Putative modifier genes in mevalonate kinase deficiency

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Abstract. Mevalonate kinase deficiency (MKD) is an autosomal recessive auto-inflammatory disease, caused by impairment of the mevalonate pathway. Although the molecular mechanism remains to be elucidated, there is clinical evidence suggesting that other regulatory genes may be involved in determining the phenotype. The identification of novel target genes may explain non-homogeneous genotype-phenotype correlations, and provide evidence in support of the hypothesis that novel regulatory genes predispose or amplify deregulation of the mevalonate pathway in this orphan disease. In the present study, DNA samples were obtained from five patients with MKD, which were then analyzed using whole exome sequencing. A missense variation in the *PEX11γ* gene was observed in homozygosis in P2, possibly correlating with visual blurring. The *UNG* rare gene variant was detected in homozygosis in P5, without correlating with a specific clinical phenotype. A number of other variants were found in the five analyzed DNA samples from the MKD patients, however no correlation with the phenotype was established. The results of the presents study suggested that further analysis, using next generation sequencing approaches, is required on a larger sample size of patients with MKD, who share the same *MVK* mutations and exhibit 'extreme' clinical phenotypes. As *MVK* mutations may be associated with MKD, the identification of specific modifier genes may assist in providing an earlier diagnosis.

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Abbreviations: MVK, mevalonate kinase gene; MK, mevalonate kinase; MKD, mevalonate kinase deficiency; HIDS, hyper IgD syndrome; MA, mevalonic aciduria; WES, whole exome sequencing; UTR, untranslated; SNVs, single nucleotide variants; INDELs, small insertion/deletions; VCF, variant call format

Key words: gene, correlation genotype-phenotype, orphan disease, mevalonate kinase deficiency, whole exome sequencing

Introduction

Mevalonate kinase deficiency (MKD; 610377) is an autosomal monogenic recessively-inherited disease, caused by mutations in the *MVK* gene (12q24.11) coding for mevalonate kinase (MK). MK is a key enzyme of the mevalonate pathway, which is essential for the biosynthesis of isoprenoids and the decrease of which is considered to lead to an overproduction of the specific marker of disease, interleukin (IL)-1β (1-3). These mutations lead to a shortage of intermediate compounds and final products of the metabolic route of cholesterol (4-6).

The residual activity of MK defines different degrees of MKD severity, ranging between an auto-inflammatory phenotype (hyper IgD syndrome/HIDS; OMIM #260920) and severe clinical presentation (mevalonic aciduria/MA; OMIM #610377) (6). HIDS is characterized by fever, which re-occurs every 2-8 weeks, variably accompanied by malaise, headache, diarrhea, abdominal pain, vomiting, skin rash, arthralgia, arthritis, tender lymphadenopathy, anemia normocytic, hepatosplenomegaly, and as oral and genital ulcers. Urinary mevalonic acid (UMA) is increased predominantly during inflammatory episodes, whereas increased levels of IL-1β, IL-1α, IL-6, IL-10, IL-18, tumor necrosis factor (TNF)-α and IFN-γ cytokines are also observed between fever episodes (7,8). Laboratory analyses show increased neutrophil counts and high acute phase reactants, often associated with persistently high serum IgD and IgA (9).

Patients with MA exhibit the above-mentioned symptoms associated with dysmorphic features, cataracts, uveitis, neurological impairments, and failure to thrive. UMA excretion is also high between attacks (10). There have been >70 different disease-causing mutations identified in the *MVK* gene. The majority of these exhibit poor genotype-phenotype correlation with respect to clinical presentation and biochemical derangements (11). Notably, it has been found that >95% of patients with HIDS are compound heterozygous for the V377I *MVK* allele, whereas a second mutant allele, I268T, is specific to patients with MA (12). Other mutations have been described in patients with HIDS and MA without a reliable genotype/phenotype correlation (13), although patients carrying the same mutation

often exhibit substantial variability in symptoms and respond differently to treatment (14).

Of note, previous studies have described a correlation and/or an overlap between the MKD phenotype and /or the *MVK* genotype with other diseases, including inflammatory bowel diseases (15,16), possibly due to shared genetic background (17), and retinitis pigmentosa, adding novel features to the wide range of the already known MKD phenotypes (18,19).

