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Molecular Genetics of Neuroendocrine Tumors

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Abstract
Through insights into the molecular genetics of neuroendocrine tumors (NETs), the genes predisposing to multiple endocrine neoplasia (MEN) syndromes were identified. In MEN1, tumors occur in the parathyroids, endocrine pancreas, anterior pituitary, adrenal glands and thymic neuroendocrine tissues. The *MEN1* gene encodes a putative growth-suppressor protein, *menin,* binding JunD, a transcriptional factor belonging to the AP-1 complex. However, new partners binding menin remain to be found. The *MEN1* gene might be involved in 1–50% of sporadic NETs. Another critical mechanism involved in NETs is the deregulation of the RET-signalling pathways by oncogenic point mutations responsible for MEN2 syndromes. MEN2 refers to the inherited forms of medullary thyroid carcinoma. The *RET* proto-oncogene, a tyrosine-kinase receptor, is activated by missense mutations occurring either in the extracellular dimerization domain or intracellular tyrosine kinase catalytic regions. In both cases the receptor is constitutionally activated in the absence of natural ligands. Endocrine tumors also belong to the clinical pattern of Recklinghausen (NF1) and von Hippel-Lindau (VHL) diseases. The genes for both syndromes have been characterized and provide new pathways for endocrine tumorigenesis related to G-protein physiology *(NF1)* and transcriptional regulation and/or endothelial cell proliferation *(VHL)*, respectively. Here, we propose a basic overview of recent data on genetic events leading a normal endocrine cell towards a fully malignant phenotype.

Basic Pathways Related to Genetic Predisposition

Neuroendocrine tumors (NETs) occur mainly in five independent autosomal dominant inherited syndromes for which the genetic pathways have recently been characterized. Multiple endocrine neoplasia (MEN) types 1 and 2 are the most common forms with high penetrance of various neuroendocrine proliferations. NETs were less commonly observed in von Hippel-Lindau (VHL) disease, Recklinghausen neurofibromatosis (NF1) and tuberous sclerosis (TSC). Other genetic syndromes characterized by single or multiple endocrine tumors were identified and mapped in the last decade, but the genes related to these diseases, such as Carney complex, non-MEN1 familial isolated hyperparathyroidism (FIHPT), Conn adenoma, and pituitary tumors, remain to be identified.

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NET Related to Inactivation of a Growth-Suppressor Gene, MEN1

MEN1 (OMIM 131100) is an inherited disease predisposing parathyroid hyperplasia/adenoma, pancreatic endocrine tumors, pituitary tumors, adrenocortical secreting or nonfunctional tumors and thymic NETs [1, 2]. Recent observations suggested that a MEN1 patient could also be affected by cutaneous or visceral (angio)lipoma and fibrosarcoma [3] and central nervous system tumors such as meningioma or ependymoma [4, 5]. The diversity of MEN1-related lesions and the embryonic origins of affected tissues suggest that the *MEN1* gene might play a critical role in early embryogenesis. Larsson et al. [6] first localized the *MEN1* gene in 1988 on the long arm of chromosome 11, band q13. Comparative genetic analysis of tumoral and constitutional genotypes with polymorphic DNA markers in 11q13 showed somatic loss of heterozygosity (LOH) suggesting that development of MEN1-associated tumors was a two-step process, a germline mutation affecting the first *MEN1* allele, and a somatic inactivation of the unaffected allele occurring by LOH [6, 7]. This suggests that tumorigenesis in MEN1 involves loss of function of a growth-suppressor gene according to the two-hit model by Knudson [8]. The *MEN1* gene was finally cloned in 1997 after 10 years of physical and genetic mapping [9, 10]. The gene spans 9 kb of the genomic DNA and contains 10 exons encoding a 610-amino acid protein, *menin*. The first exon and the 3′ 832-bp part of exon 10 are untranslated. A 2.8-kb major *MEN1* transcript was detected in all human tissues tested, including the pancreas, thymus, adrenal glands, thyroid, testis, leukocyte, heart, brain, lung, muscle, small intestine, liver and kidney [9, 10]. A large 4-kb transcript was identified in the pancreas, thymus and stomach and suggests a tissue-specific alternative promotion [10].

