Whole genome sequencing of apparently mutation-negative MEN1 patients

Samuel Backman[1], Duska Bajic[2], Joakim Crona[2], Per Hellman[1], Britt Skogseid[2] and **Peter Stålberg[1]**

Departments of [1]Surgical Sciences and [2]Medical Sciences, Uppsala University, Uppsala, Sweden

Correspondence should be addressed to S Backman
Email
samuel.backman@surgsci.uu.se

Abstract

Objective: Multiple endocrine neoplasia type 1 (MEN1) is an autosomal dominant syndrome usually caused by loss-of-function mutations in the *MEN1* gene. However, a minority of patients who fulfill the criteria for MEN1 are not found to harbor *MEN1* mutations. Besides, some of these individuals, present with a subtly different phenotype suggestive of sporadic disease. The aim of the present study was to investigate the genetic architecture of mutation-negative MEN1.
Design: Fourteen patients with a clinical diagnosis (n = 13) or suspicion (n = 1) of MEN1 who had negative genetic screening of the *MEN1* gene were included.
Methods: Constitutional DNA from the included patients, as well as tumor DNA from six of the patients, was subjected to whole genome sequencing. Constitutional variants were filtered against population databases and somatic variants were studied under a tumor-suppressor model.
Results: Three patients carried pathogenic variants (two splice-site variants, one missense variant) in *MEN1* that had not been detected during routine clinical sequencing, one patient carried a pathogenic variant in *CASR* and one patient carried a gross deletion on chromosome 1q which included the *CDC73* gene. Analysis of matched tumor DNA from six patients without mutations did not detect any recurrent genes fulfilling Knudson's two-hit model.
Conclusion: These results highlight the possibility of germline mutations being missed in routine screening, the importance of considering phenocopies in atypical or mutation-negative cases. The absence of apparent disease-causing mutations suggests that a fraction of *MEN1* mutation-negative MEN1 cases may be due to the chance occurrence of several endocrine tumors in one patient.

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Introduction

Multiple endocrine neoplasia type 1 (MEN1) is an autosomal dominant disease characterized hyperparathyroidism, pituitary adenomas, and neuroendocrine tumors of the pancreas. Additionally, patients may develop tumors in a range of tissues, including the lungs, adrenals and the thymus (1). The penetrance is near-complete penetrance by the sixth decade of life (2). The prognosis of each individual patient depends on his or her particular manifestations of the disease, but more than 70% of deaths in MEN1 patients are directly related to the disease (3). In 1988 the *MEN1* gene was mapped to a narrow region on chromosome 11q13 (4), and in 1997 the gene was cloned, enabling genetic testing of patients (5). More than 1100 mutations have been reported to date, spanning all nine coding exons of the gene as well as splice sites. Since genetic testing became available, it has been demonstrated that not all patients carry a *MEN1* mutation. It is currently estimated that 5–25% of all patients fulfilling clinical criteria for the MEN1 diagnosis have negative genetic tests for the MEN1 gene (1).

In theory, several explanations for this finding may be considered. Firstly, the sensitivity of conventional genetic

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testing is likely less than 100%, which may account for a small number of missed true cases. Secondly, sporadic forms of two manifestations may co-occur in a patient due to mere chance, mimicking the genetic MEN1 syndrome. Moreover, mutations in another gene may phenocopy MEN1. For instance, inactivating mutations in *CDKN1B* have been demonstrated to cause a MEN1 phenotype in a small number of patients (6), while syndromes such as familial isolated hyperparathyroidism (FIHP) (7), familial isolated pituitary adenoma (FIPA) (8) and hyperparathyroidism-jaw tumor syndrome (HPT-JT) (9) have partially overlapping features with MEN1. Finally, epigenetic inactivation of the *MEN1* gene, or postzygotic mutations occurring in a subset of an individual's cells may cause the phenotype without being detected on routine clinical genetic testing.