Whilst several reports suggest that the shortage of isoprenoid intermediates, including geranylgeraniol, is involved in the autoinflammatory phenotype of MKD, further data has led to a focus of attention on the end products of this pathway, including 25-deoxycholesterol (CH25) (20-22). Of note, 25-hydroxylcholesterol can act by modulating the mevalonate pathway itself by suppressing sterol regulatory element-binding proteins (SREBPs), thus highlighting a complex feedback network, which may be relevant in the modulation of MKD phenotypes (23,24). The present study hypothesized that MKD may require consideration a polygenic, rather than a monogenic, disease. The overlap and the wide range of clinical signs shared by MKD and other auto-inflammatory diseases can cause delayed or incorrect diagnosis. In order to verify this hypothesis, the presents study performed whole exome sequencing (WES) analysis on DNA samples obtained from patients with mild or severe MKD phenotypes to determine any more or less pathogenetic variants, which may be involved in determining the phenotype of the patient.

Materials and methods

Study design. Initially, the present identified the small nucleotide variants (SNVs)/small insertion/deletions (INDELs), which had a minor allele frequency (MAF) <0.05, as reported in the NHLBI Exome Sequencing Project (ESP) Exome Variant Server (http://evs.gs.washington.edu/EVS/) database and referred to the general population. In order to be considered, the variants also had to be harbored by genes belonging to the cholesterol biosynthetic pathway (Table I). The hypothesis underlying this approach was that the variants harbored by these genes modulate overall cholesterol biosynthetic activity, possibly affecting the overall availability of cholesterol and/or its biosynthetic intermediates, thus contributing to the modification of MKD clinical phenotypes.

The present study also investigated an alternative analytical strategy, selecting only the variants that are poorly represented in the general population and, at the same time, predicted as potentially pathogenic. For this purpose, the SNVs/INDELs were selected, according the following inclusion criteria: (a) variants with an MAF <0.03 in the general population, as reported in the ESP database, (b) SNVs leading to a non synonymous amino acid substitution, (c) SNVs/INDELs carried in homozygous state, (d) SNVs/INDELs predicted as pathogenic by the following *in silico* algorithms: Polyphen-2 (25), Mutation Taster (26) and likelihood relation test (27), according to scores recorded in the dbNSFP v2.0 database (http://sites.google.com/site/jpopgen/dbNSFP); (e) SNVs/INDELs considered phylogenetically conserved, based on genomic evolutionary rate profiling (GERP)++ scores reported in the dbNSFP v2.0 database. The first analytical approach examined the hypothesis that a number non-rare variants are involved in modifying the MKD clinical phenotype, acting along the same biochemical pathway, whereas the second was intended to identify rare and pathogenic variants, carried in a homozygous state and possibly associated to certain, more severe, MKD clinical phenotypes.

Table I. Cholesterol biosynthetic pathway genes used for analysis.

Abbreviation	Gene
HADHB	Hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase, β subunit
ACAT2	Acetyl-CoA acetyltransferase 2
ACAT1	Acetyl-CoA acetyltransferase 1
HMGCS1	3-hydroxy-3-methylglutaryl-CoA synthase 1
HMGCS2	3-hydroxy-3-methylglutaryl-CoA synthase 2
HMGCR	3-hydroxy-3-methylglutaryl-CoA reductase
MVK	Mevalonate kinase
PMVK	Phosphomevalonate kinase
MVD	Mevalonate (diphospho) decarboxylase
IDI2	Isopentenyl-diphosphate δ isomerase 2
IDI1	Isopentenyl-diphosphate δ isomerase 1
GGPS1	Geranylgeranyl diphosphate synthase 1
FDPS	Farnesyl diphosphate synthase
CYP51A1	Cytochrome P450, family 51, subfamily A, polypeptide 1
EBP	Emopamil binding protein (sterol isomerase)
SC5DL	Sterol-C5-desaturase
DHCR7	7-dehydrocholesterol reductase
NSDHL	NAD(P) dependent steroid dehydrogenase-like
HSD17B7	Hydroxysteroid (17-β) dehydrogenase 7
SQLE	Squalene epoxidase
LSS	Lanosterol synthase
DHCR24	24-dehydrocholesterol reductase
LBR	Lamin B receptor
TM7SF2	Transmembrane 7 superfamily member 2
SC4MOL	Methylsterol monooxygenase 1
FDFT1	Farnesyl-diphosphate farnesyltransferase 1

