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Original article

PHENOTYPE AND GENOTYPE OF PATIENTS WITH MULTIPLE ENDOCRINE NEOPLASIA TYPE 1 STUDIED IN ARGENTINA

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Abstract

Introduction: Multiple Endocrine Neoplasia type 1 (MEN1) is an autosomal dominant inherited disease with an estimated prevalence of 2-10:100 000. The main locations of tumors are parathyroid glands (HPT), gastroenteropancreatic tract (GEPT), and anterior pituitary gland (PT).

The aim of our investigation was to describe the phenotype and genotype of Argentinian patients with MEN1.

Methods: A total of 68 index patients diagnosed with at least two of the three main tumors or one tumor and a relative with MEN1, and 84 first-degree relatives were studied. We sequenced the coding region (exons 2-10); the promoter, exon 1; and the flanking intronic regions of the MEN1 gene, following the Sanger method. We used MLPA in index patients without mutation.

Results: Prevalence of tumors: HPT 87.5%, GEPT 49% ($p< 0.001$). No statistical differences in the prevalence of HPT vs. PT (68%). Prevalence of pathogenic variants: 90% in familial cases and 51% in sporadic cases. Of the different 36 pathogenic variants, 13 (36.2%) were frameshift micro-rearrangement, 8 (22.2%) were missense, 9 (25%) were nonsense, 3 (8.3%) were mutations in splicing sites, 2 (5.5%) were large deletions and, 1 in-frame micro-rearrangement. We found 7 novel pathogenic variants. Thirty-nine percent (n = 33) of first-degree relatives of 23 families were found to be mutation carriers.

Conclusion: The phenotype and genotype of Argentinian patients was similar to other MEN1 populations. A high frequency of PT and the identification of seven novel mutations are underscored.

Key words: multiple endocrine neoplasia type 1, MEN1, MEN1 pathogenic variants, primary hyperparathyroidism, pituitary adenomas, pancreatic neuroendocrine tumors

Resumen

Fenotipo y genotipo de pacientes con neoplasia endocrina múltiple tipo 1 estudiados en Argentina

Introducción: La neoplasia endocrina múltiple tipo 1 (NEM1) es una enfermedad hereditaria autosómica dominante con una prevalencia estimada de 2-10:100 000. Las localizaciones principales de los tumores son glándulas paratiroides (HPT), tracto gastroenteropancreático (TGEP) y glándula pituitaria (TP). El objetivo de nuestra investigación fue describir el fenotipo y genotipo de pacientes argentinos con NEM1.

Métodos: Estudiamos 68 casos índices diagnosticados por presentar al menos dos de los tres tumores principales, o un tumor y un pariente con NEM1, y 84 familiares de primer grado. Secuenciamos la región codificante (exones 2-10); el promotor, exón 1; y las re-

giones intrónicas flanqueantes del gen *MEN1* siguiendo el método de Sanger. Utilizamos MLPA en pacientes índice sin mutación.

Resultados: Prevalencia de tumores: HPT 87.5%, TGEP 49% ($p < 0.001$), sin diferencias estadísticas entre las prevalencias de HPT vs TP (68%). Prevalencia de variantes patogénicas: 90% en casos familiares y 51% en esporádicos. Hallamos 36 variantes patogénicas, 7 (20%) fueron noveles. Fueron 13 (36.2%) microarreglos con cambio en el marco de lectura, 9 (25%) variantes sin sentido, 8 (22.2%) con cambio de sentido, 3 (8.3%) en sitio de unión de empalme, 2 (5.5%) grandes deleciones y 1 microarreglo sin cambio en el marco de lectura. El 39 % (n = 33) de los parientes de primer grado en 23 familias fueron portadores de mutaciones.

Conclusión: El fenotipo y genotipo de los pacientes argentinos con NEM1 fue similar al de otras poblaciones. Destacamos una alta frecuencia de TP y de variaciones patogénicas noveles.

Palabras clave: neoplasia endocrina múltiple tipo 1, NEM1, mutaciones del gen *MEN1*, hiperparatiroidismo primario, adenomas hipofisarios, tumores neuroendocrinos pancreáticos.

PUNTOS CLAVE

Conocimiento actual

- Multiple endocrine neoplasia type 1 (MEN1) is a rare disease of autosomal dominant inheritance. Young patients develop tumors in the parathyroid glands, endocrine pancreas, and pituitary gland (3P). Genetic diagnosis in the tumor suppressor gene *MEN1* allows early diagnosis of tumors and improved life prognosis.

Contribución del artículo al conocimiento actual

- We studied the prevalence of tumors and germline variations of the *MEN1* gene in 68 index cases and 84 first-degree relatives. We highlight a high frequency of pituitary tumors and novel pathogenic variants.