Mutation-negative MEN1 has recently in a Dutch national cohort study been demonstrated to be qualitatively and quantitatively different from classical *MEN1*-mutation-related MEN1 (10). The age at diagnosis of the first disease manifestation is higher in mutation-negative patients. Moreover, they are less likely to develop a third manifestation of the disease and live longer than mutation carriers. These data suggest that mutation-negative MEN1 patients have another milder syndrome or that they do not have a genetic syndrome but rather the chance co-incidence of two sporadic neuroendocrine tumors or one familial tumor and another sporadically developed. This is further supported by multiple studies which have demonstrated that patients without a family history of MEN1 are less likely to carry a *MEN1* mutation (11, 12).

Whether mutation-negative MEN1 is due to sporadic co-occurrence of several neuroendocrine tumors or due to mutations in a different gene is currently an open question. We hypothesized that whole genome sequencing of constitutional DNA from *MEN1* mutation-negative MEN1 patients would enlighten us by allowing detection of other disease-causing mutations in these patients or demonstrating their absence, suggesting other mechanisms for disease development, including the possibility that these patients may have developed several sporadic tumors. Consequently, we performed a whole-genome sequencing (WGS) study of germline DNA from 14 individuals with clinically diagnosed or suspected multiple endocrine neoplasia type 1. For a subset of patients, we also performed WGS of matched tumor DNA, hypothesizing that a novel MEN-gene would behave like a tumor suppressor and conform to Knudson's two-hit model (13).

Methods

Patients

Patients with MEN1 treated at the Endocrine Surgery and Endocrine Oncology units at Uppsala University Hospital in Uppsala, Sweden between 1984 and 2012 were reviewed. The diagnosis was performed according to standard clinical criteria (1) by experienced physicians. Briefly, MEN1 was diagnosed if a patient had two of the three main MEN1-associated lesions: hyperparathyroidism, pituitary adenoma or pancreatic neuroendocrine tumor. Hyperparathyroidism was diagnosed when hypercalcemia and inappropriately elevated PTH was present. Pituitary adenomas were diagnosed on the basis of radiological findings and hormonal screening. Pancreatic NETs were diagnosed on the basis of histopathology. All patients had undergone routine clinical genetic sequencing, Briefly, the coding exons (2, 3, 4, 5, 6, 7, 8, 9, 10) of the *MEN1* gene are amplified by polymerase chain reaction, followed by bidirectional Sanger sequencing as outlined in (12). Patients without a detected mutation in *MEN1* were identified. The selection of patients for inclusion was performed by the senior authors and included a consideration of the available biomaterials. All patients provided written informed consent and ethical approval was obtained from the Regional Ethical Review Board (*Regionala etikprövningsnämnden i Uppsala*, 2014/413). Further, all patients were informed of the results of this study by their physicians and were offered clinical genetic counselling.

DNA extraction

Genomic DNA was extracted from whole blood using Qiagen DNEasy Blood&Tissue kit in accordance with the manufacturer's instructions. For patients without a clear disease-causing mutation in the constitutional DNA, from whom fresh frozen tumor material was available, DNA was extracted from matched neoplastic tissue (parathyroid or pancreatic neuroendocrine tumors) using the same kit.

Whole genome sequencing

Extracted DNA was subjected to library preparation and whole genome sequencing on an Illumina HiSeq2500 at the SNP&Seq-platform, Science for Life Laboratory (Uppsala node). Generated reads were mapped to the reference genome (human_g1k_b37) using BWA, followed by removal of duplicate reads and base quality score recalibration using GATK.

Data availability

There are currently no data repository accepting whole genome sequencing data that is fully compliant with the General Data Protection Regulation (GDPR). Once such a repository is available, the sequencing data will be appropriately deposited. In anticipation of this, requests for data access can be directed to the corresponding author.

Germline variant calling

Germline variants were called using HaploTypeCaller from the GATK. Variants were annotated with predicted impact using SnpEff (14), and for gnomAD (15) filtering allele frequency (16) and SweGen (17) allele count using vcfanno.

Somatic variant calling

Somatic mutations were called using FreeBayes (18) in parallel on ten CPUs using freebayes-parallel with a region size of 1 Mb. Mutations were called if they had a minimum alternate count of 4 and a minimum alternate allele fraction of 5%. Mutations were annotated as somatic using vcfsamplediff. Somatic mutations were extracted using a custom script.