The two strategies were performed taking into consideration the phylogenetic nucleotide evolutionary conservation, based on PhyloP (28) and GERP++ scores, as reported in dbNSFP v2.0; and only substitutions of conserved nucleotides were considered for their potential pathogenic role. This type of alteration is a predictor of deleteriousness, being a variation that reduces organism fitness, which is a property closely associated with molecular pathogenicity (29).

DNA extraction. Genomic DNA (gDNA) was extracted from 1-2 ml EDTA-anticoagulated blood from the proband and their parents using an EZ1 DNA Blood kit (Qiagen, Milan, Italy), according to the manufacturer's protocol.

Whole exome sequencing and bioinformatics analysis. The technical and scientific review board of the Institute for Maternal and Child Health-IRCCS 'Burlo Garofolo' (Trieste,

Table II. Mevalonate kinase deficiency phenotypes based on the typical clinical signs and symptoms observed in the patients.

Patient	Gender	Abdominal pain	Frequency of febrile attacks	Diarrhea	Rash	Associated condition	Phenotype (mild/severe)
P1	M	1	0	0	0	None	Mild
P2	M	2	1	2	2	Normocytic anemia scheletric pain	Severe
P3	F	2	1	1	2	Normocytic anemia	Mild
P4	F	1	0	0	1	None	Mild
P5	M	2	1	1	1	Normocytic anemia	Severe

Abdominal pain/diarrhea/rash: 0, never; 1, sometimes or often; 2, always. Frequency of febrile attacks: 0, 1/month; 1, >1/month. M, male; F, female.

Italy; no.185/08; 19/08/2008) approved the present study. For a child to be eligible, informed consent had to be obtained from parents or caregivers. Furthermore, patients with MKD of any age were excluded from the study if they had an acute or chronic infectious disease, any clinically significant disorder, or if they were currently on any medication with known effects on immunological factors, including corticosteroids. Blood was collected by venepuncture from five patients with mild or severe MKD phenotypes, determined by a physician using a visual analogue scale score (Table II). For each patient, a specific control of the identical sex and the age was used. Each control sample was collected, preserved and analyzed in an identical method of the patient to precisely identify phenotypic differences.

The samples obtained from the patients diagnosed with MKD were analyZed using whole exome sequencing (WES). Starting with 3 μg gDNA, collected from each subject, a TruSeq™ Exome Enrichment 62 Mb kit (Illumina, Inc., San Diego, CA, USA) was used to capture the whole exome; of which the target size was 62 Mb, comprising the overall genes coding sequence, the 5'-untranslated regions (UTRs) and 3'-UTRs. A fragment exome library was constructed, according to the manufacturer's protocol, and a 100 bp paired-end sequence was analyzed using the Illumina HiSeq 1000 platform (Illumina, Inc.). Raw sequencing data were collected as unmapped reads in fast Q format. CLC Genomics Workbench ver. 6.5 software was used to assess the quality of reads, to map reads back to the human reference genome, hg19, to calculate the overall coverage, to perform local realignment and base quality recalibration, and to identify SNVs and INDELs, all of which were collected into standardized Variant Call Format version 4.1 (30). The SNVs/INDELs were annotated using ANNOVAR software (31) referring to the following public databases: refGene, NCBI dbSNP build137 (http://www.ncbi.nlm.nih.gov/SNP/), 1000 Genomes Project (http://www.1000genomes.org/), NHLBI ESP Exome Variant Server (http://evs.gs.washington.edu/EVS/), dbNSFP v2.0 (27) and NCBI ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/).

