universität wien

DIPLOMARBEIT

Evaluation of MEN1 risk in individuals bearing the R171Q polymorphism

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Abstract

Menin, a 610-amino-acid tumor suppressor protein, is ubiquitously expressed and predominantly found in the cell nucleus. It has been identified in connection with the familial multiple endocrine neoplasia syndrome type I (MEN1). Mutations and alterations of this gene located at chromosome 11 have been the focus of investigations in individuals developing tumors in the parathyroid, the pituitary gland and enteropancreatic endocrine tissues. Approximately 500 different germline mutations that cause loss of the tumor suppressor function have been identified. Tumor formation occurs after loss of the remaining wild- type allele. Not only mutations but also several nucleotide polymorphisms have been found. One of them represents the non-synonymous nucleotide exchange of G to A in codon 171 in exon 3. This exchange leads to integration of the amino acid glutamine instead of arginine.

Based on their suspected diagnosis 98 MEN1- and 128 MEN2-patients were analysed in comparison to a control population (141 CAH and 33 IDDM patients) with respect to the R171Q variation in the *MEN1* gene. In addition, 100 different endocrine tumors derived from potential MEN1 or MEN2 target organ sites were studied.

400 individuals were tested at the germline level. 171Q was found in 11 individuals in heterozygous form and was not linked to a specific disease. Loss of heterozygosity was identified in 2 tumors and - most important - both alleles were alternatively undergoing LOH.

The R171Q polymorphism is found in about 3% of the tested individuals in patients with suspected MEN1 or MEN2 as well as in individuals of our control population. This percentage is not increased in tumors.

Previous literature indicates a possible role of the R171Q polymorphism in the development of a MEN1 syndrome. The results present in this diploma thesis underline that the R171Q alteration rather represents a polymorphism with lower relevance than a MEN1-causing mutation. But since some of the subjects carrying the R171Q polymorphism might be currently too young to develop a MEN1 related tumor, a final conclusion cannot be drawn yet. Clinical follow up of those carriers could reveal further knowledge in this respect and is suggested.

Zusammenfassung

Menin ist ein 65 kDa großes Tumorsuppressor- Protein, das ubiquitär exprimiert wird und hauptsächlich im Zellkern lokalisiert ist. Mutationen und andere Veränderungen dieses Gens sind mit dem familiären multiplen endokrinen Neoplasie Syndrom Typ 1 (MEN1) assoziiert. Personen mit diesem Syndrom entwickeln hauptsächlich Tumore der Nebenschilddrüse, Hypophyse und in enteropankreatischen endokrinen Geweben. Zurzeit sind bereits etwa 500 verschiedene Mutationen bekannt die alle zum Verlust der Tumorsuppressorfunktion von Menin führen. Nach Verlust des gesunden Allels entwickeln sich Tumore in mehreren Organen des Patienten. Aber nicht nur Mutationen, sondern auch viele Polymorphismen konnten bis jetzt entdeckt werden. Eine dieser Veränderungen ist ein Nukleotidaustausch von G zu A im Codon 171 im Exon 3 des *MEN1*- Gens. Dieser Austausch führt zum Einbau der Aminosäure Glutamin anstelle von Arginin.

Basierend auf ihrer klinischen Diagnose wurden 98 MEN1- und 128 MEN2- Patienten auf den R171Q- Polymorphismus untersucht. Als Kontrollgruppe wurden 141 CAH- und 33 IDDM- Patienten herangezogen. Zusätzlich zu den genomischen Proben wurde auch Material von 100 endokrinen Tumoren analysiert.

Insgesamt wurden 400 Personen untersucht. 171Q wurde 11-mal in heterozygoter Form detektiert (R171Q). „Loss of heterozygosity“ wurde in 2 von 100 Tumorproben identifiziert. In einem neuroendokrinen Pankreastumor wurde das 171Q- Allel verloren, wohingegen in einem Phäochromozytom das 171R- Allel fast nicht mehr detektierbar war.

Der R171Q Polymorphismus wurde in ca. 3% der getesteten Personen detektiert und zwar sowohl in der MEN- als auch in der Kontrollpopulation. Dieser Prozentsatz ist in Tumorproben nicht erhöht. Bisherige Publikationen lassen einen Zusammenhang zwischen dem R171Q Polymorphismus und der Entwicklung eines MEN1- Syndroms vermuten. Die Ergebnisse der vorliegenden Diplomarbeit indizieren allerdings, dass R171Q eher einen Polymorphismus mit sehr niedriger Relevanz, als eine MEN1-auslösende Mutation darstellt. Da aber einige der untersuchten Patienten, die diese genetische Veränderung tragen, vielleicht noch zu jung sind um MEN1- assoziierte Symptome aufzuweisen, kann noch keine endgültige Aussage über die Tragweite dieses Polymorphismus getroffen werden.

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1. Introduction

1.1 Tumor and cancer development

Like all cells of the human body, cancer cells arise from mitotic cell divisions and acquire intrinsic genomic alterations during this specification process. There are several different ways how cells can develop genomic changes, summarized as somatic mutations. A wide range of exogenous sources like radiation, chemicals, tobacco smoke, infections or dietary habits causes these mutations (1,4,6,7,8,9). They initiate or promote cancer development, which is a process of many years (2,3,).

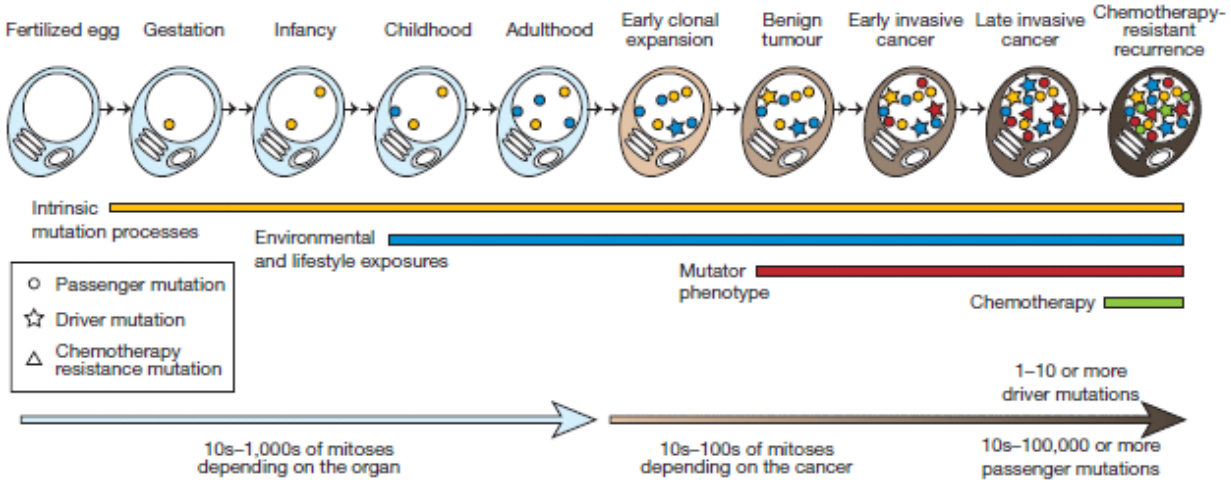


Figure 1 The process of tumor formation and cancer development involves different changes in the genetic code of the cell (Stratton M, Campbell P, Futreal PA, Nature Reviews 2009). Intrinsic mutations are acquired during normal cell division and environmental influences. "Passenger" mutations do not show any affects on cancer progression while "driver" mutations positively contribute to the survival of cancer cells.

There are certain differences between benign and malignant tumors. While benign tumors do not invade neighbouring tissue or become metastatic, malignant cells can spread to any part of the human body to develop further neoplasms. Benign tumors normally only affect physical healths when they become too large or secrete specific hormones like some neuroendocrine tumors do. In some cases, if a benign tumor undergoes further mutational events it can also evolve into cancer (2,5).

1.2 Oncogenes and tumor suppressor genes

It is believed that the development of tumors is a multistep process that involves different alterations in proto- oncogenes and tumor suppressor genes. These alterations are usually acquired mutations, although inherited germ-line mutations can predispose a person to some kinds of cancer.

Proto- oncogenes are normal genes that are involved in cell growth and differentiation encoding proteins for the control of apoptosis, trancsription factors, growth factors, growth factor receptors, chromatin remodelers, signal transducers or nuclear transcription factors. Upon alterations, proto- oncogenes promote tumor formation and cancer development ("gain-of-function") and are then termed oncogenes (10,11,13).

Tumor suppressor genes prevent the developmental process from a normal cell to cancer. Like proto- oncogenes, tumor suppressor genes regulate multiple cellular activities, including cell cycle control, DNA repair, protein degradation, differentiation and proliferation. Mutations affecting functions of tumor suppressor genes or proto-oncogenes deregulate particular signaling pathways ("loss-of-function") and can contribute to tumor formation or cancer development (12,13).

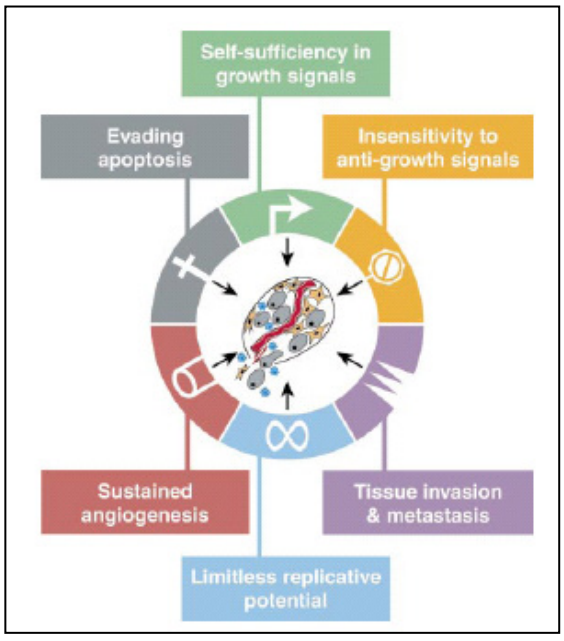


Figure 2 Acquired capabilities of cancer (Hanahan D, Weinberg RA, Cell 2000) Self-sufficiency in growth signals, insensitivity to anti-growth signals, evasion of programmed cell death, limitless replicative potential, sustained angiogenesis and tissue invasion and metastasis contribute to the transformation from a normal cell to malignant neoplasms.

The tumor suppressor activity can still be functional with only one wild type copy of the gene. According to the "two-hit" model, inherited germline mutations and a second, somatic, mutation are required for inactivation of the tumor suppressor gene and tumor formation (15,16,17,18). Inheritance of a single mutant allele accelerates tumor development and makes tumor formation more likely because only one additional mutation is required for loss of the suppressor function. Non-hereditary tumors and cancer require also two hits, but both are somatic. Often the same genes are inactivated in sporadic and inherited cancer (12).

1.3 Endocrine tumors

Hormones are secreted by several endocrine glands in different parts of the body. Because endocrine tumors arise from hormone- secreting structures they frequently also produce hormones and can lead to serious health problems. Non- functioning endocrine tumors do not secrete any hormones, but can become a problem if they push against other structures. Endocrine tumors can be benign or malignant and there are several distinct types of neoplasms.

The neuroendocrine system is the combination of the endocrine system and the nervous system. Many tumors arise from neuroendocrine cells. The vast majority are carcinoids and pancreatic endocrine tumors (19).

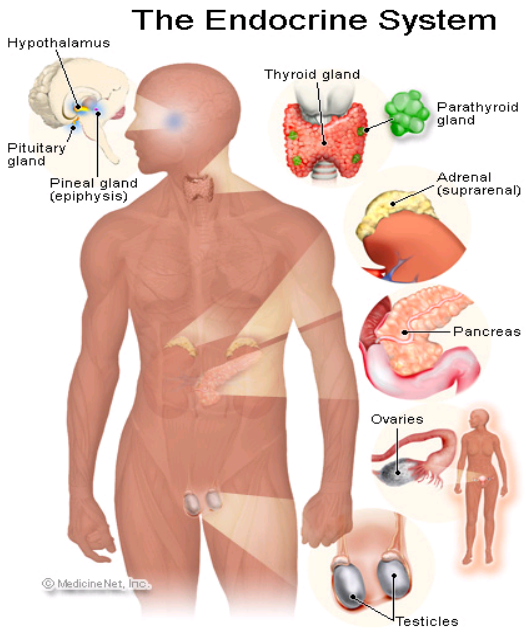


Figure 3 The endocrine system of the human body (©MedicineNet) Several glands produce specific hormones and thereby regulate important physiological functions.

1.3.1 Adrenal gland tumors

The adrenal glands are endocrine structures on top of both kidneys. They consist of two functional parts, cortex and medulla. The medulla produces adrenaline and noradrenaline, the cortex aldosterone, cortisol and dehydroepiandrosterone (20).
Adrenocortical adenomas are benign neoplasms and normally do not secrete hormones or cause any symptoms. They are frequently discovered accidentally and often no further treatment is required. About 15% are functioning and lead to endocrine disorders like Cushing´s syndrome, Conn´s syndrome virilization or feminization. They can be surgically removed (21,27,28). Adrenocortical carcinomas are malignant, but very rare neoplasms that can be functioning or non-functioning (22).
Most cases of the phaeochromocytoma arise from chromaffine cells of the adrenal medulla and secrete large amounts of catecholamines. About 10% of this type of tumors are malignant (23) and up to 25% seem to be related to different inherited genetic predisposition syndromes. Phaeochromocytomas secrete mainly catecholamines (adrenaline and noradrenaline) and their metabolites. Symptoms of phaeochromocytomas are elevated heart rate and blood pressure, panic attacks, weight loss, headaches, excessive sweating, paleness and elevated blood glucose levels (24,25).

1.3.2 Parathyroid gland tumors

Parathyroid glands are small structures behind the thyroid gland and produce the calcium and phosphorus regulating parathormone. There are usually 4 parathyroid glands found in humans. The parathyroid glands produce and secrete the calcium level regulating parathormone (29). Parathyroid tumors can be benign or malignant and often cause elevated levels of blood calcium (hypercalcemia). Malignant forms of parathyroid tumors are very rare but benign neoplasms can also cause severe health problems. Symptoms are abdominal pain, depression, vomiting, excessive urination and muscle weakness. The tumor can be surgically removed or the elevated level of parathormone is treated with antagonists such as calcitonin (30,31).

1.3.3. Pituitary gland tumors

The pituitary gland, also called hypophysis, is connected to the hypothalamus and located at the base of the brain. It is composed of two functionally different parts, the anterior pituitary (adenohypophysis) and the posterior pituitary (neurohypophysis) (32). The adenohypophysis synthesizes and secretes a panel of important hormones including ACTH, FSH, LH, THS, prolactin, endorphins and the growth hormone. The neurohypophysis is directly connected to the hypothalamus and stores and releases oxytocin and ADH (33).
Tumors of the pituitary gland are frequently benign (adenomas). Pituitary carcinomas are very rare but can spread into other areas of the brain or spinal cord and even outside of the central nervous system. Pituitary tumors can also be functioning or non-functioning, but most neoplasms overproduce some type of pituitary hormones. It is also possible that the tumor destroys parts of the pituitary gland resulting in decreased hormone levels or push against structures of the visual system. The consequences of a pituitary tumor correlate with the hormone that is overexpressed or decreased and no general symptoms can be specified (35,36). Overxpression of ACTH causes the Cushing´s syndrome. Tumors of the pituitary gland can be initially treated with medication or surgically removed.

1.3.4 Endocrine pancreatic tumors

The islets of Langerhans are the hormone producing parts of the pancreas and are made up of five different types of cells that are all secreting different hormones: Alpha cells (glucagon), beta cells (insulin), delta cells (somatostatin), PP cells (pancreatic polypeptide) and epsilon cells (ghrelin). The main part of the islets consists of insulin producing beta cells (37,38,39).
Endocrine pancreatic cancer is rare, 95% of malignant pancreatic tumors arise from the exocrine part of the pancreas that produces digestive fluids. Over 70% of pancreatic tumors are functioning.
Insulinomas are mostly benign, insulin secreting tumors of the beta cells. After eating, insulin is physiologically secreted to regulate glucose uptake into cells and influence metabolism. Tumor secreted insulin is not proper regulated and the blood glucose can decrease to a life threatening low level (hypoglycaemia) (48). Symptoms include headache, double visions, lethargy, seizures or coma. Because Insulinomas can be very small and may not be detected

by radiological examination, measuring the serum glucose and insulin levels over 48 or 72 hours during fasting is critical for detection of the tumor (40,41,42).

A gastrinoma is characterized by hypersecretion of the hormone gastrin and is frequently the cause of Zollinger- Ellison syndrome. Gastrin stimulates the secretion of gastric acid and overproduction often leads to multiple gastrointestinal ulcers (43). Around 90% of the gastrinomas are located in the pancreas or duodenum and over 60% are malignant (44). Detection occurs via measuring the serum gastrin level or secretin provocative test (39).

VIPomas arise from vasoactive intestinal peptide (VIP) producing cells of the pancreas and cause massive watery diarrhea. VIP stimulates secretion of water and electrolytes and inhibits gastric acid production. Beside diarrhea, hypokalemia and dehydration occur (39). The symptoms are treated with fluid and electrolyte replacement and controlling diarrhea. The tumor can be benign or malignant, surgically removement is curative in 50% of patients (45,46,47).

Glucagonomas result in hypersecretion of glucagon and cause the opposite effect of insulinomas, a hyperglycaemia. Glucagon stimulates gluconeogenesis (generation of glucose from non-carbohydrate carbon sources), glycogenolysis (cleavage of glycogen and release of glucose) and lipolysis (breakdown of fat) to elevate the blood glucose level. A vast amount of glucagonomas is malignant. Symptoms are necrolytic migratory erythema, weight loss, diarrhea, anemia, decreased blood amino acid level, insulin resistance and also development of diabetes mellitus (39,49,50)

Somatostatinomas are frequently malignant neoplasms of the pancreas or duodenum. Elevated levels of somatostatin often leads to diabetes mellitus, steathorrhea and gall stones. Because somatostatinomas frequently consist of many different endocrine cells, other hormone levels can be elevated as well, which makes diagnosis very difficult. Somatostatin inhibits the release of GHRH, TSH, gastrin, CCK, VIP, GIP, secretin, insulin and glucagon (39,51).

1.3.5 Carcinoid tumors

Carcinoid tumors are malignant but slow growing tumors that arise from neuroendocrine tissues. Only about 10% of carcinoids secrete hormones (54). Carcinoid tumors are found in the small intestine, rectum, appendix, respiratory tract, colon, pancreas, stomach, gonads and liver (52). The carcinoid syndrome can be the consequence of metastasis leading to elevated level of specific hormones (e.g. serotonin), diarrhea, vomiting, abdominal pain and skin

flushing. The tumor-secreted hormones can be metabolized in the liver, and no symptoms are noticeable. During metastasis, the liver can be damaged and not be able to remove the hormones anymore, leading to elevated levels of specific substances. This can cause diverse symptoms (53).

1.4 Multiple endocrine neoplasia syndromes

The multiple endocrine neoplasia (MEN) syndromes were first characterized in the early 20th century (55) and named by Steiner *et al.* in 1968 (56). They are classified into MEN1, MEN2A and MEN2B based on different genetic backgrounds and affected organs. The MEN syndromes share certain characteristics and conform to specific criteria. The syndromes are autosomal dominantly inherited. Tumor formation occurs in at least two different endocrine tissues and the tumor is made of specific polypeptide- and biogenic amine-producing cell types (APUD). In some cases also non- endocrine tumors can arise, like lipomas or colonic polyps. Progression from hyperplasia to adenoma and sometimes even to carcinoma is commonly found (57,58). Although MEN syndromes are frequently inherited, sporadic mutations in the affected genes can also promote tumor formation.

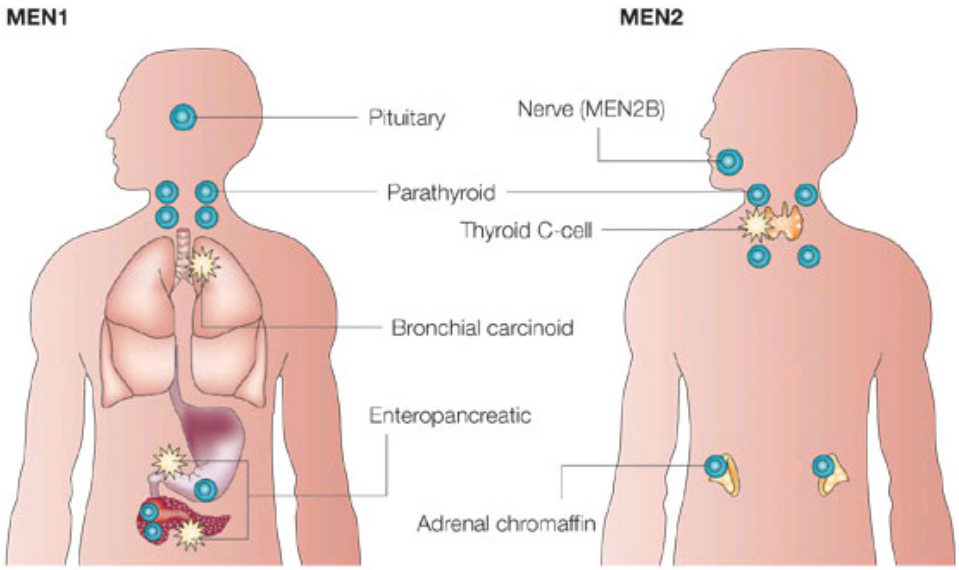


Figure 4 Most common endocrine tumors expressed in multiple endocrine neoplasia types 1 and 2 (Marx SJ, Nature Reviews 2005) Benign tumors are shown in blue, tumors with malignant potential in yellow.

