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Missense mutation in the *MEN1* gene discovered through whole exome sequencing co-segregates with familial hyperparathyroidism

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Summary

Familial isolated hyperparathyroidism (FIHP) can be encountered in the context of multiple endocrine neoplasia type 1 (MEN1), hyperparathyroidism and jaw tumour syndrome (HPT–JT) and in familial hypocalciuric hypercalcaemia (FHH). In these syndromes, germline mutations in the relevant genes (*MEN1*, *HPRT2* and *CaSR*, respectively) are detected. In some FIHP cases, the causative gene is still elusive. The objective of this study is to define the genetic basis of FIHP in a Georgian Jewish family with FIHP using whole exome capture and sequencing. DNA extracted from two sibs and one offspring from a single family all affected with multiglandular hyperparathyroidism was subjected to whole exome capturing and sequencing using the Roche NimbleGen V2 chip and the Illumina HiSeq2000 sequencing platform. Genetic variants were detected and annotated using a combination of the Genome Analysis Tool Kit and in-house scripts. Subsequent confirmation of the mutations and co-segregation analyses were carried out by Sanger sequencing in additional affected and unaffected family members. Whole exome capture and sequencing revealed the collection of variations common to the three-sequenced patients, including a very rare previously described missense mutation (c.T1021C: p.W341R) in the *MEN1* gene. The p.W341R mutation in the *MEN1* gene showed complete co-segregation in the family. Whole exome capture and sequencing led to the discovery of a missense mutation in the *MEN1* gene and ruling out of the additional candidates in a single experiment. The limited expressivity of this mutation may imply a specific genotype–phenotype correlation for this mutation.

1. Introduction

Familial isolated hyperparathyroidism (FIHP) is a rare disorder encountered in a minority (~1%) of all patients with primary hyperparathyroidism (Simonds *et al*., 2002). At times, FIHP is associated with a syndromic clustering of other endocrine tumours, jaw tumours, or is associated with hypocalciuria. In these cases, germline mutations in the multiple endocrine neoplasia type 1 (*MEN1*) gene (MIM#613733) (Chandrasekharappa *et al*., 1997; Bassett *et al*., 1998), the *HPRT2* gene (MIM# 145001) that causes hyperparathyroidism and jaw tumour (HPT–JT) syndrome (Carpten *et al*., 2002; Howell *et al*., 2003; Cavaco *et al*., 2004; Cetani *et al*., 2004), or the calcium sensing receptor *CaSR* gene (MIM# 601199) that leads to familial hypercalcaemic hypocalciuria (FHH; MIM #145980) (Pollak *et al*., 1993) can be encountered. Rarely, mutations in additional genes (e.g. *CDKN1B* – MIM# 600778) have been noted in families with a MEN1 phenotype (Pellegata *et al*., 2006). However, in some FIHP families the causative gene remains elusive (Marx, 2000). FIHP is a diagnosis of exclusion made in kindreds with familial clustering of hyperparathyroidism in the absence of any clinical,

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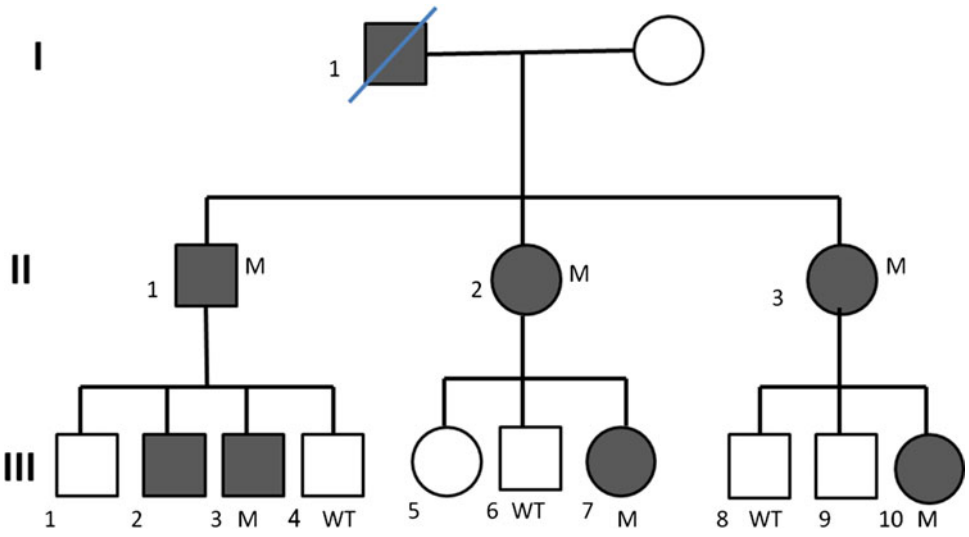