Menin is a nuclear protein containing two nuclear localization signals (NLSs) at codons 479–497 (NLS-1) and 588–608 (NLS-2) [11]. NLS-1 and NLS-2 have been defined by in vitro functional studies using *menin* deletion constructs and epitope tagging with enhanced green fluorescent protein in different subcellular fractions. Even if mostly present in the nucleus, *menin* was recently found to translate from the nucleus to cytoplasm during the cell cycle, the cytoplasmic transfer being observed during mitosis [12, 13]. No nuclear export signal or DNA-binding transmembrane or transactivation domains have been identified to date, even though protein structure software (PROSITE, SOPMA) predicted high hydrophobicity in the NH_2-terminal half of the protein, suggesting at least three leucine-zipper and helix structures in the primary/secondary organization of *menin* [9, 10].

The murine orthologue of human *MEN, Men1,* was mapped to the pericentromeric region of MMU19 (murine chromosome 19) and consequently shown to have a similar organization as the human gene [14]. Two major transcripts of 3.2 and 2.8 kb, with and without intron 1, respectively, were detected in both embryonal and adult tissues. The predicted murine protein is 611 amino acids in length. Overall, it is 97% homologous to the human *menin*. The murine *Men1* gene was subsequently cloned by 3 independent groups and the rat *Men1* gene also identified [15–17]. In situ mRNA hybridization and Northern blot studies showed the pattern of *Men1* expression during mouse development. The expression of *Men1* was detected at gestational day 7 in the whole embryo and a strong expression in the thymus, liver, CNS and testis at day 17. Hence, *Men1* expression was not only confined to endocrine organs [14, 15]. The high expression of *Men1* in the testis and mainly in Sertoli cells could assess a critical function of *menin* in reproduction.

Menin binds *JunD,* a transcription factor acting through the activator protein-1 (AP1) complex [18]. This interaction appeared specific and *menin* did not bind to other members of the AP1 family. Wild-type *menin* repressed transcriptional activation mediated by JunD by an in vitro cotransfection assay. Interacting regions of *menin* and JunD were defined by deletion mutants. Three major domains were crucial for the *menin*-JunD interaction, the 40 amino acids from the N-terminus of *menin,* and two central regions at positions 139–242 and 323–428. Four amino acid residues at positions 139, 160, 176 and 242 should be conserved for a normal *menin*-JunD interaction. A putative role of the C-terminus end of *menin* remains controversial [19]. *Menin* -JunD binding required the N-terminus region of JunD, which supports the activation domain of the protein through an interaction with a co-activator, JAB1 [20]. Recently, it has been shown that *menin*-mediated repression of JunD transcriptional activity is relieved by a specific inhibitor of deacetylase, suggesting that deacetylation of histones could play an essential role in this pathway [19]. Lastly, *menin* has been shown as a true in vitro growth suppressor after overexpression in *Ras*-transformed NIH3T3 cells [21].

More than 300 different *MEN1* germline mutations have now be identified in several independent national studies based on large series of MEN1 patients [22–27]. Figure 1 shows the spectrum of most *MEN1* mutations identified in France and recurrent mutations described by other groups. Unequivocally, the mutations were spread