1.4.1 Multiple endocrine neoplasia 2A syndrome

In 1959, John Sipple first reported the MEN2A syndrome and characterized some of its symptoms. The clinical syndrome includes multicentric medullary thyroid carcinomas (MTC), thyroid C cell hyperplasia and phaeochromocytomas as well as parathyroid hyperplasia or adenomas. Phaeochromocytomas are identified in about 50 % of the cases and parathyroid neoplasms occur in 10 to 20% (58). Variations in the *RET* proto-oncogene promote the development of this type of MEN syndromes. Mutations lead to a gain-of-function variant of the Ret protein, which then functions as an oncogene (57). The normal proto-oncogene is involved in TGF-beta signaling pathways at different sites of the body.

Screening for MTC can be performed by measuring the serum calcitonin level after pentagastrin application. Calcitonin is secreted by C cells of the thyroid gland and can be stimulated with pentagastrin. Patients with manifest MTC have a highly elevated plasma calcitonin level (59,60). Screening should begin very early in life because C cell hyperplasia in MEN2A has been reported at the age of 20 months, MTC at the age of 3 (61) and metastasis at the age of 6 (62). Another screening method for hereditary MTC is sequencing exons 10- 16 of the *RET* gene to look for mutations.

1.4.2 Multiple endocrine neoplasia 2B syndrome

Medullary thyroid carcinomas and phaeochromocytomas are associated with both, MEN 2A and MEN2B. In MEN2B, normal parathyroid glands but multiple mucosal neuromas, a nonendocrine manifestation, can be found. MEN2B manifests during the first decade of life (58), starting with benign neoplasms in mouth, eyes and the submucosa of different organs. In early adulthood, tumors of the adrenal and thyroid glands followed by MTC and phaeochromocytomas occur. Metastatic carcinomas of the thyroid can even be found in little children (63,64). In MEN2B, mutations in the *RET* proto-oncogene are frequently found too. A mutated *RET* gene indicates a 90% probability to develop this syndrome. The most common point mutation causing MEN2B is the replacement of methionine to threonine at position 918. Different mutations in the *RET* gene correlate with diverse severities of MEN2 syndromes (57). The most important clinical aspect of MEN2 is that, in contrast to MEN1, because of good screening methods, early diagnosis and surgery of cancerous progenitors can prevent cancer formation and early stages of cancer can be cured very well (57).

1.4.3 Multiple endocrine neoplasia 1 syndrome

Wermer first reported different manifestations of MEN1 as homogenous syndrome in 1954 (65). Clinical characteristics of the MEN1 syndrome are tumors in the parathyroid, anterior pituitary and enteropancreatic endocrine tissues. The first symptoms appear in the third or fourth decade of life (58). The estimated penetrances of the different MEN1 tumors are: Parathyroid tumor- 95%, enteropancreatic tumor- 70%, pituitary tumor- 50%, lipoma, angiofibroma, collagenome- 30 to 70%, thymic and bronchial carcinoid- 20%, adrenocortical tumor- 20% (66). The prevalence of MEN1 is estimated as 1 in 30 000 to 1 in 50 000.

The *MEN1* gene is located to chromosome 11q13 and consists of 10 exons (67). The ubiquitously expressed protein menin is a 610-amino-acid tumor suppressor protein and is predominantly located to the cell nucleus (68). More than 400 mutations are already known. The consequence of a mutation in the *MEN1* gene is a truncated protein that is not able to act as tumor suppressor anymore ("loss-of-function") (69). MEN1 patients normally inherit one deficient allele and tumor formation begins after loss of the remaining wild type allele ("two-hit" model) (18). Sequencing of the *MEN1*gene and regular screening of affected organs can detect early stages of tumor formation. No clear genotype phenotype correlation could be determined so far (57,75).

a) *RET* b) *MEN1*

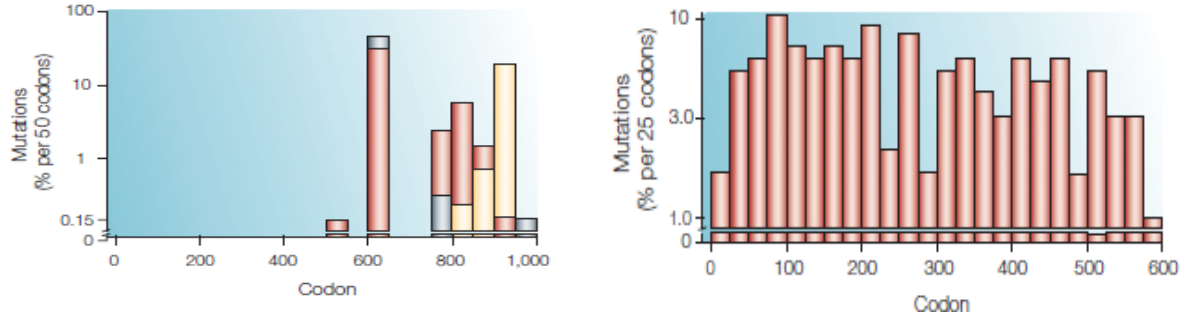


Figure 5 Logarithmic distribution of mutations in the *RET* and *MEN1* gene (Marx SJ, Nature Reviews 2005) a) In the RET gene only 15 out of 1114 codons are affected (1.3%). MEN2B is shown in yellow, MEN2A in blue and familial MTC (another variant of MEN2) in red. b) In the MEN1 gene 304 out of 610 codons (56%) can carry mutations and no mutational hot spot can be determined.

1.4.3.1 The MEN1 tumor suppressor gene

The tumor suppressor gene was first identified in 1997 (67) as the gene causing multiple endocrine neoplasia type1. Loss of the MEN1 gene function correlates with the development of several endocrine tumors. The inactivation follows the "two-hit" model, after germline mutation in one allele; a second, somatic mutation causes loss of the remaining wild type allele ("loss of heterozygosity") (70), which then frequently leads to tumor formation (18).
The MEN1 gene maps to chromosome 11q13 (67,70) and comprises 10 exons. Exons two to ten encode the 610-amino-acid protein menin. It does not show any sequence similarities to any other known protein (67). Through the identification of several interaction proteins of known functions, some kind of inference about its molecular function can be made but it is not yet clear (69). Because of the loss of the wild type allele in endocrine tumors, menin is believed to be a tumor suppressor protein. It is ubiquitously expressed even though its expression levels vary between different developmental stages and specialized tissues (71,72). Menin is primarly a nuclear protein and is able to bind DNA via its nuclear localization signals (NLS) on its C- terminus. Three NLSs are known so far, termed NLS1, NLS2 (68) and NLSa (73). Mutations in NLS1 and NLS2 lead to reduced nuclear localization. Nuclear localization signals are also important in controlling target gene transcription (74).

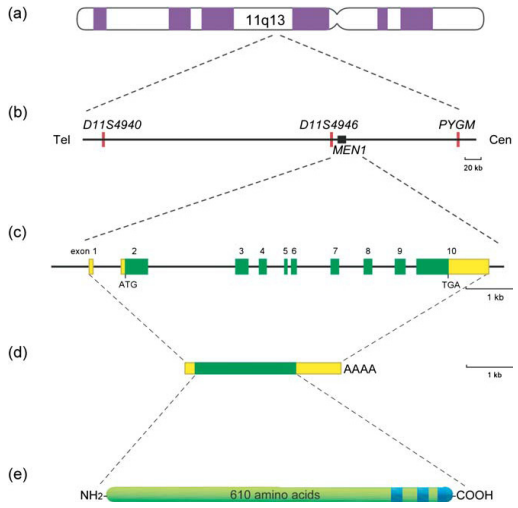


Figure 6 Localization and organization of the human MEN1 gene (Tsukada T, Nagamura Y, Ohkura N, Cancer Science 2008) a) Chromosome 11. b) The approximately 300kb genomic region containing the MEN1 gene. c) MEN1 gene showing coding (green) and non-coding regions (yellow). d) Menin mRNA with translated (green) and untranslated (yellow) regions. e) Menin protein, nuclear localization signals in blue.

1.4.3.2 Interaction partners and possible functions of menin

Although little is known about the function of menin, during the last decade a lot of different interaction partners could be identified. Menin seems to be involved in many different cellular pathways regulating DNA repair, cell cycle control or chromatin remodeling.

In mice, the *Men1* gene maps to chromosome 19 and shows 97% identity to the human menin protein. It is also ubiquitously expressed in all tissues and developmental stages (71,76). In 2000, a MEN1 mouse model was generated via homologous recombination. Mice that are homozygous null for *Men1* die at embryonic day 11.5 to 13.5, but heterozygous *Men1* mice develop multiple endocrine tumors and symptoms very similar to patients with MEN1. With an early age, the mice developed hyperplastic lesions in the pancreas and at around 12- 13 months of age tumors in the pancreas, parathyroid, pituitary and adrenal glands. Multiple endocrine tumors could be observed in almost every mouse older than 13 months (78). Loss of the complete or parts of the Men1 wild type allele could be found in all tumors that were analyzed. The full range of different stages in tumor progression could be observed, starting with hyperplasia and development to adenoma and carcinoma including metastases (78).

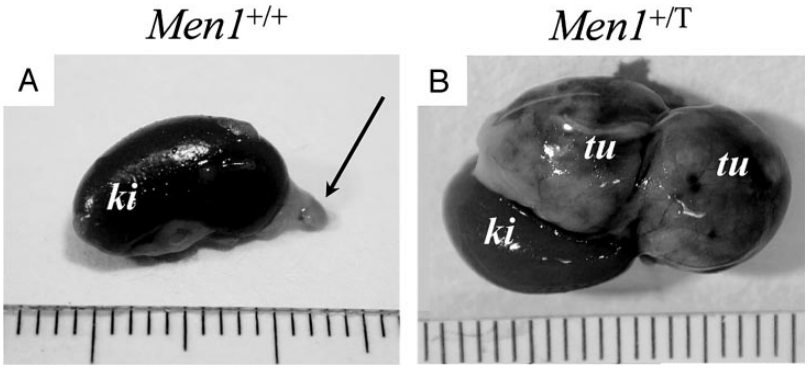


Figure 7 Example of adrenal gland tumors developed in a heterozygous mouse (Bertolino P *et al*., Molecular Endocrinology 2003) a) Normal adrenal gland from a wild type mouse, marked with an arrow. b) Tumors of the adrenal gland in heterozygous $Men^{+/T}$. *ki,* kidney

In 2003, conditional knock out of *Men1* in mice beta cells of the pancreas could be established. After homozygous deletion of exons 3 to 8 in the beta cells, more than 80% of the subjects developed pancreatic islet adenomas, which then led to increased serum insulin levels and hypoglycemia. Because it took up to 60 weeks after deletion of *Men1* to develop these tumors, it is believed that further somatic events are required for tumorigenesis(79).

Menin is expressed ubiquitously in the human body but loss of function correlates with tumor formation mainly in endocrine tissues. This tissue specificity is not restricted to *Men1*, in fact,

the loss of many other important, ubiquitously expressed tumor suppressor proteins leads to tumor formation in only a certain range of tissues. Conditional knock out of *Men1* in liver cells was performed to elucidate tumor formation in tissues normally not affected in multiple endocrine neoplasia type 1. Two out of 14 homozygous *Men1*$^{-/-}$ mice developed hepatocellular adenomas and one developed a small liver tumor. Because these tumors developed in a very late stage of life and moreover, a low occurrence of liver tumors have been reported in this mouse strain before, the authors assumed that this tumorigenesis is not connected to the deletion of *Men1* in liver cells (80).

Interestingly, around 50% of these homozygous mice developed multiple pancreatic islet cell tumors after 65 weeks and the majority of these tumors produced insulin. After PCR- testing, it was shown that not only in liver but also in islet cells *Men1* was, because of a "leaky" knock out system, slightly deleted and therefore contributed to the formation of these tumors (80). Several hypotheses have been made to explain tissue specificity of tumor suppressor genes (80,81,82) but no clear explanation could be found so far.

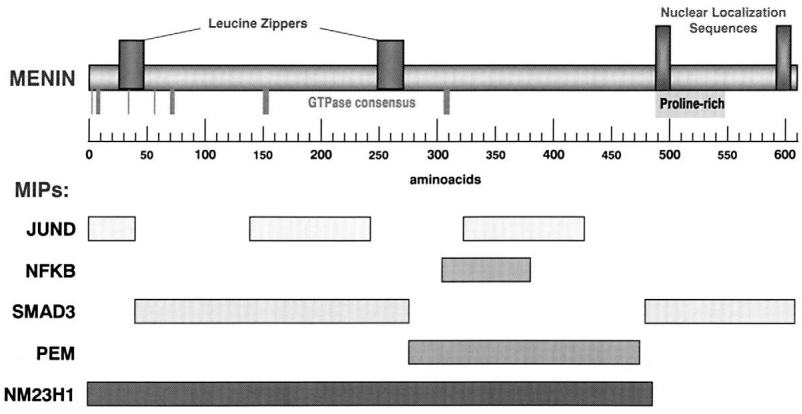


Figure 8 Interaction of menin and its partners at specific binding sites of the suppressor protein (Poisson A *et al.*, Cancer Letters 2003) Schematic representation of the structure of menin and its role in binding to certain interaction partners.

1.4.3.3 Menin and JunD

JunD belongs to the AP-1 transcription factor complex family and can act as growth suppressor or growth activator, depending on its interaction with menin (85). Different factors of the AP-1 family have distinct effects on cell proliferation, apoptosis und tumorigenesis. Via a yeast two-hybrid screen, the transcription factor JunD could be identified as one of the first

interaction partners of menin. N- and C- terminal deletions of menin revealed important binding sites of JunD at the N- terminus. Other members of the AP-1 complex family did not show any interaction with menin (86).

It was shown, that JunD acts as a growth suppressor when it is bound to menin. Menin inhibits the trancriptional activity of JunD by inhibiting MAPK-dependent phosphorylation (87) of JunD as well as through recruitment of histone deacetylase complexes (88). Luciferase assays with wild type or truncated menin revealed transcriptional repression of the luciferase activity when menin was able to bind JunD. Truncated menin lost its ability to attach to JunD and luciferase activity was highly increased (86). Addition of histone deacetylase inhibitor trichostatin A (TSA) almost completely abolished menin´s repression of JunD and luciferase activity was several folds higher with than without TSA (88).

1.4.3.4 Menin and NF- KappaB proteins

The mammalian nuclear factor-kappa B family consists of five members: NF-κB1 and NF-κB2 (NF-κB subfamily) as well as RelA, RelB and c-Rel (Rel subfamily). These proteins are involved in stress response, inflammation and cell proliferation and survival (89,90,91,92). Deregulation is linked to cancer development, immune deficiencies or autoimmune diseases.

When NF-κB1 and NF-κB2 form dimers with members of the Rel family, they become transcriptional activators. Homo- and heterodimers can be formed, which then bind different sites of DNA. NF-κB transcription factors are well regulated by inhibitory κB proteins (IκBs) (89).

The NF- κB proteins, NF- κB1 (p105/p50), NF- κB2 (p100/p52) and RelA (p65) directly interact with menin in vivo and in vitro. P65 homodimer and p50/p65 or p52/65 heterodimers are strong transcriptional activators on NF- κB binding sites. P50 and p52 homodimers are transcriptional repressors (90). Menin inhibits p65-mediated transactivation in a very significant and specific way. It could be, that repression of NF- κB pathways contributes to the tumor suppressor function of menin.

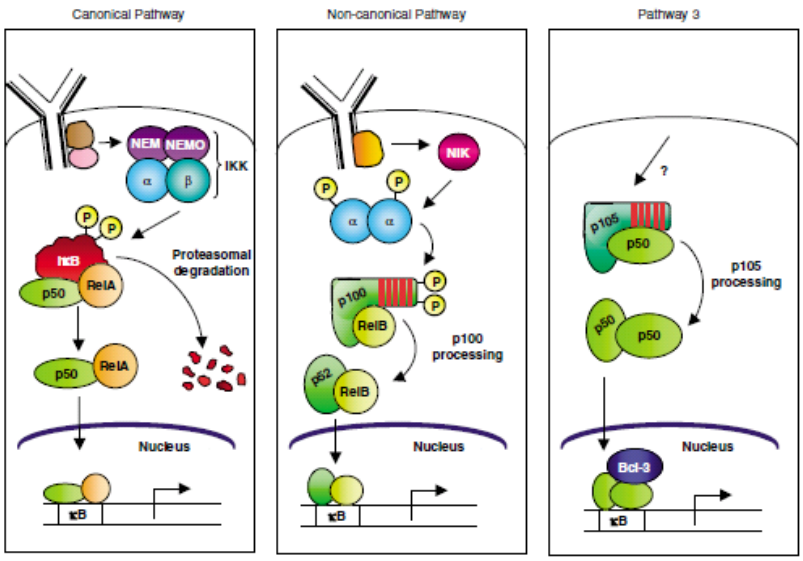


Figure 9 NF- κB signal transduction pathways (Gilmore TD, Oncogene 2006) In the classical pathway, NF-κB dimers are complexed with inhibitors until the binding of a ligand to its cell surface receptor activates an IKK (kinase) complex, which then phosphorylates the inhibitor. This leads to degradation of the inhibitor and translocation of the dimer into the cell nucleus, where several genes can be activated. The alternative pathway is for NF-κB2/RelB dimers involved in B- and T-cell organ development. Only certain receptor signals can activate this pathway. Activation of the NF-κB inducing kinase NIK leads to phosphorylation of IKK, which in turn phosphorylates NF-κB2 (p100), leading to partial proteolysis and release of p52/RelB dimer. Pathway 3 is one of many other additional pathways existing.

It could be shown, that in low menin- expressing cells the cyclin D1 is overexpressed. Activation of cyclin D1 is regulated through several factors including the NF- κB pathway. Overexpression of cyclin D1 is found in many different types of tumors (93). Theillaumas *et al.* (94) demonstrated that inhibition of the NF-κB pathway lead to normal expression of cyclin D1 in those low menin-expressing cells.

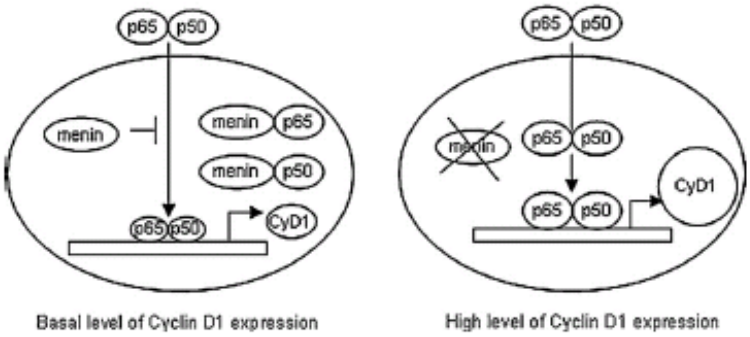


Figure 10 Cyclin D1 expression in normal and low menin-expressing cells (Theillaumas et al., Molecular and Cellular Endocrinology 2008) Normally, menin regulates cyclin D1 expression by binding to NF-κB proteins. An activated NF- κB pathway leads to elevated levels of cyclin D1, which then stimulates proliferation.

Inhibition of the binding activity of NF-κB to its DNA binding sites normalized cell proliferation. The authors suggested that overexpression of cyclin D1 in low menin-expressing cells is a consequence of NF- κB dependent activation of cyclin D1 transcription because NF-κB pathway is activated in low menin-expressing cells.

1.4.3.5 Menin and the TGF- beta family

Transforming growth factor- beta facilitates apoptosis and controls G1 cell cycle progression. It is therefore a negative regulator of cell proliferation and inactivation of the TGF-beta pathway can result in uncontrolled growth and tumor formation (95,127). Inactivation of menin neutralizes the inhibitory function of TGF-beta. Menin can interact with the TGF-beta signaling pathway protein Smad3 (97). Through binding of TGF-beta to its receptor, Smad 2 and Smad 3 are phosphorylated and associate with Smad 4. The Smad3/4 complex then activates specific target genes (96). Inactivation of menin abolishes the DNA- binding activity of the Smad complex (97). Sowa H. *et al.* demonstrated that TGF-beta negatively regulates cell proliferation of the parathyroid and PTH production. This regulation is lost when menin is inhibited (98).