Fig. 1. Pedigree phenotype and genotype in the FIHP family. Filled symbols, clinical and biochemical diagnosis of HPT; M, mutant allele; WT, wild-type allele.

biochemical or radiological evidence of MEN1, HPT–JT or FHH. Over the past 15 years we have identified (Olchovsky *et al*., 2001) and followed a multigenerational Jewish family of Georgian origin with a seemingly autosomal dominantly inherited syndrome hallmarked by multiglandular FIHP, hypercalciuria and nephrolithiasis with no clinical, biochemical or imaging modality evidence of additional endocrinopathies. The present study aimed to define the genetic basis of FIHP in that family, using a whole exome capture and massive parallel sequencing approach with Sanger sequencing confirmation and co-segregation analyses.

2. Materials and methods

General – The study was approved by the institutional review board, and each participant gave an informed consent.

Case report – A multigenerational FIHP kindred was studied (Fig. 1), the specific pedigree and relevant medical history were previously reported by us (Olchovsky *et al*., 2001), and the main clinical features of this family are reiterated in the results section.

(i) *Genetic analyses*

Whole exome sequencing (WES) – DNA extracted from two sibs and one offspring from a single family, all affected with multiglandular hyperparathyroidism was subjected to whole exome capturing and sequencing using the Roche NimbleGen V2 chip (Madison, WI) and the Illumina HiSeq2000 sequencing platform (Hayward, CA).

Variant calling and annotation – For each sequenced sample, raw sequence files were prepared using the Genome Analysis Tool Kit (GATK) suggested practice (McKenna *et al*., 2010). Briefly, each fastq file was aligned against the human hg19/GRCh37 reference genome. PCR duplicates were removed using Picard (http://picard.sourceforge.net/), reads around known and detected indels were realigned, and base quality was recalibrated using GATK. In order to call variants from the processed BAM files, we employed the GATK-recommended variant calling pipeline. Regional information including whether a variant is up/down stream of a known gene, whether it is located in a gene's intron, exon, splice junction or untranslated regions (UTRs) was added using ANNOVAR (Wang *et al*., 2010) and the Ensembl gene annotation (Flicek *et al*., 2011). Information regarding other functional regions such as miRNA and other non-coding RNA sites was gathered from the wgRNA table downloaded from the UCSC Genome Browser site (Kent *et al*., 2002). Information about the known and predicted miRNA binding sites was downloaded from TargetScan (Lewis *et al*., 2005). Allele frequencies were retrieved from designated public databases such as dbSNP (Day, 2010), the 1000 genomes project (Nothnagel *et al*., 2011), the NHLBI Exome Sequencing Project (http://evs.gs.washington.edu/EVS/) and the Complete Genomics database (Drmanac *et al*., 2010). Additional allele frequency information was added using our own database, which includes past exome sequencing data of 50 unrelated individuals. Variants were annotated with their conservation levels and their inferred constraint levels using GERP++ (Davydov *et al*., 2010), PhyloP (Siepel *et al*., 2005) and PhastCons (Cooper *et al*., 2005). Information regarding the predicted severity of each variant (namely, how deleterious a variant is predicted to be) was gathered using SIFT (Ng & Henikoff, 2003), PolyPhen2 (Adzhubei *et al*., 2010) and Mutation Taster (Schwarz *et al*., 2010).

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Clinical associations were gathered from the National Human Genome Research Institute catalogue of published Genome-Wide Association Studies (NHGRI-GWAS catalogue) (Hindorff *et al*., 2009), the Online Mendelian Inheritance in Man database (OMIM; http://omim.org/) and Uniprot's Human polymorphisms and disease mutations index (www.uniprot.org/docs/humsavar).

Gene annotation – After all variants were gathered we reviewed and annotated them using the following sources: (i) Pathway annotation, which includes all the pathways in which a given gene product has reportedly been involved in. Pathway information was gathered from the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa *et al*., 2012), Biocarta (http://www.biocarta.com) and REACTOM (Matthews *et al*., 2009). Gene biological process properties were gathered from the Gene Ontology (GO) database (Ashburner *et al*., 2000). (ii) Gene–gene and protein–protein interactions: Interactions annotations were gathered from the BioGRID interaction database (Stark *et al*., 2011), STRING protein functional interactions database (Szklarczyk *et al*., 2011) and the human protein–protein interaction prediction database (PIPs) (McDowall *et al*., 2009). Following the aforementioned steps of variant and gene annotation and classification, we reviewed the data manually in order to produce the final candidate gene table and summarize any supporting evidence for their putative involvement in familial hyperparathyroidism.

Validation of the sequencing data – Validation of the sequencing data was carried out using Sanger sequencing of PCR products, using primers that flank the mutation site. This approach was employed in order to ascertain co-segregation of any seemingly pathogenic mutation with the hyperparathyroidism phenotype in this family.

