cd4*. Based on the information available in the National Center for Biotechnology Information,

NCBI (http://www.ncbi.nlm.nih.gov) using search options 'UniGene' and 'LocusLink', the following data on the genes mentioned above was collected (Table 17).

***Rattus norvegicus* official gene symbol and name (RGD)**	**Product**	**Function**	**Expression**
Vamp1: Vesicle-associated membrane protein 1	Vesicle-associated membrane protein 1	mRNA is transiently decreased during nerve regeneration of the facial motor nucleus after axotomy	Dorsal root ganglion; mixed tissue; brain; ovary; muscle
Kcna5: potassium voltage gated channel, shaker related subfamily, member 5	Potassium voltage gated channel, shaker related subfamily, member 5	Regulation neurotransmitter release, heart rate, insulin secretion, neuronal excitability, epithelial electrolyte transport, smooth muscle contraction, and cell volume (human)	Brain; chondrosarcoma
Cd4: CD4 antigen	CD4 antigen	Testicular macrophages expressed the markers CD45 and MAC387 and most also expressed CD4	Blood cell

Table 17. The characteristics of the genes mapped to the region of interest on rat chromosome 4 in the radiation habrid experiment (source: National Center for Biotechnology Information). RGD- Rat Genome Database (www.rgd.mcw.edu).

The above genes were not analyzed further, since their expression and function were of no relevance to MENX syndrome according to the information obtained from the database.

4.5 Genotyping using the physical distances on mouse chromosome 6

Before the rat genome sequence was publicly available in an assembled form the relative position of the markers/ genes with respect to each other along the chromosome 4 was estimated by blasting their sequences against the mouse genome (http://www.ensembl.org/Mus_musculus/blastview).

Blast results revealed single rat genome Bac clones matching the gene sequence, and in the next step Bac clones containing those rat genome fragments relevant to this study were screened manually (by eye) for the presence of the microsatellite repeats in the close distance to the genes. The sequences of the new markers together with the regular sequences surrounding them were blasted against the mouse genome and in this way their approximate position has been determined. Microsatellites and genes were then arranged schematically along the rat chromosome 4. The discontinuity of the rat sequence was indicated by breaks separating single bac clones. These data were further used to perform the haplotype analysis with two recombinant animals (one affected and one unaffected) identified in previous genotyping experiment (Fig. 18).

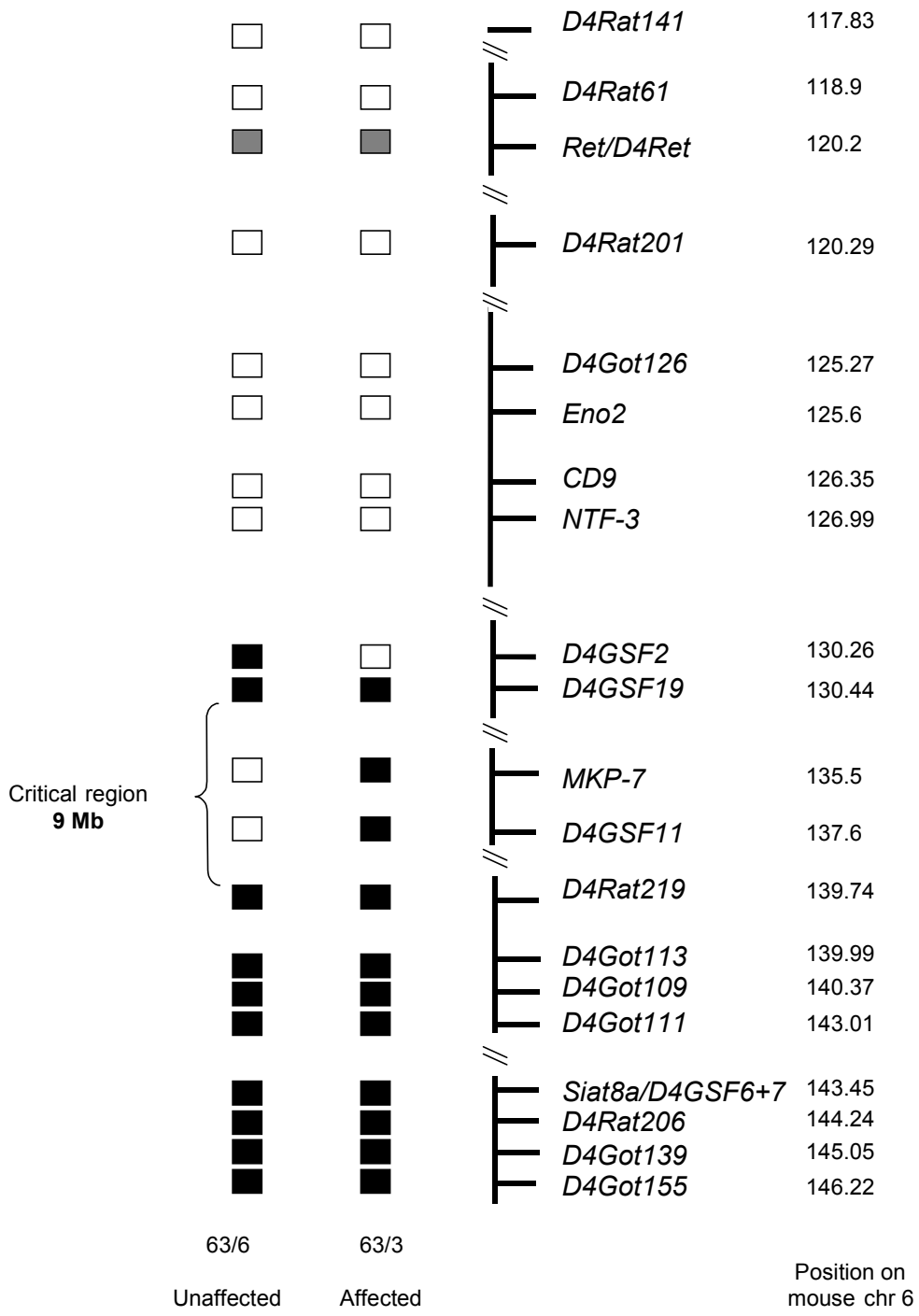


Fig. 18. Haplotype analysis of the two recombinant animals (Tab. 9b) identified previously. Names of the genes and microsatellite markers are indicated in the middle, whereas their position on mouse chromosome 6 is shown on the right. Filled squares mean homozygosity, empty mean heterozygosity. All '*GSF*' markers were self- designed.

The above haplotype analysis allowed for the determination of the 9cM large critical region on rat chromosome 4 using the unaffected animal, which is a recombinant for the markers: *MKP-7* and *GSF11* (Fig. 19).

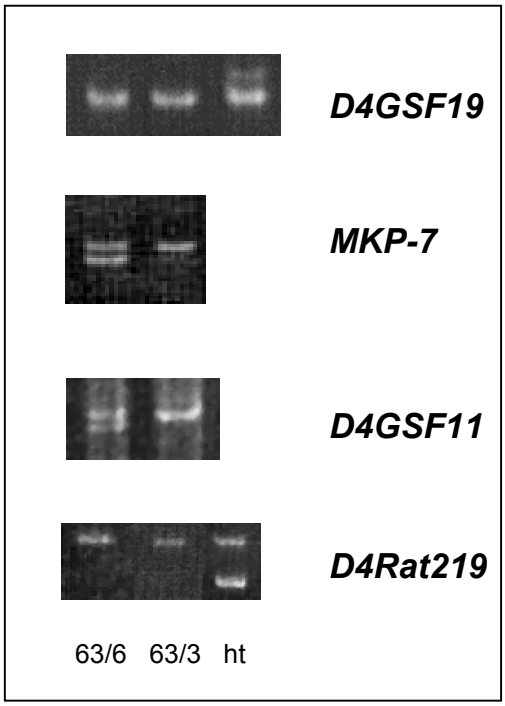


Fig. 19. Haplotypes of the rats: 63/6 (unaffected) and 63/3 (affected) analysed for the 9cM large region of interest on chromosome 4. ht – heterozygous haplotype shown for comparison.

In the result of the haplotype analysis shown in Fig. 18 it was possible to restrict the region of interest on rat chromosome 4 to about 9Mb (according to distance information inferred from the mouse genome sequence). This is the only region where the unaffected animal is heterozygous for all markers, and at the same the affected animal is homozygous, which is consistent with the recessive trait of *MENX*.