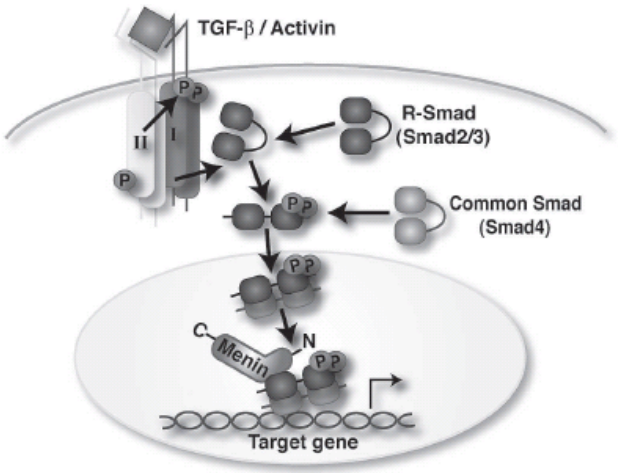


Figure 11 Menin and the TGF-beta signaling pathway (Hendy GN *et al*., Hormone and Metabolic Research 2005) Menin binds to Smad3 and facilitates target gene transcription.

1.4.3.6 Menin and nm23H1

In 2001, Ohkura *et al.* identified the metastasis suppressor protein nm23H1 (which is identical to NDP kinase A) as interaction partner of menin (99). NDP kinases are major suppliers of NTPs; ATP is the phosphate donor (100). Five isoforms, nm23H1, nm23H2 (101), nm23H4, nm23H6 and *DR*-nm23 (103), have been identified so far. Yaguchi *et al.* revealed an unexpected feature of menin, a GTP-hydrolyzing activity in the presence of nm23H1. The authors uncovered similarities between GTPases and menin. Specific consensus sequences are important for GTP binding and hydrolysis and could be also, slightly modified, found in menin although the homology to known GTPases is weak. Menin as itself does not show any GTPase activity, but in co-incubation with nm23H1, the GTP- hydrolyzing rate highly increased (102).

Roymans *et al*. identified the nm23H1 rat homologue as an association partner of centrosomes in dividing and non-dividing glioma cells. Antibody staining showed overlapping nm23H1 and γ-tubulin, which is localized to the centrosome, signals during division and also in differentiated cells. Further investigations using different human and rat cell lines, showed the same colocalization of nm23H1 and γ-tubulin. Several reports showed the association between different tumor suppressor genes with centrosomes at different stages of the cell cycle (103), suggesting that interaction of menin and nm23H1 plays an important role in genomic stability. Moreover it is known that MEN1 tumors show higher rates of genome alterations than the same tumors in non-MEN1 patients (69,104).

1.4.3.7 Menin and CDK inhibitors

Cyclin- dependent kinases are important positive regulators of the cell cycle. Dependent on mitogenic or antiproliferating signals from outside, CDKs can stimulate cell cycle progression or are inactivated. Inhibition leads to the stop of cell proliferation, the cell will be arrested (105). Two subfamilies of CDK inhibitors are known so far, the INK4 (106) and the CIP/KIP family (107). The INK4 family (p15, p16, p18, p19) inhibits CDK4 and the CIP/KIP family (p21, p27, p57) CDK2.

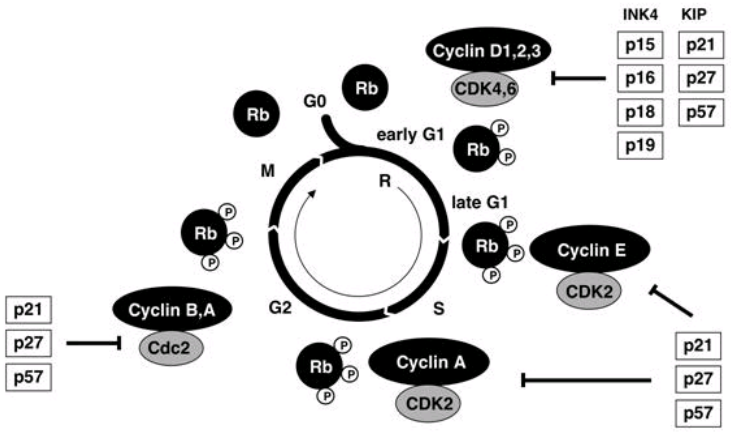


Figure 12 Regulation of the cell cycle in dependence on CDKs and their inhibitors (©Dilek Güner) Specific cyclins interact with CDKs. Activated CDKs phosphorylate Rb, which leads to progression of the cell cycle. After mitosis, Rb will be phosphorylated. CDK inhibitors can arrest proliferation at any stage of the cell cycle.

Menin was shown to be part of MLL (mixed lineage leukemia) chromatin remodelling complexes, recruiting histone methyltransferases and activating anti-proliferative genes, like those for p18 and p27. Loss of menin or MLL leads to down-regulation of those two CDK inhibitors and deregulation of the cell cycle. p18 and p27 are expressed in neuroendocrine tissues and in the nervous system. p18- p27 double mutant mice develop multiple endocrine neoplasias, while p18 or p27 null mice did not exhibit any connection to increased tumor formation. p18 null mutants showed to be highly responsive to mitogenic signals and expansion of T- lymphocytes. Comparison of the pituitaries of $p18^{-/-}$, $p27^{-/-}$ and p18-p27 double mutants revealed striking differences to wild type organs. Pituitaries of the single mutant mice were always enlarged (due to hyperplasia) and double mutants developed tumors at the age of three months. No subject survived longer than 3.5 months, dying from multiple adenomas (110). Further experiments with p18-p27 double mutants lead to tumor formation in different endocrine tissues like the thyroid, adrenal glands, pituitary, pancreas duodenum, testes and parathyroid (111).

1.4.4 Alternative splicing and splicing defects

In eucaryotic genes, exons are separated by non-coding introns that will be removed during mRNA maturation. After assembly of the spliceosome on pre-mRNA, introns are eliminated and exons join to form mature mRNA. During alternative splicing, different combinations of exons can assemble, leading to multiple protein variants encoded by only a single gene.

Alternative splicing has four main mechanisms, exon skipping, intron retention, alternative acceptor and donor splice signal selection. Enhancer or silencer complexes regulate splice sites (117, 118).

In cancer cells, abnormal splicing has been shown in connection with altered protein functions that stimulate cancer development (119). Cancer specific splice variants are absent from normal tissue and the splicing machinery works less effective than in normal cells, leading to lower levels of splice events. It could also be shown that alternative splice patterns differ between normal and cancerous cells (120).

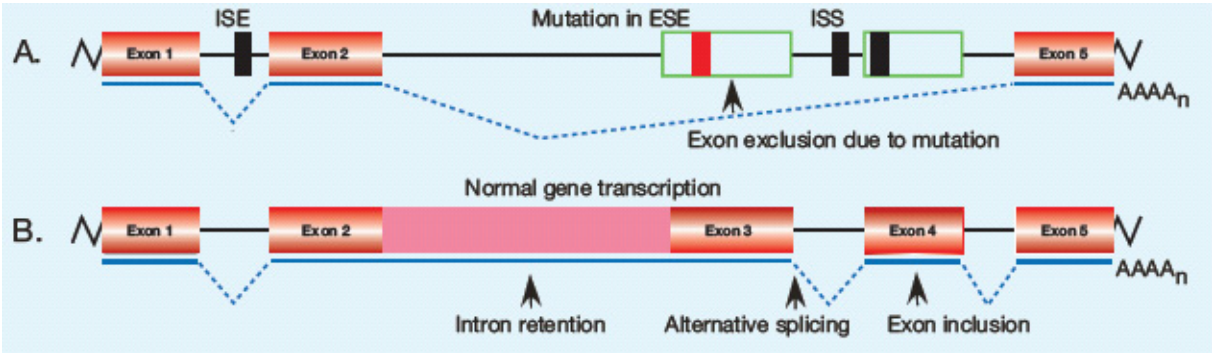


Figure 13 Aberrant splicing in cancer development (Fackenthal JD et al., Disease Models & Mechanisms 2008) A) Exclusion of one exon due to a mutation in the exon splicing enhancer sequence. B) No splicing of the second intron, it remains in the mature mRNA.

MEN1 mRNA is translated into menin protein from exon two to exon 10; exon 1 remains untranslated. In 1997, the ubiquitously expressed 2.9 kb sized transcript and an additional 4.2 kb variant detected in the pancreas and thymus could be identified (112). Due to the fact that the 2.9 kb transcript was visualised more as a smear than a clear band, Khodaei-O´Brien *et al*. looked for similar-sized transcript isoforms and reported six splice variants, exons 1A- 1F, in different tissues, including pancreas, thymus and kidneys. Since no evidence for alternative splicing within the coding region or alternative polyadenylation at the 3´end could be demonstrated, the authors focused on the 5´end of *MEN1*. All transcripts detected contain the same open reading frame not affecting the protein sequence. The heterogenity of the 5´end seems to originate from alternative splicing and distinct transcription initiation sites (113).

Mutations that alter splicing of MEN1 mRNA were identified in 20% of multiple endocrine neoplasia type 1 patients. These alterations eliminated splice donor sites or created new splice acceptor sites leading to truncated menin proteins. No other mutations could be found in those patients (114).

1.4.5 The R171Q polymorphism

At position 171 in exon three of the *MEN1* gene, the nucleobase guanine is changed into adenine, which results in transition of amino acid arginine (R) to glutamine (Q). This polymorphism occasionally occurs in the general population with a frequency between 1% and 5%. It has been previously detected in MEN1 patients and their family members as well as in sporadic MEN1 tumors (115). Balogh *et al*. reported the R171Q polymorphism in six out of 32 familial and sporadic Hungarian MEN1 patients. Disease causing mutations could be only detected in three subjects. The patients developed tumors of the parathyroid and pituitary glands (116). De Carlo *et al.* detected the R171Q polymorphism in 5 Italian family members but only one had clinical signs of MEN1 syndrome. In another family, 4 relatives bearing this polymorphism, two of them with specific symptoms, could be reported. No tumor tissue was available. In the control population, 50 healthy Italians, the R171Q polymorphism could not be demonstrated (115).

Two variants of this polymorphism, R171Q and R176Q, exist. This is due to an alternative splice donor located 15 nucleotides downstream of exon two. These five additional amino acids keep the reading frame intact and the menin protein functions normally (121). Which variant occurs more frequently is not known.

2. Aim of the Study

The multiple endocrine neoplasia syndrome type 1 gene is sequenced and analysed in routine work. Since there are several hundred disease-causing mutations known so far, this is an excellent tool to confirm a diagnosed MEN1 syndrome. Analysis of peripheral blood samples derived from suspected MEN1 patients give rise to accidentally identified new mutations and polymorphisms. Such findings are not further investigated in routine work but can become part of specific research projects. Identification of such alterations is very important since the MEN1 syndrome is dominantly inherited to the offsprings.

In 2008, the already known R171Q polymorphism was detected as being the only DNA alteration in the MEN1 gene of a patient, treated at the General Hospital of Vienna, carrying multiple endocrine neoplasms including a neuroendocrine pancreatic tumor. Evaluation of the potential tumorigenic effect of the R171Q polymorphism became the focus of my diploma thesis.

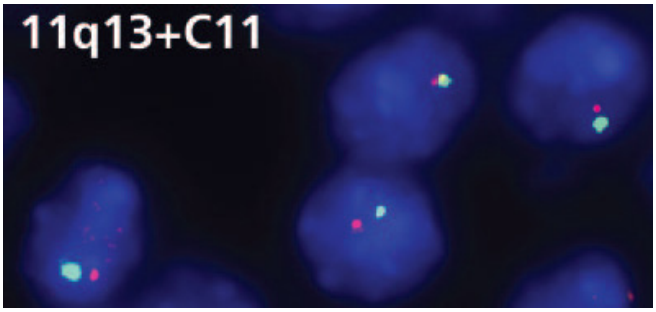


Figure 14 FISH analysis of MEN1 region (red) and centromere 11 (green) (Perren A *et al*., Journal of Clinical Endocrinology and Metabolism 2003) Clear loss of heterozygosity (LOH) in endocrine cells of a microadenoma (a normal cell would have two copies of each chromosome which results in two green and two red points). LOH of centromere 11 indicates complete loss of a large region or maybe even the entire chromosome 11.

3. Materials and Methods

3.1 Human blood and tumor samples

Samples were derived from patients exhibiting symptoms typical for one of the investigated diseases (Multiple endocrine neoplasia types 1 and 2, congenital adrenal hyperplasia and insulin dependent diabetes mellitus). Genetic analyses of involved genes were performed to confirm the suspected diagnoses. This material was then used for detection of the R171Q polymorphism. Patients not affected from MEN1 represented the control population. Peripheral blood samples were stored for at least one day at –80°C. Leucocytes carrying the genetic information were lyzed and the DNA could be isolated. Tumor DNA was isolated from surgically removed tumors that were stored in liquid nitrogen. A large variety of MEN1- and MEN2 related tumors could be analysed.
Personal informations like names, dates of birth or diagnoses of the patients were anonymised. Identification of one of the patients due to the data present in this diploma thesis will not be possible. Written informed consent for genetic analyses was obtained from all subjects studied.

3.2 Tissue homogenisation and isolation of DNA, RNA and proteins

Different methods have been used for DNA isolation of tumor tissue and blood samples. The procedures were varying in effort and effectiveness. Parallel isolation of DNA, RNA and protein could be only performed with the TRIzol method.

3.2.1 Homogenisation of tumor tissue

Frozen solid tumors were put on dry ice and small pieces of tissue were cut off. Approximately 50 to 100 mg tumor material was then homogenised in 1 mL TRIzol Reagent

(Roche) at 20 000 rpm (Polytron PT 3100 homogeniser). As the TRIzol looked cloudy and no large pieces could be visualised, the solution was kept at room temperature for 10 minutes to permit the complete dissociation of nucleoprotein complexes. After incubation, 0.2 mL of chloroform per 1 mL of TRIzol was added to the samples and the tubes were vigorously vortexed for 15 seconds. The samples were then incubated at room temperature for 3 minutes and centrifuged at 12 000 rpm for 15 minutes at 4°C. The solution separated into a lower red, phenol-chloroform phase, an interphase and a colourless upper aqueous phase. RNA was isolated from the aqueous phase, DNA and protein were found in the interphase and lower phase.

3.2.2 DNA isolation with TRIzol

Before DNA isolation, the aqueous phase was removed and saved in fresh tubes for following RNA isolation. 0.3 mL of 100% ethanol per 1 mL of TRIzol Reagent were added and the samples were mixed by inversion. After 3 minutes of incubation at room temperature, the samples were centrifuged at 4900 rpm for 5 minutes at 4°C. The phenol-ethanol supernatant was separated from the precipitated DNA at the bottom of the tube. It was saved for subsequent protein isolation. The DNA pellets were washed three times in a solution containing 0.1 M sodium citrate in 10% ethanol. Per 1 mL of TRIzol, 1 mL of this solution was used. The pellets were stored in the washing solution for 30 minutes at room temperature (shaking). After each wash, the samples were centrifuged at 4900 rpm for 5 minutes at 4°C. After washing, the supernatant was removed and the DNA was suspended in 75% ethanol. 1.5 mL of ethanol has been used for 1 mL of TRIzol. The samples were then incubated for 10 minutes at room temperature (shaking) and centrifuged at 4900 rpm for 5 minutes at room temperature. The DNA was air dried for 15 minutes in an open tube. The pellets were then dissolved in 300 µL of 8 mM NaOH. The samples were adjusted with 1 M HEPES solution (Gibco) to pH 7 to 8. For an amount of 300 µL, 9.6 µL of 1 M HEPES have been used. The DNA was stored at –20 to –80 °C.

3.2.3 RNA isolation with TRIzol

After DNA isolation, the aqueous phase was used for RNA precipitation. 0.5 mL of isopropyl alcohol was used per 1 mL of TRIzol Reagent. Samples were incubated for 10 minutes at room temperature and subsequently centrifuged at 12 000 rpm for 10 minutes at 4°C. The RNA pellets were then washed in 1.5 mL of 75% ethanol. After vortexing, the samples were centrifuged at 9500 rpm for 5 minutes at 4°C. The pellets were air-dried and dissolved in RNase-free water. At the end, the solution was incubated for 10 minutes at 55°C. The RNA was stored at –80°C.

3.2.4 Protein isolation with TRIzol

Proteins were precipitated from the phenol-ethanol supernatant obtained during DNA isolation. First, 1.5 mL of isopropyl alcohol per 1 mL of TRIzol Reagent was added to the solution. The samples were then incubated for 10 minutes at room temperature and centrifuged at 12 000 rpm for 10 minutes at 4°C. The supernatant was removed and the pellets were washed three times in 2 mL of a solution containing 0.3 M guanidine hydrochloride in 95% ethanol. The pellets were stored for 20 minutes in the washing solution (shaking) and centrifuged at 9500 rpm for 5 minutes at 4°C after each washing step. Afterwards, the samples were vortexed in 2 mL of 100% ethanol. They were then stored in ethanol for 20 minutes and centrifuged at 9500 rpm for 5 minutes at 4°C. After the washing procedure, the pellet was air-dried for 10 minutes and dissolved in 1x sample buffer. The protein extract was then stored at –80°C.

3.2.5 DNA isolation with DNAzol

Approximately 50 mg of tumor tissue was homogenised in 1 mL DNAzol. The tumor cells were dispersed and lysed by hand using a pestle from the Sample Grinding Kit (Amersham Biosciences). The homogenate was stored for 10 minutes at room temperature. After incubation, the samples were centrifuged at 11 000 rpm for 10 minutes at 4°C. The supernatant was transferred into a fresh tube. 0.5 mL of 100% ethanol per 1 mL of DNAzol initial used were added. The samples were then mixed by inverting the tubes 10 times and

incubated at room temperature for 3 minutes and the precipitated DNA was transferred into a fresh tube. It was then washed twice with 1 mL of 75% ethanol. The tubes were inverted 5 times, ethanol was removed and the DNA pellets were dissolved in 200 µL of 8 mM NaOH solution. The pH was adjusted with 24 µL of 1 M HEPES.

3.2.6 DNA isolation with phenol

Approximately 50 mg of tumor tissue was homogenised in 600 µL 1x PBS. The tumor cells were again dispersed and lysed by hand using a pestle from the Sample Grinding Kit. 32 µL of 10% SDS (Bio-Rad) solution and 30 µL of proteinase K (Invitrogen) were added to the homogenate. Dependent on the amount of tumor tissue, the samples were incubated for 30 to 60 minutes at 55°C (water bath). Afterwards, 900µL of saturated phenol were added to each sample. This mixture was then vortexed for 5 seconds with maximum speed and centrifuged at 12 000 x g for 5 minutes at room temperature. After centrifugation, the mixture was separated into 3 phases. Only the clear, top layer was used for further experiments. This procedure was performed two times. The samples were then extracted twice with 900 µL of a phenol-chloroform-isoamyl alcohol solution and again vortexed and centrifuged like before. After 4 times of extraction, the top layer was transferred into glas tubes filled with 2 mL of cooled 100% ethanol. The DNA precipitated after panning the tubes. It was transferred into a fresh tube and dissolved in ddH_2O. The amount of added ddH_2O was dependent on the size of the DNA.

3.2.7 DNA isolation from blood samples with the QIAamp DNA Blood Maxi Kit

The sample material was whole blood treated with sodium citrate or EDTA as anticoagulants. It was stored at –80°C and warmed up at 35°C in a water bath shortly before isolation. At the beginning, 500 µL of protease solution were pipetted into 30 mL sterile plastic tubes, 5 mL of the whole blood samples added and mixed by inverting. 6 mL buffer AL was added to each sample and inverted again. After 1 minute of vortexing, cells should be lysed. The tubes were incubated at 70°C in the water bath for 10 minutes. 5 mL of 100% ethanol were then added and samples were inverted again.

The samples were then transferred into fresh tubes containing columns. The tubes were closed and centrifuged at 1850 x g for 4 minutes at 20°C. The filtrate was discarded. 5 mL of buffer AW1 were pipetted onto the columns and centrifuged at 4500 x g for 4 minutes at 20°C. 5 mL of buffer AW2 were transferred to the columns and centrifuged at 4500 x g for 17 minutes at 20°C. All filtrates were discarded and the columns were then transferred into fresh collection tubes. 650 µL of aqua ad injectabilia were added to the collection tubes, incubated for 5 minutes at room temperature and centrifuged at 4500 x g for 7 minutes at 20°C. The filtrates were added to the columns again, incubated for 5 minutes at room temperature and centrifuged at 4500 x g for 7 minutes at 20°C. The filtrates were then transferred into glas tubes filled with 2 mL of cooled 100% ethanol. The precipitated DNA was removed and dissolved with aqua bidest. in a fresh tube. The amount of added aqua bidest. was dependent on the size of the DNA precipitate.

3.2.8 Cell lysate

Tissue samples of human pituitary adenomas, ovarian carcinomas and carcinoids were surgically removed and frozen in liquid nitrogen. The samples were then cut into smaller pieces (on dry ice) and stored in cryotubes in liquid nitrogen. For DNA extraction, approximately 200 mg of tissue fragments were homogenized in 300 µL homogenization buffer (precooled). The homogenate was then centrifuged at 10 000 x g for 10 minutes at 4°C and stored at –80°C.