3. Results

Case report – Briefly, affected individuals from two generations from a non-consanguineous, Georgian Jewish family were the focus of this study. In three cases, uni-glandular parathyroidectomy undertaken at ages 18–45 years was performed (indicated by recurring nephrolithiasis and hypercalciuria). This intervention resulted in normocalcaemia that lasted 3–5 years. In all three cases, hypercalcaemia recurred and additional parathyroid adenomas were located prior to second surgery. Subsequent surgery, indicated by high levels of hypercalcaemia, was subtotal parathyroidectomy. In one case, recurrence of hypercalcaemia within 9 months led to finding a fifth intramediastinal parathyroid gland that was subsequently surgically removed. Analysis of all parathyroid glands removed from these three patients ($n=13$) showed parathyroid adenomas with no cystic features or evidence of malignancy. Affected individuals from the same family who were evaluated ($n=6$) all had hypercalcaemia (ranging from 13·1 to 15·3 mg%), hypercalciuria (ranging from 576 to 898 mg/24 h), 4/6 patients had recorded evidence of nephrolithiasis at age range from 18 to 33 years. Initial evaluation in 1998 and subsequently in 2010 in all six affected individuals, revealed no biochemical evidence of abnormal hormone secretion or other biochemical abnormalities (PRL, GH, IGF-1, TSH, ACTH, cortisol, FSH, LH, insulin, gastrin, vasoactive intestinal peptide (VIP), glucagon, serotonin, adrenalin, noradrenalin and glucose concentrations), no pituitary abnormalities by MRI, no pancreatic or adrenal masses by CT ultrasound and MRI, normal chest X-rays and jaw orthopentography. Of note one affected woman (III-10 in Fig. 1) had a transient hyperprolactinaemia that was spontaneously resolved within 12 months. Thus, the diagnosis of FIHP was made in this family based on the evidence of primary hyperparathyroidism in three generations with a seemingly autosomal dominant mode of transmission, histological evidence for multiglandular disease and the lack of any clinical, biochemical or radiological evidence of additional endocrinopathies.

Genetic analysis results – Variant calling from WES phase of the three genotyped patients resulted in a total of 762 791 detected variants, of which 732 235 (96%) were found in all three patients: 9539 of these shared sequence variants were either amino acid (AA) (8659) or splice junction (880) altering mutations. Of these, 324 were considered rare (allele frequency $<1\%$ in all of the tested databases). Of these rare variants, eight were considered deleterious since they either led to a truncated protein (as a result of a premature stop codon) or were predicted to be deleterious by all prediction tools employed. Finally, we compiled a list of genes associated with hyperparathyroidism using a combination of the OMIM (http://www.ncbi.nlm.nih.gov/omim) and HuGENet (http://www.cdc.gov/genomics/hugenet/) databases (Supplementary Table S1). The average coverage in these genes was 21·6×, 25·8× and 22·6× for individuals II-1, II-2 and III-3, respectively (Fig. 1). Of the eight deleterious variants, only one mutation altered one of the HPT-associated genes (*MEN1*). The mutation (chr11: 64573732: c.T1021C: p.W341R) was found in a heterozygous state in all three patients and resulted in a change from a large size and aromatic AA (tryptophan) to a large size and basic AA (arginine). Additionally, the mutation was found in a region highly conserved among mammals and predicted to deleteriously alter protein function by all prediction tools employed. Therefore, this variant presented the most likely causative variant. Since this was the only HPT-associated gene

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Table 1. *Deleterious mutations following all filtration stages*

Chromosome	Position	Reference	Alternate	Gene	CDS	Protein	Rs number	dbSNP AF	1KG AF	Exome Project AF
chr17	36484901	—	A	GPR179	c.4551_4552insT	p.M1517fs	.	.	.	.
chr7	138827025	C	T	TTC26	c.C373T	p.R125C	.	.	.	0.000142
chr19	33390785	T	C	CEP89	c.A1853G	p.E618G	.	.	.	.
chr11	64573732	A	G	MEN1	c.T1036C	p.W346R	.	.	.	.
chr3	184007478	C	G	ECE2	c.C1523G	p.P508R	rs144817798	.	.	0.000285
chr9	34611127	A	C	C9orf23	c.T167G	p.V56G	.	.	.	.
chr2	110323415	C	T	SEPT10	c.G385A	p.V129M	.	.	.	.
chr4	126240968	A	T	FAT4	c.A3402T	p.E1134D	rs144768563	0.0023	0	0.001658

mutation, we elected not to further pursue co-segregation analysis for the other seven seemingly pathogenic variants (Table 1). Supplementary Table S1 details non-synonymous mutations in other putative HPT-associated genes.