Polymerase chain reaction (PCR) and sanger analysis. PCR amplification was performed for the specific gene coding sequence of the *MVK* and *RAB40AL* genes in 15 μl total volume. Each reaction contained 50 ng/μl genomic DNA and KAPA 2 G Fast Hot Start Readymix (RESNOVA, Rome, Italy). PCR amplification was performed using a common annealing temperature in a touchdown thermocycler with a two-step cycle: Initial denaturation at 96˚C for 3 min, followed by a gradiant (0.5˚C reduction per cycle) of 10 cycles at 96˚C for 15 sec, 63˚C for 10 sec and 72˚C for 1 second. The second step was 28 cycles of 96˚C for 15 sec, 53˚C for 15 sec and 72˚C for 1 sec. The PCR products were purified with 2 μl ExoSAP (USB Corporation, Cleveland, OH, USA) by incubation at 37˚C for 20 min and 85˚C for 10 min. All thermal cycling, PCR amplifications, ExoSAP purifications and sequencing reactions were performed in a 2720 Thermal Cycler (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The amplification products were directly sequenced by the Sanger method using an ABI PRISM 3130XL automated DNA sequencer (Applied Biosystems; Thermo Fisher Scientific, Inc.). The sequences were analyzed using Seqman II software (DNASar I Lasergene, 7.0; DNAStar, Inc., Madison, WI, USA). The PCR amplification and Sanger sequencing primers used to confirm the variations identified in the PEX11 G gene were as follows: PEX11GcDNA, forward 5'-TGAAACTGAGACAGAGGCTG-3' and reverse 5'-AGT GTCAGGGGGTAGTGG-3; and Pex11gcDNA, forward CCT GTGGACAATGCTGAAG and Pex11gcDNA, reverse TCA TCAAGG GCTGTCTGC.

The PCR amplification and Sanger sequencing primers used to confirm the variations identified in the uracil-DNA glycosylase (*UNG*) gene were as follows: *UNG* exon2, forward 5'-CTGTCCGCTTTTGCTGGG-3' and reverse 5'-CCGGCT ACACTAACAAGAC-3'.

Urinary mevalonic acid measurement. Mevalonic acid levels were determined in the patients with MKD using standard procedures, as described by Shoemaker *et al* (32). The urine samples were collected over 24 h and stored at 4˚C until analyzed.

Urinary mevalonic acid concentrations were determined using gas chromatography/mass spectrometry. Spectra were

Table III. Mevalonate kinase deficiency genotype and levels of mevalonic acid.

Patient	Mevalonic acid (μg/ml)	Mutation	Genotype	dbSNP v137
P1	5,000	c.G394A; p.V132I	Het	rs104895336
		c.G1129A; p.V377I	Hom	rs28934897
P2	2,638	c.T803C; p.I268T	Het	rs104895304
		c.G1129A; p.V377I	Het	rs28934897
P3	>20,000	c.16_34del; p.6_12del	Het	rs104895334
		c.G1129A; p.V377I	Het	rs28934897
P4	11,054	c.G1129A; p.V377I	Hom	rs28934897
P5	>20,000[a]	c.G1006A; p.G336S	Hom	rs104895358

[a]Also high outside of fever episodes. Het, heterozygous; Hom, homozygous.

obtained using a Hewlett Packard gas chromatograph 6890 system (Hewlett Packard, Palo Alto, CA, USA), equipped with a Hewlett Packard 5973 quadrupole, operating in electron-impact mode at 70 eV, as described previously (32). UMA is considered to be within a normal range between 34 and 323 μg/ml (internal laboratory reference).

B lymphocyte phenotype. The analysis of B lymphocyte phenotype was performed using the whole peripheral blood samples from the patients and controls. Briefly, 100 μl of blood was washed twice in phosphate-buffered saline (PBS), and then stained with anti-IgD/IgM fluorescein isothiocyanate (2.5 μl/test; cat. no. 348206/314506; BioLegend, Inc., San Diego, CA, USA), anti-CD27 phycoerythrin (PE; 5 μl/test; cat. no. 130093185), anti-CD19 PE-Vio770 (5 μl/test; cat. no. 130096641); and anti-CD45 (5 μl/test; cat. no. 130092880), all from Miltenyi Biotec GmbH (Bergisch Gladbach, Germany). Data were acquired using a Cyan ADP cytometer (Beckman Coulter, Brea, CA, USA), and analysis was performed using FlowJo software v7.6 (Tree Star, Inc., Ashland, OR, USA). B-switched memory cells were identified as CD45/CD19/CD27 positive and IgM/IgM negative.