<u>Homogenization buffer</u>

Tris-buffered saline
1% Triton X-100
10µg/mL aprotinin
1µg/mL pepstatin
10µg/mL leupeptin
0.8 mM Pefabloc

3.3 Genotyping of the R171Q polymorphism

3.3.1 Polymerase chain reaction of tumor and genomic DNA

After DNA isolation, optical density of the DNA samples was measured at the BioPhotometer (Eppendorf). Dependent on those results, different amounts of DNA were added to the PCR reaction. From samples with an optical density of 1000 µg/mL, 1 µL was used for analysis. Since the R171Q polymorphism is located to exon 3 of the *MEN1* gene, only this part of the gene was amplified with PCR reaction. The DNA was mixed with following reagents from the Expand High Fidelity PCR System (Roche) as well as additional components:

Per reaction

5 µL of buffer 2 (12 mM)
2 µL of dNTPs (0.2 mM/µL)
1 µL of primer 1 (1:4 dilution)
1 µL of primer 2 (1:4 dilution)
3 µL of DMSO
0.8 µL of HF- enzyme
12.8 µL of master mix

At last, aqua bidest. was added to reach a final reaction volume of 50 µL.

PCR primers (MWG)

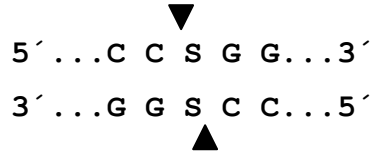
Forward primer (NIN 3 up): 5´- AGG AGA ATC TGA GGT TGG GT -3´
Reverse primer (NIN 4 down): 5´- GCC GAA GGA GAG AGT TAT G - 3´

PCR program

94°C	4 min	
94°C	45 sec	**25 x**
56°C	45 sec	
72°C	1 min	
94°C	45 sec	**10 x**
56°C	45 sec	
72°C	45 sec	
10°C	∞	

3.3.2 Restriction enzyme digest with NciI

The restriction endonuclease NciI (New England Biolabs) has following recognition sites (S stands for a C or a G):



Per 10 reactions

30 µL of NEBuffer 4
4 µL of NciI
116 µL of aqua bidest.

1x NEBuffer 4

50 mM potassium acetate
20 mM Tris- acetate
10 mM magnesium acetate
1 mM DTT
pH 7.9

15 µL of each PCR amplified sample and 15 µL of the restriction digest master mix were put together and incubated for 1 hour at 37°C.

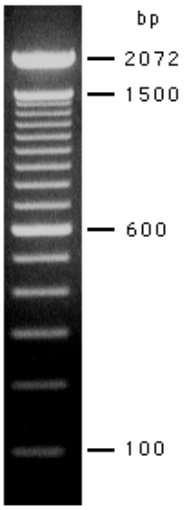
3.3.3 Agarose gel electrophoresis

1% agarose (Bio-Rad) was boiled in 50 mL of 1x TBE buffer and mixed with 2.5 µL of a 1µg/mL ethidium- bromide stock solution (Sigma). It was then poured into a gel tray and cooled down for 20 minutes. 13 µL of the samples and 2 µL of sample loading buffer (Elechrom Scientific) were mixed and loaded onto the gel. The 100 bp DNA Ladder (Invitrogen) was used as marker. The gel was running for 60 minutes at 100 V. The bands were visualised with the Lumi- Imager F1 (Roche).

1x Tris- borate- EDTA buffer (Sigma)

89 mM Tris
89 mM borate
2 mM EDTA
pH 8.3

100 bp DNA Ladder



3.3.4 Real Time PCR

The other part of the samples was analysed via real time PCR, using two different probes for both alleles. Real time PCR operates like a commonly used PCR but in addition, specific labeled probes for each allele are used. The DNA- based probe has a fluorescent reporter dye at one end and a quencher dye on the other end. The quencher prevents detection of the fluorescent signal. The included DNA polymerase has a 5´-3´exonuclease activity. During each cycle, primers and probes bind specifically to DNA. The polymerase cleaves the probes and therefore separates the quencher from the fluorophore; the fluorescent signal can be detected and measured. Amplification of the target DNA results in increased fluorescence (122).

Allelic discrimination of the R171Q polymorphism was performed with TaqMan SNP Genotyping Assay. This was purchased from Applied Biosystems (C_632994_10) for the polymorphic site of residue 171 (rs607969) according to NCBI db SNP cluster identification.

The assay consists of a mix of sequence- specific, unlabeled forward and reverse PCR primers and a TaqMan MGB probe. Each MGB probe contains a reporter dye at the 5´end of each probe. VIC® dye (2_-chloro-7_-phenyl-1,4-dichloro-6-carboxyfluorescein) is linked to the probe for allele one and FAM™ (6-carboxy-fluorescein) dye is specific for the second allele. The DNA samples were diluted 1:80 with aqua bidest. and additionally mixed with 10.25 µL of aqua bidest. to a final concentration of 1 to 20 ng of DNA. The TaqMan Universal PCR Master Mix, the DNA sample and the TaqMan SNP Genotyping Assay Mix were pipetted into a 96-well plate and sealed with a MicroAmp Optical Adhesive Film (Applied Biosystems). Samples were analysed with ABI Prism 7000 and ABI Prism 9500 Sequence Detection System and SDS software (Applied Biosystems).

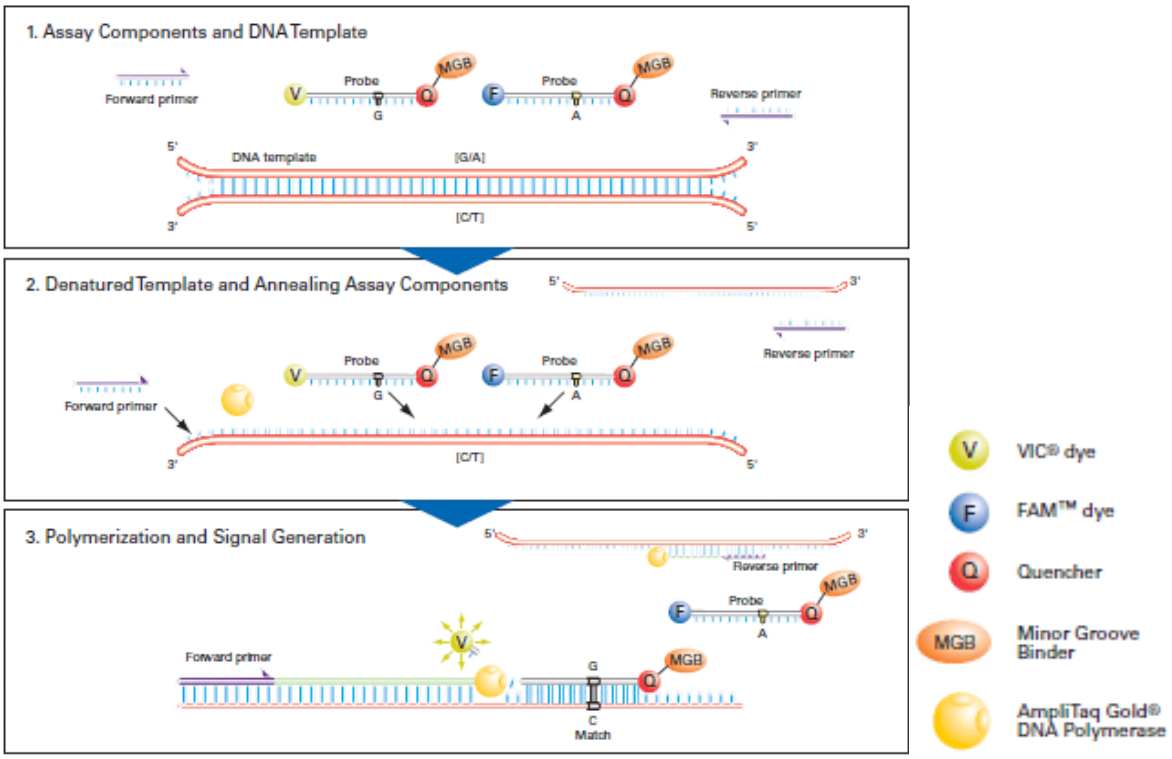


Figure 15 5´Nuclease assay process (©Applied Biosystems) During PCR, the following steps occur: Each oligonucleotide MGB probe anneals to its complementary sequence. The fluorescence signal of the reporter dye is inhibited by the quencher dye. AmpliTaq Gold DNA polymerase extends the primers and additionally cleaves the annealed probes. Cleavage separates the quencher from the reporter dye and the fluorescent signal can be detected. A substantial increase of either one of the signals or both at the same time indicate for homozygosity or heterozygosity of the tested genetic locus.

Volume per well

12.5 µL of 2x TaqMan Universal PCR Master Mix
1.25 µL of TaqMan SNP Genotyping Assay Mix
11.25 µL of diluted DNA sample
25 µL of reaction volume

Thermal cycler conditions

Hold 10 min 95°C
Denature 15 sec 92°C
Anneal 1 min 60 sec
} **40x**

Context sequence ([VIC/FAM])

CTCAGACAGGGCGAGGTGGACATCC[C/T]GGAGACCCAGGGCCTGGCAGGCCCC

3.4 Sequencing of the R171Q polymorphism

3.4.1 Polymerase chain reaction

After DNA isolation, 1 µL of DNA is mixed with 36.2 µL of water bidest. and following reagents from the Expand High Fidelity PCR System (Roche) as well as additional components:

Per reaction

5 µL of buffer 2 (12 mM)
2 µL of dNTPs (0.2 mM/µL)
1 µL of primer 1 (1:4 dilution)
1 µL of primer 2 (1:4 dilution)
3 µL of DMSO
0.8 µL of HF- enzyme
12.8 µL of master mix

The primers are diluted 1:4 to a final concentration of 25 pMol. The reaction volume is 50 µL per tube.

PCR primers (MWG)

Exon 2

forward	5´- AGG GGT CTC ACC GAC AAA - 3´
reverse	5´- AAG AAG TGG GTC ATG GAT AA - 3´

Exon 3/4

forward	5´- AGG AGA ATC TGA GGT TGG GT - 3´
reverse	5´- GCC GAA GGA GAG AGT TAT G - 3´

Exon 5/6

forward	5´- ATA GGC TAA GGA CCC GTT CT - 3´
reverse	5´- GAC TGG ATG GGC GAT A - 3´

Exon 7

forward	5´- TTC TGC CAC TGC TTA CTG T - 3´
reverse	5´- GGG AAA GAT GTG ACA CCT TA - 3´

Exon 8/9

forward	5´- AGA CCC CTT CAG ACC CTA CA - 3´
reverse	5´- AGG TGA GAG CAA GGT TGC C - 3´

Exon 10

forward	5´- CGG GCT TGT CAG ACT TT - 3´
reverse	5´- CTG TGC TCC CTT GGG TTA AG - 3´

PCR programs

Exons 2, 5, 6

94°C	4 min	
94°C	45 sec	**25x**
57°C	45 sec	
72°C	60 sec	
94°C	45 sec	**10x**
57°C	45 sec	
72°C	90 sec	
4°C	∞	

Exons 3, 4, 7

94°C	4 min	
94°C	45 sec	25x
56°C	45 sec	
72°C	60 sec	
94°C	45 sec	10x
56°C	45 sec	
72°C	90 sec	
4°C	∞	

Exons 8, 9

94°C	4 min	
94°C	45 sec	25x
58°C	45 sec	
72°C	60 sec	
94°C	45 sec	10x
58°C	45 sec	
72°C	90 sec	
4°C	∞	

Exon 10

94°C	4 min	
94°C	45 sec	25x
55°C	45 sec	
72°C	60 sec	
94°C	45 sec	10x
55°C	45 sec	
72°C	90 sec	
4°C	∞	

The samples were then loaded onto a 1% agarose gel (for detailed description see 3.3.3).

3.4.2 DNA extraction from agarose gel

After gel electrophoresis, the DNA was visualised by a UV transilluminator. The bands were cut out of the gel and put separately into fresh tubes. The DNA was purified with the High Pure PCR Purification Kit (Roche). Each PCR product was mixed with 600 µL of binding buffer and incubated at 56°C for 10 minutes in a water bath until all of the agarose gel was dissolved. 100 µL of 2- propanol was added to each sample and vortexed. The samples were transferred to High Pure filtration columns and were centrifuged at 13 000 rpm for 1 minute at 20°C (Biofuge fresco). The filtrate was discarded, 600 µL of washing buffer were added to the column and the samples were then centrifuged at 13 000 rpm for 1 minute at 20°C. This step was repeated using 200 µL of washing buffer. At last, only the column was centrifuged at

13 000 rpm for 1 minute at 20°C to air- dry the samples. The columns were put into fresh tubes and 50 µL of elution buffer were added. After 30 seconds of centrifugation at 13 000 rpm, the DNA was collected in the tubes.

3.4.3 Cycle- sequencing

DNA sequencing starts with a modified PCR reaction. In addition to a template DNA, standard deoxynucleotides, primers and DNA polymerase, fluorescently labeled dideoxynucleotides (ddNTPs) are used as DNA chain terminators. ddNTPs lack the 3´OH-group which is essential for the formation of a phosphodiester bound between two nucleotides; DNA synthesis stops (126). DNA strands of many different lengths will be obtained. Each ddNTP reaction is transferred to one of four individual slots. The fragments are separated by gel electrophoresis on a polyacrylamide gel. A laser located at the bottom of the sequencer detects the DNA fragments and they are visualised on a screen.

For DNA sequencing of exons 2- 10, the Thermo Sequenase Cycle Sequencing Kit was used.

Reaction mix

13 µL of the PCR product
2 µL of reaction buffer
1 µL of primer (25 pMol)
1.5 µL of Thermo Sequenase DNA polymerase
17.5 µL total amount

The nucleotides were separately pipetted into 4 different reaction tubes (one tube for dGTP, one tube for dATP and so on). 4 µL of the reaction mix were then added to 3.7 µL of dGTP, dATP, dTTP and dCTP.

Sequencing program

94°C	4 min	
94°C	30 sec	} **40x**
59°C	30 sec	
72°C	60 sec	
4°C	∞	

After the sequencing reaction, 4 µL of stop solution were added to each tube.

Sequence primer

Exon 2

NIN-2-IR	5´- CTG AAG AGG GTG GGG AAG - 3´
NIN-22-IR	5´- CTC TAT GCC CGC TTC AC - 3´
NIN-2-down IR	5´- AAG AAG TGG GTC ATG GAT AA - 3´

Exon 3

NIN-3-IR	5´- GGA GTG GGA GGG AGT GTG - 3´

Exon 4

NIN-4-IR	5´- TGT GGC TGA GCG GGT ATT - 3´
NIN-4-down IR	5´- GCC GAA GGA GAG AGT TAT G - 3´
NIN-4-IRrv	5´- AAT ACC CGC TCA GCC ACA - 3´

Exon 5

NIN-5-IR	5´- ATA GGC TAA GGA CCC GTT CT - 3´

Exon 6

NIN-6-re-IR	5´- CTC AGC CAC TGT TAG GGT C - 3´
NIN-6-down IR	5´- GAC TGG ATG GGC GAT A - 3´

Exon 7

NIN-7-re-IR	5´- GTG GTT GGA AAC TGA TGG A - 3´
NIN-7-down IR	5´- GGG AAA GAT GTG ACA CCT TA - 3´

Exon 8

NIN-8-IR	5´- CCC CTT CAG ACC CTA CAG - 3´

Exon 9

NIN-9-IR	5´- CCT GTG CCC TCT GCT AAG - 3´
NIN-9-seq IR	5´- GGA CAC GGG AGA CGA TTC -3´
NIN-9-IRrv	5´- AGG TGA GAG CAA GGT TGC C - 3´

Exon 10

NIN-10-re-IR	5´- ACA TGG GCT CAG AGT TGG - 3´
NIN-10-up-IR	5´- CGG GCT TGT CAG ACT TT - 3´

3.4.4 Sequencing gel and sequence analysis

Per gel (41 cm long)

12.6 g of urea (Bio-Rad)
3.3 mL of Long Ranger Gel Solution (Cambrex)
3 mL of 10x TBE
15 mL of ddH_2O

The reagents were continuously mixed on a magnetic stirrer until the urea was completely dissolved. The sequencing gel plates, spacers and the comb were cleaned with 70% ethanol and dried with towels. Spacers and gel plates were assembled and fixed with clamps. When everything was fixed, 20 µL of TEMED and 200 µL of APS were added to the gel solution. It was then poured between the two gel plates and properly dispersed. At the end, the gel comb was inserted at the top of the plates. Polymerization of the gel took around 50 minutes.
The comb was then removed and the slot as well as the plates were carefully cleaned from gel pieces. The comb was turned around and put back into the gel slot.
The gel was put into the LI-COR 4300 DNA analyzer and 1000 mL of 1x TBE were added to the upper and lower buffer tank. After connecting the analyzer to the power supply, the pre-run was started. The samples were incubated at 75°C for three minutes. When the pre- run was completed, 0.6µL of each sample was loaded into the slots and the running process was started. Analysis of the visualized bands was performed with e-Seq DNA sequencing software (LI-COR).

3.5 Multiplex Ligation- dependent Probe Amplification

Chromosomal aberrations are very common features of tumor and cancer development. Deletions or duplications of whole chromosomes, millions of base pairs or single exons lead to deregulated levels of specific DNA fragments, influencing gene expression. Several well established methods for the detection of changes in chromosome structure are available, but only few can detect deletions or duplications in single exons. Those assays are often expensive, time-consuming and high amounts of DNA have to be applied (123).

Multiplex ligation- dependent probe amplification (MLPA) is a new method for relative quantification of DNA sequences. It is a variation of PCR but using different probes for specific DNA targets and one pair of primers. The hybridised probes-but not the target DNA-are amplified. The probes are split into two oligonucleotides. One oligonucleotide contains the sequence recognised by the fluorescent-labeled forward primer, the other one is recognised by the reverse primer. They can be only amplified, when both parts of the probe are bound to their target sites.

The MEN1 MLPA kit contains 29 different probes with variable lenghts ranging between 144 and 391 base pairs. Each of those probes has its unique length and can be separated by capillary electrophoresis. Seven out of ten exons of the *MEN1* gene can be tested. In addition, several probes for genes located close to the *MEN1* gene as well as on unrelated chromosomes are included. The reference genes located on other chromosomes should not be affected. For one MLPA reaction, only 20ng of DNA is required. Heterozygous deletions of target DNA sequences should give a 35-50% reduced signal of that probe in comparison to healthy individuals. Blood analysis in respect to mutations in the *MEN1* gene is performed in routine diagnosis. With MLPA, relative copy number of different exons can be easily determined at the same time (123,124). Hochstenbach *et al.* compared 527 DNA samples derived from amniocytes of pregnant women analysed via MLPA and karyotyping. In summary, specificity of MLPA was determined as 99.8% and sensitivity as 100% (125).

For MLPA analysis, a DNA amount of 50- 200 ng was used. One µL of genomic DNA or 4 µL of tumor DNA were mixed with TE buffer to a total amount of 5 µL. The samples were incubated at 98°C for 5 minutes and subsequently cooled down to 25°C. 1.5 µL of SALSA Probemix and 1.5 µL of SALSA MLPA buffer were added to each reaction. After mixing the samples, they were first incubated at 95°C for one minute and then at 60°C for 16- 18 hours.
The cycler was cooled down to 54°C and 32 µL of the Ligase mix were added to each tube. The samples were first incubated at 54°C for 15 minutes followed by 98°C for 5 minutes.
For the PCR reaction, 10 µL of the ligase reaction were pipetted into fresh tubes and mixed with 4 µL of SALSA PCR buffer and 24 µL of aqua bidest. The cycler was heated to 60°C, 10 µL of the PCR mix were added to each sample and the PCR reaction was started.
One µL each sample was then pipetted into a microtiter plate and mixed with 0.5µL of Rox Standard and 8.5 µL of formamide. The plate was then covered with a Microseal A Film (Bio-Rad) and incubated at 94°C for 3 minutes.

After incubation, the plate was placed in the ABI-3130XL Genetic Analyser and the run was started. Empty slots were filled with 10 µL of HPLC water (Merck).

For this experiments, the **SALSA MLPA Kit P244 AIP-MEN1 (MRC-Holland)** was used.

Ligase mix for 18 samples

54 µL of Ligase-65 Buffer A
54 µL of Ligase-65 Buffer B
450 µL of ddH_2O
18 µL of Ligase-65

PCR mix for 18 samples

36 µL of SALSA sMLPA PCR Primer mix
36 µL of SALSA Enzyme dilution buffer
99 µL of H_2O
9 µL of SALSA Polymerase

PCR program

95°C	30 sec	} **35x**
60°C	30 sec	
72°C	60 sec	
72°C	20 min	
4°C	∞	

PCR primer (5´-3´)

Forward primer (labelled) *GGG TTC CCT AAG GGT TGG A
Reverse primer (unlabelled) GTG CCA GCA AGA TCC AAT CTA GA

TE buffer

10 mM Tris-HCl pH 8.2
1 mM EDTA

3.5.1 MLPA data analysis

Visual examination was performed by analysing the results with Genemapper V4.0 (ABI). Within this first analysis, the capillary electrophoresis peak profiles could be visualised. Further analysis was performed using the Coffalyser MLPA software (MRC-Holland). The Excel-based program normalised the electrophoretic results obtained through sequencing analysis. With this tool, deletions and duplications were visualised and also different parameters like DNA concentration or ligase activity could be checked.
MRC-Holland defined relative probe signals by dividing [the peak area of each amplification product] by [the total peak area of only the reference probes in the probe mix]. A probe ratio between 0.7- 1.3 could be seen as normal.