Multiple endocrine neoplasia type 1 (MEN1, OMIM #131100) is a rare disease of autosomal dominant inheritance with an estimated prevalence of 2-10:100 000 inhabitants. MEN1 predisposes patients to various types of tumors, most of them endocrine tumors. The three most frequent tumor locations are the parathyroid glands, with their functional expression as hyperparathyroidism (HPT); the gastroenteropancreatic tract (GEPT); and the anterior pituitary gland (PT). In the context of MEN1, HPT has a penetrance greater than 90%, GEPT tumors between 30% and 80%, and PT between 30% and 50%. Additionally, a significant number of patients have tumors of the adrenal cortex, neuroendocrine tumors of the thymus, facial angiofibromas, collagenomas, and lipomas[1-7].

The gene involved in this disease is *MEN1*, a tumor suppressor gene located in chromosome 11q13. It consists of 10 exons that cover approximately 1.83 kb of coding DNA and determines the synthesis of menin, a 610-amino acid protein[8]. Menin is a nuclear protein with several molecular functions: it interacts with DNA, chromatin, and several proteins; regulates transcription and repair of DNA, genome stability, cytoskeletal components, and telomerase activity; and, perhaps most importantly, exerts a negative regulation of cell proliferation[9-11].

More than 1300 germline and somatic pathogenic variants have been identified throughout the coding region, flanking intronic and splicing sites[12]. The spectrum of variants found includes those that produce frameshifts and nonsense variants that predict a truncated protein, changes in splicing sites that result in loss of exons or inclusion of introns and altered coding sequence, and missense variants that could partially or completely alter menin's regulatory functions[10-13].

The finding of germline *MEN1* pathogenic variants is necessary to confirm the clinical diagnosis in index cases, defines carrier status among at-risk relatives, and enables preimplantation and prenatal genetic diagnosis. Identification of mutation carriers together with early detection of tumors would result in decreased morbidity and mortality in these patients[14-22].

Between 5% and 30% of patients with MEN1 might not harbor a mutation in the coding region of *MEN1*. These could be phenocopies, that is, patients with the MEN1 phenotype in whom no mutation is found in the *MEN1* gene. Mutations may or may not be found in other genes, such as *CDKN1B*[20-22].

The aim of our work was to determine the prevalence of the main tumors, the rate of detection of pathogenic variants, and the spectrum of genomic variants in a population of patients with a clinical diagnosis of MEN1 born in Argentina.

Materials and methods

Patients

We studied 152 subjects with clinical MEN1 or suspected MEN1 from January 2004 to January 2019. We included 68 apparently unrelated index cases from a variety of reference populations in our country, (35 female; mean age 40 ± 16 years; and 84 first-degree relatives (41 female; 34 ± 19 years). Genetic screening of *MEN1* was performed at the Molecular Biology Laboratory of the Unit of Endocrinology and Nuclear Medicine of the Italian Hospital of Buenos Aires (HIBA). The HIBA Ethics Committee and the Faculty of Medicine of the University of Buenos Aires approved the study. The patients, or their parents, and controls provided written consent to participate in the study.

We included 66 healthy subjects with no personal or first-degree family history of MEN1-related tumors or any other cancer (51 female; 34 ± 6 years) as controls.

The patients' age was registered as their age at the time of diagnosis of their first tumor or, if that information was not available, their age at the time of referral. The index case was defined as the first family member referred to the medical consultation and who underwent molecular testing.

We searched for the patient's clinical data in the HIBA electronic database or received it from professionals from other medical centers.

The clinical diagnosis of MEN1 was established according to international guidelines for the diagnosis and treatment of MEN1.[14,20]

1- Patients with at least two of the three MEN1-associated tumors and no known living or deceased first-degree relatives harboring MEN1-associated tumors were defined as sporadic index cases (S-MEN1).

2- Patients with at least one MEN1-related tumor and at least one first-degree relative with at least two major MEN1-related tumors were defined as familial index cases (F-MEN1).

3- Patients with a positive genetic diagnosis without clinical or biochemical manifestations of MEN1 were considered asymptomatic carriers.

Patients presenting only atypical clinical signs of MEN1 were not included.

Methods

DNA was extracted from peripheral blood leukocytes using the commercial Illustra blood genomic Prep Mini Spin Kit (General Electric Healthcare, UK). The entire coding region (exons 2-10), promoter, exon 1, and the flanking intronic regions of the *MEN1* gene (RefSeq NM_130799.2) were amplified and sequenced.

The amplified products were separated by electrophoresis in 1.0% agarose gel in TBE buffer, stained with ethidium bromide, and visualized under UV light. Then, the fragments were cut with a scalpel for their subsequent purification using the Promega Wizard SV Gel and PCR Cleanup System kit. Primers and cycling conditions are available upon request.

Sequencing was performed with the Sanger method. From 2006 to 2009, 33 P-labeled dideoxynucleotides (Thermo Sequenase Radiolabeled Terminator Cycle Sequencing Kit, Amersham Bioscience) were used. The sequencing products were separated by PAGE under denaturing conditions and visualized by autoradiography. From 2010 to 2019, the amplified fragments and their primers were sent for automatic sequencing to Macrogen Inc. Samples of patients in whom no mutation was identified by Sanger sequencing were studied with multiple ligand-dependent probe amplification (MLPA) technique (MRC Holland, Amsterdam, The Netherlands, EK1-FAM SALSA MLPA Reagent kit, probes P244-025R *AIP-MEN1-CDKN1B*). A genetic analyzer was used (Applied Biosystem 3500xL).