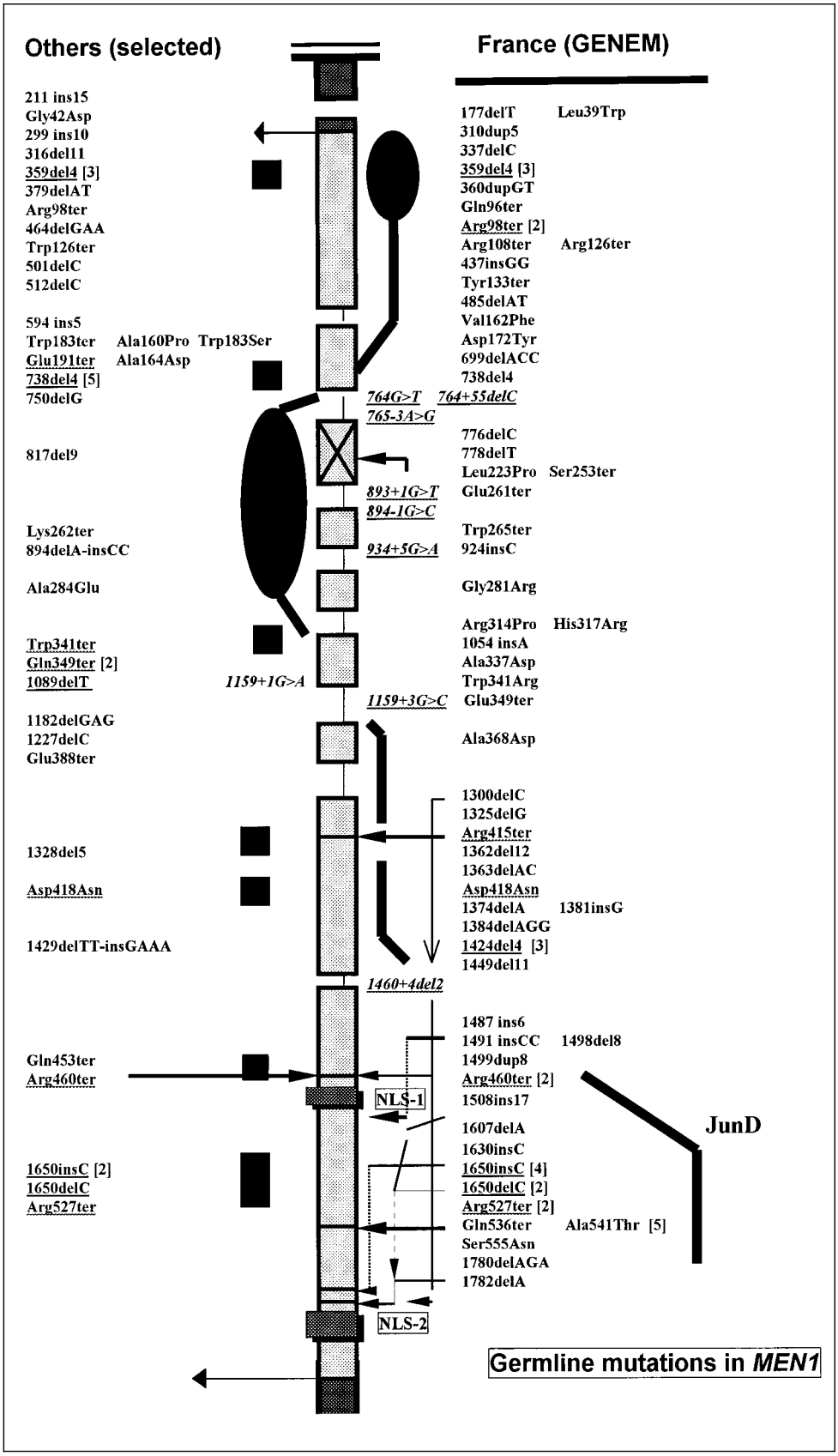

Fig. 1. Germline mutations in the *MEN1* gene according to the menin–JunD binding domains. Most mutations were identified in French families through the GENEM network. Mutations are represented according to the international nomenclature, i.e Val162Phe is a missense at codon 162; Arg460ter is a nonsense at codon 460; 1149del11 is a frameshift deletion beginning at nucleotide 1449. ins = Insertions; splice-mutations are in italics. The underlined mutations and black boxes at the left of the gene represent relative hotspots observed in the French series of mutations and by other groups [22–27]. The JunD protein is represented by a bold line and the interacting regions with menin by large circles near the N terminus and the central region of the *MEN1*-encoded sequence. NLS1 and NLS2 designate the two nuclear localization signals identified in exon 10. The introns are not fully represented.

