

Deregulation of genetic pathways in neuroendocrine tumors

Alain Calender

Department of Genetics, Hôpital Edouard Herriot, Lyon and University of Lyon,
School of Medicine

Place d'Arsonval 69437 Lyon Cedex 03 France

E-mail : alain.calender@chu-lyon.fr

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Introduction

Neuroendocrine tumors (**NET**) are uncommon diseases occurring sporadically or in a familial context of autosomal dominant inherited syndromes such as Multiple Endocrine Neoplasia (**MEN**). During the last decade, at least six major genes involved in initial steps of NET have been characterized and one would expect that clinical screening syndromic forms of NET will include now genetic studies as a common tool for presymptomatic diagnosis [1]. Four major MEN syndromes are [MEN1](#), [MEN2](#), [von Hippel Lindau disease \(VHL\)](#), and Carney Complex (CC) which represent the most common forms of inherited predisposition to NET with variable but high penetrance of proliferations in various neuroendocrine tissues. Less commonly, endocrine tumors of the pancreas, parathyroids and adrenal glands have been observed in phacomatosis such as [Recklinghausen disease \(NF1\)](#), Tuberous sclerosis (TSC). Lastly, familial occurrence of a single endocrine lesion, such as primary hyperparathyroidism or pituitary adenoma have been identified as putative new genetic diseases for which genetic pathways remain to be identified. Most NET predisposing diseases were related to inactivation of growth suppressor genes, according to the Knudson model, except MEN2, an inherited form of medullary thyroid carcinoma, which occurs through dominant activation of a proto-oncogene, the RET tyrosine kinase receptor. Nevertheless, recent knowledge on the biological roles of most genes involved in cancer predisposition teaches us that a single gene might have pleiotropic effects at various stages of the cell physiology as for instance both a negative and a positive regulator function depending on the cellular context and sometimes the type of mutation [2].

Cloning of genes involved in genetic diseases predisposing to NET has led to a better insight in the molecular nature of tumor initiation of (neuro)endocrine tissues in most endocrine glands. Even if the genes and related syndromes are presented separately, we would expect that genetic studies in patients with NET will now help to the differential diagnosis of syndromic diseases and that a single type of endocrine tumor may be related to the deregulation of distinct genes and related pathways. Major syndromes predisposing to NET and overlapping symptoms leading in some

patients and/or families to address the differential diagnosis by molecular genetic tools are presented in **Figure 1**.