We compared the sequences obtained to the reference sequence of the *MEN1* gene (OMIM 613733). The genetic variants were named according to Human Genome Variation Society (http://www.hgvs.org/mutnomen) with position +1 in ATG of RefSeq NM_130799.2 and classified according to the American College of Medical Genetics and Genomics (ACMG) as benign variant (BV), likely benign variant (LBV), variant of uncertain significance (VUS), likely pathogenic variant (LPV), or pathogenic variant (PV) based on clinical segregation and population data [23,24].

The impact and effects of variants were analyzed using the following databases: dbSNP (http://www.ncbi.nlm.nih.gov/SNP/), Ensemble (http://www.ensembl.org/index.html), the Human Gene Mutation Database (http://www.hgmd.cf.ac.uk/ac/index.php), ClinVar-NCBI-NIH (https://www.ncbi.nlm.nih.gov/clinvar), the UMD-MEN1 database (http://umd.be/MEN1/), the Leiden Open Variation Database (https://www.lovd.nl/), GnomAD (https://gnomad.broadinstitute.org/) and the software Human Splicing Finder 3.0 (http://www.umd.be/HSF3/) and Mutalyzer (https://mutalyzer.nl).

Novel missense variants that had not been described in the literature or in genetic databases were assessed for their clinical significance and pathogenicity by sequencing 132 alleles from a population of normal controls. Also, predictive algorithms used for *in silico* analysis such as Mutation Taster, Polyphen 2, and SIFT were applied. We performed multiple alignments of the protein sequence to observe the conservation of the mutation site throughout the evolution of the species using the NCBI BLAST and CLUSTALW2.

Statistical analysis

In the descriptive analysis, quantitative data were presented using the mean ± standard deviation or the median and interquartile range (25-75%), depending on their distribution. Qualitative data were expressed in terms of absolute and relative frequencies. Prevalence of tumors was presented with its relative frequency and its 95% confidence interval (IC 95%).

To assess the normal distribution of the quantitative data, histograms, and the Shapiro-Wilk hypothesis tests were used. For the comparison between groups, the t-test was applied to the normally distributed quantitative data. Additionally, the Bartlett test was used to assess homoscedasticity. In non-normally distributed quantitative data, the Wilcoxon test was employed.

The chi-square test or Fisher test based on assumptions was used to compare qualitative data.

To compare age at diagnosis between groups of patients without pathogenic variants, and different types of pathogenic variants, Kruskal Wallis test and the Dunn post hoc test were used. We considered p values < 0.05 statistically significant. The analysis was conducted using R software version 4.2.3.

Results

Clinical features

The prevalence of HPT was 87.5 % (95% CI 73 to 93) whereas the prevalence of GEPT was 49% (95% CI: 36 to 63; p < 0.001). We found no statistical differences between the prevalence of PT (68%) (95% CI: 54 to 79) and HPT or GEPT (p > 0.05). Fourteen patients (20%) presented the 3 main tumors. HPT and PT was the most frequent association but was not significantly more common than the other associations.

Familial MEN1 (F-MEN1) was diagnosed in 42.6% of patients and sporadic MEN1 (S-MEN1) in the remaining 57.4. Patients with F-MEN1 were younger, although not significantly ($p = 0.051$), were more frequently male, and had a higher frequency of mutations (Table 1).

Patients carrying pathogenic variants (46/68) were significantly younger than those without pathogenic variants ($p < 0.001$) (Table 2). We found no significant differences in the prevalence of main tumors between familial and sporadic groups (Table 1) or among patients with and without mutations (Table 2).

Genetic features

- *Frequency of mutations*

We found germline pathogenic variants in 67.6 % (46/68) of the index cases and in 39.3 (33/84) first-degree relatives in 23 families (Tables 3 and 4).

In the F-MEN1 group, 26/29 (89.6 %) patients were carriers of pathogenic variants. Twenty-

Table 1 | Comparison between familial MEN1 index cases (F- MEN1) and sporadic MEN1 index

	F-MEN1	S-MEN1	Test de Fisher *p*
n	29	39	
With mutation; n (%)	26 (89.7)	20 (51.3)	0.002
Female; n (%)	10 (34.5)	25 (64.1)	0.030
Age; mean ± SD	36.7 ±16.4	44.3 14.8	0.051
HPT; n (%)	24 (82.8)	36 (92.3)	0.27
PT; n (%)	16 (37.1)	30 (76.9)	0.15
GEPT; n (%)	16 (55.2)	17 (43.6)	0.48

SD: standard deviation; HPT: hyperparathyroidism; GEPT: gastroentero-pancreatic tumor; PT: pituitary tumor

Table 2 | Comparison of MEN1 index cases with and without MEN1 gene mutations

	With mutation	Without mutation	*p*
n	46	22	
Female; n (%)	23 (50.0)	12 (54.5)	0.927
Age; mean ± SD	36.7±14.7	50.0±14.8	0.001
S-MEN1; n (%)	20 (43.5)	19 (86.4)	0.002
HPT; n (%)	39 (84.8)	21 (95.5)	0.260
PT; n (%)	29 (64.4)	17 (77.3)	0.434
GEPT; n (%)	25 (54.3)	8 (36.4)	0.259

SD: standard deviation; S-MEN1: MEN1 sporadic index cases; HPT: hyperparathyroidism; GEPT: gastroenteropancreatic tumor; PT: pituitary tumor

three (88.4 %) of the mutations were detected by Sanger sequencing, and the other 3, were large deletions found by MLPA (11.5%). Five patients presented novel pathogenic variants (Table 3).