Validation of the whole exome sequencing data – Sequencing the W341R *MEN1* missense mutation showed that all tested individuals with the primary HPT phenotype exhibited the mutation, and three unaffected family members showed the wild-type allele only. Notably, 6/7 affected family members who agreed to participate in the study were all mutation carriers. The seventh affected member (Patient III-2 in Fig. 1) and three unaffected family members (III-1, III-5 and III-9 in Fig. 1) all refused participation.

4. Discussion

In this study, we combined WES with prior information regarding HPT-associated genes in order to effectively pinpoint the causative mutation in a proband with FIHP. Although WES focuses on the functional regions of the genome, each sequencing run was associated with tens of thousands of variations per sequenced individual. In order to narrow down the list of candidate variants, we initially annotated them using various data sources that enabled the stratification of variants by frequency, coding sequence effects and predicted functional alterations. Focusing our search only on rare (allele frequency $<1\%$ in all of the databases) AA altering variations that are either non-sense mutations or predicted to alter protein function, resulted in eight final variants. Comparing the list of genes affected by these variants with our compiled list of HPT-associated genes, only one variant matched: a rare change from tryptophan to arginine in position 341 of the MEN1 protein. The same mutation was previously reported in two non-familial cases displaying an MEN1 phenotype: one individual of French origin presented with primary HPT, pancreatic endocrine tumour and a non-secreting adrenal tumour (Giraud *et al*., 1998) and another patient of Chinese ancestry who harboured the same mutation, was diagnosed with primary HPT, endocrine pancreatic, thyroid and pituitary tumours (Tso *et al*., 2003). An additional study, reviewing 170 probands with at least one MEN1 syndrome-associated feature, reported the same mutation in one proband with FIHP (Wautot *et al*., 2002). The reasons for the different phenotypes of the family reported herein from two other individuals who carry the same mutation remain elusive. Although some studies (e.g. Kassem *et al*., 2000) suggested that mutations in exons 3–7 of the *MEN1* gene result in isolated HPT without additional endocrinopathies, this claim of an established genotype–phenotype correlation in

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MEN1 is not universally accepted (Lemos & Thakker, 2008).

Given the diverse ethnic origin of the previously reported individuals who harbour the mutation (French, Chinese and Jewish-Georgian) it seems unlikely that this mutation represents a founder mutation, and in all likelihood reflects a mutational hot spot. Although the W341R**MEN1* gene mutation is in all likelihood the causative mutation in the family presented herein, the final proof of causation is still lacking. Only functional analyses of the effect that this mutation has on the MEN1 protein function may enable firm establishment of the pathogenic role of this mutation in the FIHP phenotype. Other genes that may be associated with the same phenotype do not show co-segregation and are thus unlikely to harbour any deleterious mutation relevant to the phenotype.

Reviewing the additional seven candidate mutations, none of the genes that harbour these mutations had a clear association with HPT, and their interacting proteins or the biological processes and pathways they are involved with are not associated with the pathogenesis of HPT (Table 1).

We also reviewed the mutation spectra in other HPT-associated genes. One interesting variant, detected in a heterozygous state in all three patients, was found in the calcium-sensing, G-protein-coupled cell-surface receptor (*CASR*; c.G2956T: p.A986S). The variant is a known polymorphism (rs1801725) with a known association with increased extracellular calcium levels (Cole *et al*., 1999). Another HPT-associated polymorphism shared by all three patients was a synonymous heterozygous variant in the parathyroid hormone gene (*PTH*; c.C247A:p.R83R; rs6256) associated with lower levels of serum PTH (Kanzawa *et al*., 1999). Yet both variants are polymorphic and thus unlikely to contribute to the observed FIHP phenotype.

Given the limited number of genes that have been implicated in familial hyperparathyroidism phenotype, the question of the screening approach used for detecting the causative mutation, namely whole exome sequencing, needs to be addressed. From the cost-effectiveness analysis aspect, the costs of genotyping each of the three genes known in three affected individuals would be ~$15 000 on a commercial basis. Thus, it made more sense to try and encompass the known genes and yet unknown genes in a single, less expensive experiment. Moreover, as exemplified by the recent report by Nesbit *et al*. (2013), novel genes may still be detected as underlying a closely related phenotype.

In conclusion, a combined approach of WES and targeted candidate gene-filtering approach facilitated uncovering the most likely causative mutation in the *MEN1* gene in a family with FIHP in addition to a comprehensive characterization of the mutation spectra in the entire coding region which may modulate the MEN1 mutation phenotype.

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5. Supplementary material

To view supplementary material for this article, please visit http://dx.doi.org/10.1017/S0016672313000141.

6. Conflict of interest statement

All authors declare that they have no conflict of interest regarding the data published.

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