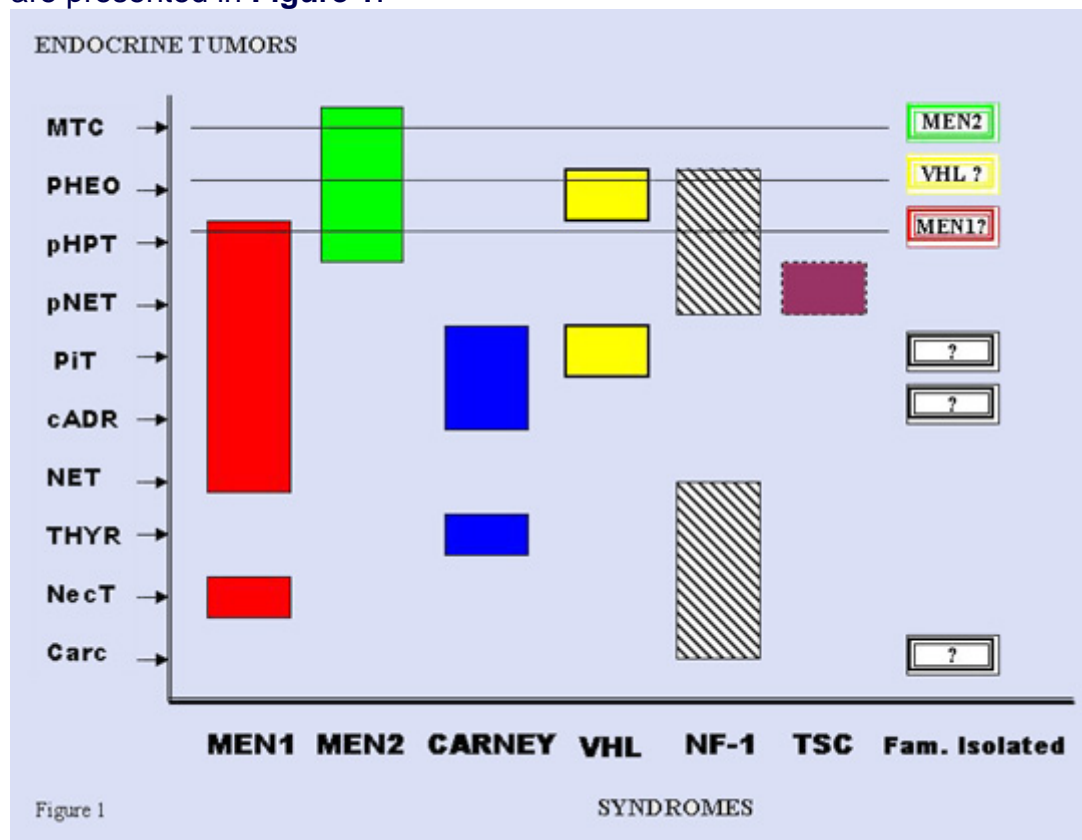


Figure 1 : Overlapping lesions in genetic syndromes predisposing to neuroendocrine tumors. Major endocrine lesions described are **MTC** (medullary thyroid carcinoma), **PHEO** (pheochromocytoma), **pHPT** (primary hyperparathyroidism), **pNET** (pancreatic endocrine tumors), **PIT** (anterior pituitary tumors), **cADR** (adrenal cortical tumors), **NET** (bronchic or thymic 'carcinoids'), **THYR** (thyroid papillary or follicular carcinoma), **NecT** (neuroectodermic-derived tumors such as meningioma, paraganglioma, ependymoma), **Carc** midgut and hindgut carcinoids). Major genetic syndromes showed are MEN1 (Multiple Endocrine Neoplasia type 1), MEN2 (Multiple Endocrine Neoplasia type 2), VHL (von Hippel Lindau disease), NF-1 (Recklinghausen disease), TSC (Tuberous Sclerosis) and Fam. Isolated which represent genetic diseases with occurrence of a single and homogeneous lesion in probands and first or second degree relatives, such as familial isolated MTC or hyperparathyroidism. The most probable diagnosis suggested for each lesion are indicated inside the boxes. When designed by ? , the genes involved in these familial occurrences are unknown to date.

MEN1, a major genetic context predisposing to NET

From the disease to the gene

Multiple Endocrine Neoplasia type 1 (MEN1, OMIM 131100) is an autosomal dominant syndrome characterized by hyperplasia and/or multiple tumors of the parathyroid, endocrine pancreas, anterior pituitary, foregut-derived neuroendocrine tissues and adrenal cortical glands [3]. Less common lesions have been associated with MEN1 including cutaneous proliferations such as angiofibroma, collagenoma, [lipoma](#) and melanoma [4,5] and peripheral or central nervous system tumors such as [ependymoma](#) [6,7]. The *MEN1* locus has been localized to chromosome 11q13 in 1988 by linkage studies and the [MEN1](#) gene itself was identified in 1997 after a 9 years-long period of extensive positional cloning [8,9,10]. The *MEN1* gene consists of 10 exons, the first of which is untranslated, spanning around 7-10 Kb of genomic sequence and encoding menin, a protein of 610 amino acids. A major menin 2,8 Kb transcript and the protein were expressed in most human tissues analyzed, the

menin RNA presenting a striking 4,2 Kb large form suggesting alternative promotor triggering and/or alternative splicing in 5', a question remaining to date unresolved [10].