All SALSA MLPA probe mixes contain four DNA Quantity control fragments (Q-fragments) and three DNA Denaturation control fragments (D-fragments). The small Q-fragments are not ligation-dependent and their appearance is coupled to the DNA concentration used. Can enough DNA be detected during analysis, the Q-fragments are visualised as very small peaks, which means that MLPA reaction was successful. When all four Q-fragments are bigger than half the size of the peaks of the D-fragments and MLPA probes, either the ligation reaction was not succesfull or the amount of DNA was too small. The D-fragments are ligation-dependent and should be amplified like all of the MLPA probes.

11q13 probes arranged according to chromosomal location

Length (nt)	SALSA MLPA probe	Gene/exon	Ligation site	Partial sequence (20 nt adjacent to ligation site)	Distance to next probe
			NM_000244.2		
247	1164-L00720	MEN1 exon 11	1855-1856	CGCCATCAAGCT-GCAACTCACGGC	0.7 Kb
154	3404-L02795	MEN1 exon 10	1390-1391	CGGCATCTGCAA-ATGGGAGGAGGG	0.6 Kb
337	3403-L09561	MEN1 exon 9	1218-1219	ACAAGGAGTTCT-TTGAAGTAGCCA	0.6 Kb
301	1666-L01245	MEN1 exon 8	1092-1093	ACCCCTACATGT-ACCTGGCTGGCT	1.6 Kb
274	1665-L01244	MEN1 exon 4	672-673	AGGATCATGCCT-GGGTAGTGTTTG	2.0 Kb
220	1664-L01243	MEN1 exon 3b	250-251	CGTGGAGCATTT-TCTGGCTGTCAA	0.7 Kb
193	1663-L01242	MEN1 exon 2b	34-35	GAGATCCCAGAA-GCCACAGCGCAG	216.9 Kb
328	1667-L09560	SNX15 gene		CGAAGGATGACT-TCCTGCGGCACT	545.9 Kb
355	4157-L03512	FAM89B gene		ACAAACACCTGT-GCCAAGACCTGA	88.3 Kb
229	1120-L00060	RELA gene		AAAGGACTGCCG-GGATGGCTTCTA	305.6 Kb
382	4159-L03514	SART1 gene		CCGCAAGAAGGA-GAAGGAGGTAGT	374.9 Kb
160	4155-L03510	BRMS1 gene		CAGAAGAGATGG-AAGCAGAGGGTG	1140.9 Kb
			NM_003977.1		
292	7379-L09558	AIP exon 1	89-90	GAGTCCGGAAGT-TGCCGAAAGGGA	0.1 Kb
175	7380-L09559	AIP exon 1	170-171	AAAACGTGTGAT-ACAGGAAGGCCG	3.9 Kb
238	7381-L07028	AIP exon 2	304-305	CCATGGAGCTCA-TCATTGGCAAGA	2.3 Kb
364	7382-L09069	AIP exon 3	503-504	ACAGATGCGTGA-ACACAGCTCCCT	0.7 Kb
211	7383-L07030	AIP exon 4	660-661	GCAGTGCCACTT-ATCCACCAGGAG	0.3 Kb
265	7384-L09556	AIP exon 5	824-825	GCTGCTGCTCAA-CTACTGCCAGTG	0.5 Kb
319	7385-L09557	AIP exon 6	981-982	CAGGCTGACTTT-GCCAAAGTGCTG	2157.1 Kb
310	5403-L04809	CCND1 gene		TCCGCCCTCCAT-GGTGGCAGCGGG	

Figure 16 (© MRC-Holland 2009) All MEN1 and reference probes located at the chromosomal region 11q13.

Lenght (nt)	Chromosomal position
64-70-76-82	Q-fragments; only visible with less than 100 ng sample DNA
92	Ligation dependent control fragment at 2q14
144	3q29
166	9p22
184	6p21.3
202	2p13
256	7q11
283	13q12
346	1p34
373	17q11
391	7q11

Table 1 Reference probes that are not located at chromosomal region 11q13.

3.6 Menin protein expression

3.6.1 Polymerase chain reaction

For this PCR reaction, tumor cDNA was used for amplification. Because the full length *MEN1* gene could not be amplified, only the first 725 (R171Q) and 740 (R176Q) nucleotides were analysed, respectively.

Per reaction

5 µL of buffer 2 (12 mM)
2 µL of dNTPs (0.2 mM/µL)
1 µL of primer 1 (1:4 dilution)
1 µL of primer 2 (1:4 dilution)
3 µL of DMSO
0.8 µL of HF- enzyme
12.8 µL of master mix

The primers were diluted 1:4 to a final concentration of 25 pMol. At last, aqua bidest. was added to reach a final reaction volume of 50 µL.

PCR Primer

Forward primer 5´- ATG GGG CTG AAG GCC G -3´
Reverse primer 5´- GCA CAC ACC ATG AAC CGC C -3´

PCR program

94°C	4 min	
94°C	45 sec	**25 x**
56°C	30 sec	
72°C	1 min	
94°C	45 sec	**10 x**
56°C	45 sec	
72°C	45 sec	
10°C	∞	

The result of the PCR reaction was checked on 1% agarose gel. For detailed gel preparation see 3.3.3. 8 µL of the PCR product and 2 µL of DNA loading buffer were loaded.

3.6.2 Ligation with the pGEM- T Easy Vector (Promega)

For this ligation reaction, 5 µL of 2x Rapid Ligation Buffer, 1 µL of pGEM- T Easy Vector, 3 µL of PCR product and 1 µL of T4 DNA ligase were mixed and incubated for one hour at room temperature.

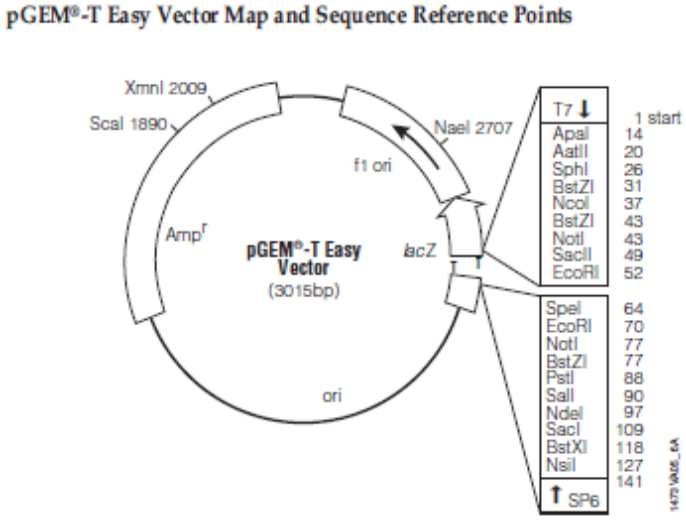


Figure 17 (©Promega) The pGEM- T Easy Vector with its restriction enzyme recognition sites, origin of replication, ampicillin resistance gene and the lacZ gene with the multiple cloning site within its reading frame.

3.6.3 E. coli transformation

50 µL of competent cells and 3 µL of the ligation reaction were mixed and incubated for 10 minutes on ice. After this procedure, the cells were heat shocked for 40 seconds at 42°C and put back on ice for one minute. While mixing with 400 µL of SOC medium (Gibco BRL) and incubation for 45 minutes at 37°C, 2x YT medium was prepared.

2x YT medium

16g/L tryptone
10g/L yeast extract
5 g/L NaCl
dd H_2O

This mixture was autoclaved for 10 minutes and then 15g/L agar were added and autoclaved again. After cooling down, 1000 µL/L ampicillin (Sigma) was added. The medium was then poured into petri dishes. 50 µL of X-Gal (Promega) were dispersed on the solid medium. 100 µL of the transformation reaction was then plated on the selective medium and incubated at 37°C over night.
On the next day, white colonies were inoculated into liquid LB medium+ ampicillin and incubated at 37°C over night (shaking). After that, cells were centrifuged and freezed at –20°C.

The white colonies were also checked for an insert through colony PCR.

Colony PCR for one reaction

5 µL of buffer 2 (12 mM)
2 µL of dNTPs (0.2 mM/µL)
1 µL of forward primer (1:4 dilution of 100 pMol)
1 µL of reverse primer (1:4 dilution of 100 pMol)
3 µL of DMSO
0.5 µL of HF polymerase
17.5 µL of H_2O

A small part of one colony was added to the reaction tube and the PCR reaction was then loaded onto a 1% agarose/TBE gel. See 3.3.3 for detailed description.

PCR Primer

Forward primer 5´- ATG GGG CTG AAG GCC G -3´

Reverse primer 5´- GCA CAC ACC ATG AAC CGC C -3´

PCR program

94°C	4 min	
94°C	45 sec	**25 x**
56°C	45 sec	
72°C	1 min	
94°C	45 sec	**10 x**
56°C	45 sec	
72°C	45 sec	
10°C	∞	

3.6.4 DNA extraction from agarose gel

Since it was not clear if the DNA concentration in the PCR product was high enough for sufficient transformation, the remaining PCR product was loaded onto 1% agarose/TBE gel. The DNA fragment was excised from agarose gel and extracted with the NucleoSpin Extract II Kit (Clontech Laboratories).

First, the weight of the gel slice was determined. For each 100 g of agarose gel 200 µL of Buffer NT were added. The total amount of the gel slice was 210mg. The fragments were then incubated at 50°C until all of the gel was completely dissolved. A NucleoSpin Extract II Column was placed into a Collection Tube and samples were loaded. The tubes were centrifuged for 1 minute at 11 000 x g. The flow-through was discarded and the column was placed in the Collection Tube.

600 µL of Buffer NT3 were added and centrifuged for 1 minute at 11 000 x g. The filtrate was discarded and the column was placed back into the Collection Tube. The samples were then centrifuged for 2 minutes at 11 000 x g to remove all of the Buffer NT3. The column was then placed into a fresh tube and DNA was eluted with 25 µL of aqua bidest. The samples were incubated for one minute at room temperature and finally centrifuged for one minute at 11000 x g. The DNA was stored at –80°C.

3.6.5 Isolation of plasmid DNA (mini preparation)

The pellets were resuspended in 3000 µL of 1x PBS solution. 1500 µL of the cells were centrifuged at 9000 rpm for 10 minutes. The supernatant was discarded and the pellet was mixed with 150 µL of P1 buffer from the QIAprep Spin Miniprep Kit (Qiagen) and vigorously vortexed. 200 µL of P2 buffer were added and the tubes were inverted several times. At last, 200 µL of P3 buffer were added and the samples were inverted again. The tubes were then centrifuged at 13 000 rpm for 10 minutes. The supernatant was transferred into a fresh tube and 1000 µL of 96% EtOH were added. The samples were then centrifuged at 13000 rpm for 10 minutes. The pellets were washed with 70% ethanol and then air-dried. The DNA was mixed with 30 µL of ddH_2O.

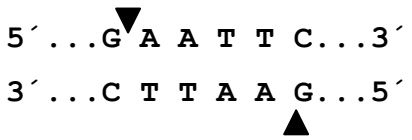
<u>10x PBS</u>

9 g of Na_2HPO_4 x $2H_2O$
1.5 g of KH_2PO_4
40.92 g of NaCl

ddH_20 was added to reach a final volume of 500 mL.

3.6.6 Restriction digest with EcoRI

The restriction endonuclease EcoRI (Roche) has following recognition sites:



<u>Per 12 reactions</u>

127 µL H_2O
33 µL buffer H
5 µL of EcoRI

15 µL of each PCR amplified sample and 13 µL of the restriction digest master mix were put together and incubated for 1 hour at 37°C. The restriction digest was then loaded onto 1% agarose/ TBE gel. See 3.3.3 for detailed description. Three of the samples were then subject to sequence analyses.

3.7 Immunohistochemistry of endocrine tumors

For the detection of menin protein, RIN-5F cells were obtained from ATCC (CRL-2058). RIN-5F is a secondary clone of the rat islet tumor cell line RIN-m (ATCC CRL-2057). Aliquots of approximately 2 x 10^4 cells were loaded by centrifuge onto glass slides (cytospin preparation). The slides were then stored at –80°C and could be directly used for immunofluorescene analyses. Tumors were frozen and cut with a microtome into thin sections (cryosections, 4µm).

The slides were fixed in cold aceton (the aceton was at –20°C for 20 minutes) and incubated for 10 minutes at –20°C. 2 µL of the first antibody, a polyclonal rabbit menin antibody (abcam, ab2605) was diluted with 400 µL of 1x PBS (1:200). The slides were washed three times with 1x PBS and air- dried. 100 µL of the antibody dilution was applied on each slide. The slides were incubated for 2 hours at room temperature or over night at 4°C in a wet chamber.
4 µL of the second antibody, Alexa- Fluor 488 goat anti-rabbit antibody (A11070, Invitrogen), was mixed with 800 µL of 1x PBS. The slides were washed three times with 1x TBPS. The antibody dilution was centrifuged for 5 minutes at 13 000 rpm, then applied on the slides and incubated for one hour at room temperature. The slides were then washed with 1x TBPS and sealed with Vectashield (Vector Laboratories) and cover slides.

The second method used, started with 3 minutes of fixation in aceton (at room temperature). Afterwards the cells were marked and 100 µL of RPMI-1640 + L-glutamine medium was added (blocking reaction). The slides were then washed twice with 1x PBS by directly pipetting PBS on the cells. 50 µL of PBS and 100 µL of the diluted menin antibody (1:250) were added on the slides. The reaction was then incubated for 2 hours at room temperature (in a wet chamber, slightly shaking). The slides were then washed in 1x PBS for 10 minutes. The second antibody, Alexa- Fluor 488 goat anti- rabbit antibody, was diluted 1:200 with PBS and

centrifuged for 5 minutes at 13 000 rpm at 4 °C, then applied and incubated for one hour at room temperature. Then 30 µL of TO-PRO-3 reagent (Molecular Probes) was added to each slide and further incubated for 10 minutes. Afterwards, the samples were washed in 1x PBS, mounted with Vectashield and sealed with a coverglass.

1x TBPS

9 g of Na_2HPO_4 x $2H_2O$
1.5 g of KH_2PO_4
40.92 g of NaCl
1% Tween 20

3.8 Western blot analysis

10 µL of each protein extract was mixed with 20 µL of 1x sample buffer and denaturated at 95°C for 2 minutes. 10% precast tris glycin gels (Anamed) were used for electrophoresis. 20 µL of the samples were loaded into the slots and the gel was run at 200 V and 60- 100 mA for 1 hour.

For western blot transfer (semi dry), six Whatman filterpapers and the nitrocellulose membrane were soaked in transfer buffer (4°C). Three Whatman papers were put on the semi dry blotter. Then the membrane, the gel and three additional Whatman filter papers were put on top. Blotting was performed with 25 V and 100 mA for 1 hour. After incubation of the membrane with blocking solution for 50 minutes, the first antibody was added (1.5 µL of menin antibody (abcam, ab2605) were diluted with 4000 µL of 1x PBS) and put on 4°C over night. On the next day, the membrane was washed two times with 1x TPBS, ten minutes each. 1 µL of the second antibody (goat anti- rabbit, P0448, Dako) was diluted with 4000 µL of 1x PBS and incubated for 50 minutes at room temperature. The membrane was washed two times with 1x TPBS, ten minutes each. At last, 1 mL of Chemiluminescence (Roche) Solution 1 and 10 µL of Chemiluminescence Solution 2 were added to the membrane. The result was visualised with the Lumi- Imager F1 (Roche). Afterwards, the first antibody against beta-actin (Novus Biologicals, NB 600-501) was added. 1 µL of beta- actin antibody was diluted with 4000 µL of 1x PBS and incubated for 50 minutes at room temperature. The membrane was then washed two times with 1x TPBS, ten minutes each. 1 µL of goat anti- mouse second

antibody (P0447, Dako) was diluted with 4000 µL of 1x PBS and incubated for 40 minutes at room temperature. The membrane was again washed two times with 1x TPBS and finally 1 mL of Chemiluminescence Solution 1 and 10 µL of Chemiluminescence Solution 2 were added. The bands were then visualised with the Lumi- Imager F1 (Roche).

4x Sample Buffer (10 mL)

617 mg of DTT
4 mL of SDS (8%)
2.4 mL of Tris/HCl pH 6.8
5.1 mL glycerol
0.4 mL bromphenol-blue
aqua bidest. was added to a final volume of 10 mL

10x Running Buffer

30.2 g Tris
144 g Glycine
10 g SDS
ddH_2O was added to a final volume of 1000 mL

Blocking Solution

9 mL 1x PBS
1 mL 10% BSA Blocking Solution (KPL)

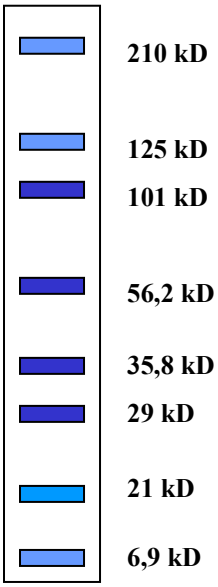
10x Tris/ Glycine buffer

30. 2 g Tris
144 g Glycine
ddH_2O was added to a final volume of 1000 mL

Transfer Buffer

50 mL of 10x Tris/ Glycine buffer
350 mL of ddH_2O
100 mL methanol

Natural Prestained SDS- PAGE Protein Standard, Broad Range (Bio-Rad)



3.9 Reverse Transcription

After RNA extraction and purification, reverse transcription was performed. 2 µg of total RNA was mixed with DNase I (Invitrogen) and incubated for 15 minutes at room temperature. The reaction was terminated using 1 µL of EDTA. The RNA was then reverse transcribed into complementary DNA using 1 µL of the SuperScript II™ reverse transcriptase (Invitrogen) and further reagents:

1 µL of hexamer random primers
1 µL of dNTPs (10 mM)
0.5 µL of RNaseOUT
2 µL of DTT
4 µL of 5x First-Strand Buffer (Invitrogen)

Aqua bidest. was added to a final reaction volume of 20 µL.

The reaction parameters were defined as: 65°C 15 min, 4°C 2 min, 25°C 4 min, 42°C 50 min and 65°C 10 min.

3.10 Quantitative real time polymerase chain reaction

cDNA of insulinomas and unaffected islets of Langerhans were analysed in respect to their relative *MEN1* expression. Expression analysis was performed with TaqMan Gene Expression Assay (ID Hs00365720_m1) supplied from Applied Biosystems.

Volume per cDNA sample

25 µL of 2x TaqMan Universal PCR Master Mix
2.5 µL of TaqMan SNP Genotyping Assay Mix
2 µL of cDNA sample
20.5 µL of aqua bidest.
50 µL of reaction volume

Quantitative real time PCR was performed in duplicates, each well containing 22 µL, using the ABI Prism 7000 cycler (Applied Biosystems).

Thermal cycler conditions

Hold 10 min 95°C
Denature 15 sec 92°C } **40x**
Anneal 1 min 60 sec }

3.10.1 Analysis of relative gene expression

Relative quantification describes the change in expression of the *MEN1* gene relative to the expression of the housekeeping gene *GAPDH*. The $2^{-\Delta\Delta CT}$ method has been used for calculation (128). The C_T values provided from the real time PCR reaction analysis were

imported into Microsoft Excel. The samples were loaded as duplicates and the number of cycles needed for the detection of the fluorescent signal was counted for each duplicate. Then the mean of both results was determined. This was done for the *MEN1* gene expression as well as for the internal control gene *DAPDH.* Then the difference between cycle numbers of the control gene and the target gene was calculated. The standard derivation of the duplicates was also determined. It is important, that both duplicates have very similar cycle numbers or otherwise the results could be falsified. One sample was taken as a reference for all other results; it was defined as 100 %. The reference sample was one of the healthy islets of Langerhans. The relative expression of *MEN1* could then be visualised in a histogram.