Variants with an allele frequency of less than 15% or an alternate allele count of less than six were subsequently filtered out, as were variants with a mean quality of the alternate allele of less than 20. Variants were annotated with SnpEff.

Somatic copy number analysis

A file with loci to include was generated from the 1000 Genomes phase 1 high confidence SNPs by extracting the locations of SNPs with allele frequencies of 0.3 or greater from the VCF file using a custom python script. BAF and LogR files for the samples were generated using alleleCount and convertAlleleCounts.R (from ASCAT (19, 20)). ASCAT v 2.3 was applied to the BAF/LogR files.

Analysis of germline variants

Coding variants and variants in splice sites in selected genes (*MEN1*, *CDKN1B*, *CASR*, *CDC73*, *RET* and *AIP*) were manually inspected. In order to exclude false-negative results due to insufficient coverage, the coverage of all exons in the longest transcript of each of these genes, with a padding of eight bases upstream and downstream of each exon, was extracted using the igvtools (21) count function. The mean and minimum coverage for each gene was calculated using custom scripts.

For patients without credible germline driver variants in *MEN1* or known phenocopy genes, all missense (or equivalent) and truncating (or equivalent) mutations in a set of 96 genes associated with DNA repair or cancer syndromes (list extracted from a recent publication on pancreatic neuroendocrine tumors (22)) with a PHRED genotype quality of 20 and a read depth of at least 10 reads were extracted and manually curated.

For the analysis of potential novel MEN genes, all missense (or equivalent) and truncating (or equivalent) mutations with a PHRED genotype quality of 20 and a read depth of at least 10 reads were extracted. These were filtered against pre-calculated filtered allele frequencies from gnomAD. The maximum credible allele frequency was calculated as $\frac{\text{Prevalence} \times \text{maximum allelic contribution}}{\text{Penetrance}}$ (16). The parameters were generously estimated to MEN1 prevalence of 1/10 000, maximum allelic contribution 0.25 and penetrance 0.7, which resulted in a cutoff of 3.6×10^{-5}.

Potential novel MEN genes were studied under a tumor suppressor model in which a missense/truncating somatic variant was required in the presence of a missense/truncating germline variant.

Results

Cohort description

Out of 128 patients in a local MEN1 database comprising the patients with a MEN1 diagnosis between 1984 and 2012 at a tertiary referral center, 33 did not have a known mutation in *MEN1*. Fourteen were included in this genetic study (Fig. 1). Characteristics of the included patients, including fulfilled MEN1 critera are presented in Table 1, Supplementary Table 2 and Supplementary materials (see section on supplementary materials given at the end of this article). One patient had only one disease manifestation, while 12 of the patients had 2 main MEN1 manifestations (hyperparathyroidism, pancreatic neuroendocrine tumors and pituitary adenomas), and one patient had three major manifestations. All fourteen patients had hyperparathyroidism, seven had pituitary adenomas (the majority of which were prolactinomas), and seven had pancreatic neuroendocrine tumors. The average age at the diagnosis of the first manifestation was 47 years (range 24–69 years). Of the patients, seven were female and seven were male.

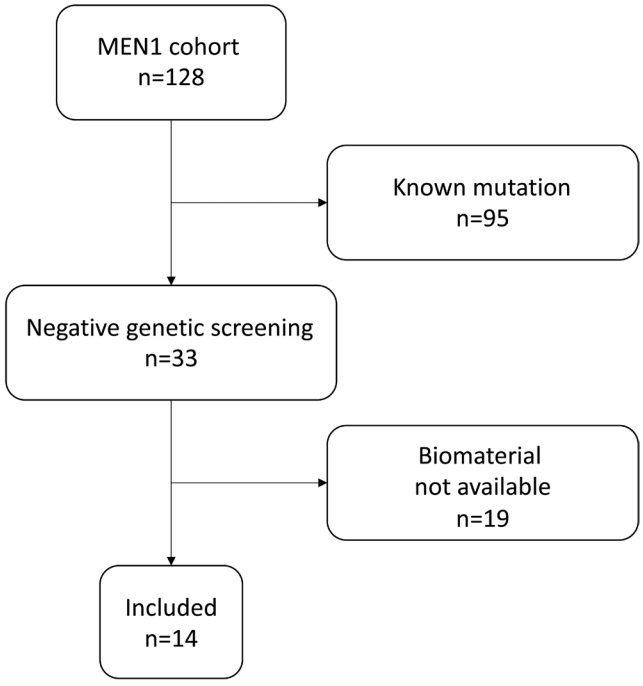

Figure 1
Flow chart of patient inclusion. The initial MEN1 cohort includes patients treated at a tertiary referral center between 1984 and 2012.