Results

A total of ~14 Gb of sequence data per sample were produced using WES analysis, corresponding to 53X overall median coverage, among all the samples. The overall target percentage covered at least 20X, and was 80.7, 71, 73.6, 79, 75.3% of the whole exome, in patients 1, 2, 3, 4 and 5, respectively. On average, 59.404 variants per sample were identified.

As shown using Sanger Sequencing, the patients with MKD in the present study carried at least one mutation in the *MVK* gene. Data are present, together with the patients' mevalonic acid levels, in Table III.

By selecting the SNVs/INDELs with an MAF <0.05, the present study identified 10 exonic SNVs, listed in Table IV, six of which were harbored by *MVK*, as already known, with the remaining four variants identified in the *SQLE*, *IDI1* and *NSDHL* genes, even when none of them were in a homozygous or a compound heterozygous state.

Subsequently, the SNVs/INDELs were selected, according to the second filtering criteria, which can define SNPs that are hard to confirm. The results of this filtering identified four variants in patient 2 (P2) and two variants in patient 5 (P5), as shown in Table V.

Variants in P2. The analyses revealed an SNV in the *PEX11γ* gene (19p13.2), carried in a homozygous state by patient P2. A missense variation, rs11668511 (NM_080662 c.C646T; p.L216F), was found in exon 5 of the *PEX11γ* gene. Several *in silico* tools, including Polyphen-2, MutationTaster and LTR, predicted this variant to be pathogenic or disease causing (Table V).

The present study also found the following variants in different genes: rs146931399 (NM_145246.4 c.G241A; p.V81I) on the *FRA10AC1* gene, rs145520946 (NM_031302.3 c.A817G; p.M273 V) on the *GLT8D2* gene and, finally, rs202215334 (NM_001080489 c.G304A:p.D102 N) on the *GLOD5* gene. The functions of the proteins encoded by these genes remain to be elucidated and limited previous data exists. Therefore, it is difficult to correlate these variants with the clinical phenotype of P2.

Variants in P5. The present study detected a variant in the *UNG* gene (12q23-q24.1), which was carried in a homozygous state in patient P5. In this patient, the missense variation, rs151095402 (NM_080911.2 c.262C>T; p.R88C), in exon 2, was found in homozygosis for the first time, to the best of our knowledge.. Despite being rare (MAF, ~0,0012), this missense mutation had no effect on the IgG, IgA or IgE concentrations. In addition, a normal percentage of B-switched memory lymphocytes was observed (Fig. 1).

Discussion

Several studies have documented the phenotypic heterogeneity of patients carrying mutations in the *MVK* gene (15-19).

In the patients recruited in the present study, a correlation was observed between *MVK* genotype and UMA urine levels, and, of note, p.V132I has already been (33) described as being associated with lower UMA levels. However, the present study found a poor correlation between the' clinical phenotypes of

Table IV. Variants carried by genes belonging to the cholesterol biosynthetic pathway.

Refgene	Chr	AA change	MAF (esp6500)	MAF (1,000 g)	dbSNP 137	CVar	PolyPhen2	LRT	Mutation Taster	GERP++	PhyloP	P1	P2	P3	P4	P5
SQLE NM_003129	8	exon6:c.A937T:p.N313Y	0.0130	0.01	rs118130263	N	D	D	P	0.08 (C)	0.139 (N)	Het	WT	WT	WT	WT
		exon6:c.A1042G:p.I348V	0.0016	0.0005	rs199608260	N	B	P	P	-4.43 (N)	-1.574 (N)	WT	WT	Het	WT	WT
IDI1 NM_004508	10	exon1:c.G38A:p.C13Y	0.0346	0.02	rs7091756	N	B	U	P	-3.54 (N)	-1.854 (N)	WT	WT	WT	Het	WT
MVK NM_000431	12	exon2:c.16_34del:p.6_12del	NA	NA	rs104895334	N	NA	NA	NA	NA	NA	WT	WT	Het	WT	WT
		exon5:c.G394A:p.V132I	NA	NA	rs104895336	N	B	P	P	-7.14 (N)	-1.322 (N)	Het	WT	WT	WT	WT
		exon9:c.T803C:p.I268T	0.0003	NA	rs104895304	Y	D	D	D	4.60 (C)	1.930 (C)	WT	Het	WT	WT	WT
		exon10:c.G1006A:p.G336S	NA	NA	rs104895358	N	D	D	D	3.61 (C)	1.095 (C)	WT	WT	WT	WT	Hom
		exon11:c.G1129A:p.V377I	0.0017	NA	rs28934897	Y	B	P	D	3.17 (C)	0.489 (N)	Hom	Het	Het	Hom	WT
NSDHL NM_015922	X	exon4:c.G356A:p.R119K	0.0003	NA	rs200930841	N	B	P	D	3.97 (C)	0.567 (N)	Het	WT	WT	WT	WT