The above haplotype analysis also excludes neurotrophin-3 (*NTF-3*) from the further analysis. *NTF-3* is a neurotrophic factor required for the development of the enteric nervous system (Chalazonitis et al., 2001) and therefore it was the 'hottest' candidate gene for MENX.

After the rat genome was sequenced and assembled it was possible to find new markers mapping in the critical region identified in result of the haplotype analysis.

Rat genome sequence published by the National Center for Biotechnology Information was used to identify new markers. First, the relevant fragment of the rat genome i.e. distal part of chromosome 4 was identified inside the whole genome sequence by blasting the partial sequences of genes known to be found in the region of interest. Subsequently, a ca. 13Mb contig was screened manually for repetitive sequences to design new primers. In the following haplotype analysis four recombinant animals were used. Out of nearly 100 self-designed microsatellites only 12 appeared to be informative and therefore were included in the analysis. In addition a microsatellite marker for *MKP-7* was designed and included in the mapping analysis. *MKP-7* codes for a protein involved in a signal transduction patway, hence is a candidate (Fig. 20).

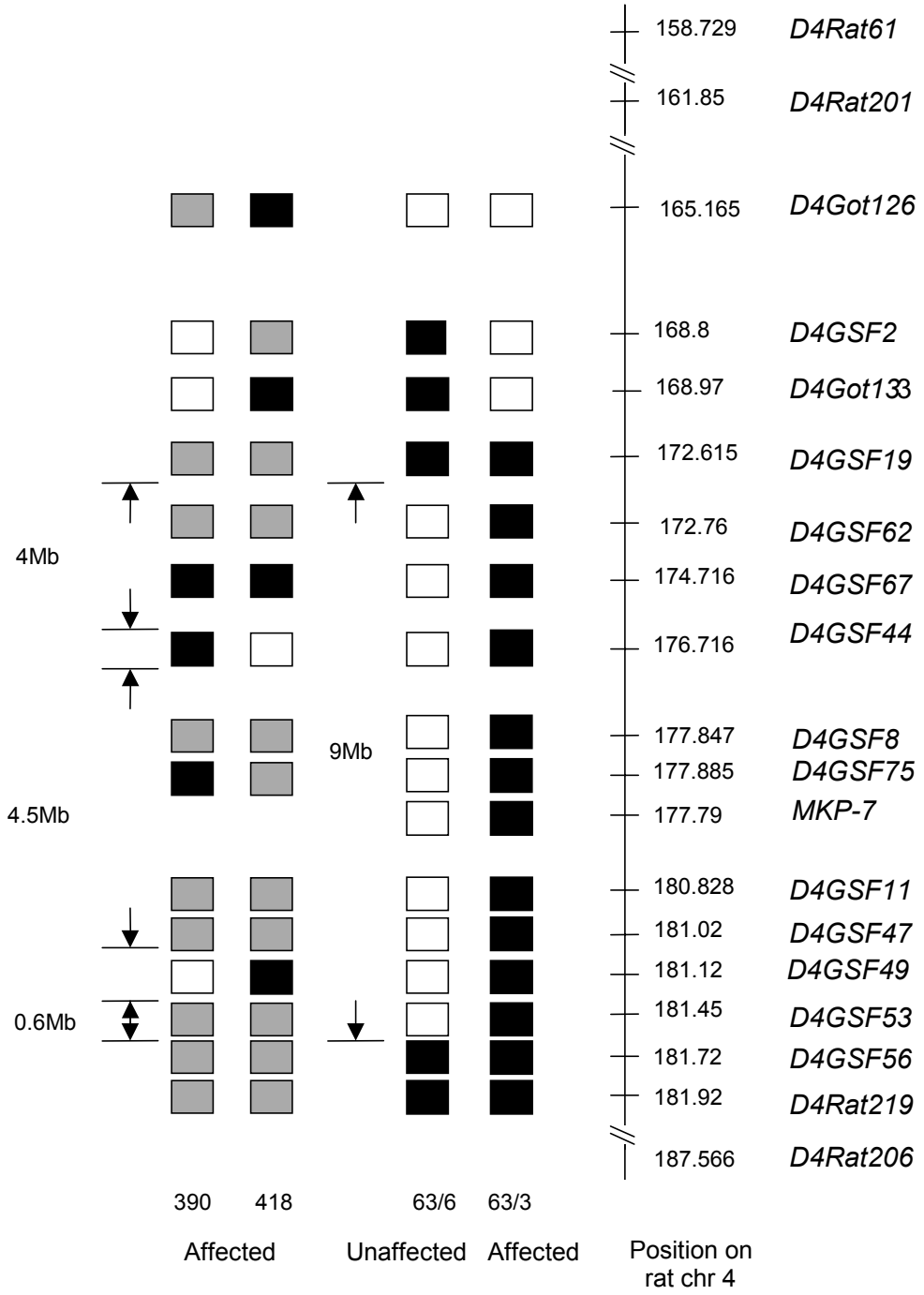


Fig. 20. Haplotype analysis of five recombinant animals. Names of genes and microsatellite markers, as well as their position on rat chromosome 4 in Mb are indicated on the right. Filled squares mean homozygosity, empty- heterozygosity, grey- not determined. Arrows point to the newely identified fragments potentially harboring *MENX* locus. '*GSF*' markers were self-designed. Animals 390 and 418 belong to two different families (A. Fritz, GSF - Institute of Pathology).

The haplotype analysis shown in Fig. 20 shows three candidate regions on rat chromosome 4: 4Mb, 4.5Mb, and 0.6Mb large. The genes found in those regions include:

Klrc1: killer cell lectin-like receptor subfamily C

Klrd1: CD94 antigen

Ly49: lymphocye antigen 49 complex

Cdkn1b: cyclin-dependent kinase inhibitor 1B

Ptpro: protein tyrosine phosphatase, receptor type, O

LOC297682: similar to MAP kinase phosphatase-7

LOC297695: similar to WW domain binding protein 11

Pde6g: phosphodiesterase 6G, cGMP-specific

For more information on the above genes see Table 18:

Gene symbol	**Function**	**Reported to be expressed in**
Klrc1	Member of the natural killer NKG2 group; expressed primarily in natural killer (NK) cells and encodes a family of transmembrane proteins characterized by a type II membrane orientation (extracellular C terminus) (**human**)	Pooled germ cell tumors; hypernephroma; natural killer cells, cell line; placenta; lymphocyte (**human**). Thymus; lymph node; mammary gland; Hematopoietic Stem Cell (Lin-/c-Kit-/Sca-1-) (**mouse**).
Klrd1	Member of the natural killer *NKG2* family; antigen preferentially expressed on NK cells and is classified as a type II membrane protein because it has an external C terminus (**human**)	Heart (**rat**). Placenta; liver; lung; testis; pooled pancreas and spleen; leukocyte (**human**). Spleen; thymus; mammary gland; lymph node; aorta and vein (**mouse**).
Ly49	*Ly-49* stimulatory receptor 3 is a promiscuous activating rat NK cell receptor for nonclassical MHC class I - encoded target ligands (**rat**).	Spleen; lymph node (**mouse**).
LOC 297695	Similar to WW domain binding protein 11; Npw38-binding protein NpwBP; SH3 domain-binding protein SNP70. Mediates protein- protein interactions	Dorsal root ganglia; ovary; kidney; brain; placenta embryo; lung, liver, heart, muscle, spleen; mixed tissue; hypothalamus; peneal gland brain; ventral prostate; anterior pituitary