Menin does not reveal homologies to any other known proteins nor includes consensus motifs from which the putative function of the protein could be deduced. Nevertheless, menin was characterized as a nuclear protein containing two nuclear localization signals (NLS) in exon 10 [11] which have been functionally defined by *in vitro* expression of deletion constructs labelled by epitope-tagging with enhanced green fluorescent protein (EGFP). Despite the nuclear localization suggesting a major role of menin in the regulation of genome expression, the MEN1 protein was recently shown to move from nucleus to cytoplasm during the cell cycle and mainly the mitotic process [12]. These data contrast with the absence of any variation of size and amount of menin throughout the cell cycle as studied in synchronized cells [13].

Major function of menin in normal and MEN1-related conditions

Major function of the *MEN1* gene product might be related to the ability of menin to bind JunD, a transcription factor belonging to the multimeric AP1 transcription regulation complex [14]. Deletion constructs and natural mutants of menin have shown that interaction with JunD needs three major domains of the menin protein, the first 40 aminoacids of the N-terminal region and two central sequences at positions 139-242 and 323-428 respectively. The menin-JunD interaction seemed to be specific and established through the N-terminal sequences (amino acids 1-70) of JunD and a co-activator, JAB1, involved in the transcriptional regulation activity of JunD in the AP1 complex [14]. Specific JunD missense mutants in the N-terminal domain failed to bind menin with a subsequent activation of their transcriptional activity suggesting escape of menin control [15]. Wild-type menin repressed transcriptional activation mediated by JunD upon *in vitro* co-transfection assays. Independantly, some authors have confirmed these data and showed that menin mediated repression of JunD activity was related to histone deacetylation mechanisms which are involved in chromatin-mediated regulation of gene expression [16].

Despite extensive characterization of menin-JunD interaction, the physiopathogeny of MEN1-related endocrine and non- endocrine lesions remains poorly understood, mainly considering the fact that AP1 is an ubiquitous factor in all tissues which regulates most of cellular process such as, mitosis, DNA replication, transcription, apoptosis and response to physical or chemical stress [1]. JunD was considered as a transcriptional activator but paradoxical observations showed that overexpression of JunD in NIH3T3 cells suppresses cell growth. This suggests that JunD might be both a co-repressor and a co-activator in AP1 depending the cellular context. Recent studies showed that overexpression of menin in *ras*- transformed NIH3T3 cells inhibits cell growth supporting the hypothesis that menin is a growth-suppressor through complex interactions within the JunD-AP1 complex [17].

More than 300 unique germline mutations have now been identified in MEN1 family probands analyzed throughout the world [18-24]. Somatic mutations have also been reported in sporadic forms of endocrine tumors with variable incidence of 20-30% in parathyroid, endocrine pancreas (gastrinomas, insulinomas), lung carcinoids and less than 1% in pituitary and adrenal cortical tumors [25-33]. All these mutations were recently included in an unpublished mutation database developed in the frame of UMD (Universal Mutation database) software allowing an easy evaluation of genotype-phenotype correlations [34]. Over 70% of germline mutations related to the MEN1 disease are nonsense and frameshifts predicting truncation and/or absence of

the abnormal protein. Missenses, in-frame deletions or insertions and splice-site alterations account for the remaining 30% mutations described in the clinical context of MEN1. A putative effect of truncating mutations might be a premature degradation of the truncated menin through the protein catabolism pathway as suggested by the failure of Western-Blot detection of mutant protein in most cases analyzed [13]. This observation do not exclude a transient and abnormal effect of the truncated menin within the nucleus and cytoplasm, a question which will be further adressed by immunohistochemical competent monoclonal antibodies against menin. Intronic and splice-site mutations were shown to alter RNA splicing with abnormal exon skipping and/or intronic retention [24,35,36].