over the entire coding and noncoding sequences of the *MEN1* gene without significant clustering related to known functional domains of the protein. Approximately 70% of the *MEN1* germline mutations were nonsense and (deletional or insertional) frameshift-truncating mutations. Missense substitutions and in-frame deletions/insertions represented ≈20–25% of the mutations. Intronic and splice-junction mutations were reported in a few families and some of them were shown to alter RNA splicing with an abnormal exon skipping or intronic retention [28, 29]. Most mutations occurred once, while some of them were observed twice or more in apparently unrelated families [22–27]. Haplotype analysis with 11q13 polymorphic markers demonstrated that most recurrent mutations were not related to a founder effect assessing the data from two independent linkage disequilibrium studies performed before the *MEN1* gene was cloned [30]. Recently, two independent groups analyzed the 11q13 haplotypes in families with recurrent mutations and showed that the same mutation occurs commonly in genetically unrelated families [31, 32]. Conversely, a common founder effect characterized by a single mutation and common haplotypes has been reported in four kindreds from Newfoundland expressing the prolactinoma variant of MEN1 ($MEN1_{BURIN}$) [33]. In specific regions of the gene, hot spots involving a cytosine or a guanine might be explained by replication errors related to slipped-strand mispairing in unstable and/or repetitive motifs with an high GC content, such as the 1650del/insC in exon 10 [34]. Based on exhaustive analysis of patients with sporadic MEN1 and both their parents, Bassett et al. [24] estimated the rate of neomutations occurring in *MEN1* to 10–15%. Most, if not all reported *MEN1* germline mutations which alter or delete a single amino acid in humans occurred at residues highly conserved between human and rodents, suggesting thereby a functional/pathogenic significance. No genotype-phenotype correlations have been established to date. Despite extensive studies, we and others did not succeed to find a relation between the type and location of *MEN1* mutations and the clinical features of MEN1 in probands and families [22–27]. However, most patients with aggressive phenotypes share truncating mutations. Secondly, MEN1-related FIHPT, a genetic variant of MEN1, has mostly been related to missense mutations occurring between exons 3 and 7 [35, 36]. One would suggest that FIHPT-related *MEN1* mutations could only be missenses and restricted to the central region of *menin*. Nevertheless, in MEN1, lesions occur both metachronously and synchronously and the clinical use of such data will be restricted by the fact that any patient with a *MEN1* germline mutation might in the future develop pancreatic, pituitary and adrenal tumors [Calender et al., unpubl. observ.]. Families derived from a common ancestor and sharing common mutations and haplotypes have heterogeneous clinical expression of the disease, an observation which assesses the absence of genotype-phenotype correlation and might suggest an important role of modifier genes and/or environmental factors [37].

From a biological point of view, all truncating mutations, either nonsenses or frameshifts, affect one or both NLSs in the C-terminal region of *menin*. Curiously, the two NLSs were never affected by missense mutations indicating that these sequences could play a critical role in *menin* function and metabolism. Based on the mutation data in MEN1 patients, we could suggest two distinct mechanisms impairing the function of *MEN1;* the first one by deleting NLSs might affect the nuclear localization of *menin* and/or induce fast degradation pathway(s) of truncated protein. Our recent data indeed show that truncated forms of *menin* were not detectable by Western blot analysis in MEN1 patients with nonsense mutations [38]. As observed in parathyroid tumors [39], complete loss of a *MEN1* allele or short truncation by nonsense mutations did not result in reduced transcription and protein expression levels, suggesting the upregulation of the wild-type allele. The second mechanism might concern the functional properties shared by internal domains of the protein that would be selectively impaired by missense or splice site mutations. In such cases, in vitro functional tests will be needed to assess the mutagenic properties of a specific amino acid substitution and distinguish it from a rare polymorphism. Around 5–10% of MEN1 families do not show germline *MEN1* mutations even after full sequence analysis of exonic and intronic sequences. Clinical criteria used for the diagnosis of MEN1 are crucial and one would expect a ≈95% mutation detection rate when familial MEN1 was defined as a patient with three (or more) first-degree relatives with two (or more) MEN1-related major lesions. Most families without *MEN1* mutations display an atypical clinical pattern, with one or two lesion(s) affecting the proband and one major or uncommon MEN1-related tumor(s) in the relatives. This might also reflect the genetic heterogeneity of the syndrome or the occurrence of phenocopies which are mainly due to lesions commonly observed in the non-MEN1 population, such as pHPT and prolactinoma [40, 41]. In some MEN1 families, *MEN1* mutations could also have been missed. The *MEN1* gene structure is not fully known in the 5′ region, and promoter(s) and/or regulatory regions

could be affected by unknown mutations. Chromosomal or intragenic rearrangements such as large germline deletions, either within or encompassing the *MEN1* gene, might have been missed by PCR-based sequencing. A *MEN1* deletion has recently been suggested in a Japanese pedigree by RFLP-gene dosage analysis and quantitative PCR [42] and demonstrated in a large French MEN1 family using molecular cytogenetic tools [Lespinasse et al., submitted]. Finally, the complexity and diversity of *MEN1* mutations show us the need of clinical screening as a prerequisite for molecular diagnosis. In clinical practice, genetic analysis is useful to assess syndromic diagnosis of MEN1, but to date we still do not exclude the diagnosis of MEN1 when the mutation was not found.