In the S-MEN1 group, 20/39 (51.3%) cases were carriers of pathogenic/likely pathogenic variants, all of them detected by Sanger sequencing. We found no large rearrangements by MLPA. Two patients presented novel pathogenic variants (Table 4).

Of the 14 patients carrying 3 tumors (6 in F-MEN1 and 8 in S-MEN1), 10 presented pathogenic variants.

- *Most frequently affected exons and recurrent mutations*

We found 36 independent pathogenic/likely pathogenic variants affecting the coding exons (2 to 10) and the *splicing* introns 3, 4, and 9. Twenty-one (21/36; 58%) of the mutations found were in exons 2, 3, 9, and 10 (Figure 1).

Fifteen apparently unrelated index patients shared 6 recurrent pathogenic variants: c.249_252delGTCT, c.792delC, c.1102delG, c.1328C>A, and c.1340T>C (distributed in exons 2, 5, 8, and 9) and a full deletion of the *MEN1* gene. Reconstruction of family history was incomplete, and we could not exclude a common founder in familial and/or sporadic cases.

- *Analysis of variants patterns*

Of 36 pathogenic variants, 13 (36.2%) were frameshift microrearrangements (11 small deletions, 1 small duplication, and 1 small insertion), 9 (25) were nonsense variants, 8 (22.2) were missense variants, 3 (8.3) were variants at a splice junction, 2 (5.5) were large deletions, and 1 was an in-frame microrearrangement. Twenty-two (61.1 %) of those variants (frameshift rearrangements and nonsense) are considered truncating.

Because they are not described in our available databases (detailed in the Methods section), 7 pathogenic variants (19.4%) were considered novel: 6 frameshift rearrangements (c.244delG, c.471delG, c.483_495del13, c.487delG, c.672delA, and c.1060_1063dupTGCC) and 1 missense mutation (c.1664G> T). *In silico* predictions suggested that c.1664G>T is a pathogenic variant. This variant was not present in 132 alleles of our normal control subjects and absent in 1000 genomes project.

We detected 10 benign and probably benign variants according to ACMG, in 29/68 patients (42.6%) localized in exons 1, 2, 3, 8, 9, and 10 and introns 1, 2, and 9. The most frequently found benign variant was c.1254 C>T (31.7%), followed by c.-533T>A (24.4), IVS1-39C>G (9.8), IVS2+168G>A (9.8), c.512G>A (7.3), IVS9+103C>G (4.8), IVS1-17C>G (4.8), and c.435C>T, c.1621G>A, and c.1080 C>A (2.4 % each). Two of them, c.512G>A and c.1080 C>A, are considered LBV.

- *Correlation genotype/phenotype*

To study the correlation genotype/phenotype and ensure that sample sizes were adequate, patients were divided into three groups depending on the presence and types of variants: 1- Absence of pathogenic variants, (n = 22); 2- Mild variants (missense), (n = 11) and 3- Severe variants (nonsense, frameshift rearrangements and large deletions), (n = 34). No differences were found among the groups and the presence of tumors (HTP, PT, and GEPT; p = 0.47, p = 0.57, and p = 0.28, respectively).

Table 3 | Phenotypic and genotypic characteristics of familial MEN 1 patients (F- MEN1)