Homologs of MEN1 sequences in other species

The murine *Men1* gene was mapped and cloned in the pericentromeric region of murine chromosome 19 which is syntenic to human 11q13 [37]. The genomic organization of *Men1* is similar to that of the human gene including 10 exons and a noncoding region covering 6,7 Kb of genomic DNA. Suggestive of what might happen in humans, two major transcripts of 2,8 and 3,2 Kb were detected in most embryonic and adult tissues, resulting from alternative splicing of intron 1 [38]. The predicted protein is 611 amino acids in length and characterized by 97% homology with the human sequence. Two independant teams subsequently cloned the murine and rat *Men1* and showed similar data on structure and expression [39,40]. The expression of *Men1* is detected as earlier as gestationnal day 7 in the whole embryo, and a strong expression limited to thymus, liver, nervous system and gonads at day 17. Hence, the expression of *Men1* was not confined to organs affected in MEN1. In testis, *Men1* expression was found to be higher in Sertoli cells than in germ cells [37]. These data assess independant observations suggesting that menin-JunD related pathways within the AP1 complex might directly control the transition of granulosa cells to terminally differentiated, non-dividing luteal cells in ovarian gland [41]. Lastly, the zebrafish and drosophila homologs of *MEN1* have been recently identified and show respectively 75% and 47% homologies with the human sequence [42,43]. Among the amino acid residue substitutions reported as disease-associated missense mutations, most of them (70%) were completely conserved either in zebrafish and drosophila *menin*, indicating an evolutionnary conserved protein with a fundamental role in biological processes.

Animal model of MEN1 and pathogenic effect of MEN1 gene inactivation

According to the Knudson model, *MEN1* appears to be a growth suppressor gene with tumors in MEN1 affected patients showing somatic loss of the wild-type allele, so-called loss of heterozygosity (LOH) at 11q13 [44,45]. Functionnally, all truncating mutations affect one or both NLS in the C-terminal domain of menin. Conversely, missense mutations have never been observed inside the menin NLSs sequences, suggesting that the fonctionnal role of these sequences might be critical for cell survival and mainly at the embryonic level. Recently, the first mouse model of MEN1 was produced through homologous recombination of the mouse *Men1* gene [46]. A major mice strain lacking exons 3-8 of the *Men1* gene was produced and heterozygous *Men1*^{+/-} were bred to generate *Men1*^{-/-} homozygotes. Homozygous inactivation of the *Men1* gene was lethal at around days 10-14 of embryogenesis with developmental delay, defects in cranial and facial developments. Interestingly, *Men1*^{+/-} heterozygotes develop hyperplastic pancreatic islets and first small tumors after 9 months of age and significantly larger tumors with capsular invasion after 16 months of age. Other tumors observed in heterozygous *Men1*^{+/-} mice were parathyroid hyperplasia/adenoma, pituitary adenoma and adrenal cortical carcinomas

in 24, 26 and 20% of cases analyzed throughout the course of the time study. Most of the tumors observed in this animal model showed loss of heterozygosity on the wild-type allele [46].

Clinical implications of MEN1 gene analysis

Taken together, updated data on MEN1 suggest that inactivation or absence of both *MEN1* alleles is a critical factor in MEN1-related endocrine tumors initiation. Identification of nonsense or any other truncating mutations in a MEN1 suspected patient clearly assess the diagnosis. With missense or intronic mutations, we might consider that pathogenic effects may be related to disturbances in menin-JunD (or forthcoming new functional interactions) related pathway or abnormal splicing respectively. In such cases, *in vitro* functional tests will be required to assess for instance that an amino acid substitution must be considered as a mutation and not a rare polymorphism. Clinical criteria used for diagnosis of MEN1 are crucial and in our experience, *MEN1* germline mutations were found in 95% of probands/families with as a patient sharing at least three major lesions of the syndrome and a first-degree relatives affected by one (or more) MEN1-related lesions [21]. Most families without demonstrable *MEN1* mutations display an atypical clinical pattern, which might suggest genetic heterogeneity of the disease or the occurrence of phenocopies with lesions which are commonly observed in the non-MEN1 individuals, such as primary hyperparathyroidism and prolactinoma [47,48]. Lastly, when MEN1 is strongly suspected, mutations might occur in unknown part of the *MEN1* sequence, as the 5' regulatory region for which functional characterization is in progress [49]. Large intragenic rearrangements and/or deletions, either within or encompassing the *MEN1* gene might have been missed by routine PCR and sequencing procedures. A *MEN1* deletion has been suggested in a Japanese MEN1 pedigree by quantitative Southern-Blot analysis and shown recently in a large French MEN1 family using molecular cytogenetics procedures [50, Lespinasse et al, in preparation]. Finally, we might conclude that clinical screening of patients remains a prerequisite of genetic analysis and that functional knowledges on menin-related pathways must be kept in mind of clinicians following MEN1 affected patients.