Cdkn1b	Posttranscriptional regulation; Cyclin kinase inhibitor p27 plays a role on TNF-alpha induced mesangial cell proliferation; physiologically relevant regulator of cyclin D-Cdk4/6 activity as well as mechanistically a target of c-Myc action (**rat**). Binds to and prevents the activation of cyclin E-CDK2 or cyclin D-CDK4 complexes, and thus controls the cell cycle progression at G1. The degradation of this protein, which is triggered by its CDK dependent phosphorylation and subsequent ubiquitination by SCF complexes, is required for the cellular transition from quiescence to the proliferative state (**human**).	Brain E15; Mixed tissues (**rat**) Placenta; Thymus; Liver; adenocarcinoma; Uterine; carcinoid; brain, pineal gland; anaplastic oligodendroglioma; Prostate; kidney ; B-cell, chronic lymphotic leukemia; Adipose; human lung epithelial cells; fetal brain; prostate; squamous cell carcinoma; medulloblastoma; stomach; Pooled human melanocyte, fetal heart; glioblastoma; Primary Lung Epithelial Cells; head, neck; cervix; breast normal; hepatocellular carcinoma; Adrenal gland; Hypothalamus; pituitary; Bone marrow; muscle (skeletal); breast; mammary gland; melanocyte; skin, normal; neuroblastoma; placenta; multiple sclerosis lesions; cartilage; hippocampus; hypothalamus; adenocarcinoma, cell line; Islets of Langerhans; human retina; ovary; insulinoma; uterus; Chondrosarcoma; Metastatic Chondrosarcoma; pineal gland; muscle; blood; lens; Primary Lung Cystic Fibrosis Epithelial Cells; cerebellum; ovarian tumor; adrenal adenoma; colon; colon tumor; lung; parathyroid tumor; metastatic prostate bone lesion; kidney tumor (**human**). Spinal cord; hypothalamus; aorta and vein; sympathetic ganglion; thymus; cerebellum; kidney; head; eyeball; cortex; intestine; lung; retina; mammary gland; ovary; embryo; inner ear; heart; lymph node; adipose; liver tumor; spleen; colon; brain (**mouse**).
LOC 297682	Similar to MAP kinase phosphatase-7	Pituitary; placenta embryo; lung; testis, pooled; prostate tissue (**rat**)
Pebp-2	Phosphatidylethanlomine binding protein 2; *pebp-2* is a testis-specific 21-kDa protein found within late meiotic and haploid germ cells and colocalizes with members of the MAP kinase pathway in late spermatocytes and spermatids	No information

Pde6g	Visual transduction protein **(rat)**. *PDEG* functions to link c-Src and G-protein-coupled receptor kinase 2 in a signaling unit that regulates p42/p44 mitogen-activated protein kinase by epidermal growth factor **(human)**.	Ovary; mix - brain, ovary, placenta, kidney, lung, liver, embryo, heart, muscle, spleen **(rat)** Retina; skeletal muscle; lung; whole brain; pineal gland; metastatic chondrosarcoma; pooled germ cell tumors; blood; uterus; pineal body; liver and spleen **(human)** Retina **(mouse)**
LOC 297699	Similar to UNR-interacting protein (WD-40 repeat protein PT-WD) (MAP activator with WD repeats)	Kidney; brain; placenta; prostate; dorsal root ganglion; chondrosarcoma; cartilage; placenta embryo; brain motor neuron; pituitary; adipose tissue; hypothalamus; whole embryo; lung, pooled; heart, pooled; testis, pooled; cortex; spleen; ovary **(rat)**
Ptpro	expression reflects podocyte injury induced by puromycin aminonucleoside (**rat**). Transcript variant 1 and 2 may play a role in regulating glomerular epithelial cell (podocyte) structure and/or function. Variant 3, 4, and 6: in B cells, its overexpression promotes G0/G1 growth arrest. *PTPRO* is involved in the differentiation and axonogenesis of central and peripheral nervous system neurons, where it is in a position to modulate intracellular responses to neurotrophin-3 and/or nerve growth factor (**mouse**).	Mixed tissue; dorsal root ganglia; hypothalamus normal (**rat**). kidney; fetal brain; breast; pooled germ cell tumors; leukopheresis; brain; colon; frontal lobe; bone marrow; neuroblastoma; adenocarcinoma cell line; hypothalamus; fetal eyes, lens, eye anterior segment, optic nerve, retina; human skeletal muscle; heart; adenocarcinoma; normal prostate (**human**). Whole brain; retina; stomach; cortex; cerebellum; thymus; vagina; testis; skin; kidney; adrenal gland; spinal cord; bone marrow; spinal ganglion; heart; head; visual cortex; pituitary gland; infiltrating ductal carcinoma; egg; newborn brain; sympathetic ganglion; colon; neural retina (**mouse**).

Table 18. The characteristics of the genes, which were found in the regions of interest on rat chromosome 4. Source: NCBI.

From all the genes listed in Table 16. *similar to MAP kinase phosphatase-7,* and *PTPRO* as the genes potentially involved in signal transduction patways (similar to *RET*) were obviously the most interesting candidate genes Sequencing of *similar to MAP kinase phosphatase-7* did not reveal any mutations in this gene.

The above study presents the mapping of the gene responsible for the novel variant of the multiple endocrine neoplasia (MEN2X). The project was carried out from August 2000 to July 2003 in the Institute of Pathology, GSF Reasearch center for Environment and Health. Because of the limited time resources it was impossible to further investigate the subject of this work within the frames of the doctoral thesis.

5 Discussion

This study presents the mapping of the gene locus responsible for a previously unrecognized form of multiple endocrine neoplasia (MEN) developing as a sporadic mutation in the rat. The new variant of MEN (MENX), in contrast to the disease forms in man, is inherited in a recessive fashion (Fritz et al., 2002), which suggests that a novel gene is responsible for MENX.
Genome-wide linkage analysis was used to map the *MENX* locus to rat chromosome 4. The analysis was performed on a group of 41 backcross animals (Wistar/Nhg x SD^{we}) x SD^{we} (SD^{we} indicates affected SD white eye rats). The *MENX* locus was initially mapped to an approximately 22cM interval that included the *RET* gene, which is responsible for MEN type 2A and 2B. However, further mapping with the use of additional informative microsatellite markers allowed the exclusion of *RET* from the critical region. This was ultimately defined as a region of about 9Mb on chromosome 4. This region includes three potential candidate genes which are discussed below.

Sporadic cancers of the neuroendocrine tissues are recognized, leading for example to pituitary adenoma, parathyroid adenoma, adrenal pheochromocytoma, and C-cell carcinoma. Here the cause is most probably an initial gene mutation, either inherited via germ line or acquired during tissue development.
In addition to sporadic disease there are a number of genetic factors causing malignancy of neuroendocrine cells. These include familial cancer syndromes, defined as an increased specific cancer risk of blood relatives arising through germ line transmission of a gene mutation, e.g. Li Fraumeni syndrome and Retinoblastoma (*p53*/*Rb1* mutation respectively; Srivastava et al., 1990; Benedict et al., 1983). In neuroendocrine tissues at least three such syndromes are recognized: multiple endocrine neoplasia type 1 (MEN1), 2A (MEN2A) and 2B (MEN2B). Related syndromes involving only one neuroendocrine cell type include familial medullary thyroid carcinoma (FMTC) and familial pheochromocytoma. Whilst the latter two only affect single tissues (C-cell and adrenal) the first three are responsible for cancers of multiple neuroendocrine

tissues (Raue and Zink, 1992). MEN1 syndrome (OMIM 131100) is characterized by tumors of parathyroid, pituitary, and pancreatic islet cells; Tissues afected in MEN2A (OMIM 171400) include thyroid medulla, adrenal (pheochromocytoma) and parathyroid; The phenotype of MEN2B (OMIM 162300) resembles that of MEN2A (presence of medullary thyroid carcinoma, pheochromocytoma), additionally MEN2B patients develop neuromas of the mucous membranes (Table 19).

Disorder	**Gene muta-tion**	**Phenotype**						
		Pituitary adenoma	Insuli-noma	Parathyroid adenoma	Pheochro-mocytoma	MTC	Paragan-glioma	Neuroma
MEN1	*MENIN*	+	+	+				
MEN2A	*RET**			+	+	+	+	
MEN2B	*RET**				+	+		+

Table 19. Characteristics of multiple endocrine neoplasia syndromes. MTC-medullary thyroid carcinoma. * Different *RET* mutations are responsible for MEN2A and MEN2B.

MEN syndromes are rare disorders. For MEN1 the incidence is estimated at 0.2-1 cases per 100,000 in the general population (Trump et al., 1996; Karges et al., 2000), MEN2 was identified in about 1000 kindreds (Brandi et al., 2001). One of the two genes known to be mostly involved in the development of MEN syndromes is *MENIN*. *MENIN*, responsible for multiple endocrine neoplasia type 1, was first identified in 1997 (Chandrasekharappa et al., 1997) and mapped in human to chromosome 11 (Larsson et al. 1988). It encodes a protein predominantly localized in the nucleus (Guru et al., 1998). A role in gene transcription is suggested by the proteins found to interact with MENIN, which include the transcription factors JunD (Agarwal et al., 1999) and NF-kappaB (Heppner et al., 2001). Agarwal et al. showed that *MENIN* repressed the transcriptional activation mediated by JunD, and that missense *MENIN* mutations disrupted *MENIN* interaction with JunD. This latter study suggests that the tumor suppression action of *MENIN* is based on the direct binding to JunD, and resultant inhibition of JunD activated transcription. Gallo et al. suggested that *MENIN* acts downstream of MAP kinase activation (Gallo et al., 2002).