Sequencing quality

Characteristics of the germline sequencing data are presented in Supplementary Table 1. The mean coverage was 37.8X (range 28.9X to 45.2X). The coverage of the *MEN1*, *RET*, *CDKN1B*, *CASR*, *CDC73* and *AIP* coding regions and splice sites was comparable to the genomic coverage and none of the sequenced germline samples lacked coverage of any coding region in these genes. Coverage statistics for the genome and selected genes are presented in Supplementary Table 3.

MEN1 mutations

We first scrutinized variants in the *MEN1* gene in order to detect any mutations that had been missed in clinical genetic screening. Three of the included patients were found to carry pathogenic (according to ACMG-AMP criteria (23)) variants in the *MEN1* gene (Table 2). One patient carried c.1186-2A>G, a splice-site variant disrupting the canonical GU-AG splice motif. Another patient carried the synonymous p.Arg223Arg variant, also located in a splice site. Finally, a third patient carried a p.Pro12Leu missense mutation. None of the variants were present in the gnomAD database. All variants were classified as pathogenic (Supplementary Table 2). In order to identify larger structural variants affecting the *MEN1* gene, candidate germline structural variants and copy number alterations were interrogated. No variants overlapped the *MEN1* locus.

Known MEN1 phenocopies

We subsequently analyzed variants in genes causing known MEN1 phenocopies. Other than a common and benign missense variant (rs2066827, gnomAD allele frequency 0.2701) found in two patients, no protein-altering variants were found in *CDKN1B*. No protein-altering variants were found in the *GCM2* gene. Other than two common missense variants (rs641081 and rs4930199) found in all cases, no protein-altering variants were detected in the *AIP* gene. The common polymorphism rs1799939 (p.G691S, gnomAD allele frequency 0.205) in *RET* was detected in four patients. Additionally, one patient carried the rs201740483 (p.T1038A) variant, which is classified as Likely benign ($n=4$) and Uncertain significance ($n=1$) in ClinVar and has an allele frequency of 0.0006 (0.0019 in Non-Finnish Europeans) in gnomAD. All of these variants were excluded as causes of the disease on the basis of allele frequency and/or prior classifications.

One patient carried a *CASR* p.Ile555Val variant, previously reported in a Danish patient with familial hypocalciuric hypercalcemia (FHH). The MEN1 diagnosis in this patient was based on primary hyperparathyroidism and the presence of a neuroendocrine tumor metastasis in the liver, with an unknown primary tumor.

Patient 14 had a first-degree relative with parathyroid cancer and had been diagnosed with primary hyperparathyroidism. Copy number analysis revealed a heterozygous germline deletion of a segment on chromosome 1, spanning the *CDC73* locus (Fig. 2). No other protein-altering variants were found in *CDC73*.

Variants in cancer-associated genes

In the patients without clear pathogenic mutations, we specifically interrogated genes with known involvement in DNA damage repair or cancer syndromes, as such mutations have previously been described in patients with pancreatic neuroendocrine tumors (22). Twenty-eight variants with allele frequencies less than 1% in gnomAD were detected (Supplementary Table 4). The majority (18/28) were classified as likely benign on the basis of ClinVar entries. The majority of the remaining variants were missense variants of unknown significance. There

Table 1 Overview of the included patients.