Reagents

Product	Company	Cat. No.
10% BSA Blocking Solution	KPL	50-61-00
10% Tris-Glycine gels	Anamed	TG10110
100 bp DNA ladder	Invitrogen	15628-019
10x TBE buffer	Sigma	T-4415
Agarose	Bio-Rad	161-3101
Alexa Fluor 488 Goat Anti-Rabbit AB	Invitrogen	A11070
Ampicillin	Sigma	A-2804
APS	Bio-Rad	161-0700
Aqua bidestillata	Pharmacy, AKH	-
BM Chemoluminescence Blotting Substrate	Roche	11500708001
Different primers	MWG	-
DNA Blood Maxi Kit	QIAGEN	51194
DNaseI	Roche	104159
DNAzol	Molecular Research Center	DN 127
dNTPs	Roche	1814362
EcoRI	Roche	11175084001
Ethidium bromide	Sigma	E-1385
Expand High Fidelity PCR System	Roche	1732650
Gene Scan-500 ROX Size Standard	Applied Biosystems	401734
Goat anti mouse antibody	Dako	P0447
Goat anti rabbit antibody	Dako	P0448
HEPES Buffer Solution, 1 M	Gibco	15630056
High Pure PCR Purification Kit	Roche	1732376
IR2 Stop Solution	Li-Cor	830-04997
LiChrosolv water	Merck	1153331000
Long Ranger Gel Solution	Cambrex	50611
Mouse monoclonal anti- Beta-actin AB	Novus Biologicals	NB 600-501
NciI	New England Biolabs	R0196S
Nucleo Spin Extract II Kit	Macherey-Nagel	740609.10
pGEM- T Easy Vector System	Promega	A1360
Phenol-chloroform-isoamyl alcohol	Gibco	15593-031
Polyclonal rabbit anti menin antibody	Abcam	ab2605
Proteinase K	Roche	3115879
QIAprep Spin Miniprep Kit	QIAGEN	27104
Rin- 5F cells	ATCC	CRL-2058
RPMI-1640+ L-glutamine	Gibco	11879-020
Salsa MLPA Kit P244	MRC-Holland	P244A1P
Sample Grinding Kit	Amersham Biosciences	80-6483-37
Sample Loading Buffer	Elechrom Scientific	P/N 3034
Saturated phenol	Gibco	15513-039
SDS, 10%	Bio-Rad	161-0416
SDS-PAGE Protein Standard, broad range	Bio-Rad	161-0318
SOC medium	Gibco	15544-034
Super Script II reverse transcriptase	Invitrogen	18064-014
TaqMan Gene Expression Assay	Applied Biosystems	Hs00365720_m1
TaqMan SNP Genotyping Assay	Applied Biosystems	C_632994_10

TaqMan Universal PCR Master Mix	Applied Biosystems	4318157
TEMED	Bio-Rad	161-0800
Thermo Sequenase Cycle Sequencing Kit	USB	US78500
TO-PRO-3	Molecular Probes	T3605
TRIzol Reagent	Invitrogen	15596-026
Tween 20	Bio-Rad	170-6531
Urea	Bio-Rad	161-0731
Vectashield	Vector Laboratories	H-1000
X-Gal	Promega	V3941

4. Results

4.1 Genotyping of the R171Q polymorphism

Two distinct methods have been used for detection of the single nucleotide polymorphism at position 171 in exon three of the *MEN1* gene. DNA was isolated from leucocytes and endocrine tumor tissue. Several control populations have been analysed to reveal the incidence of the R171Q polymorphism in the general population. Based on their suspected diagnosis, 98 MEN1, 128 MEN2, 33 insulin dependent diabetes mellitus (IDDM) and 141 congenital adrenal hyperplasia (CAH) patients were analysed in respect to the R171Q variant. In addition, 100 MEN1- and MEN2- related tumor tissues have been tested whether they carried this polymorphism and were undergoing loss of heterozygosity (LOH) at the same time. Loss of heterozygosity, which means that only the mutated allele of the *MEN1* tumor suppressor gene can be found in tumor tissue (the functional wild type allele is lost), is a characteristic feature of tumor formation (18,70).

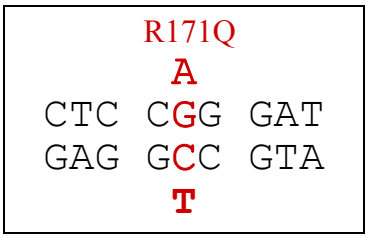


Figure 18 Polymorphic sequence of position 171 in exon three of the *MEN1* gene The more common variant is homozygous CGG, around 3% of the analysed subjects carry the heterozygous CGG/ CAG variant.

One part of the samples was analysed via polymerase chain reaction of exon three followed by restriction digest with the endonuclease NciI. The restriction enzyme will not cut the CAG variant of this codon; the full length PCR product of 900 bp will be obtained. The more common CGG variant is cut into two fragments, 700 bp and 200 bp long.
Loss of heterozygosity is visualized as two lanes sized 700 and 900 bp, whereas the lost allele looks faded. This is due to the fact that not only tumor DNA but also DNA from surrounding tissues and blood have been isolated, where both alleles can be detected normally. Two out of 100 tumor samples (2%) simultaneously were undergoing LOH and were positively tested for

the R171Q polymorphism. One LOH was identified in a pancreatic endocrine tumor, another in a phaeochromocytoma, the latter not being a typical MEN1-tumor but occasionally found in MEN1 patients. Interestingly, both 171 variants were undergoing LOH. In the pancreatic tumor, the altered allele 171Q and in the phaeochromocytoma the wild type variant 171R was lost.

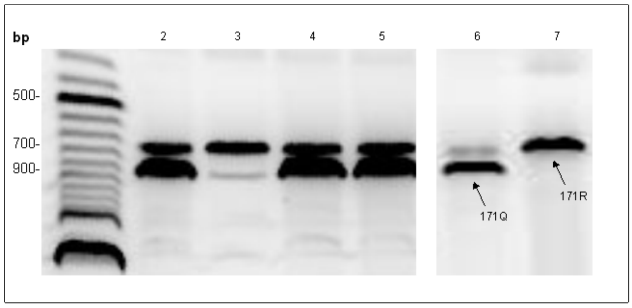


Figure 19 PCR and restriction enzyme digest of locus 171 in exon 3 DNA derived from healthy individuals, endocrine tumor bearing patients and tumor DNA have been chosen as template. Lane 2: heterozygous individual. Lane 3: LOH of 171Q. Lane 4 and 5: siblings bearing the R171Q polymorphism. Lane 6 LOH of 171R. Lane 7: healthy individual, homozygous for 171R.

The other part of the samples was analysed via real time PCR, using two different probes for both alleles. The DNA probes are labeled with fluorescent reporter dies at the 5´end. Allele 1 (CGG) is labeled with VIC dye, which is then detected as a green signal. Allele 2 (CAG) is labeled with FAM dye; it appears red. The more common genotype is homozygous for allele 1, the FAM labeled signal will not cross the threshold. In subjects carrying the R171Q polymorphism, both alleles are present and will be amplified; the green and the red signal are equally detected. In tumor cells that were undergoing LOH, detection of the lost allele is difficult. DNA from surrounding tissues contributes to the amplification profile. The concentration of the lost allele can be high enough to cross the threshold, which leads to a poor signal of it. Or if it is not, then the LOH will appear like the common homozygous CGG variant. Therefore, all analysed tumor samples were also checked for LOH through PCR followed by restriction enzyme digest. Genomic DNA samples carrying the variant CAG in homozygous form could not be found. The ROX standard is a reference dye to which VIC and FAM can be compared during analysis. Such comparison is necessary for correcting variations between different wells.

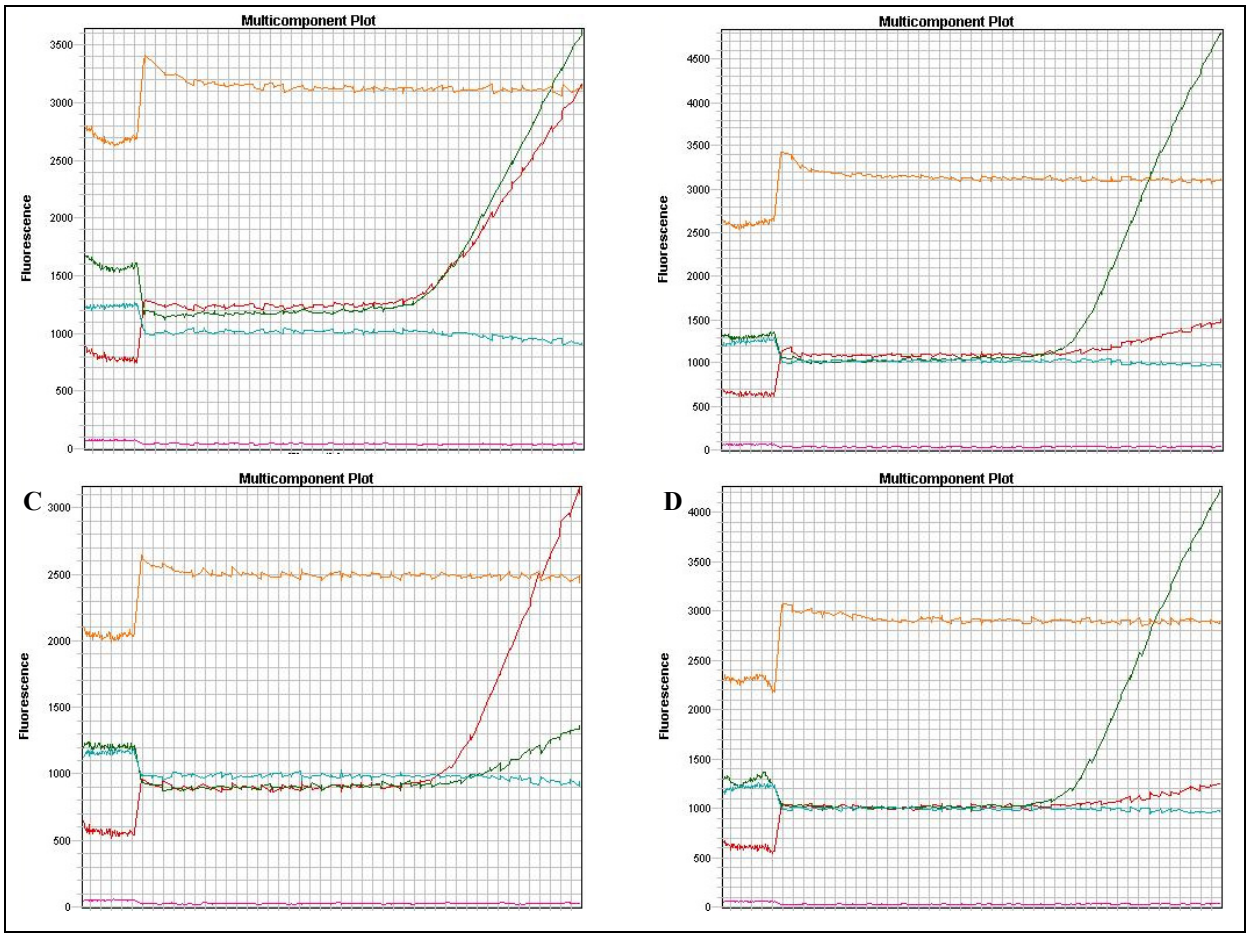


Figure 20 Amplification plots of specific samples analysed via real time PCR A) The R171Q polymorphism detected in genomic blood of a patient bearing multiple endocrine tumors. B) LOH in pancreatic tumor tissue; the wild type 171Q allele is lost. Samples A) and B) are from the same patient. C) LOH in a phaeochromocytoma; the altered allele 171R is lost. D) Healthy individual homozygous for the normal variant CGG. B) and D) look very similar; further experiments for detection of LOH in tumor tissues were carried out. Orange line, ROX. Green line, VIC. Red line, FAM. Blue line, background.

Altogether, 500 DNA samples were analysed in respect to the R171Q polymorphism. 12 out of 500 (2.4%) were tested positive for this alteration. Three out of 98 (3.1%) MEN1 diagnosed patients carried R171Q. The control population, composed of three differently diagnosed groups of patients, showed an incidence of 2.7% (8 out of 302) for this polymorphism. 3.2% of MEN2, 1.4% of CAH and interestingly high 6.1% of IDDM patients carried the R171Q polymorphism, the percentage being not increased in MEN1 patients.

suspected diagnosis	total	heterozygous A/G	homozygous G/G	LOH
CAH	141	2	139	0
IDDM	33	2	31	0
MEN1	98	3	95	0
MEN2	128	4	124	0
tumors	100	1	97	2
total	**500**	**12**	**486**	**2**

Table 2 Summary of all samples tested for R171Q Genomic DNA derived from patients diagnosed with MEN1, MEN2, CAH and IDDM as well as endocrine tumor tissues and neoplasms. Altogether, the R171Q polymorphism was detected in 2.4% of the analysed DNA samples.

Tumor	homozygous (G/G)	heterozygous (G/A)	LOH
Adenomas	4	0	
Bladder carcinoma	1	0	
Carcinoids	6	0	
Cell lines	3	0	
CMLs	2	0	
Gastrinoma	1	0	
Glomus tumor	1	0	
Graves´diseases	8	0	
Insulinomas	7	0	
Islet cell carcinoma	1	0	
Kidney cell carcinoma	1	0	
Liver metastases	1	0	
Macro adenomas	2	0	
MTCs	8	0	
multifocal adenoma	1	0	
neuroendocrine carcinoma	1	0	
neuroendocrine pancreas tumors	4	0	1
neuroendocrine tumors	5	0	
Ovarian carcinomas	3	0	
Paragangliom	1	0	
Parathyroid carcinoma	1	0	
Phaeochromocytomas	9	0	1
Pituitary adenomas	16	0	
Strumas	5	1	
T-cell leukemias	2	0	
Thymus carcinoid	1	0	
Thyroid carcinomas	2	0	

Table 3 Summary of all 100 analysed tumor DNA samples Tumor tissues were derived from different MEN1- and MEN2-specific tumors, thyroid tissue from patients suffering from Graves´ disease or struma and cell lines as well as some non-typical MEN tumors. One neoplasm was detected to carry the R171Q polymorphism; two tumors were undergoing LOH in different alleles.

In addition, 100 tumors were analysed. 13 samples were derived from patients diagnosed with Graves´ disease and struma, both enlargements of the thyroid gland, and thus not typical

tumor tissue. 2 out of those 100 samples were undergoing LOH (2%), one struma was tested positive for the R171Q polymorphism.

4.2 Multiplex ligation- dependent probe amplification

MLPA was used for the detection of LOH in endocrine tumor DNA. The *MEN1* gene is located at chromosome 11q13 and consists of 10 exons. MLPA probes are available for exons 1, 2, 3, 7, 8, 9 and 10. Two tumors that carry the R171Q polymorphism and were undergoing LOH could be identified through polymerase chain reaction followed by restriction digest. To get more information about the dimension of this loss, multiplex ligation- dependent probe amplification was performed on genomic DNA samples as well as on tumors undergoing LOH and reference tumors not affected in 11q13.

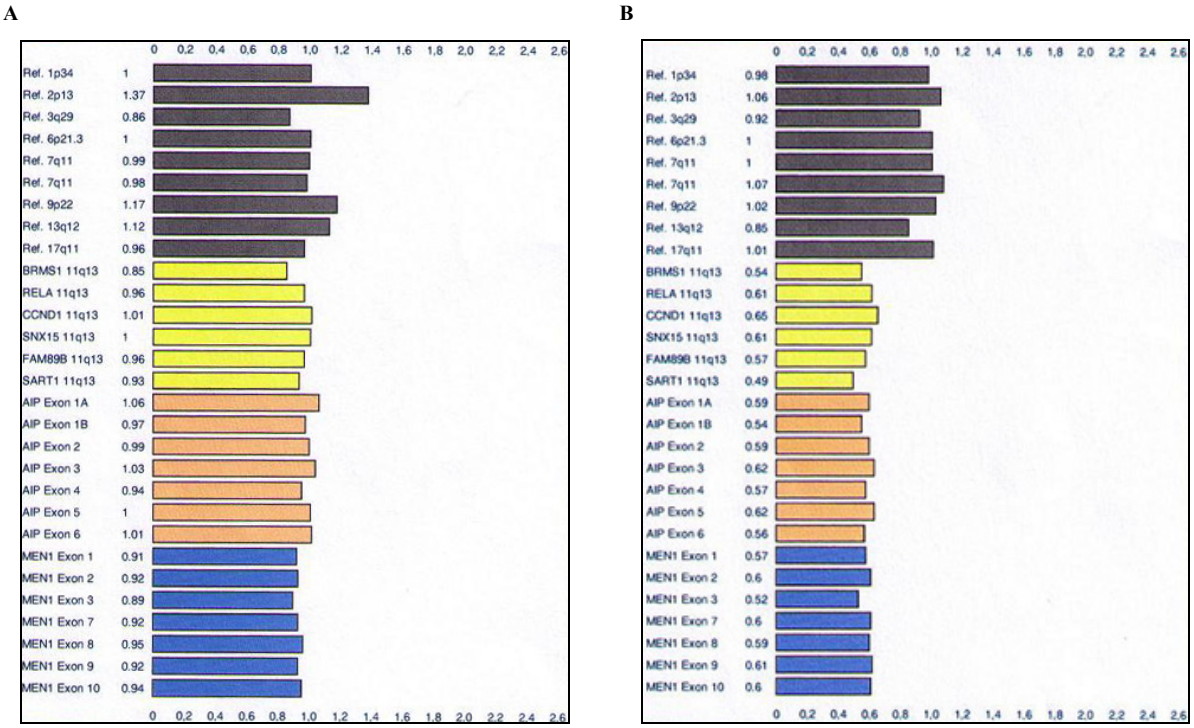


Figure 21 Genomic and tumor DNA isolated from a MEN1 patient bearing the R171Q polymorphism

A) DNA derived from the blood. No deletions or duplications can be detected in the *MEN1* gene. The elevated signal of 2p13 is most likely false positive since all of the samples analysed in the test series showed this increase. B) Pancreatic tumor DNA. Large deletions of several genes at 11q13, including the *MEN1* gene, could be identified.

One patient, heterozygous for the 171 locus, developed multiple endocrine neoplasms including a tumor of the pancreas. This tumor was undergoing LOH and lost the altered allele

171Q. Blood and tumor tissue samples were available and both were analysed with MLPA method. No chromosomal aberrations could be identified in the patient´s genomic DNA. In the tumor DNA, large deletions of multiple genes at the 11q13 locus were detected. Reduction of the *MEN1* probe signals ranged from 40% to 48%. All of the seven reference genes located at 11q13 were also undergoing deletion, resulting in highly reduced probe signals between 35% and 46%.

Another patient, bearing a phaeochromocytoma, lost the wild type allele 171R and showed similar results. Probe signals for *MEN1* were significantly reduced (35% to 47%). Reference genes located at 11q13 also showed reduced probe signals ranging from 35% to 44%. Control genes not located at chromosome 11 were not affected. No blood sample was available from this patient but because of the co-isolation of surrounding tissue not affected from the LOH in the tumor, it could be shown that this patient also carried the R171Q polymorphism.

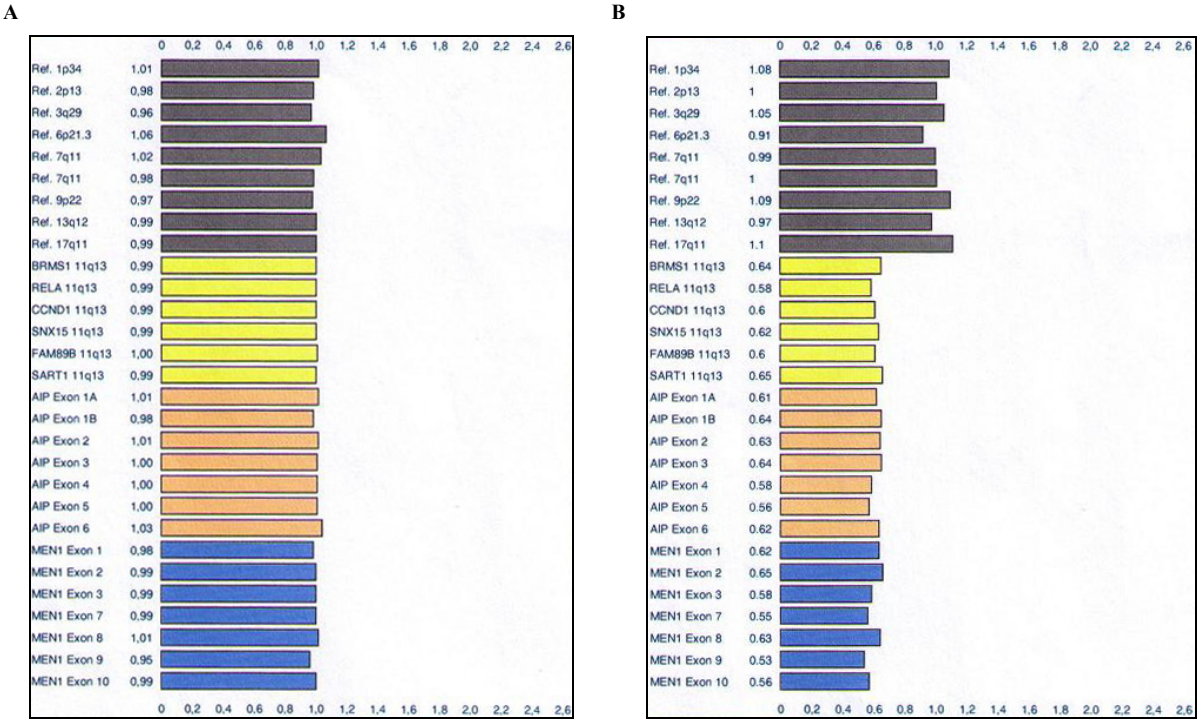


Figure 22 MLPA analysis of a healthy individual and a phaeochromocytoma undergoing LOH A) DNA derived from blood of a healthy individual not affected in the *MEN1* gene. B) LOH in a phaeochromocytoma. Significantly reduced probe signals of genes located at 11q13 indicate large deletions of this region in one chromosome 11.