The second gene involved in the genesis of multiple endocrine neoplasias is *RET*. This codes for a tyrosine kinase, a membrane molecule transducing signals for cell growth and differentiation (Putzer and Drosten, 2004). Mutations in *RET* are associated with MEN2A, MEN2B, and Hirschsprung disease, OMIM 142623 (Hansford and Mulligan, 2000). Genetic syndromes with the involvement of either *MENIN* or *RET* share an apparently dominant mode of inheritance, however, their biologies differ. Loss of function of *MENIN*, typically for a tumor suppressor gene, proceeds as predicted by Knudson's 'two hit hypothesis' (Knudson, 1971). Knudson proposed that the loss of function of both alleles of a tumor suppressor gene is needed for tumor formation. In the paradigm of Retinoblastoma used by Knudson, the first hit is present in the germline, followed by a second (somatic) mutation leading to loss of both alleles. Although MEN1 is trasmitted as a dominant trait, inheritance of one defective copy of *MENIN* is not sufficient to transform a normal cell into a tumor cell; MEN1 develops as a result of a second mutation leading to the loss of the remaining normal allele. In case of *RET* a gain of function event underlies tumorigenesis, where only one hit is required for activation. Moreover, *RET* can undergo oncogenic activation *in vivo* and *in vitro* by cytogenetic rearrangement creating chimeric transforming oncogenes, *RET/PTC*, which have frequently been found in thyroid papillary carcinomas (Salabe, 2001).

There are a number of neuroendocrine malignancies where neither *MENIN* nor *RET* are involved. Carney Complex (CNC1: OMIM 160980; CNC2: OMIM 605244) is an autosomal dominant disease characterized by cardiac and cutaneous myxomas and the appearance of endocrine tissue tumors. Genetic linkage analysis of families affected with Carney complex have identified two loci for the disease: 17q24 for the CNC1 variant, and 2p16 for the variant designated CNC2. The chromosome 17 form (Carney complex type I) has been shown to be due to mutations in the *PRKAR1A* (R1alpha regulatory subunit of cAMP-dependent protein kinase A) gene (Stratakis et al., 2001). The *PRKAR1A* gene encodes the inhibitory subunit type 1A of protein kinase A, which is known to be an important effector molecule in many endocrine signaling pathways (Stratakis, 2002). Sandrini et al. (2002) suggested that PRKAR1A acts as a tumor-suppressor gene in sporadic thyroid cancer. Groussin et al. (2002)

demonstrated an inactivating PRKAR1A mutation expressed at the protein level and leading to loss of function of the inhibitor, causing uncontrolled stimulation of the PKA pathway in patients with Carney complex. This presumably mimics trophic hormone/ growth factor action on the endocrine cells.

CNC2 remains so far unidentified. Matyakhina et al. (2003) stated that cytogenetic changes of the 2p16 chromosomal region that harbours the CNC2 locus are mostly amplifications that overlap with an amplicon found also in sporadic thyroid cancer, and that map to an area often deleted in sporadic adrenal tumours. Both thyroid and adrenal tumors are part of Carney complex indicating that the responsible gene(s) in this area may indeed be involved in both inherited and sporadic endocrine tumour pathogenesis and/or progression.

A mutation in *SDHB* (succinate dehydrogenase complex, subunit b, OMIM 185470) mapped in human to chromosome 1p35-36 (Leckschat et al., 1993) was reported in a family with both extra adrenal pheochromocytoma and cervical paraganglioma, as well as in one patient with extra adrenal pheochromocytoma (Astuti et al., 2001). Astuti et al. predicted that the mutation of an arginine to a termination codon in *SDHB* gene would result in a truncated *SDHB* protein lacking the C-terminal 191 amino acids. Young et al. (2002) identified a missense point mutation detected in the *SDHB* gene: a G-to-A transition in exon 7, which alters a conserved arginine to a histidine.

Niemann and Muller (2000) reported a patient with familial nonchromaffin paragangliomas type 3) with a G-to-A transition in the *SDHC* (succinate dehydrogenase complex, subunit c, OMIM 602413) gene. Niemann also described a case of malignant paraganglioma characterized by a G-to-T transversion of intron 5 of *SDHC* (Niemann et al., 2003).

A number of mutations which lead to familial paragangliomas have been detected in the *SDHD* (succinate dehydrogenase complex, subunit d, OMIM 602690) gene mapped to 11q23. They include numerous C-to-T transitions (Baysal et al. (2000), a missense mutation in exon 4 (tyr114 to cys), and a deletion of a G at position 13838 in exon 4 (Milunsky et al., 2001). Astuti et al. (2001) investigated the *SDHD* gene in patients with familial pheochromocytoma and identified a germline frameshift mutation in exon 2: a 2-bp deletion

predicting a truncated protein. However, no experiments with the knockout mice have been carried out to confirm the effect is indeed due to this mutation.

Neurofibromatosis type 1 (NF1, OMIM 162200) is an autosomal dominant disorder characterized primarily by neurofibroma tumors of the skin, but where pheochromocytoma can occasionally occur (Hirsch et al, 2001). Ledbetter et al. (1989) mapped the neurofibromatosis type 1 gene (*NF1*) to 17q11. Vogel et al. (1999) generated a mouse model for neurofibromatosis type 1 and concluded that an additional mutation in the *p53* tumor suppressor gene is required to predispose *Nf1+/-* mouse neural crest-derived cells to malignant transformation. Cichowski et al. (1999) demonstrated that chimeric mice partially composed of *Nf1*-/- cells develop neurofibromas, which demonstrated that loss of the wildtype *NF1* allele is rate-limiting in tumor formation in *Nf1*+/- mice.

Von Hippel-Lindau syndrome (VHL: OMIM 193300) is a familial cancer syndrome inherited in a dominant manner, observed in the population with a frequency of 1:36,000 (Lonser et la., 2003). The most frequent symptoms of the disease are retinal, cerebellar, and spinal hemangioblastoma, renal cell carcinoma, pheochromocytoma, and endocrine pancreatic tumors. The syndrome develops as a result of a germline mutation in the *VHL* tumor suppressor gene, which has been mapped to 3p25 (Seizinger et al., 1991). Kanno et al. (2000) showed that the *VHL* gene plays an important role in neuronal differentiation and transcription; expression of the VHL protein was correlated with neuronal differentiation in CNS progenitor cells, and the *VHL* gene transduction induced neuronal differentiation.
Additionally, Lui et al. examined VHL-related pheochromocytomas and found the loss of chromosome 11, which appeared to be specific for those malignancies (Lui et al., 2002).

Predisposition to pheochromocytoma occurs also in MENX, another syndrome where neither *RET* nor *MENIN* are involved. It could be possible that the *MENX* gene mutation increases the susceptibility to phaeochromocytoma tumorigenesis. The existence of an additional loci for pheochromocytoma predisposition was previously suggested by Woodward et al. (1997). The group

analyzed patients with no evidence of von Hippel Lindau disease, MEN2, or neurofibromatosis. No *VHL*, *RET* or *GDNF* (ligand for *RET*) mutations were identified, however, a *GDNF* sequence variant was identified in 1 sporadic tumor.

The phenotypic characteristics of the multiple endocrine neoplasia syndrome that developed spontaneously in Sprague-Dawley rat (MENX), which is presented in this work, is different from those described in man (Table 5). Thus the MENX syndrome is difficult to classify as one of the known neuroendocrine disorders.

More importantly, MENX is a recessive disease, whereas MEN1, MEN2A, MEN2B, Carney complex, neurofibromatosis type 1 and Von Hippel-Lindau syndrome are all inherited in a dominant manner. This suggests that the new variant of multiple endocrine neoplasia is either developed due to a different genetic mechanism of neuroendocrine tumorigenesis, or represents a rat-specific inheritance pattern.