Case ID	*MEN1* variant WGS	Other variant WGS	Sex	Family history	HPT	Pituitary tumor	PNET type	Adrenal	Tumor sequenced
1			M	No	Yes	GH-producing			HPT
2			F	No	Yes	Prolactinoma		Bilaterally enlarged	HPT
3			M	No	Yes		Insulinoma		Insulinoma
4			M	No	Yes	Prolactinoma		Right adrenal enlarged	HPT
5			F	No	Yes		Non-functioning		
6		*CASR* p.Ile555Val	F	Yes	Yes		Non-functioning*		
7	c.1186-2A>G		M	No	Yes		Gastrinoma	Bilateral hyperplasia	
8	p.Arg223Arg		F	No	Yes	Non-functioning	Non-functioning		
9		*ATM* p.Val2886fs	F	Uncertain	Yes	Prolactinoma			
10			M	No	Yes	Prolactinoma		Left adrenal adenoma	HPT
11			F	No	Yes		Insulinoma		Insulinoma
12			F	Yes	Yes	Prolactinoma with GH secretion			
13	p.Pro12Leu		M	N/A	Yes		Insulinoma, gastrinoma	Adenoma	
14		*CDC73* deletion	M	Yes**	Yes				

More detailed clinical information is included in Supplementary Table 1.
*The patient had a liver metastasis with unknown primary, reportedly positive for Chromogranin A and pancreatic polypeptide. **Sibling afflicted with parathyroid carcinoma.

were no nonsense variants, and only a single frameshift variant: Patient 9 was found to harbor a p.Val2886fs variant in *ATM* which is reported as 'likely pathogenic' in ClinVar. However, the patient was heterozygous for this variant which is associated with the autosomal recessive *ataxia telangiectasia* syndrome, and its involvement in the tumors of this patient remains uncertain.

Somatic mutations

Tumor DNA from patients with no apparently disease-causing germline mutation, from whom adequate tumor tissue was available, was subsequently subjected to whole genome sequencing (Table 3). After filtration for quality, read depth and effect, the tumors had on average 25.8 (range 16–34) putative protein-altering or splice-affecting mutations. Variants in Cancer Gene Census genes are presented in Table 4 and the full list of mutations are presented in Supplementary Table 5.

Analysis of potential novel MEN genes

In order to identify potential novel MEN genes, germline variants with sufficient quality that had a filtering allele frequency of 3.6×10^{-5} or less were investigated. On average 110 (range 94–129) such variants were present in

Table 2 *MEN1* variants.

Case ID	Location	Ref	Alt	ENST00000337652	Protein	Classification*
7	chr11:64572290	T	C	c.1366-2A>G		Pathogenic
8	chr11:64575363	C	G	c.669G>C	p.Arg223Arg	Pathogenic
13	chr11:64577547	G	A	c.35C>T	p.Pro12Leu	Pathogenic

*Classification according to ACMG-AMP criteria (23).

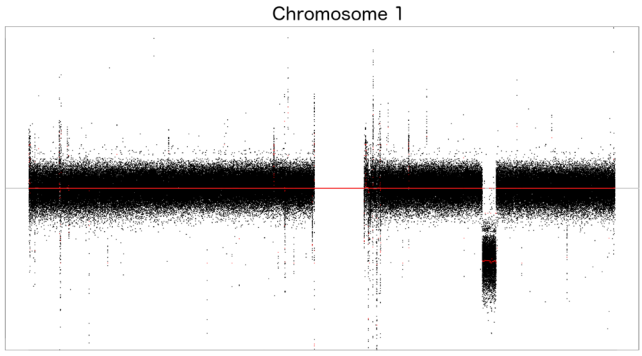

Figure 2
Gross deletion on chromosome 1 including *CDC73* in patient 14.

each germline sample. No such variants occurred in the genes included in the Cancer Gene Census. Each tumor-normal pair was assessed individually under a tumor-suppressor model which required independent somatic and germline hits affecting the same gene. Variants in pseudogenes and germline variants present in more than a single instance in SweGen were excluded. After filtration, only a poorly studied lincRNA (*AL390778.1*), found mutated in a single patient, remained. Numerous frameshift variants in this gene are present in gnomAD, rendering it a highly unlikely candidate gene.

Somatic copy number variants

Since a somatic second-hit to a tumor suppressor gene could also be caused by a copy number alteration, we analyzed the copy number profiles of the lesions. Four of the six interrogated tumors were diploid (Supplementary Figs 1, 2, 3, 4, 5 and 6). One carried a deletion of part of chromosome 1, and amplification of part of chromosome 9 (Fig. 3). Another tumor had a low-amplitude alteration, indicative of either low tumor purity or a subclonal event.