Oncogenic activation of RET, a tyrosine-kinase membrane receptor, induces MEN2

Clinical expression of RET germline mutations

The two major variants of Multiple Endocrine Neoplasia type 2 (MEN2A or Sipple's syndrome, OMIM 171400 ; MEN2B or Gorlin syndrome, OMIM 162300) result from missense activating (or oncogenic) mutations of RET , a gene localized on chromosome 10q11-2 and encoding a transmembrane tyrosine-kinase (TK) receptor [51,52]. MEN2 might be considered as the inherited form of medullary thyroid carcinoma (MTC) , a constant lesion in MEN2, associated or not with pheochromocytoma and/or primary hyperparathyroidism. MEN2B is a rare variant characterized by an early-occurrence and aggressive MTC, pheochromocytoma, mucosal neuromas on the gastrointestinal tract and a marfanoid habitus. FMTC (Familial isolated Medullary Thyroid Carcinoma) might be considered as the classical MENA syndrome with very low penetrance of pheochromocytoma and hyperparathyroidism [53,54].

Functional insights on the c-ret protein

RET genomic size is 60 Kb and the gene contains 21 exons [55]. The c-ret protein is characterized by an extracellular cysteine-rich homodimerization domain and an intracellular TK catalytic site. The N-terminal part of the extracellular region contains

a cadherin-like domain which mediates calcium-dependant cell-cell adhesion. This receptor binds at least four ligands, GDNF (Glial cell line Derived Neurotrophic Factor), neurturin, artemin and persephrin, all of them inducing homodimerization of the c-ret protein through the cysteine-rich region, thereby triggering the TK catalytic site [56,57]. Intracellular events after ligand binding and c-ret dimerization involve cross-phosphorylation of TK domains and the downstream activation of Ras-MAP-kinase and PI3/AKT transduction pathways [58,59]. Biological properties of c-ret and ligands is related to the genesis of the peripheral and central nervous system and renal excretory tract. Strikingly, both GDNF *-/-* and c-ret *-/-* knock-out mice share the same phenotype with an early death after birth, lack of neurons in the whole digestive tract and kidney agenesis [60,61,62]. The c-ret protein is highly expressed in many normal endocrine tissues, developing kidney and in human tumors of the neural crest derivatives, such as MTC, and pheochromocytoma [63,64]. Recently, it has been proven that a constitutively activated *RET*-MEN2A allele promotes cell survival *in vitro* in the absence of any growth factors and that this effect might be controlled by a specific domain around c-ret tyrosine 1062 through the PI3/AKT / MAP kinase pathways [65].

Germline RET mutations in MEN2 and downstream deregulated pathways

All germline mutations of *RET* observed in MEN2 were missense mutations affecting either the cysteine-rich extracellular dimerization domain (exons 8 to 13) or intracellular TK catalytic sites (exons 15 and 16) [66,67]. Genotype- phenotype correlations have been clearly established, mutations occurring in exons 8 to 14 being mostly related to MEN2A and FMTC variants, mutations observed in exons 15 and 16 being always related to a MEN2B phenotype. Conversely, transgenic mice expressing an activating MEN2A-related mutation in exon 11 develop bilateral MTC as in humans but never pheochromocytoma [68,69]. Mutations occurring in exons 8 to 11 induce spontaneous homodimerization of the c-ret protein in absence of the ligand(s) [70]. Mutations described in exon 13 and 14 affect the catalytic site by inducing an inappropriate binding to substrates of the intracellular signalling pathway [70,71]. Lastly, MEN2B-related mutations in exons 15 and 16 switch the substrate specificity of the c-ret TK from a membrane receptor towards an intracellular tyrosine-kinase, thus inducing abnormal signalling pathways [70,72].