To gain insight into the identity of potential candidate genes we have reviewed previously described animal model where neuroendocrine tumors appear (Lee et al., 1982; see 1.1.2). The spectrum of affected organs in MENX and the Long-Evans rats is not identical, and the severity of the symptoms is different, for example, medullary thyroid carcinoma in Long-Evans rats, versus medullary thyroid hyperplasia in SD rats, or parathyroid hyperplasia in the former strain compared with parathyroid adenoma in the latter. This may be either due to a more severe character of MENX, leading to death of the animals before appearance of carcinoma, or differences in diagnostic procedure. Moreover, multiple endocrine neoplasia in the Long-Evans rats is not a recessive disorder. Another difference is the lack of cataract in the animals described by Lee et al., but the presence of cataract in MENX - affected animals, as well as the onset of the disease: Long-Evans rats develop symptoms at older age, whereas SD rats become affected in the early adolescence. Some of the differences mentioned above may be due to the different genetic background of the animals. Another possibility is that the two syndromes are allelic variants.

The involvement of tumor supressor genes in multiple endocrine neoplasia syndromes was studied by Nikitin et al. (1999), and Franklin et al. (2000). The group of Nikitin et al. described a multiple endocrine neoplasia- like phenotype associated with *Rb* deficiency in mice. $Rb^{+/-}$ mice displayed pheochromocytomas, as well as hyperplasias of Langerhans cells and lung neuroendocrine cells, which are incidentally absent in MENX rats. Furthermore, the animals described by Nikitin et al. did not exhibit cataract. In the study presented by Franklin et al. (2000) it was also shown that mice lacking *p18* and *p27* ($p18^{-/-}$- $p27^{-/-}$) also developed neuroendocrine tumors. The spectrum of affected tissues overlapped MEN1 and MEN2; the most frequently observed symptoms in $p18^{-/-}$ $p27^{-/-}$ animals were pituitary adenoma and carcinoma, adrenal medullary hyperplasia and pheochromocytoma, and thyroid C-cell hyperplasia. In addition to the endocrine tumors that are also found in patients affected with MEN, the $p18^{-/-}$ $p27^{-/-}$ double mutant mice also developed hyperplasia of the testis. Franklin et al. examined *p18-p21* ($p18^{-/-}$- $p21^{-/-}$) mice. They observed that in a $p18^{-/-}$ background, loss of *p21* increased the frequency of pituitary pathology, as well as the progression from hyperplasia to adenoma, however the spectra of tumors in *p18-p21* mutant mice differed from those developed by *p18-p27* mutants: symptoms observed in p18-p27 mutant animals overlap in the same cell types as seen in MEN patients (Table 20).

Phenotype	$p18^{-/-}$ $p27^{-/-}$	$p18^{-/-}$ $p21^{-/-}$	**MEN1 and MEN2**
Pituitary adenoma	+	+	+
Pituitary carcinoma	+		+
Adrenal medullary hyperplasia	+	+	+
Pheochromocytoma	+		+
Thyroid C-cell hyperplasia	+	+	+
Testis hyperplasia	+	+	
Parathyroid hyperplasia		+	+

Table 20. Comparison of the tissue involvement in double mutants *p18-p27* and *p18-p21*, and MEN. Based on Franklin et al., 2000.

The molecular process responsible for the pathological similarities between *p18-p27* mutant mice and humans affected with MEN is not clear, however one possibility is that *p18* and *p27* (*CDK1b*) interact functionally with *MENIN* and/or *RET* signal pathways. This leads to the speculation that there may be tumor suppressor genes involved in a mixed multiple endocrine syndrome, like *MENX* that interact with common points in *MENIN* and *RET* signal transduction pathways. Another aspect is the involvement in the regulation of the cell cycle by *Rb1*, *p27*, and *p21*. Neuroendocrine cells may undergo a continuous influence by feedback loops, which in absence of cell cycle control can lead to uncontrolled proliferation. A stable cell cycle arrest mediated by *Rb1* would prevent tumor development, whereas normal growth factor levels upon the loss of cell cycle control may lead to endocrine tissue hyperplasia and subsequently to tumor development. Inactivation of the *Retinoblastoma* pathway could lead to endocrine tumorigenesis. It was previously shown that the germline inactivation of the mouse *Rb* gene predisposed the animals to pituitary tumors (Hu et al., 1994).

To examine the role of *MEN1* in tumor formation, a mouse model of MEN1 was generated by Crabtree et al. (2001). The tumor incidence and pattern of tissue distribution in the MEN1 mice correlated closely with the human MEN1 phenotype. Homozygous mice died *in utero* (presumably due to complete loss of *Men1*), whereas heterozygous mice developed features very similar to those observed in man. At the age of 9 months hyperplasia and tumors of insulin-producing islet cells (insulinoma), as well as parathyroid adenomas were observed. Later on, tumors of adrenal (bilateral pheochromocytomas), and pituitary were also seen, as well as thyroid abnormalities including cysts and follicular adenoma. All of the tumors tested showed loss of the remaining wild-type *Men1* allele, consistent with its function as a tumor suppressor gene. Although the animals display a phenotype similar to MEN1, they do not develop paraganglioma or cataract, which are observed in the SD^{we} rats.

Perhaps one of the most interesting studies was carried out by Smith-Hicks et al., where a mouse model was generated to study the tumorigenesis and tumor progression in MEN2B (Smith-Hicks et al., 2000). A single amino acid

substitution of threonine for methionine was introduced in the *RET* gene (*RET*^{MET918THR}) by site-directed mutagenesis. This mutation is known to alter the pattern of *RET* autophosphorylation in man leading to MEN2B (Hansford and Mulligan, 2000). Heterozygous mutant mice displayed C-cell hyperplasia and chromaffin cell hyperplasia/ pheochromocytoma. Mutant homozygotes resembled the human syndrome in that they developed earlier C-cell and chromaffin cell hyperplasia and the symptoms were of increased severity; however, they differed from humans with MEN2B in two respects: they did not develop medullary thyroid carcinoma or ganglioneuromas specifically localized in the gastrointestinal tract and mucosa. However, they did display neuromatous enlargement of sympathetic ganglia. These ganglioneuroma-like areas caused large anatomic malformation not present in human MEN2B. As opposed to MENX-affected rats, where heterozygotes remain essentially tumor free, in the model described by Smith-Hicks et al., both homozygous and heterozygous animals develop symptoms.

In the attempt to identify the chromosomal location of the mutant *MENX* locus linkage analysis was performed on a group of 41 backcross rats (WIST/Nhg x SD^{we}) x SD^{we}. In the initial screening with a set of microsatellite markers spanning the whole rat genome a very strong linkage ($p<<0.001$) was found for chromosome 4 (LOD 14.3). The markers located on other chromosomes showed no statistical significance.

Rodent (mouse and rat) homologs of *MENIN* have been mapped by Karges et al. (1999). The region of human chromosome 11 harboring *MENIN* is homologous to rat chromosome 1 (http://www.informatics.jax.org/searches/homology_report.cgi?_Marker_key=38676). In the absence of linkage to chromosome 1 the *MENIN* gene can be excluded as a candidate for MENX. The haplotype analysis of the microsatellite markers selected for chromosome 1, among them those flanking *MENIN* (*D1Rat132* and *D1Rat90*), showed no association to *MENX*. It was therefore concluded that *MENIN* was not involved in the new variant of multiple endocrine neoplasia. Previous studies by Fritz et al. (2002) show no mutation in *MENIN* coding sequence in MENX rats.

After exclusion of *MENIN* as a candidate gene it was necessary to also consider the potential role of *RET* in the development of MENX. As previously reported by Fritz et al., (2002) no mutations in *RET* had been found in the SD^{we} animals, but since *RET* was mapped in rat to the distal part of chromosome 4 (Canzian et al., 1995), and strong linkage was found for this chromosome in our study, it was necessary to confirm the hypothesis that a gene other than *RET* was responsible for MENX.

After the initial haplotyping using only a few markers on chromosome 4, a set of additional chromosome 4 microsatellites were examined to narrow down the region of interest. The haplotype analysis enabled the definition of a critical region on chromosome 4, spanning a 22cM fragment. Radiation hybrid mapping subsequently revealed that *RET* was located outside this region (in a proximal position to the chromosomal fragment defined by markers *D4Rat201* and *D4Rat206*).