The tumor sample with loss of part of chromosome 1 was screened for deleterious somatic mutations on the remaining copy. Two mutations remained after filtering for effect and filtering allele frequency: A missense variant in *KIF1B* and a missense variant in *KIAA2013*. Allele frequency data suggested that the tumor cells were homozygous for both variants. SIFT classified the *KIF1B* variant as tolerated and the *KIAA2013* variant as damaging, while PROVEAN classified the *KIF1B* variant as deleterious and the *KIAA2013* variant as neutral. No somatic mutations were detected in this region.

In total, five out of the fourteen patients had identifiable causative constitutional genetic variants, while the remainder could not be demonstrated to carry disease-causing mutations in their germline DNA.

Discussion

We describe an in-depth genetic characterization of a series of patients with a clinical diagnosis or suspicion of MEN1, who were negative for *MEN1* mutations in clinical screening. Three patients were found to carry *MEN1* mutations by whole-genome sequencing. Two of these mutations affected splice sites, while one was a missense mutation. All patients with *MEN1* mutations developed pancreatic neuroendocrine tumors and were the only three patients with two or more pancreatic lesions. One patient had a *CDC73* deletion, and one patient had a *CASR* mutation. For the remaining nine patients, no causative genetic event was identified.

There are several possible reasons for these mutations being missed in clinical screening. No technique is perfectly sensitive, and variations in the protocols used occur over time. All patients had undergone Sanger sequencing of the coding exons of the *MEN1* gene, which theoretically is a very sensitive method for heterozygous germline mutations. However, false-negative genetic tests are not a novel concept and may occur due to user error, technical issues, or sequence characteristics leading to for example, allelic dropout (24, 25). Moreover, detected mutations (i.e. mutations present in the sequencing traces) may be missed during analysis due to human error or poor sequencing data. Variant interpretation represents

Table 3 Sequenced tumor lesions.

Case ID	Sequenced tissue	Mean coverage	Number of somatic mutations	Somatic copy number aberrations
1	HPT	74.2X	34	No
2	HPT	74.7X	29	No
3	Insulinoma	72.1X	26	No
4	HPT	69.9X	28	No
10	HPT	70.3X	22	Yes
11	Insulinoma	72.4X	16	Yes

Table 4 Somatic mutations in Cancer Gene Census genes.

Case ID	Location	Ref	Alt	Gene	Protein impact
2	chr22:24175838	CT	CTTT	*SMARCB1*	p.Thr366fs
3	chr4:87968589	T	C	*AFF1*	p.Val301Ala
3	chr11:108119812	T	G	*ATM*	p.Asp406Glu
4	chr5:38490309	C	A	*LIFR*	p.Gly717Val
10	chr11:534286	C	G	*HRAS*	p.Gly13Arg
10	chr20:31022485	AGG	AG	*ASXL1*	p.Gly658fs
11	chr10:81470419	G	A	*NUTM2B*	p.Gly545Ser

an additional potential caveat. Indeed, two of the three additional mutations were located in or near splice-sites, and one of these also leads to a synonymous change of an amino acid-encoding codon. The interpretation of these variants may be more difficult than for example nonsense or frameshift mutations, meaning that they may be more likely to be missed. There is to the best of our knowledge no data establishing the analytical sensitivity of Sanger sequencing for *MEN1* gene detection, although Sanger sequencing and targeted NGS have been shown to have identical sensitivity (26).