Case	Gender	Age (y)	Pheno-type	Exon	Codon	Genetic variant	Type of variant	Predic-tive effect	Pro-tein variant	Novel	Positi-ve rela-tives ratio
1	F	25	HPT/PT	2	8	c.22A>T	P	NS	p. K8X		2/2
2	M	33	HPT	2	82	c.244delG	P	FSµRGT	p.D82Yfs	*	
3	M	9	PT/TGEPT	2	96	c.286C>T	P	NS	p.Q96X		1/1
4	M	61	PT /HPT/GEPT	2	83-84	c.249-252delGTCT	P	FSµRGT	p.I85Sfs		5/12
5	F	39	PT /HPT/GEPT	3	156	c.466G>T	P	MS	p.G156C		
6	F	15	PT	3	157	c.471delG	P	FSµRGT	p.A158Pfs	*	2/5
7	M	35	HPT/GEPT	3	161-165	c.483_495del13	P	FSµRGT	p.V162Rfs	*	
8	F	20	GEPT	3	163	c.487delG	P	FSµRGT	p.A164Pfs	*	0/3
9	F	42	PT/HPT/GEPT	3	171	c.512G>A	LBV	MS	pR171Q		
10	M	44	PT/HPT/GEPT	3	184	c.551T>A	P	MS	p.V184E		1/4
11	F	58	HPT/GEPT	3		c.593G>A/	P	NS	p.W198X		1/1
				1		c.-533T>A	B				
				int1		IVS1-39C>G	B				
				int2		IVS2+168G>A	B				
12	M	16	HPT/ PT	Int 3	—	IVS3-1 G>A	P	SJ	—		1/1
13	M	32	HPT/Thymoma NET	5	264	c.791T>C	P	MS	p.L264P		2/5
14	M	33	HPT/ PT	7	349	c.1045C>T	P	NS	p.Q349X		
					418	c.1254C>T	B		p.D418D		
15	M	70	HPT/GEPT	8	368	c.1102delG	P	FSµRGT	p.A368Pfs		3/11
16	F	34	HPT/ PT	8	368	c.1102delG	P	FSµRGT	p.A368Pfs		1/1
17	M	40	HPT/GEPT	8	354-355	c.1060_1063dupTGCC	P	FSµRGT	p.R355Lfs	*	2/6
				3	171	c.512G>A	LBV	MS	p.R171Q		
18	M	2	AC	9	415	c.1243C>T	P	NS	p.R415X		
19	M	56	HPT/GEPT	9	443	c.1328 C>A	P	MS	p.S443Y		
				1	—	c.-533 T>A	B	—	—		
				int 1	—	IVS1-17 C>G	B	—	—		
				int 2	—	IVS2+168 G>A	B	—	—		
20	M	28	HPT/ PT	9	447	c.1340T>C	P	MS	p.F447S		3/4
21	M	38	HPT/GEPT	9	447	c.1340T>C	P	MS	p.F447S		2/2
22	M	34	HPT/GEPT	9	447	c.1340T>C	P	MS	p.F447S		1/1
23	M	35	GEPT	int 9	—	IVS9+1G>A	P	SJ	—		1/3
24	F	28	HPT/ PT			Total deletion allele	P	HI	—		1/3
25	F	32	HPT/ PT	7-10		Partial deletion allele	P	HI	—		
26	M	70	HPT/ PT	—	—	—	—	—	—		
27	M	53	HPT/ PT/ GEPT	9		c.1252G>A	P	MS	p.D418N		3/6
28	M	45	HPT/GEPT	1		c.-533T>A/	B				
				9		c.1254C>T	B		p.D418D		
29	F	37	PT /HPT/GEPT			Total deletion allele	P	HI			0/3

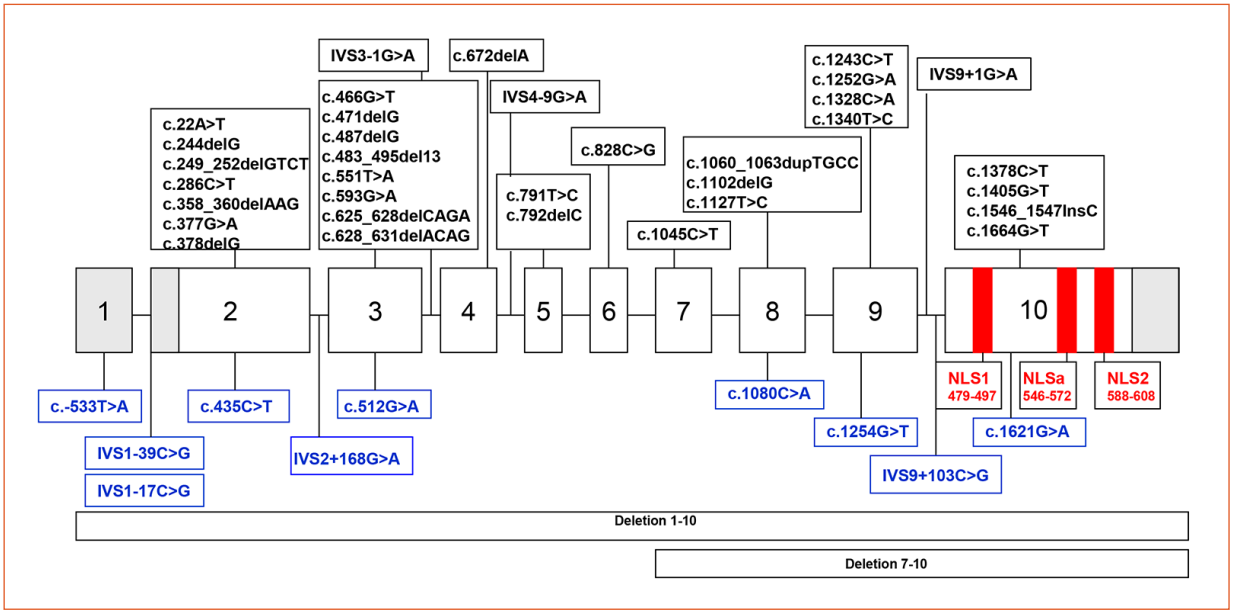
HPT: hyperparathyroidism; GEPT: gastroenteropancreatic tumor; PT: pituitary tumor; B: benign variant; P: pathogenic variant; LBV: likely benign variant; LPV: likely pathogenic variant; FSµRGT: frameshift micro-rearrangement; NS: nonsense; MS: missense; In-frame microRGT: in-frame micro rearrangement; SJ: splice junction; HI: haploinsufficiency, AC: asymptomatic carrier, first and only member of a family with a clinical diagnosis of MEN1 undergoing genetic diagnosis