A synteny map of rat, mouse, and human was created to identify the genes located within the region of interest on rat chromosome 4. Synteny for the distal part of rat chromosome 4 was found on mouse chromosome 6 and portions of human chromosomes 3, 10p, and 12p. According to the OMIM database, no obvious candidate genes implicated in neuroendocrine syndromes, nor cataract formation has been mapped to these regions. Obviously, the information obtained from the synteny map was not complete since the mouse and rat genome were at the time not fully annotated.

Cataract is known to be associated with a number of metabolic diseases such as: galactosaemia, galactokinase deficiency, diabetes, and Lowe syndrome. Lowe syndrome (OMIM 309000) is a human genetic disorder associated with an inborn bilateral cataract. However, apart from cataract there are no other similarities with MENX: Lowe syndrome is X-linked (Mueller et al., 1991) and the affected organs are brain and kidney. A list of genes involved in the development of cataract in humans, are listed in Table 21.

Gene	Chromosomal location	Mutation
LIM2 (MP19)	19q13	Dominant
MIP (MIP26)	12q13	Dominant
GJA1	6q21	Dominant
GJA3	13q21	Dominant
GJA8	1q21	Dominant
CRYAA	21q22	Dominant and recessive
CRYAB	11q22	Dominant
CRYBA1	17q11	Dominant
CRYBA2	2q34	Not determined
CRYBA4	22q11	Not determined
CRYBB1	22q12	Dominant
CRYBB2	22q12	Dominant
CRYBB3	22q11	Dominant
CRYGA-F	2q33	Dominant
CRYGS	3q15	Dominant and recessive
PITX2	4q25	Dominant
PITX3	10q25	Dominant

Table 21. Genes contributing to the cataract development (modified from Graw, 2003).

Since all affected animals used in this project develop an inborn cataract, only genes involved in human congenital cataract could be of interest for this study. So far only four loci associated with recessive inheritance of cataract have been described. A nonsense mutation, a G-to-A substitution resulting in the formation of a stop codon in the alpha-cristallin gene (*CRYAA*) located on chromosome 21q causes autosomal recessive cataract (Pras et al., 2000), but unlike MENX no other symptoms are known to accompany the cataract formation. Also, an unknown gene causing autosomal recessive cataract in three inbred Arabian families was mapped to chromosome 3p (Pras et al., 2001). Two other loci: on chromosome 9q, described in a Swiss family by Heon et al. (2001), as well as a mutation in *LIM2* gene on human chromosome 19 (Pras et al., 2002) are associated with the formation of an autosomal recessive cataract, which is however age-related, as opposed to the inborn cataract observed in SD^{we} rats.
None of the above genes maps to regions of the human genome (chromosomes 3, 10p, and 12p), which are syntenic with the region on rat

chromosome 4 harboring the *MENX* locus. Furthermore, none of the above genes are known to be related to any neuroendocrine disorder. This leads to the conclusion that the *MENX* gene is not one of the known cataract genes. Although there are a number of neuroendocrine diseases known in man, there are no reports of any such tumor syndromes accompanied by the development of cataract. Indeed an informal review of both the German MEN1 and MEN2 patient colectives failed to find any cases of cataract at the time of presentation (Siggelkow H., Raue F., Atkinson MJ., personal communication). However, it cannot be excluded that there exists a human disorder caused by the same gene that is involved in the novel variant of multiple endocrine neoplasia observed in *SD^{we}* rats.

In case of MENX it is worth considering whether there are modifying genes involved in influencing the development of the disease. Modifiers might affect the protein interactions, or clinical factors associated with disease progression. It is possible that due to the action of a modifying gene or genes the spectrum of affected organs in human is different, or the symptoms are less severe from those observed in rat. Although no phenotypic diversity has been observed in SD^{we} rats, it is possible that the crossing of SD^{we} animals with rats of other than Wistar genetic background would reveal a variably severe phenotype, which could be tested in future experiments.

To gain insight into potential candidates, human genes located within the region of interest on rat chromosome 4 were analyzed. Among them there are three genes that may be of some importance for the development of the MENX syndrome, and therefore should be considered as candidates (Fig. 21): *similar to MAP kinase phosphatase-7*, as well as *PTPRO* code for proteins involved in signal transduction pathways, similar to *RET*, which is responsible for MEN2A and MEN2B, whereas *CDKN1B* (*p27*) was shown to be involved in the development of MEN-like tumors (Franklin et al., 2000). Although in some cases the rat homologues of genes identified in human are not easily recognized because the homology between the two species is low (in the case of rat and human MKP-7 it is 87.9%, according to the NCBI database), it can be assumed that the rat genes correspond to the given homologues in human. This assumption can be supported by the fact that the order of the genes in rat and

its syntenic equivalent in human is the same. Moreover, the distances between the corresponding genes in both species are very similar.

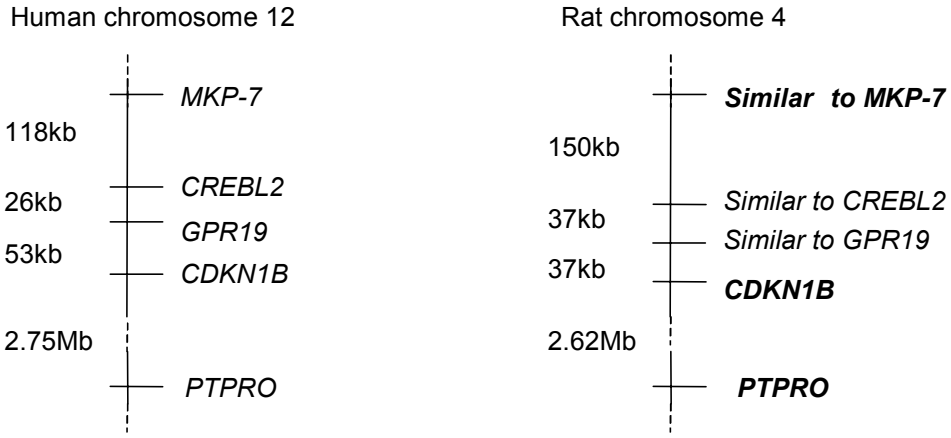


Fig. 21. Position of some of the genes localized within the region of interest in human and in the rat (candidates shown in bold). *CDKN1B*- cyclin-dependent kinase inhibitor 1B; *CREBL2*- cAMP responsive element binding protein-like 2; *MKP-7*- MAP kinase phosphatase 7; *PTPRO*- protein tyrosine phosphatase, receptor type, O. (Source: NCBI).

One of the candidate genes is the rat gene *similar to human MAP kinase phosphatase-7.* The MAP kinase phosphatases (MKPs) are a family of dual specificity phosphatases that target MAP kinases (Alessi et al., 1993). *MKP-7* has been cloned by Tanoue et al. (2001). In the study conducted by Masuda et al. (2001) it was concluded that MKP-7 behaves as a nuclear shuttle as well as a phosphatase. Masuda et al stated that since MKP-7 was specific for phosphorylated JNK, it could also function as a tumor suppressor by negatively regulating the JNK pathway. In a later report (Masuda et al., 2003) the group presented the identification of the three domains in MKP-7 required for interaction with MAP kinases: D-domains I and II (possible MAP kinase docking domains) and a long COOH terminal stretch. The interaction of MKP-7 through the D-domain II leads to the inhibition of JNK1 activation. D-domain II also plays a greater role in inhibiting p38 alpha activation when compared to the D-domain I. In contrast to the study of Tanoue et al., Masuda et al. showed that MKP-7 could bind to and inhibit the activity of ERK2, and that both D-domains are important for this process.

If, as Masuda et al. speculate, substrate specificity is integrated in the D-domain II and the long COOH terminal stretch, it is possible that a mutation would

produce a conformational change in MKP-7 that alters the enzyme's specificity towards JNK, p38, and ERK, which further leads to a disequilibrium between the three pathways. Because MKP-7 is a potential negative regulator of the MAP kinase signaling pathway, it could be involved in carcinogenesis by deregulating cell proliferation and apoptosis. It has been previously shown that the activation of JNK and p38, as well as the inhibition of ERK were critical for induction of apoptosis in rat PC-12 pheochromocytoma cells (Xia et al., 1995) (Fig. 22).