The present study employed whole-genome sequencing, while routine clinical genotyping typically relies on Sanger sequencing or targeted next generation sequencing. The rationale for using WGS in the present study was to enable detection of mutations in novel disease-causing genes. WGS is not a perfectly sensitive or specific method, and there is a possibility of both false-negative findings due to for example insufficient coverage and false-positive findings due to sequencing errors, issues with alignment, and insufficiently strict criteria for variant calling. To minimize the risk for false-negative results in the present study, we calculated the coverage of each of the main genes of interest. The advantage of whole genome sequencing over exome sequencing is slightly greater power for coding variant detection and greater resolution for copy number variant detection (27), in addition to the possibility to call mutations in non-coding regions, at the cost of lower coverage and/or higher sequencing costs. As the number of genes currently implicated in multiple endocrine neoplasia are few, with relatively specific phenotypes, there is currently no rationale to use whole genome sequencing outside of a research setting. Moreover, it has been demonstrated that intronic *MEN1* mutations are a rare cause of the MEN1 syndrome, if at all (26), suggesting that sequencing of the exons and splice sites is generally sufficient. In the present study we additionally sequenced tumor DNA from a subset of the included patients in order to apply a two-hit tumor suppressor model for gene discovery. While this may be useful in a research setting, we do not consider it to add value in a routine clinical genetic setting.

Two patients presented with clear phenocopies. Patient 14 did not fulfill the diagnostic criteria for MEN1. However, at the time of the initial parathyroidectomy he was suspected to have MEN1 and underwent screening according to an MEN1 surveillance protocol for almost two decades. A thorough review reveals that his brother had parathyroid cancer, suggesting that the HPT-JT syndrome is a far more likely diagnosis. Indeed, this patient was found to carry a heterozygous deletion of the entire *CDC73* gene. Gross deletions of the *CDC73* gene have recently been described in a number of patients with HPT-JT syndrome (28, 29, 30). One patient had *CASR* mutation that had previously been described in a Danish cohort (31). The diagnosis of MEN1 in this patient was based on hyperparathyroidism and a liver metastasis of a neuroendocrine tumor. Based on the finding of a *CASR* germline mutation, it is likely that the patient had coincident familial hypocalciuric hypercalcemia or HPT and a sporadic neuroendocrine tumor. The father of this patient had received a diagnosis of HPT, and a daughter had been diagnosed with mild HPT prior to this study but has not undergone surgery. Both HPT-JT and FHH accompanied by sporadic endocrine tumors have previously been reported misdiagnosed as MEN1, and represent a diagnostic pitfall (32).

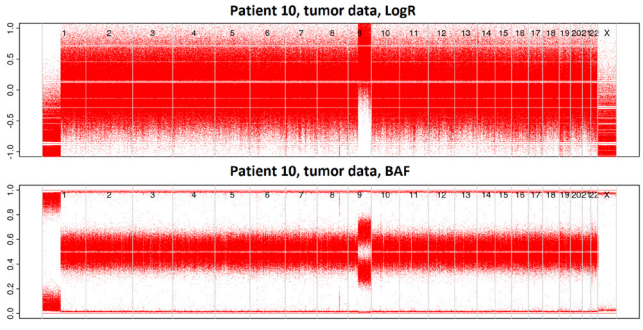

Figure 3
Somatic copy number variants.

The absence of mutations in *CDKN1B* is not surprising as these mutations have proved to be a rare cause of MEN, with approximately 20 cases described to date (33).

No recurrent somatic mutations were found in the insulinomas or parathyroid lesions, and no mutations were detected in the *MEN1* or *YY1* genes. Previous exome sequencing studies have demonstrated a relatively low prevalence of recurrent somatic mutations in these tumor types (34, 35). In the light of these studies, the present results are not surprising.

The candidate gene analyses did not identify any recurrent genes with both a somatic and a germline hit. Patient 10 carried two rare missense variants in genes located in a region of chromosome 1 that was deleted in the tumor. It cannot be excluded that one of them is related to the tumors, although in the absence of further evidence they remain variants of uncertain significance. The conflicting *in silico* predictions of their pathogenicity as well as the lack of recurrent hits in these genes suggest that they are not causative of the MEN1 phenotype.