Table 4 | Phenotypic and genotypic characteristics of sporadic MEN 1 patients (S- MEN1)

Case	Gender	Age (y)	Pheno-type	Exon	Codon	Genetic variant	Type of variant	Predic-tive effect	Pro-tein variant	Novel	Positi-ve rela-tives ratio
1	F	15	GEPT/HPT	2	83-84	c.249_252delGTCT	P	FSµRGT	p.I85Sfs		
2	F	43	HPT/Adrenal Adenoma	2	83-84	c.249_252delGTCT	P	FSµRGT	p.I85Sfs		1/5
				9	418	c.1254C>T	B		p.D418D		
3	M	34	HPT/PT	2	83-84	c.249_252delGTCT	P	FSµRGT	p.I 85 Sfs		0/1
4	F	38	GEPT/ PT	2	126	c.378delG	P	FSµRGT	p.W126Xfs		
				3	171	c.512G>A	LBV		p.R171Q		
5	M	14	HPT/ PT	2		c.358_360delAAG	P	*In frame-*	p.K120del		
				1		c.-533T>A	B	µRGT			
				int1		c.-39C>G	B				
6	F	38	HPT/ GEPT/ PT	2	126	c.377G>A	P	NS	p.W126X		
7	M	63	HPT/ PT	2	145	c.435C>T	B		p.S145S		
				9	418	c.1254C>T	B		p.D418D		
				int 1	—	IVS1-17 C>G	B	—	—		
8	M	30	HPT/ GEPT/ PT	3	210-211	C.625_628delCAGA	P	FSµRGT	p.T210Sfs		
9	F	56	HPT/PT	3		c.628_631delACAG	P	FµRGT	FS		
10	F	56	HPT/ GEPT/ PT	4	224	c.672delA	P	FSµRGT	p.G225Dfs	*	
11	F	28	HPT/ PT	int 4	—	IVS4-9G>A	P	SJ	—		
12	M	27	HPT/ PT	5	264	c.792delC	P	FSµRGT	p.W265Gfs		
13	F	34	HPT/ PT	5	264	c.792delC	P	FSµRGT	p.W265Gfs		
14	F	38	GEPT/HPT	6	276	c.828C>G	P	NS	p.Y276X		
15	F	74	HPT/ PT	8	360	c.1080C>A	LVB		p.I360I		
16	F	47	HPT/TP	8	376	c.1127T>C	P	MS	p.L376P		
17	F	54	HPT/ GEPT/ PT	9	415	c.1243C>T	P	NS	p.R415X		
18	F	68	HPT/ PT	9	418	c.1254C>T	B		p.D418D		
19	F	50	HPT/ GEPT/ PT	9	418	c.1254C>T	B		p.D418D		
20	M	46	HPT/PT	9	418	c.1254C>T	B		p.D418D		
21	F	30	HPT/ GEPT/ PT	9	418	c.1254C>T	B		p.D418D		
22	F	39	GEPT/HPT	9	418	c.1254C>T	B		p.D418D		
23	F	31	HPT/ PT	int 9	—	IVS9+103 C>G	B	—	—		
				9	418	c.1254C>T	B		p:D418D		
				1	---	c.-533T>A	B				
24	F	54	HPT/Thymoma NET	10	460	c.1378C>T	P	NS	p.R460X		
				1	—	c.-533T>A	B	—	—		
				int 9	—	IVS9+103 C>G	B	—	—		
				9	418	c.1254C>T	B	—	p.D418D		
25	M	56	GEPT /PT	9	443	c.1328C>A	P	MS	p.S443Y		
				1		c.-533T>A	B				
				int1		IVS1-39C>G	B				
				int2		IVS2+168 G>A	B				
26	F	42	GEPT /HPT	10	469	c.1405G>T	P	NS	p.E469X		0/2
27	F	42	HPT/ PT	10		c.1621G>A	B		p.A541T		
28	F	30	HPT/ GEPT/ PT	10	555	c.1664G>T	LPV	MS	p.S555I	*	0/2
29	M	48	HPT/ GEPT / PT	10	516	c.1546_1547insC	P	FSµRGT	p.R516Pfs		
30	M	76	GEPT /HPT	—	—	—	—	—	—		
31	F	47	HPT/ PT	—	—	—	—	—	—		
32	M	65	HPT/ PT	—	—	—	—	—	—		
33	F	57	GEPT /HPT	—	—	—	—	—	—		
34	F	49	GEPT / PT	—	—	—	—	—	—		
35	F	52	HPT/PT	1		c.-533T>A/	B	—			
				9		c.1254C>T	B		p.D418D		
36	M	28	HPT/ PT	—	—	—	—	—	—		
37	M	25	HPT/PT	1		c.-533T>A	B				
				int1		IVS1-39c>G	B				
				int2		IVS2+168G>A	B				
38	M	49	HPT/PT	1		c.-533T>A	B		—		
				9		c.1254C>T	B		p.D418D		
39	M	53	HPT/PT	—	—	—	—	—	—		