Clinical implications of RET sequencing in MEN2

To date, germline *RET* mutations were found in 100% of MEN2A, 90% of FMTC and 95-100% of MEN2B families. In terms of clinical use, we might conclude that routine screening of exons 8, 10, 11, 12, 13, 14, 15 and 16 of *RET* is now an useful tool for an accurate presymptomatic diagnosis in this disease. Germline *RET* mutations were also found in 5-7% of sporadic cases of MTC [73,74], mostly in patients for which clinical and genetic informations have been less informative at initial diagnosis, suggesting *de novo* mutations or missed familial cases. MEN2 syndrome represents a model in genetic predisposition to cancer by the fact that molecular genetic analysis of *RET* might be considered as an early preventive action, leading to prophylactic thyroidectomy in (young) asymptomatic gene-carriers [75].

Mutations in the VHL gene predisposes to pancreatic NET

Clinical features

In a recent series of 158 patients affected by VHL (von Hippel Lindau or OMIM 193300) disease, Hammel et al showed pancreatic involvement in 77% of cases, including true cysts, serous cystadenomas and neuroendocrine tumors in 12% of VHL patients [76]. None of the patients with pancreatic NET had symptoms of

hormonal hypersecretion suggesting that VHL-related pancreatic NET are mostly non-functional. VHL-related pancreatic NET might be distinguished from MEN1-related tumors based on 1) the absence of primitive duodenal tumors 2) frequent non-functional lesions with focal positivity for pancreatic polypeptide, somatostatin, glucagon and insulin 3) a clear-cell morphology related to intracytoplasmic lipid and myelin accumulation and 4) the frequent occurrence of microcystic adenomas around the clear-cell tumors. VHL disease is an autosomal dominant disease which main lesions are retinal hemangioblastomas, retinal hemangioblastomas and/or cerebellar hemangioblastomas, renal cancers, pheochromocytoma, pancreatic lesions and auricular endolymphatic epithelial proliferations [77,78].

VHL gene and function

The VHL gene was cloned in 1993 on chromosome 3p35-36 and contains only three exons encoding a protein with pleiotropic functions [79]. The VHL protein interacts with the elongin family involved as regulators of transcriptional elongation [80,81]. Other functions of the VHL protein have been related to the hypoxia-induced cell regulation by enhanced expression of VEGF (Vascular Endothelial Growth Factor), regulation of extracellular matrix fibronectin expression and stabilization and lastly, VHL protein interacts with Cullin-2 and Rbx1, two major components of the cellular ubiquitination or protein catabolism machinery [82,83,84]. Many naturally occurring mutations in VHL have either been shown or are predicted to abrogate assembly with elongins and Cullin-2, suggesting a functional role for these interactions in VHL-related tumors and digestive NET [85,86].

Clinical implications of VHL gene analysis

VHL gene sequencing has been useful in VHL disease presymptomatic diagnosis and in some clinical states suggesting differential diagnosis with MEN1 and MEN2. In fact, germline mutations of the *VHL* gene have been shown in patients with pancreatic endocrine tumors without any lesions suggesting MEN1 [87] and in rare cases of familial occurrence of pheochromocytomas [88].