NET Related to Oncogenic Activation of RET, a Tyrosine-Kinase Membrane Receptor

Germline mutations of the *RET* proto-oncogene encoding a transmembrane tyrosine-kinase (TK) receptor confer predisposition to clinical variants of MEN2, the inherited forms of medullary thyroid carcinoma (MTC) [43, 44]. In MEN2A (Sipple's syndrome), MTC is associated to pheochromocytoma (30–50%) and primary hyperparathyroidism (10–20%). In MEN2B (Gorlin's syndrome) the major clinical features are MTC, pheochromocytoma, mucosal neuromas and skeletal abnormalities associated with a marfanoid habitus and ganglioneuromatosis of the gastrointestinal tract [45]. The third variant of MEN2 was defined as familial MTC (FMTC), in which MTC occurs as the sole phenotype in 3 or more patients [46]. FMTC might be considered as MEN2A with low penetrance of pheochromocytoma. C-cell hyperplasia is the earliest lesion observed in hereditary MTC and is characterized by abnormal basal and pentagastrin-stimulated calcitonin values. MTC related to a MEN2 genetic predisposition is multifocal. Pheochromocytomas in MEN2A/B are bilateral in around 70% of cases. Two major issues, malignant evolution of MTC and cardiovascular failures due to latent pheochromocytoma, have to be considered for the prognosis of patients with germline *RET* mutations. MTCs in MEN2B are more aggressive and appear in young children [47]. In some FMTC, thyroid cancer is expressed only at later age and a long-term follow-up is needed in order to exclude the risk of occurrence of pheochromocytoma in a specific family [48]. The natural history of MEN2 and the risk of malignant disease underscore the need for early management of patients by surgical treatment of MTC and, when present, of pheochromocytoma.

The *RET* gene has been assigned to chromosome 10q11–2. The c-ret protein displays an extracellular cysteine-rich homodimerization domain and an intracellular TK catalytic site. The distal part of the extracellular region contains a cadherin-like domain which mediates calcium-dependent cell–cell adhesion [49]. The c-ret protein belongs to a multiprotein complex acting as a receptor for four related ligands, glial cell line-derived neurotrophic factor (GDNF), neurturin, artemin and persephrin, each of them acting through specific coreceptors, GFRα-1, 2, 3 and 4, respectively [50]. GFRα coreceptors interact with ligands and induce homodimerization of c-ret through the cysteine-rich region, thereby leading to the catalytic activation of TK domains. GDNF and neurturin promote the survival of a variety of neurons, and GDNF is required for the development of the enteric nervous system and kidney [51, 52]. Intracellular events after ligand binding and dimerization involve cross-phosphorylation of TK domains of dimerized RET and a signal-transducing complex consisting of Shc, Grb2 adapters and the subsequent activation of a Ras-MAP-kinase pathway [53]. *RET* genomic size is 60 kb and the gene contains 21 exons [54]. It is expressed in many tissues including thyroid, adrenal, neuroendocrine tissues and the developing kidney [55, 56]. The c-ret protein induces the genesis of the peripheral and central nervous system and the renal excretory tract. Nullizygote (knock-out) c-ret –/–, GDNF –/– and GFRα-1 –/– mouse strains that died soon after birth lacked neurons in the whole digestive tract and showed kidney agenesis [57–59]. The phenotype observed in knock-out models mimicked that of the human Hirschsprung disease characterized by intestinal aganglionosis. *RET* mutations, including deletions, point mutations and splice-site alterations, have been observed in some autosomal dominant forms of Hirschsprung [60]. These mutations lead to loss of function and suggest that c-ret protein might be critical for differentiation, proliferation and migration of neural crest cells. The protein encoded by RET is highly expressed in human tumors of the neural crest derivatives, such as neuroblastoma, medullary thyroid carcinoma and pheochromocytoma [61]. The *RET* gene has also been involved in sporadic papillary thyroid carcinoma (PTC) through chromosomal rearrangements producing various types of abnormal RET/PTC fusion proteins [62].

Germline mutations of RET in MEN2 are missenses occurring either in the extracellular cysteine-rich domain or intracellular TK catalytic sites. Missense mutations in codons 609, 610, 611, 618, 620, or 634 located in the extracellular dimerization domain have been detected in 98% of MEN2A and 85% of FMTC patients [63]. The most common mutations observed in MEN2A affected codon 634, either Cys634Arg, C634Tyr and Cys634Gly

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