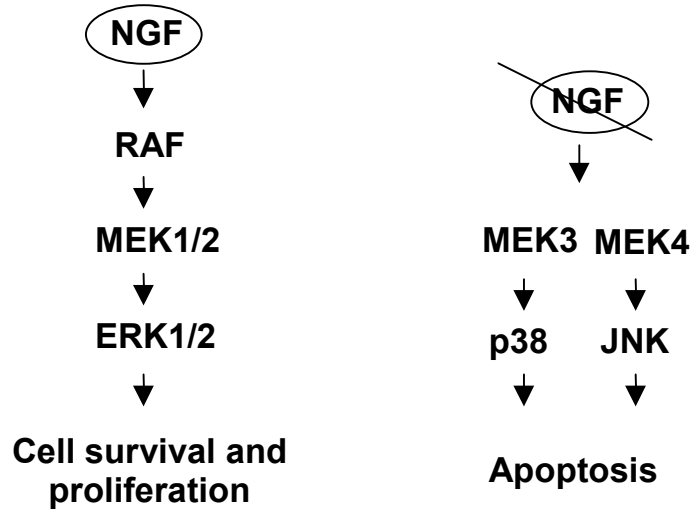


Fig. 22. Signaling pathways in PC-12 rat pheochromocytoma cells. Neuronal survival and apoptosis may be regulated by the ERK and p38/Jnk pathways depending on the presence or absence of NGF. NGF- nerve growth factor; RAF- RAF oncogene; MEK- mitogen-activated protein kinase kinase; ERK- extracellular signal-regulated kinase; p38- p38 MAP kinase; JNK- c-Jun N-terminal kinase (modified from Xia et al., 1995).

Resistance of cells to apoptosis can lead to expansion of a cell population and subsequently to accumulation of additional mutations within this population. It is known that ERK is involved in oncogenic transformation (Aguirre-Ghiso et al., 2003) and metastasis (Tanimura et al., 2003). Aguirre-Ghiso et al. showed that high ERK/p38 ratio favors tumor growth, whereas high p38/ERK ratio leads to tumor growth arrest. This suggests that the balance between the above pathways is important for determination of cell fate. Loss of MKP-7 via mutation would decrease dephosphorylation of downstream kinases leading to growth stimmulation.

The function of *MKP-7* is still unknown, there are no mouse mutants described (Mouse Genome Informatics database 3.01), and there is no evidence of *MKP-7* mutation in neuroendocrine tumors. However, it has been mapped in human to chromosome 12p12, a region that is known to be prone to deletions in several tumors (Baccichet and Sinnett, 1997; Kibel et al., 1999), which suggests that *MKP-7* might be a tumor suppressor gene. Moreover, both *RET* and *MKP-7* are involved in JNK signalling: Willoughby et al. (2003) showed that MKP-7 upon binding with JNK-interacting protein-1 (JIP-1) reduces JNK activation, whereas RET is known to activate JNK (Chiariello et al., 1998).

The second candidate gene is a cyclin dependent kinase inhibitor *CDKN1* (*p27*) identified by Toyoshima and Hunter (1994) as a negative regulator of G1 progression. Sheaff et al. (1997) showed that expression of cyclin E-CDK2 at physiologic levels of ATP results in phosphorylation of *CDKN1B*, leading to elimination of *CDKN1B* from the cell and progression of the cell cycle from G1 to S phase. At low ATP levels, the inhibitory functions of *CDKN1B* are enhanced leading to the cell proliferation arrest. *p27* was mapped in human to chromosome 12p13 (Pietenpol et al., 1995).

Low expression of *p27* occurs frequently in many types of human tumors: breast cancer (Foulkes et al., 2004), colorectal carcinomas (Noguchi et al., 2003), gliomas (Schiffer et al., 2002), or mantle cell lymphoma (Chiarle et al., 2000). Fero et al. (1996) reported on a syndrome of multiorgan hyperplasia developed in *p27*-deficient mice. *p27*-null animals developed pituitary adenomas grossly evident by 2-3 months of age. *p27* gene dosage determined animal size (*p27* –/– > *p27* +/– > *p27* +/+); the highest cell proliferation was observed in spleen, thymus, and pituitary of *p27*–/– mutants. Fero et al. concluded that continued cell proliferation in the absence of *p27* may lead do hyperplasia, and even partial decrease in *p27* levels appears to trigger cell proliferation, as could be deducted from the increased size of *p27* hemizygotes. Rivard et al. (1999) demonstrated that the activation of the MAP kinase pathway is required for *p27* down-regulation in fibroblasts and epithelial cells. Rivard et al. showed that the expression of Ras proteins in those cells was sufficient to induce a significant down-regulation of *p27* levels. Overexpression of *p27* was also shown to be associated with apoptosis in rat fibroblast cell line (Wang et al., 1997), breast

cancer cells (Katayose et al., 1997), lung cancer cell lines (Naruse et al., 2000), colorectal carcinoma (Hsu et al., 2004), and melanoma (Zhang and Rosdahl, 2005). Franklin et al. proposed a model where *p18* and *p27* mediate separate pathways to collaboratively inhibit pituitary tumorigenesis by controlling the function of Rb (Franklin et al., 1998) (Fig. 23).

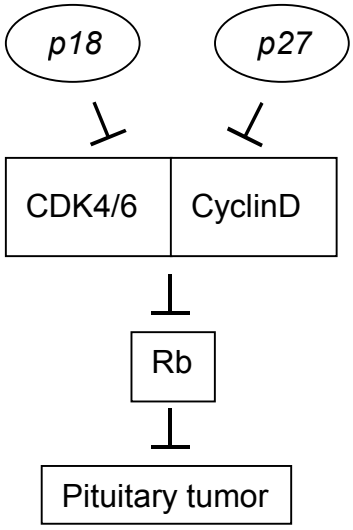


Fig. 23. Suppression of pituitary tumorigenesis by *p18* and *p27*. CDK- cyclin dependent kinase; Rb- retinoblastoma (Franklin et al., 1998).

As described above, one of the characteristics of MENX syndrome is the development of pituitary tumors. According to the model proposed by Franklin et al., it can be speculated that pituitary tumorigenesis in MENX is due to a mutation in *p27* gene. A possible mechanism could be that the conformational changes prevent *p27* from interacting with the downstream targets of the pathway.

The third candidate gene, ***Ptpro*** identified in 1994, encodes a receptor-type protein tyrosine phosphatase (Tagawa et al., 1994). Beltran et al. observed mouse *Ptpro* in neuron populations expressing TrkA nerve growth factor receptor, or TrkC, the neurotrophin-3 receptor. They suggested that mPTPRO was involved in neuron growth and differentiation and modulated intracellular responses to neurotrophin-3 (Beltran et al., 2003). Neurotrophin-3 (*NT-3*) originally represented a candidate gene for MENX because of its mapping to chromosome 4 and its function: it regulates the proliferation of cultured neural crest progenitor cells which contribute to the development of the

neuroendocrine tissue (Zhou and Rush, 1996; Chalazonitis, 2004). McGregor et al. (1999) showed that the expression of the TrkC (NT-3 receptor) increases during the progression towards medullary thyroid carcinoma, which suggests TrkC may play a critical role both in maintenance of the normal C cell phenotype and in the formation of MTC (Fig. 24). Although there is no evidence for this, it can be possible that there exists a signalling pathway common for all three candidate genes.