Forthcoming genes involved in neuroendocrine tumors through the Carney Complex

Carney complex (CC or OMIM 160980) is an autosomal dominant disease predisposing to various types of tumors including cardiac and cutaneous myxomas, spotty pigmentation of the skin and nonneoplastic hyperfunctioning endocrine states, as nodular adrenocortical hyperplasia associated with Cushing syndrome, pituitary and thyroid adenomas [89]. Clinical evaluation and genetic linkage analysis of families affected by Carney complex suggested at least two distinct loci for disease genes, the first on chromosome 2p16, the second on chromosome 17q24 [90,91]. Recent positional cloning studies demonstrate that 17q-linked Carney disease was caused by mutations in the R1 regulatory subunit of cAMP-dependant protein kinase A (PKA), so-called the PPKAR1 gene [92,93]. Most mutations were nonsense or frameshifts inducing haploinsufficiency of the PPKAR1 subunit. This protein acts as a tumor suppressor gene in most tissues by down-regulating the PKA activity. Loss of the PPKAR1 regulatory subunit promotes cell proliferation and growth of benign tumors in multiple tissues. Hyperendocrine states are likely to be a result of increased PKA activity and cAMP levels by disruption of the tetrameric inactive form of the enzyme [94]. The second gene involved in Carney complex and localized on 2p16 remains to be identified.

Discussion

Apart from well-defined genes involved in genetic predisposition to NET, we were not able to show a common pathway leading to the proliferation of neuroendocrine cells. We might expect that various mechanisms occur depending the embryological origins of the organs concerned. **Figure 2** summarizes the mechanisms related to *MEN1*, *MEN2*, *VHL*, *CC* and *NF1/TSC* mutations in the genetic syndromes described previously. The genetic mechanisms underlying malignant progression of benign neuroendocrine tumors remain unknown. Some genes, as *NF1* (Neurofibromatosis type 1) and *TSC1/2* (Tuberous Sclerosis) have been suggested as putative loci involved in tumoral progression from the fact that both genetic syndromes related to mutations in these genes, either Recklinghausen Neurofibromatosis (NF1) and Tuberous Sclerosis (TSC) might rarely predispose to endocrine tumors such as pheochromocytomas, primary hyperparathyroidism and pancreatic somatostatinomas or insulinomas [95,96]. The *NF1* (chromosome 17) and both *TSC1* (chromosome 9) and *TSC2* (chromosome 16) genes are involved in the membrane signal transduction pathway by acting as negative regulators of ras (NF1) or rab-5 (*TSC1/2*)-related small G-proteins [97,98]. *NF1*, *TSC1/2* disrupt GTP-ras or rab-5 complex and are considered as GTPase-activating proteins (GAP), an independant class of growth-suppressor genes.