HPT: hyperparathyroidism; GEPT: gastroenteropancreatic tumor; PT: pituitary tumor; B: benign variant; P: pathogenic variant; LBV: likely benign variant; LPV: likely pathogenic variant; FSµRGT: frameshift micro rearrangement; NS: nonsense, MS: missense; In frame-µRGT: in frame micro rearrangement; SJ: splice junction; Y: years

Figure 1 | Distribution of germline variants on MEN1 gene. Rectangles represent MEN1 exons numbered from 1 to 10, non-coding regions are gray and the three nuclear localization signals (NLS) in exon 10 are shown in red. Pathogenic variants are in black letters and benign variants are in blue. Truncating, missense and splice variants are shown above the gene and benign variants and large deletions are shown below.



On the other hand, a significant difference was found in the age of diagnosis (group 1: mean age 50 ± 15; group 2: 41 ± 10, and group 3: 36 ± 15 years, $p < 0.008$ (Kruskal-Wallis test). Applying Dunn's post hoc test, there was a significant difference between groups 1 and 3 ($p < 0.02$).

No similarities in the age of diagnosis and prevalence of tumors were found among most of the patients that share the same pathogenic variants (Tables 3 and 4). We have no data of the follow up of the patients to confirm this heterogeneity.

Discussion

The prognosis of patients with MEN1 could be improved with early detection of tumors, which could be achieved with an early genetic diagnosis in index cases and then in first-degree relatives, who have a 50% chance of inheriting the disease. In turn, mutation carriers should undergo periodic biochemical testing and imaging according to international expert consensus MEN1 guidelines[14-20]. Genetic studies should be performed in an accredited clinical genetics laboratory.

Before proceeding with genetic diagnosis, patients should receive genetic counseling. Currently, genetic testing of the *MEN1* gene includes sequencing of exons, intronic flanking regions, and splicing sites. If a pathogenic variant is not identified, large rearrangements need to be ruled out using the MLPA technique.

In our series of patients, we found germline heterozygous pathogenic variants in 46 of the 68 patients with MEN1. As expected, this prevalence was significantly higher in familial than in sporadic patients. These rates of prevalence are similar to those found in other studies.[14,17,20,25-29]

The fact that patients with familial disease as well as those with a pathogenic variant are younger than patients in whom we found no pathogenic variant could be explained by the theory of the first and second hit of neoplastic diseases, due to mutations in tumor suppressor genes. Therefore, endocrine tumors in the context of MEN1 have an earlier age of onset of their tumor disease[30].

On the other hand, our findings confirmed previous observations that an early age at tumor manifestation is a risk factor for finding a pathogenic variant in patients with MEN1[31, 32].

We observed a predominance of women in the S-MEN1 but not in F-MEN1 (Table 1) or in the group with pathogenic variants or mutations, which was expected to occur in a disease with an autosomal dominant inheritance pattern.

The prevalence of HPT was the highest although not significantly higher than that of PT. HPT is the most prevalent manifestation of MEN1 and the second manifestation in order of prevalence is usually GEPT[14,17,20,25-29]. However, the second manifestation in our patients was PT. The prevalence of pituitary tumors in patients with MEN1 varies from 15 to 50% in different series[20,33-35] There is an increasing prevalence of pituitary adenomas observed globally that might be due to the imaging technique's greater sensitivity[36].

The high frequency of pathogenic variants in patients harboring more than 2 tumors is also related to Knudson´s theory of the first and second hit.

Germline mutations in the gene *MEN1* are scattered throughout the 1840-bp and splice sites of MEN1 gene. This characteristic of *the MEN1 gene* of not having "hot spots" is the reason for the genetic analysis implying its complete initial study in all index cases[4-20]. On the other hand, Lemos and Thakker described warm zones in exons 2, 3, 9, and 10[12]. In accordance with these observations, we found 65% of the pathogenic and likely pathogenic variants in exons 2, 3, 9, and 10. It would be an area with repetitive DNA sites conducive to mutagenesis and, in the case of exons 2 and 10, with a proportionally greater length.

Of the 46 patients with pathogenic variants, 36 presented distinct variants. Recurrent variants were found in 15 unrelated cases. Frequent or recurrent variants may occur due to independent mutational events in the gene's warm regions or due to founder variants. Seven of the 15 unrelated cases shared variants located in exon 2 and 3, in accordance with the gene's warm zone. It was not possible for our team to conduct the haplotype segregation study to determine whether the patients with the same pathogenic variant were related.

The type of pathogenic variants found is very similar to that Lemos and Thakker et al, and Concolino et al have reported[12-37]. As described in tumor suppressor genes, most of the pathogenic variants in MEN1 are inactivating. In our study, at least 67% of the pathogenic variants lead to an absent or truncated protein.