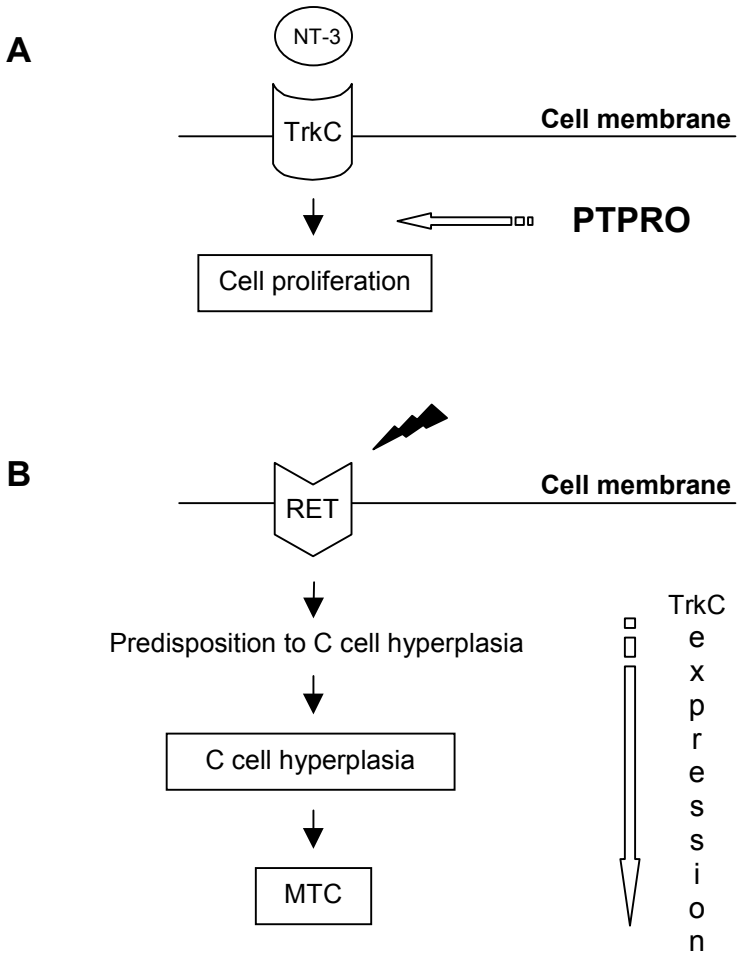


Fig. 24. Schematic representation of the involvement of the protein tyrosine phosphatase, receptor type, O (*PTPRO*) in cell proliferation. A- *PTPRO* modulates intracellular responses to neurotrophin 3 (*NT-3*), as proposed by Beltran et al. (2003). B- Formation of medullary thyroid carcinoma (MTC) after mutation of *RET*; Expression of TrkC, which is a neurotrophin 3 receptor, increases during MTC procession, suggesting TrkC's role in tumorigenesis.

Based on the haplotype analysis, *NT-3* was placed outside the critical region on rat chromosome 4, and sequence analysis revealed wild type *NT-3* sequence in both a wild type and an affected animal. However, if *Ptpro* modulates the response to neurotrophin (as Beltran et al. speculate) it can be presumed that this modulation leads to the development of the neuroendocrine tumors. Mechanisms involved in a modulation leading to tumorigenesis may include:

- malfunction of the NT-3 receptor (assuming that PTPRO interacts with the NT-3 receptor)
- disturbance of the cell signaling pathway after binding neurotrophin to its receptor (assuming that PTPRO directly or indirectly modulates the proteins involved in the signal transduction).

Although neither *MKP-7*, nor *PTPRO* are known so far to cause any neuroendocrine disorders, they can still be included as candidate MENX genes and warrant further considerations. Further efforts to identify the *MENX* gene should concentrate in the first place on sequencing cacndidates in *SD*^{we} rats and control, healthy animals to find possible mutations and to check for the expression of the candidate genes in neuroendocrine cells. It would also be useful to examine tissue-specific expression patterns of *MKP-7*, *p27* and *PTPRO* in the affected animals compared to unaffected rats. Another possibility is that the continuous updating of the information deposited in the public genetic data bases will lead to the identification of other candidates for MENX, which should also be sequenced. In case none of the above strategies prove to be successful, it will be necessary to look for additional microsatellite markers and/or SNPs, which would be used to further narrow down the critical region on rat chromosome 4. Considering that the identification of new informative microsatellite markers, able to distinguish SD and Wistar alleles, may not be an easy task, which held true in the course of this project, designing new animal crossings using the SD^{we} strain and rats of a different (non- Wistar) genetic background should also be considered.

As in rats, the human variant of MENX would be expected to be inherited in a recessive manner. In the past, the recessive character of a genetic disease was easier recognized, as family sizes were large. In modern families an affected

individual would probably remain the only one affected in the otherwise healthy family, and hence appear as a sporadic case.
Since in the rat the neuroendocrine tumors develop within the first year of life, the symptoms of a human equivalent of MENX should be observed in early adolescence. Considering that the life span of the MENX-affected rats is significantly shorter (on average 243 days compared to 519 days in healthy animals; Fritz et al., 2002), and comparing the life expectancy between rat and human, it can be assumed that the neuroendocrine tumors in humans would be developed well before the patient is in middle age. The symptoms of human MENX should be similar to those developed by affected SD^{we} rats, although the inclusion of the cataract formation is uncertain as the etiology may be secondary to endocrine changes.

After the identification of the *MENX* gene and the frequency of mutation and tumor phenotype in man, it will be possible to genetically screen the individuals at risk for *MENX* mutations. Highly accurate genetic screening using genetic markers can be done in the informative families whenever DNA is available from at least 2 family members proven to be affected. As stated in the Guidelines for Diagnosis and Therapy of MEN1 and MEN2 the *MEN1* germline mutation test for MEN1 carrier identification is recommended, and in 10-20% of cases in which those mutations fail to be detected genetic linkage analysis can identify MEN1 carriers. In MEN2 families *RET* germline mutation testing when performed rigorously, it reveals a *RET* mutation in over 95% of MEN2 cases (Brandi et al., 2001).
The use of polymorphic markers for the *MENX* gene may allow the identification of family members at high risk for developing tumors associated with the disease. Depending on the function of the *MENX* gene, it may be possible to test the affected individuals, as well as their siblings in the early stage of life for appropriate physiological deficiencies (hormone or enzyme level tests). In such case prenatal diagnosis may also be considered. After the hereditary MENX syndrome in man has been diagnosed (genetically, clinically, and biochemically) in an individual patient, systematic genetic family screening should be performed in all first degree relatives. Screening for pheochromocytoma (measurement of urinary excretion of catecholamines) and hyperparathyroidism

(serum calcium and parathyroid hormone level determination) should be considered to improve recognition and contribute to the more effective treatment as in case of MEN2 patients (Ledger et al., 1995; Brandi et al., 2001).

The purpose of this study was to identify the gene responsible for a new variant of MEN in the rat. The locus involved in the disease was mapped to an approximately 9Mb large region on rat chromosome 4. The list of candidate genes found in this region allows us to limit the potential genes considerations. The function of the candidate genes (*MKP-7* homologue, *p27*, and *Ptpro*) as signaling molecules suggests it is possible that *MENX* gene is involved in signal transduction mechanisms.

Summary

The multiple endocrine neoplasia-like syndrome (MENX) is a hereditary cancer syndrome in the rat which developes within the first year of life and which is characterized by multiple tumors affecting the neuroendocrine system. The spectrum of the affected organs overlaps the MEN1 and MEN2 syndromes in human, but, in contrast to them, the MENX is inherited as a recessive trait. Unlike the MEN variants known in man, the MENX is also characterized by an inborn bilateral cataract.

This work presents the mapping of the *MENX* locus to rat chromosome 4 by a genome-wide linkage analysis and a selection of candidate genes based on the data available in the electronic genetic resources. The mapping of the *MENX* locus was done in 41 animals obtained from a (Wistar/Nhg x SDwe) x SDwe interstrain backcross, where SDwe (Sprague-Dawley white eye) indicates the affected animals. The initial screening of the rat genome using the microsatellite markers revealed a linkage with the disease phenotype to the chromosome 4. No linkage was found on the rat chromosome 1 where the rat homologue of the *MEN1* is located – the gene known to be responsible for the multiple endocrine neoplasia type 1 in man. The MENX disease locus was initially mapped to an approximately 22cM large interval including *RET* gene known to be involved in the development of the human MEN2A and MEN2B syndromes. In order to find out about the potential involvement of *RET* in MENX, several microsatellite markers located in the proximity of *RET* were analyzed for a linkage to the disease phenotype. The result of this analysis excluded *RET* from the critical region on the rat chromosome 4. The additional informative microsatellites were used to restrict the region of interest leading ultimately to the mapping of the *MENX* gene to an approximately 9cM interval. The data presented in this work shows that the gene involved in the MENX and possibly in other endocrine tumors is located within the distal part of the rat chromosome 4. Analysis of the human syntenic regions did not identify genes involved in the structure or function of the neuroendocrine organs, neither did it identify genes playing role in the development or the pathology of the eye.

Generation of the additional recombinant animals showing crossing-over within the identified critical region should enable a more precise mapping of the gene

responsible for the MENX and a systematic mutation analysis of all candidate genes. The additional functional studies such as the expression of the candidate genes in the affected tissues or the loss-of-heterozigosity analysis in the tumors, as well as the use of online genetic tools and resources should make the identification of the *MENX* gene possible and further on contribute to our understanding of the genetic mechanisms of the neuroendocrine tissue tumorigenesis.

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