A majority of the included patients (9/14) were not found to carry disease-causing or likely disease-causing germline variants. Consequently, it may be possible that mutation-negative MEN1 is due to random coincidence of two separate and genetically unrelated endocrine lesions in a single patient. Both hyperparathyroidism and pituitary adenomas are relatively common in the population, and the occurrence of one endocrine tumor in a patient might precipitate increased laboratory work-up and higher likelihood of diagnosis of a second lesion. Moreover, common genetic variants may confer a small risk increase, although this needs to be investigated in a large cohort of patients and controls. Additionally, mutations in *MUTYH*, *CHEK2* and *BRCA2* have been described in patients with pancreatic NETs (22); however, no such mutations were detected in the patients included in this study. The prevalence of primary hyperparathyroidism in certain studies approaches 1% (36), and the prevalence of clinically apparent pituitary adenomas may be up to 0.1% (37). The prevalence of both HPT and PA increases with age and can be assumed to be even more common in older patients. Moreover, undiagnosed HPT and pituitary lesions are apparently even more common. Ten percent of healthy volunteers have detectable pituitary micro-adenomas on MRI (38) while autopsy series have demonstrated a 7 and 2.4% prevalence of parathyroid hyperplasia and adenoma (39), respectively. Given these data, we estimate that between 1% (given the clinical prevalence) and 18% (given the autopsy/imaging prevalence) of patients with sporadic pancreatic neuroendocrine tumors may meet the diagnostic criteria for MEN1 by chance alone. This interpretation is in line with the observation that mutation-negative patients develop MEN1-related tumors at a higher age and are less likely to develop additional manifestations of the disease (10), and the fraction of patients with a MEN1-like presentation who are not found to carry a mutation has recently been reported to possibly be higher than previously thought (40). Notably, the three patients in the present study who were found to carry *MEN1* mutations all had pancreatic lesions. It is well established that patients with pancreatic involvement are more likely to carry a mutation than those with only non-pancreatic lesions. Currently, it is not clear if and how mutation-negative patients with a lack of family history should be followed.

Notably, many of the patients were diagnosed with their first endocrine tumors relatively late in life. Of the nine patients without a disease-causing mutation, four received their first diagnosis after the age of 60 years, and the mean age at diagnosis was 60 years. Moreover, the majority did not have any known family history of MEN1-associated lesions. All nine had two lesions, with HPT in combination with a pituitary adenoma being the most common combination (6/9). This is concordant with previous studies showing that the combination of HPT and pituitary adenomas without pancreatic involvement is associated with a low risk of carrying an *MEN1* mutation (41).

The present study has several limitations, in addition to the small sample size. Firstly, mosaicism for *MEN1* mutations has been reported (42). Somatic mosaicism for *MEN1* mutations due to a postzygotic event causing mutations to be present in some tissues but not in others may contribute to genetic underdiagnosis of the syndrome. However, the absence of *MEN1* mutations from tumor tissues in this cohort renders the presence of mosaicism in the present material unlikely, as a mosaic mutation leading to tumor development would be readily detected in tumor tissue due to clonal expansion. However, future studies may consider epigenetic mechanisms of *MEN1* inactivation, due to for example promoter hypermethylation. This could be readily studied using bisulfite sequencing of the *MEN1* promoter region. Epigenetic germline inactivation of *SDHC* by promoter hypermethylation has been described in a patient with multiple paragangliomas (43). Such a mechanism has to the best of our knowledge not been studied in MEN1. Moreover, the analysis of potential novel MEN genes was limited to a tumor-suppressor model. While activating

germline variants in proto-oncogenes do occur in human tumor syndromes (e.g. *RET* variants in MEN2 (44)), they are rare in comparison with inactivating germline variants in tumor-suppressor genes. While a tumor-suppressor gene can be identified through bi-allelic inactivation, a putative proto-oncogene would require either thorough functional characterization or a different study setup allowing segregation analysis. Nevertheless, no gene was found to have sufficiently rare missense variants in more than two samples in the current cohort, or in any of the genes included in the Cancer Gene Census. Consequently, we conclude that if such mutations cause the MEN1 phenotype, they do so in a small fraction of patients.

Conclusion

While patients with negative genetic screening for *MEN1* mutations may occasionally be found to harbor a mutation upon rescreening, or to present as one of the known phenocopies, a majority (9/14) of patients in the present study were not found to carry a disease-causing germline variant. This highlights the possibility that a subset of mutation-negative MEN1 patients may fulfill the diagnostic criteria simply due to the sporadic co-incidence of two or more endocrine tumors.

Supplementary materials
This is linked to the online version of the paper at https://doi.org/10.1530/EJE-19-0522.

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