MLPA analysis revealed interesting chromosomal aberrations in tumors undergoing loss of heterozygosity. Patients developing those tumors were tested positive for the R171Q polymorphism. It could be shown that not only one copy of the *MEN1* gene but also of several

other genes located to this chromosomal region were lost in tumor tissues. Large deletions of this locus could also be due to complete loss of one chromosome 11 in tumor cells. Reference genes located to unrelated chromosomes were not affected. Genomic DNA of one patient showed no chromosomal aberrations whereas highly reduced probe signals of chromosomal region 11q13 could be identified in a pancreatic tumor of the same patient.

4.3 Cycle sequencing of the R171Q polymorphism

Two patients simultaneously carrying the R171Q polymorphism and bearing endocrine tumors have been identified. Since development of multiple endocrine neoplasms and loss of the *MEN1* gene could be also due to disease causing mutations, sequencing of exons two to ten of the *MEN1* gene was performed.

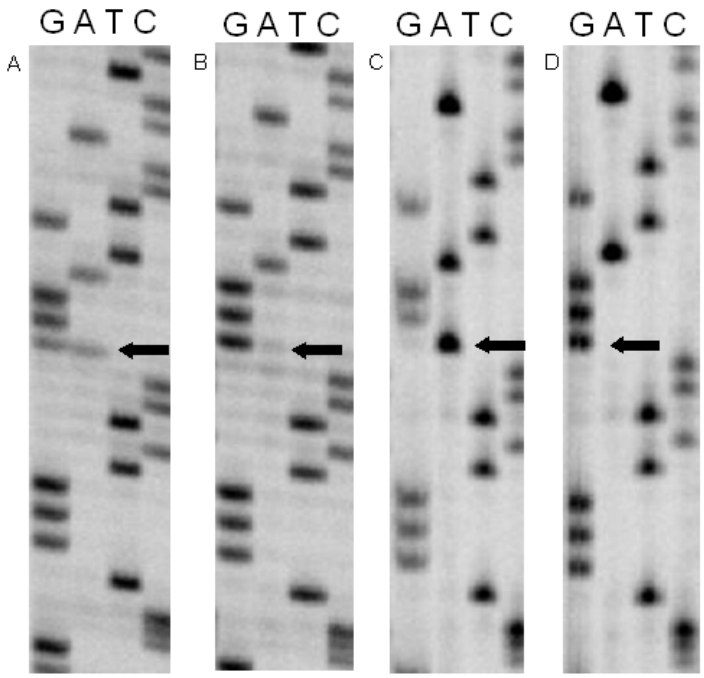


Figure 23 Sequencing of the R171Q polymorphism and loss of heterozygosity in endocrine tumors
A) Heterozygous banding of 176R and 176Q. B) LOH of 176Q visualised as faded A band next to G. Samples A and B are from the same patient C) LOH of 176R in a phaeochromocytoma with almost complete absence of the G band. D) Homozygous allele 176R in a healthy individual.

Sequencing of the genomic DNA and the two tumor samples revealed no further alterations or mutations in the tumor DNA of the phaeochromocytoma. The patient bearing the neuroendocrine pancreatic tumor was tested positively for a second polymorphism. In exon 9

at position 418, the codon GAC was changed into GAG. The polymorphic alteration at locus 171 in exon 3 is visualised as double band, indicating the existence of two nucleotides, guanine and adenine, at the same position. Therefore, the alleles of the *MEN1* gene differ from each other at this specific locus. Loss of heterozygosity could be clearly identified in the phaeochromocytoma. In this case, the tumor lost the wild type allele and the altered variant remained. In the endocrine pancreatic tumor, detection of LOH was not easy due to background staining naturally falsifying the results.

4.4 Cloning of the *MEN1* gene

At position 171 in exon three of the *MEN1* gene, the nucleotide guanine is changed to adenine, which results in the transition of amino acid L-arginine (R) to L-glutamine (Q). Like L-lysine and L-histidine, L-arginine consists of six carbon atoms and is, due to its complex guanidinum group localized to the distal end, counted among the class of basic amino acids. The guanidinum group is positively charged in neutral, acid and weak alkaline environment. The positive charge is de-localised between the amino groups, leading to the formation of multiple H-bonds. L-glutamine is the γ-mono-amide of glutamic acid and is polar and uncharged under all biological conditions. It can spontaneously cyclize and deamidate.
Since those two amino acids are quite different, an exchange could have different consequences for the functions, folding or interactions of the menin protein. The first 725 (R171Q) and 740 (R176Q) nucleotides were amplified with PCR, respectively.

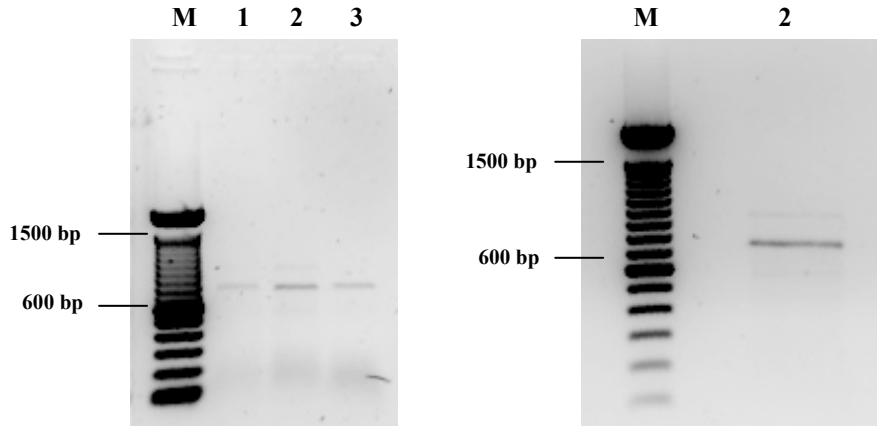


Figure 24 Tumor cDNA was amplified and used for further cloning experiments Three different tumor samples were used as template cDNA. All three could be amplified and visualised on agarose gel. In sample 2, three different bands could be seen. This may be due to differently sized transcripts and splicing variants.

After ligation of the PCR product into a pGEM vector and transformation of this construct into E.coli cells, the obtained plasmid DNA was digested with EcoRI. Two different variations could be visualised and were additionally sequenced.

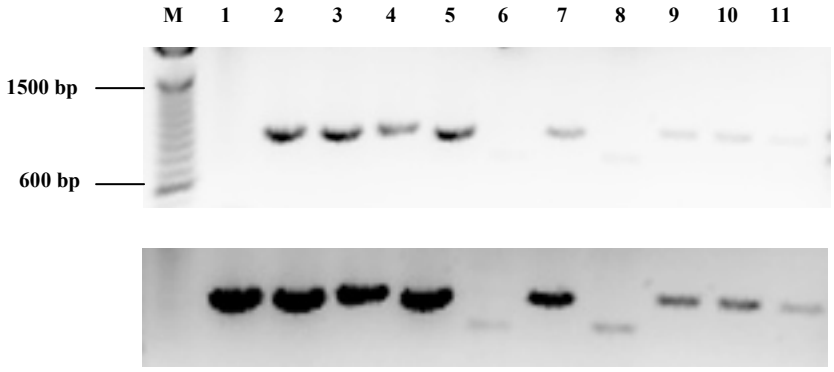


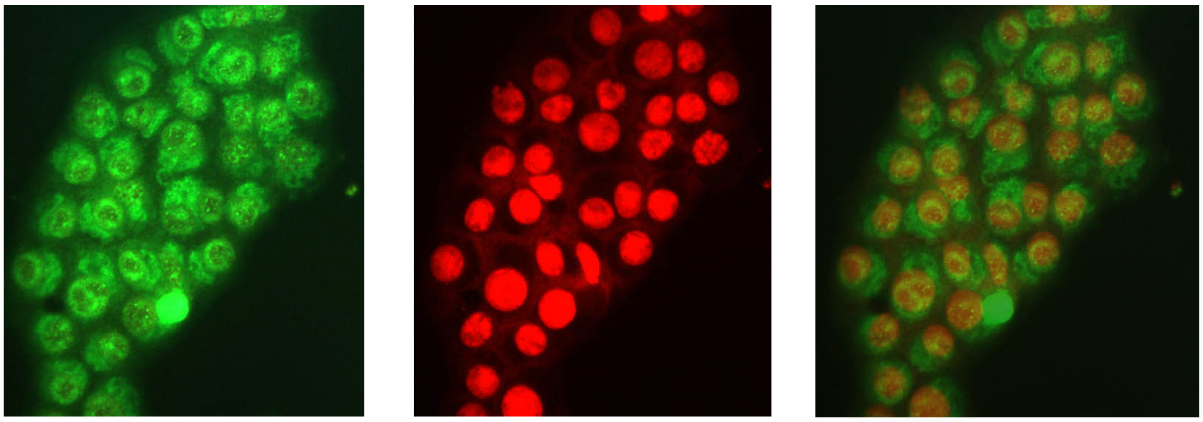
Figure 25 DNA inserts obtained from plasmid vectors multiplied in bacterial cells Sequencing of number 4, 7 and 8 revealed that the larger insert was the one we were looking for.

Based on the genetic analysis of the R171Q polymorphism, no clear evidence for a potential role in the development of a MEN 1 syndrome could be found. Therefore, cloning of the *MEN1* gene was not continued from this point.

4.5 Immunhistochemistry

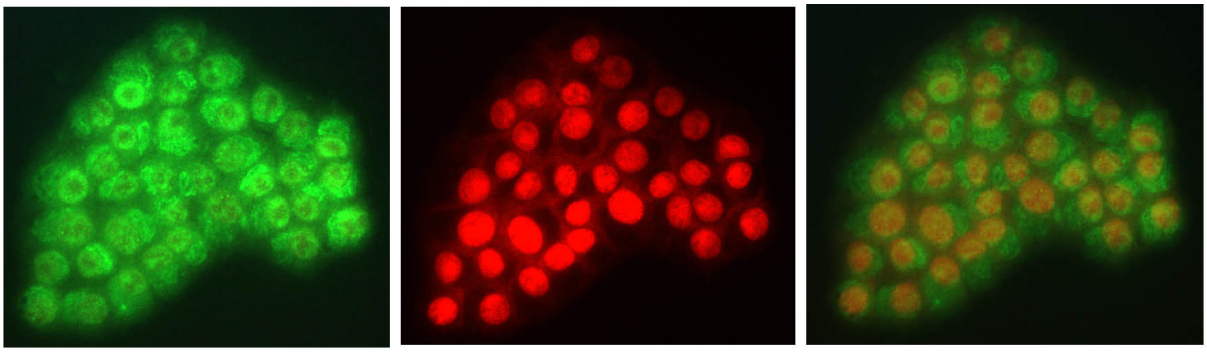
Menin protein is primarly found in the cell nucleus and is able to bind DNA via its nuclear localization signals (NLS) on its C- terminus. Three NLSs are known so far, termed NLS1, NLS2 (68) and NLSa (73). Mutations in NLS1 and NLS2 lead to reduced nuclear localization. Nuclear localization signals are also important in controlling target gene transcription (74). Although menin is seen to be predominantly a nuclear protein, it has several interaction partners localized to the cytoplasm. It remains to be established if the localization of menin is connected to cell cycle progression or if menin also has a small fraction present in the cytoplasm. Another possibility would be that menin may shuttle between the nucleus and the cytoplasm, interacting with cytoplasmic partner proteins.

A **Rat islet tumor cell line I**



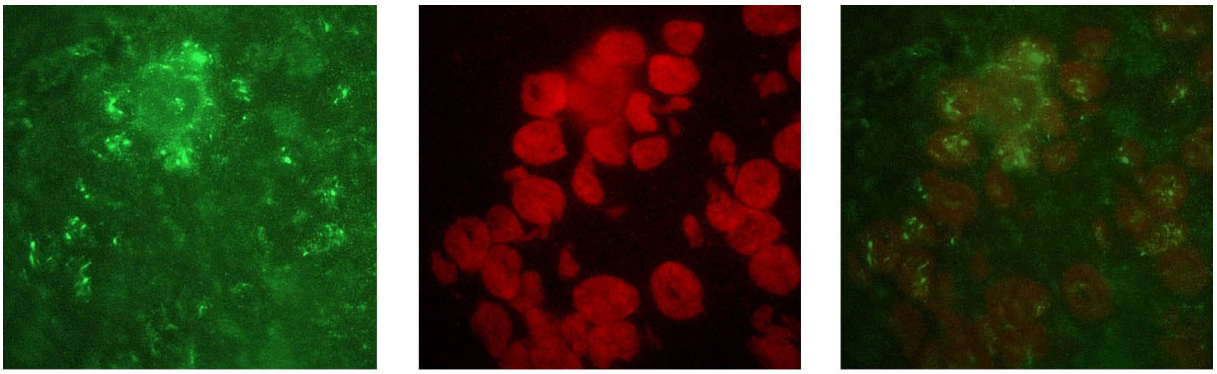
Menin | Propidium Iodide | Menin/ PI merge

B **Rat islet tumor cell line II**



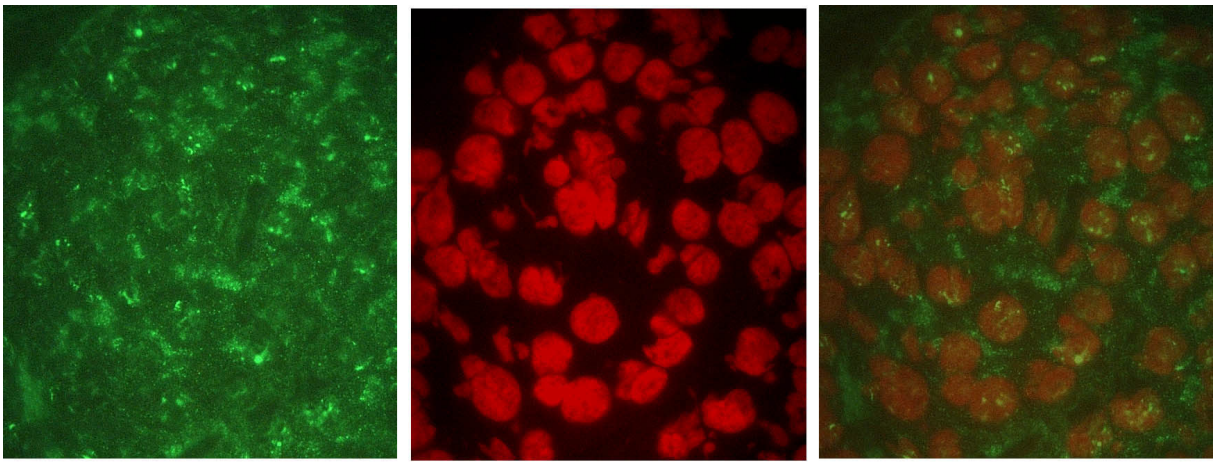
Menin | **Propidium Iodide** | Menin/ PI merge

C **Human pituitary adenoma cells I**



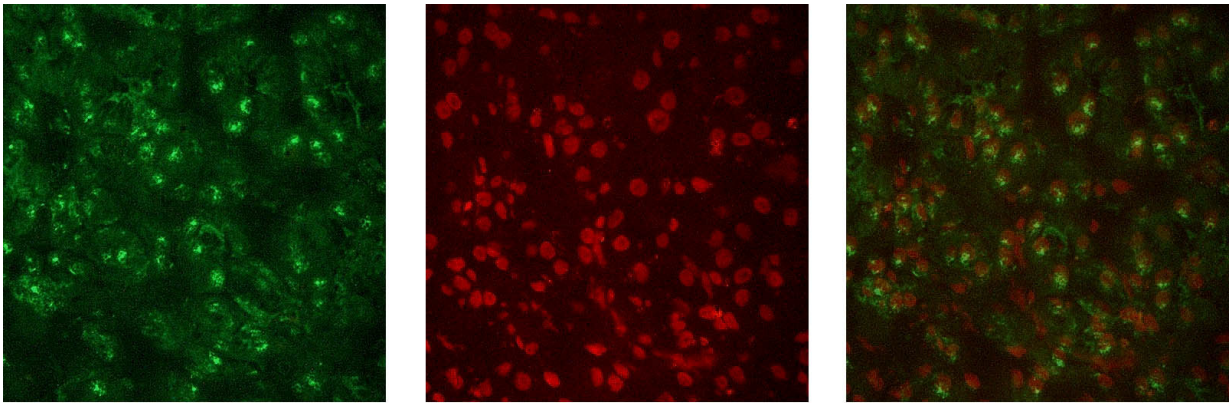
Menin | **TO- PRO- 3** | **Merge**

D **Human pituitary adenoma cells II**



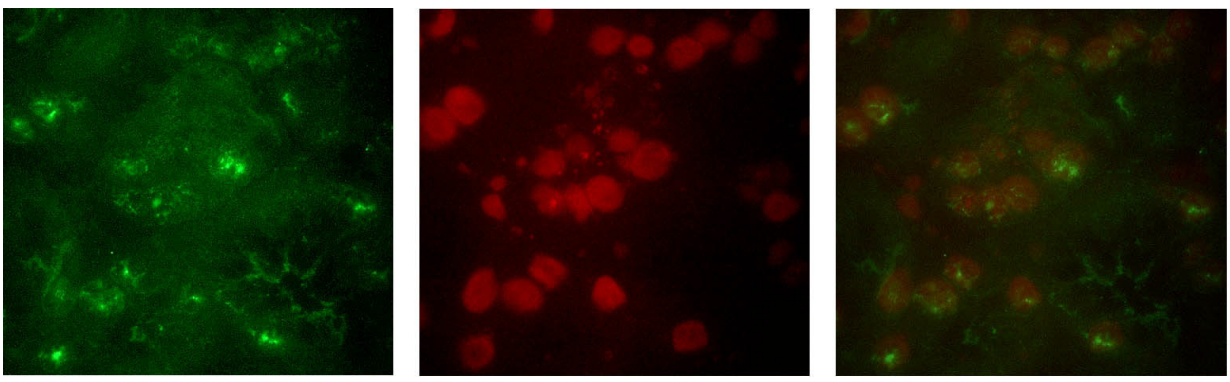
Menin **TO-PRO-3** **Merge**

E **Human insulinoma cells I**



Menin **TO-PRO-3** **Merge**

F **Human insulinoma cells II**



Menin **TO-PRO-3** **Merge**

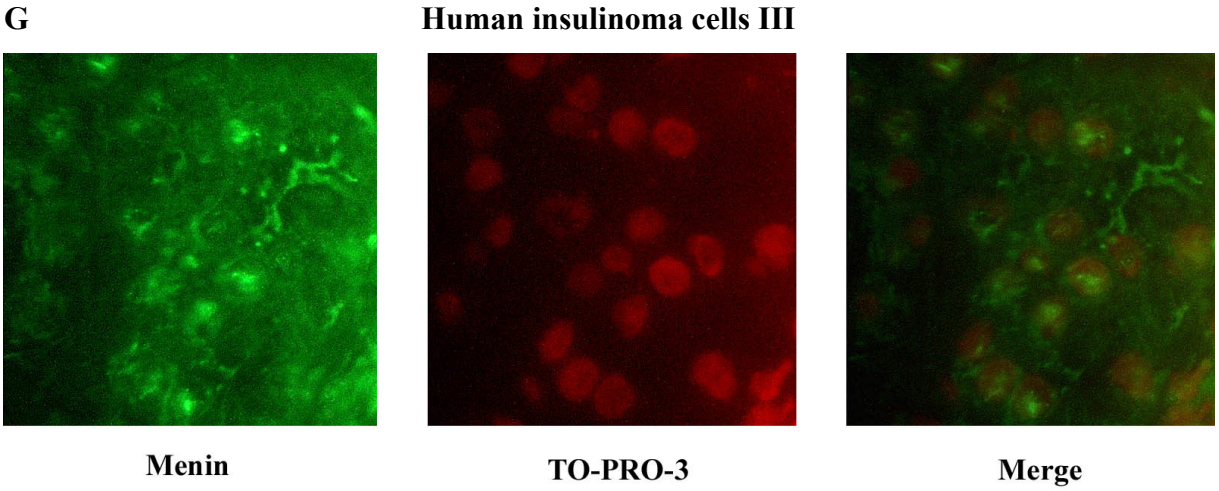
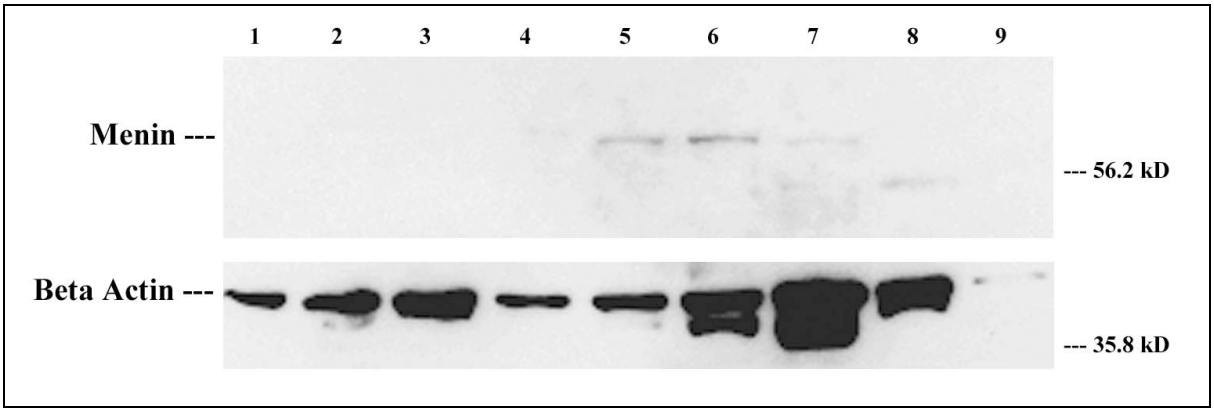


Figure 26 Localization of the menin protein in rat islet tumor cells, pituitary adenoma cells and human insulinoma cells Left panels represent the menin protein expressed in endocrine tumor cells (green), staining of DNA through propidium idode or TO-PRO-3 iodide is shown in the middle (red). Right panels represent merged figures of DNA and menin protein signals. In figures 29.A and 29.B, RIN-F5 cells were analysed, figures 29.C and 29.D represent human pituitary adenoma cells. Figures 29.E, F and G show human insulinoma cells. Original magnification: Fig. A, B and E, fourhoundredfold and Fig. C, D, F and G thousandfold.