We found large deletions in 11.5% of positive familial cases, and the application of MLPA was successful in 3 of the 6 familial index cases with no pathogenic variant identified by Sanger sequencing and in none of the 14 sporadic index cases of the same type. These data confirm MLPA's usefulness in the detection of genetic abnormalities in familial MEN1 patients without pathogenic variants found after DNA sequencing by Sanger or by full-gene next-generation sequencing, as was recently reported[5,26,28,36,38,39].

Seven of the 36 pathogenic variants found in our study were novel. We reviewed 5 databases, as we mentioned in the Methods section, and confirmed that they had not been previously described. Their pathogenic character is given by the type of variant, and all of them produce a truncated protein except c.1664G>T.

The novel nonsense mutation, c.1664G>T, corresponds to a 35-year-old female patient with HPT, metastatic gastrinoma, and prolactinoma. She had 2 asymptomatic children, who tested negative for the mutation. Other relatives were not available to perform a segregation analysis of the disease with genotype. We found the mutation in a highly conserved genetic region across species within the NLSa nuclear signaling site. In turn, a similar variant, c.1664G>A (p.S555N), is considered a pathogenic/likely pathogenic variant[40]. Therefore, we classify 1664G>T (p.S555I) as a likely pathogenic variant, according to ACMG.

Among the nonpathogenic variants, we found 2 LBVs in our series (c.512G>A and c.1080C>A).

The synonymous variant, c.1080C>A (p.I360I), did not show changes in the Splice Finder analysis and was reported in the ClinVar NIH database as likely benign.

Balogh et al and De Carlo E et al detected c.512G>A, the other LBV, as the only alteration in the *MEN1* gene sequence in Hungarian and Italian MEN1 patients, and they suggested that it could be a low-penetrance mutation. [27,41] Interestingly, we found the variant c.512G>A in 2 index patients, case 4 of sporadic and case 8

of familial MEN1 patients. In the first patient, it was associated with a pathogenic variant (Table 3). The second patient had 3 tumors (HP, PT, and GEPT) (Table 4), and we found no pathogenic variant. However, we did not find c.512G>A in 3 siblings of the index case, 1 of whom presented 2 typical manifestations of MEN1 (HPT and PT) during follow-up (data not shown). These 2 cases show evidence against the possibility of the likely pathogenic feature of c.512G>A.

In our series, 28.6% of patients with MEN1 according to clinical definitions, most of them sporadic, remain without a pathogenic variant. According to the literature, 10-30% of these patients show no evidence of a mutagenic event in the *MEN1* gene[20,22].

The reason for this proportion of patients with negative analysis of the *MEN1* gene has been sought in other genes. One such candidate gene is *CDKN1B,* some of whose genetic variants produce a phenotype with HPT, PT, and gonadal tumors, among others. This syndrome has been described as MEN4. Other genes possibly responsible for a MEN1-like phenotype are *AIP,* other *CDKIs* (p15, p18, p21), *CaSR,* and *CDC73*. However, finding pathogenic variants of these genes in MEN1-variant-negative patients is very rare (1% or fewer in each case), and does not seem to be the answer to the problem[42-44].

To determine the usefulness of the analysis of these and other genes, we will have to wait for the results of panels designed for the study of MEN1 patients.

The existence of pathogenic variants in introns far from the exon/intron junction and regulatory regions of the gene has also been postulated. Recently, Carvalho et al conducted a systematic study of 76 patients using the target next-generation sequencing technique to study the entire *MEN1* gene. The authors did not find any mutation in regulatory or noncoding areas and therefore concluded that it would be a rare and infrequent event[29].

Similarly, Backman et al studied germline DNA by whole genome sequencing of 14 mutation negative MEN1 patients and found 2 of them had mutations in the *CaSR* and *CDC73* genes. Again, no mutations in the non-coding areas of MEN1 were found[45].

Finally, de Laat et al studied the clinical course of patients with MEN1 with and without a pathogenic variant in the MEN1 gene. They found that patients without a pathogenic variant were mostly sporadic, had clinical manifestations at an older age, and had a mortality rate similar to that of the general population. The authors proposed that these patients would have a syndrome like but more benign than MEN1 or a coincidence of two sporadic neuroendocrine tumors[31].

In our study, no correlation was found between the type of mutation, age of diagnosis or tumor prevalence. Although genotype/ phenotype correlations in MEN1 are under discussion, some families harbor recurrent tumor patterns and large deletions have been associated with early disease onset[46,47]. Larger number of index patients and longer follow up of the family members is necessary to study genotype/phenotype correlations in MEN1.

Our study of 68 index cases of Argentine nationality with clinical MEN1 shows a prevalence of pathogenic genetic variants of 68%. The genetic diagnosis of index cases also allowed us to identify 39% of relatives as mutation carriers, most of them asymptomatic, who should attend an annual check-up for early detection of tumors. On the other hand, we were able to discharge 46 first-degree relatives, potentially saving medical resources. *MEN1* gene identification and the possibility of making genetic diagnosis have significantly improved the clinical management of patients and their families. This study has confirmed those achievements in our country.

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