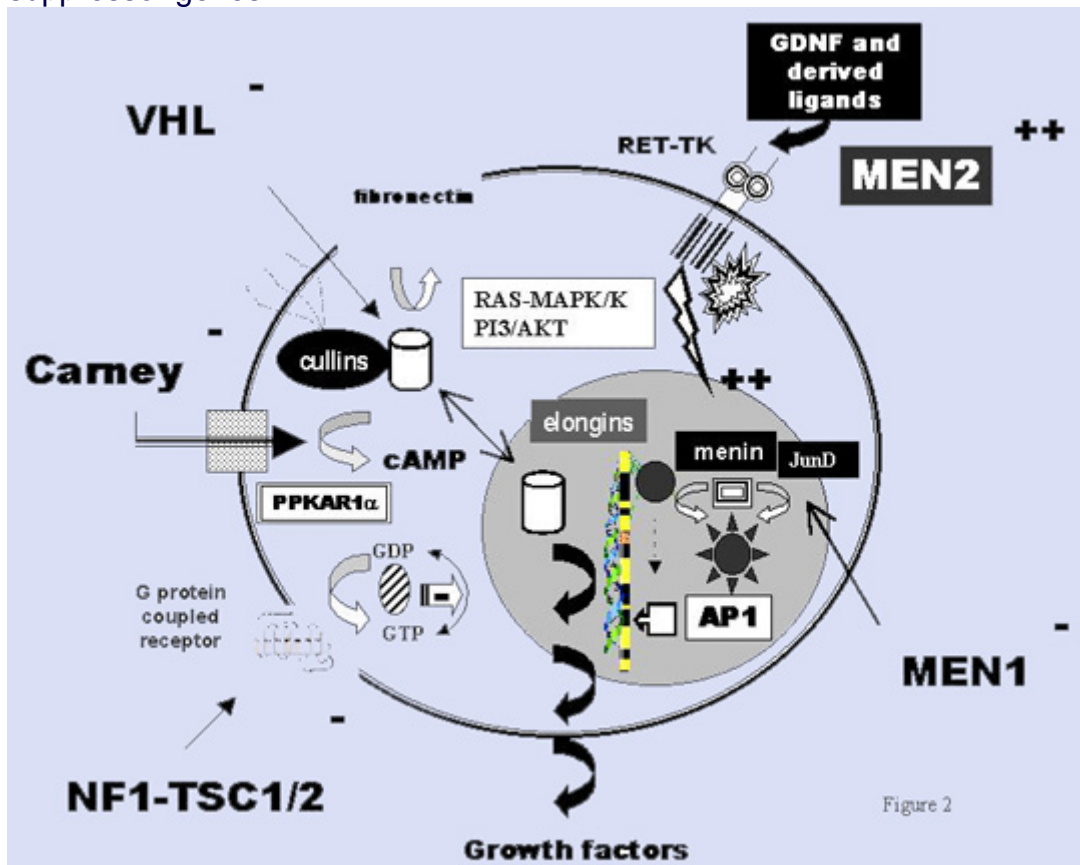


Figure 2 : Schematic summary of the complexity of pathways involved in genetic predisposition to neuroendocrine tumors. All proteins, membrane receptors, pathways and interactions are detailed in the text. When designed by +, the gene involved in the syndrome (such as *MEN2*) acts as an oncogene with a dominant mechanism. When designed by -, the genes related to syndromes (such as *MEN1*, *NF1*, *TSC*, *Carney* complex and *VHL*) are considered as growth suppressors, acting by negative regulation of various pathways shown in this figure.

Most cancers result from multistep scenarios and we might consider that many other genes are involved in the progression of benign NET towards a fully malignant phenotype. Mutations of well-known growth-suppressor or onco-genes, such as [p53](#), [Ki-RAS](#), [HER2/NEU](#), [C-MYC](#), [N-MYC](#), [N-RAS](#), C-JUN, [PRAD-1](#), have been

excluded as major events in NET progression even if some studies showed upregulation of gene expression in tumor tissues [1]. Up-regulation of genes such as bcl-2, an apoptosis-regulating function and p53 or down or de-regulation of adhesion molecules such as CD44 have been suggested to be of importance as prognostic markers in pancreatic and bronchial carcinoid tumors [99]. Nuclear nm23 and Ki-67 proliferation-associated markers have also been considered as useful tools for valuable prognostic information and identification of patients at risk of disease-related death [100,101]. Nevertheless, more hopes on this topic remain related to the identification of loci-specific loss of heterozygosity (LOH) mainly on chromosomes 1p and 1q [103], 3p25-26 [102,103], 11q13 and 18q [104, 105]. Loss of the *MEN1* loci might induce latent genomic and/or chromosomal instability as suggested by a few reports but the basic mechanism leading to this instability has not been proven to date [1]. Most of the genes involved in such LOH's remain to be identified and their implications in neuroendocrine cell proliferation must be demonstrated by *in vitro* functional tests. Malignant progression of neuroendocrine tumors might also be triggered by overexpression of growth factors involved in endocrine and endothelial cell proliferation such as TGF , EGF, NGF and VEGF/VEGF-related factors [106,107,108,109].

Finally, most of the genes involved in the initiation of endocrine tumors have been now discovered and are involved in genetic predisposition to NET. In sporadic and malignant forms of neuroendocrine tumors, other genes remain to be found and their respective roles during the multistep progression of tumors to be identified. Genetic studies have contributed to the development of animal models of neuroendocrine tumors in the context of genetic syndromes closed to what have been observed in humans. This represents a powerful tool for therapeutical assays in the future and mainly by understanding the mechanisms leading a normal endocrine cell towards a fully malignant and metastatic clone.

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Contributor(s)

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