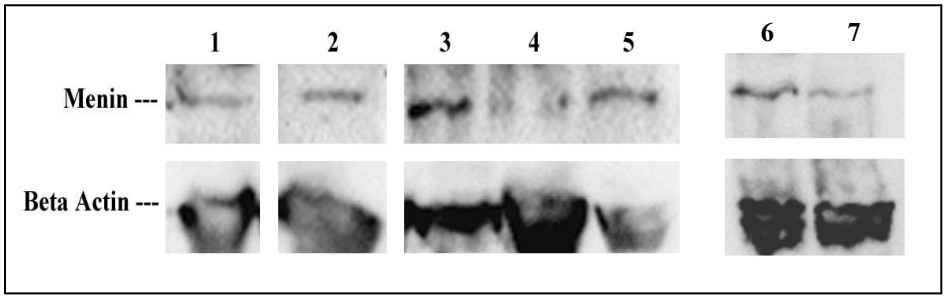
Since endocrine tissue material of human healthy individuals is not available, a rat islet cell tumor cell line, a human pituitary adenoma and human insulinoma cells were used for antibody staining. Due to the fact that mutations in the *MEN1* gene and subsequent reduction of the menin tumor suppressor protein are uncommon reasons for endocrine tumor formation, tumor tissue material could be used for the detection of menin protein. It could be shown that approximately 80- 90 % of menin is localized in the cell nucleus. Signals of menin located to the cytoplasm may be due to degradation processes or transient interaction with cytoplasmic partner proteins. Cytoplasmic menin localization seemed to be increased in rat insulinoma cells but this could also be due to specific features of this cell line or due to the different organism used for detection. Intense signals of the menin protein in human pituitary adenoma and insulinoma cells could be almost entirely detected in the cell nucleus region. Faint signals could also be due to background staining or cross-interactions with other proteins.

4.6 Western blot analysis

A



B



C

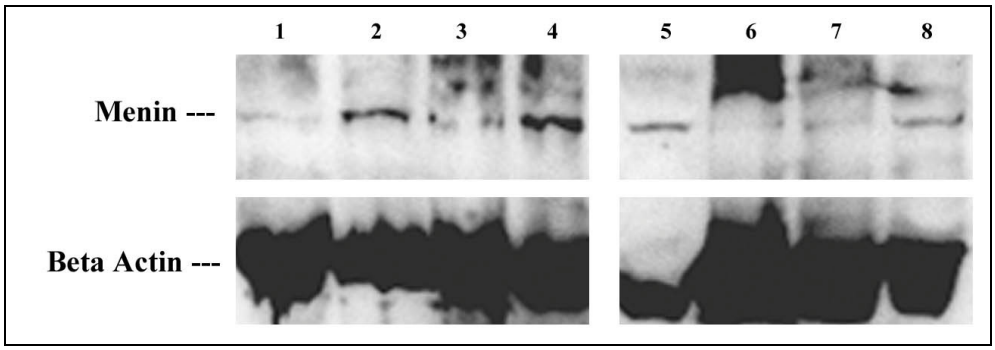


Figure 27 Menin protein detection in different endocrine tumors A) Cell lysates of pituitary adenomas (1, 4, 5, 6), carcinoids (7, 8, 9) and ovarian carcinoma (2). Protein isolation was performed according to 3.2.8; nuclear proteins cannot be detected in high quantities. **B)** Protein extracts of phaeochromocytomas (2, 5), neuroendocrine pancreatic tumors (3, 4, 6), a MTC (1) and a paraganglioma (7) were analysed. **C)** Protein extracts of neuroendocrine pancreatic tumors (2, 3, 4), a MTC (8), a pituitary adenoma (6), a paraganglioma (1) and phaeochromocytomas (5, 7) was performed. Two samples were loaded twice, B6=C3 and B7=C1. Proteins in B) and C) were extracted using TRIzol method. The molecular weight of menin is 68 kD, the molecular weight of beta- actin is 42 kD. Beta- actin, localized to the cytoplasm, was used as loading control.

Menin is a ubiquitously expressed tumor suppressor protein that can be reduced in specific endocrine tumors. Since expression of menin can strongly vary in endocrine neoplasms, expression in different MEN1- related tumors was determined using western blot analysis. Menin can be predominantly detected in the cell nucleus and therefore proteins were extracted using TRIzol reagent. This method ensures break down of the cell nucleus and release of nuclear proteins. Disadvantage of this procedure is the formation of a highly compacted pellet, making resuspension of the protein difficult. No control samples could be analysed since proteins of healthy endocrine organs were, like said before, not available.

4.7 Quantitative real time PCR

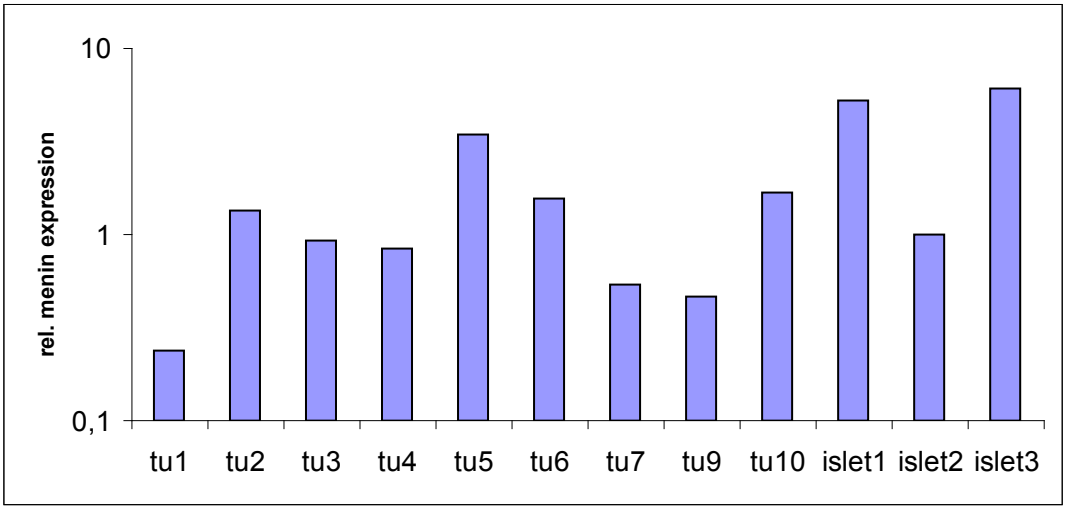


Figure 28 Relative menin expression in insulinomas and healthy islets of Langerhans Relative quantification of *MEN1* expression was performed in respect to the *GAPDH* expression. Islet sample number 2 was defined as reference. Samples were loaded as duplicates.

RNA expressed in insulinoma cells and unaffected islets of Langerhans was transcribed into complementary DNA and amplified by real time PCR. Fluorescent-labeled probes specific for *MEN1* bind their target DNA and can be detected after each amplification step. The intensity of the fluorescent signal is equal to the amount of expressed RNA. The level of expressed RNA is then comparable to the amount of expressed menin tumor suppressor protein. Nine tumors and three islets of Langerhans were analysed. The second islet was used as reference. Tumors 1, 7 and 9 have noticeable decreased levels of *MEN1* expression.

5. Discussion

5.1 The MEN1 syndrome is specified by several criteria

Multiple endocrine neoplasia syndrome type 1 is an autosomal dominantly inherited disease and is connected to the development of multiple tumors in the parathyroid, anterior pituitary gland and enteropancreatic endocrine tissue. *MEN1* is a tumor suppressor gene and mutations can lead to an altered menin protein, abolishing the protective impact of the tumor suppressor (69). MEN1 patients usually carry one mutated gene that they have inherited or acquired by a de novo mutation. Tumor formation begins after loss of the remaining wild type allele ("two-hit" model) (18). It is important to note that only a minor percentage of all endocrine gland hyperactivities and tumors are related to this *MEN1* gene associated alterations. Therefore it has become state of the art in clinical endocrinology that certain criteria have to be fulfilled before individuals bearing an endocrine tumor have to be tested at the *MEN1* locus. The main diagnostic criteria of a MEN1 syndrome are the familial association of hyperplasia or neoplasia formation at more than one of the previously mentioned endocrine sites and/ or a documented missense mutation at the *MEN1* locus in germline. In contrast, it has been well demonstrated that in sporadic tumors at typical *MEN1* sites somatic mutations and even loss of heterozygosity (LOH) might occur. However this does not represent a MEN1 syndrome as no germline alterations are present at the *MEN1* locus in those individuals.

5.2 The R171Q polymorphism is found in the general population

By genotyping tumor DNA derived from endocrine tumor tissues, two samples were shown to undergo LOH and both were positively tested for the R171Q polymorphism. Interestingly, both alleles were observed to undergo LOH. In the endocrine pancreatic tumor, the altered 171Q was lost and in a phaeochromocytoma, it was the wild type allele 171R. It is of note, that a phaeochromocytoma is not a typical tumor associated with mutations in the *MEN1* gene and the respective MEN1 syndrome, but is common in multiple endocrine neoplasia type 2 (MEN2).

The R171Q polymorphism could be identified in 2.4% of all analysed samples of blood and tumor tissue, including the control populations not affected with MEN1 syndrome. The result slightly increases to 2.75% if only the genomic samples are included. This percentage was not increased in individuals diagnosed with MEN1 syndrome by clinical criteria. These results add further expanded data (increased sample size and clinical observation period) to the report of an Italian group who claim that this particular alteration might be of relevance for the development of a MEN1 syndrome, although with low penetrance. This is discussed in detail further down. A higher incidence could be identified in patients suffering from insulin dependent diabetes mellitus (6%). To clarify whether this observation has a role in IDDM, which has so far not been reported, in depth studies will be necessary.

Analysis of 400 blood samples revealed the R171Q polymorphism to be present in the general population and not to be restricted to MEN1 patients. The medical history of control subjects tested positively for this polymorphism was analysed and no evidence of a MEN1 syndrome could be found in any of those patients. However, it should be considered that the mean age of the largest control population, the congenital adrenal hyperplasia (CAH) patients, was 35.6 years in contrast to the mean age, 52.7 years, of the MEN1 patients. Since benign tumor formation is assumed to take many years and symptoms of MEN1 occur during the second half of life, the development of future endocrine tumors in those young CAH patients cannot be excluded. In this context it is of note that medical records of hospitals other than the General Hospital of Vienna could not be accessed. The mean age of the control population MEN2 was 54.2 years.

5.3 Loss of heterozygosity is observed in two endocrine tumor samples

Analysis of 100 tumor samples revealed two endocrine tumors that were both positively tested for the R171Q polymorphism and undergoing loss of heterozygosity. LOH implicates loss of the functional wild type copy of a tumor suppressor gene in tumor cells. The altered non-functional copy remains and tumor formation is triggered. One LOH was identified in a pancreatic endocrine tumor, another in a phaeochromocytoma, the latter not being a typical MEN1-tumor but occasionally found in MEN1 patients. Interestingly, both 171 variants were undergoing LOH. In the pancreatic tumor, the altered allele 171Q (Fig. 19, lane 3) and in the phaeochromocytoma the wild type variant 171R was lost (Fig. 19, lane 6). Since both alleles, 171R and 171Q, were alternatively lost, a connection between the R171Q polymorphism and development of multiple endocrine tumors due to a MEN1 syndrome is unlikely. If this polymorphism would have impact on tumor formation, both patients would have lost the functional wild type copy and only the remaining altered allele would have been be detected.

5.4 Sequencing reveals further alterations in one tumor sample

Sequencing of exons two to ten of the *MEN1* gene was performed on tumor samples of patient one, bearing the endocrine pancreatic tumor, and patient two, bearing the phaeochromocytoma, as well as on the genomic blood sample of patient one. In the phaeochromocytoma, loss of the wild type allele could be easily identified. In this tumor, only the altered CAG signal was seen, the common CGG variant was fainted (Fig. 23.C). Analysis of the genomic DNA from patient one could clearly confirm the results obtained with genotyping methods, the subject carried the R171Q polymorphism (Fig. 23.A). Loss of heterozygosity of the altered allele was not easy to detect. Comparison to the most common variant of this locus, homozygous 171R, showed no clear results. On the one hand, the faint signal of the A band could be due to co-isolation of surrounding tissue material or on the other hand, just background staining of the sequencing reaction (Fig. 23.B). However, since the LOH was detected with genotyping methods before, there should be no doubt about its presence.

Sequencing of the genomic DNA and the two tumor samples revealed no further alterations or mutations in the tumor DNA of the phaeochromocytoma. The patient bearing the neuroendocrine pancreatic tumor was tested positively for a second polymorphism. In exon 9

at position 418, the codon GAC was changed into GAG. This led to the exchange of amino acid histidine to glutamine. Alterations of this locus were observed before (128, SNP cluster ID rs2071313) but this SNP could only be determined in that particular patient.

Furthermore, two relatives of a potential index MEN1 patient were identified as heterozygous 171 carriers. Unfortunately the individual exhibiting symptoms typical for MEN1 syndrome was not available anymore. Both tested individuals had no clinical signs of endocrine disorders at the age of 47 and 56, respectively. No further conclusion can be drawn from this medical history data. A ten year clinical follow up might provide higher certainty. However we anticipate that those two patients will not develop a MEN1 syndrome.

5.5 Chromosomal region 11q13 seems to be strongly affected

Multiplex ligation- dependent probe amplification (MLPA) was used for the detection of deletions or duplications in the *MEN1* gene. Although MLPA worked very well with genomic DNA samples, tumor DNA analysis had to be adapted until clear results were obtained.
Both samples carrying the R171Q polymorphism were undergoing complete loss of the respective *MEN1* gene (Fig. 21.B and 22.B). Furthermore, probes specific for genes located at chromosome 11q13 showed highly reduced signals as well, whereas control genes located to unrelated chromosomes were not affected. This means, that both individuals tested positive for the R171Q polymorphism might suffer from loss of a large part of chromosome 11q13. This could also mean, that not only the *MEN1* gene was lost in tumor cells but also several genes located to this chromosomal region or even the entire chromosome 11. These findings correlate with published data of Perren *et al.* (129). Fluorescence in-situ hybridisation was performed using specific probes for the centromere of chromosome 11 as well as for the *MEN1* gene. In microadenoma cells, only one signal of each probe could be detected (Fig. 14), indicating large deletions of one copy of chromosome 11.

5.6 The menin protein is variously expressed in endocrine tumors

Using western blot analysis, different expression levels of the menin protein in specific endocrine tumors could be visualized. Detection of the control protein beta- actin but not of menin could have several reasons. One possibility was, that the subject actually suffered from MEN1 syndrome and underwent complete loss of the tumor suppressor protein or just had an undetectable low expression. It could also be due to methodological problems of the preparation of tumor samples, degradation processes, unsuccessful menin isolation or weak protein redissolution. Another explanation may be the expression of different menin variants. As depicted in figure 30.A (samples 7 and 8), two different bands could be visualized. In sample 8, no expected sized menin but a protein with lower molecular weight could be detected. The same band is visualized in sample 7, in addition to the common menin protein signal.

Figures 30.B and 30.C showed menin expression using a different isolation method ensuring extraction of nuclear proteins. Especially neuroendocrine pancreatic tumors (67%) showed high levels of menin expression (Fig. 30.B, lanes 3 and 6; Fig. 30.C, lanes 2 and 4). Phaeochromocytomas and a MTC (Fig. 30.B, lane 5; Fig. 30.C, lanes 5 and 8) had also elevated levels of menin in comparison to other tumors. Very low expression could be shown in pituitary adenomas (Fig. 30.B, lane 4; Fig. 30.C, lane 6), a phaeochromocytoma (Fig. 30.C, lane 7) and a paraganglioma (Fig. 30.B, lane 7 or fig. 30.C, lane 1). Expression patterns of menin protein in endocrine tumors depend on preparation methods and antibodies used for menin detection and therefore cannot be properly compared to already published data.

It is already known (112, 113), that the transcript of the *MEN1* gene as well as the menin protein can vary in size but based on those results, it is not possible to differentiate between menin variants and degradation products.

Relative expression of the *MEN1* gene in different insulinomas as well as unaffected islets of Langerhans was determined using quantitative real time PCR. Tumors 1, 7 and 9 show significant reduction of gene expression. Relative to the expression rate of islet number 2, tumor one showed only 24%, tumor seven 54% and tumor nine 46% of gene expression. Tumors 2, 5, 6 and 10 revealed higher MEN1 expression than the reference sample (135%, 346%, 156% and 170%). Patients bearing those insulinomas were not affected from MEN1

syndrome or mutations in the *MEN1* gene. Since the two other reference islet samples showed five fold higher expression, menin seems to be unequally expressed in healthy islets of the Langerhans as well as in endocrine tumors. Differences in expression level where not age or gender related.

5.7 The menin protein is frequently found in the cell nucleus

Detection of the menin protein by fluorescent-labeled antibodies showed nuclear localization in around 90% of the cells. A human pituitary adenoma, a human insulinoma as well as rat islet tumor cells were used for detection. Cytoplasmic localization of menin in rat tumor cell line cells seemed to be increased. In human tissue samples, intense signals of the menin protein could be almost entirely detected in the cell nucleus region. Faint signals could also be due to background staining or cross-interactions with other proteins. The most evidentiary picture is shown in figure 26.E. The four hundredfold magnified part of a human insulinoma reveals strong menin signals exclusively in the cell nucleus.

5.8 Previous literature implicates possible role of R171Q in tumor formation

De Carlo *et al.* identified an Italian family affected with endocrine diseases. The family members were all tested positive for the R171Q polymorphism. A 46-year-old woman suffered from multiple endocrine tumors and a MEN1 syndrome was diagnosed. A few years earlier, her sister was undergoing surgery of a pituitary adenoma and a parathyroid adenoma, typical MEN1 associated tumors. Her father died of liver cirrhosis but was affected with recurrent gastric ulcerations and a paternal aunt died for a probable pituitary tumor. Genetic analysis of the patient, her two sisters, the brother and the brother´s daughter revealed the R171Q polymorphism in all tested individuals. The brother and his daughter showed no clinical signs. In a different family (mother, two sons and the father), the R171Q polymorphism could also be identified. The mother and one son suffered from pituitary adenomas. 50 healthy Italians were used as control population and the R171Q polymorphism could not be detected.

Balogh *et al.* analysed 32 peripheral blood derived DNA samples of patients diagnosed with MEN1 syndrome or suffering from MEN1-related neoplasms. Six out of those index patients were tested positively for the R171Q polymorphism (18.75%). Three individuals carried additional missense mutations, one carried a further polymorphism and two patients with sporadic MEN1-related state were only bearing the R171Q polymorphism. Family screening was only performed in first-degree relatives of index patients with disease-causing *MEN1* gene mutations.

5.9 Impact of the R171Q polymorphism on the development of a MEN1 syndrome

In conclusion, the results underline that the R171Q alteration rather represent a polymorphism with lower relevance than a MEN1-causing mutation. 400 genomic and 100 tumor samples were analysed and an overall incidence of 2.4% and 3% for this polymorphism could be determined, respectively. The low incidence of the R171Q polymorphism is in contrast to the small size of the control population used by De Carlo and the analysis of 50 healthy individuals is not representative. Balogh *et al.* revealed a higher incidence of 18.75% in patients bearing different endocrine tumors. This is in contrast to the MEN1 site tumor incidence of 3% revealed in the present work. Furthermore, it is of note that data from NCBI exist, showing an incidence of 1.9% for the R171Q polymorphism. 17 out of 892 analysed individuals carried this alteration, which led them to the conclusion that this protein alteration represents a polymorphism and is not to be associated with MEN1 syndrome. In these premises the results obtained for the present work seem to be in accordance with published data at the NCBI. However, we cannot eliminate the possibility that some of the NCBI individuals were below MEN1- tumor formation age.

Nevertheless, due to the published data of De Carlo and Balogh as well as the identification of a phaeochromocytoma undergoing LOH of the 11q13 locus and only bearing the R171Q polymorphism, this alteration seems to participate to endocrine tumor formation in some rare cases and perhaps in combination with other, yet unidentified, factors. Since some of the subjects carrying the R171Q polymorphism might be currently too young to develop MEN1-related tumors, a final conclusion cannot be drawn yet. A clinical follow up of those carriers could reveal further knowledge in this respect and is suggested.

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Lab experience

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