MAF in the general population is shown with reference to the Esp6500 and 1,000 g databases. CVar indicates whether there is an association between the variations and the clinical phenotype. Polyphen2, LRT and Mutation Taster refer to *in silico* variation pathogenicity predictors. GERP++ and PhyloP indicate the nucleotide evolutionary conservation. *SQLE*, squalene epoxidase; *IDI1*, isopentenyl-diphosphate δ isomerase 1; *MVK*, mevalonate kinase; *NSDHL*, NAD (P) dependent steroid dehydrogenase-like. Refgene, reference gene; Chr, chromosome; AA, amino acid; MAF, minor allelic frequency; CVar, clinical variance; Y, yes; N, no; GERP, genomic evolutionary rate profiling; P1, patient 1; P2, patient 2; P3, patient 3; P4, patient 4; P5, patient 5; D, deleterious; U, unknown; B, benign; P, possibly damaging for Polyphen 2; NA, not applicable; (C), conserved (N), not conserved WT, wild-type; Het, heterozygous; Hom, homozygous.

Table V. Rare variants, predicted to be likely pathogenic by three software packages.

RefGene	Chr	AA change	MAF (esp6500)	dbSNP 137	PolyPhen2	LRT	Mutation Taster	GERP++	PhyloP	P1	P2	P3	P4	P5
FRA10AC1 NM_145246	10	exon5: c.G241A:p.V81I	0.0008	rs146931399	P	D	D	4.1 (C)	1.095 (C)	WT	Hom	WT	WT	WT
GLT8D2 NM_031302	12	exon10: c.A817G:p.M273V	0.0085	rs145520946	D	D	D	4.26 (C)	0.869 (N)	WT	Hom	WT	WT	WT
PEX11G NM_080662	19	exon5: c.C646T:p.L216F	0.0242	rs11668511	D	D	D	5.2 (C)	2.405 (C)	WT	Hom	WT	WT	WT
GLOD5 NM_001080489	X	exon3: c.G304A:p.D102N	0.0014	rs2022334	D	D	D	4.74 (C)	2.077 (C)	WT	Hom	WT	WT	WT
UNG NM_080911	12	exon1: c.C262T:p.R88C	0.0012	rs151095402	P	D	D	4.21 (C)	2.182 (C)	WT	WT	WT	WT	Hom
MVK NM_000431	12	exon10: c.G1006A:p.G336S	NA	rs104895358	D	D	D	3.61 (C)	1.095 (C)	WT	WT	WT	WT	Hom

MAF in the general population is shown with reference to the Esp6500 database. Polyphen2, LRT and Mutation Taster describe the *in silico* variation pathogenicity prediction. GERP++ and PhyloP indicate the nucleotide evolutionary conservation. *FRA10AC1*, fragile site 10q23.3; *GLT8D2*, glycosyltransferase 8 domain-containing protein 2; *PEX11G*, peroxisomal biogenesis dactor 11G; *GLOD5*, glyoxalase domain-xontaining protein 5, *UNG*, uracil-DNA glycosylase; *MVK*, mevalonate kinase; MAF, minor allelic frequency; GERP, genomic evolutionary rate profiling; D, deleterious; P, possibly damaging; (C), conserved; (N), not conserved; WT, wild-type; Hom, homozygous; P1-5, patients 1-5.

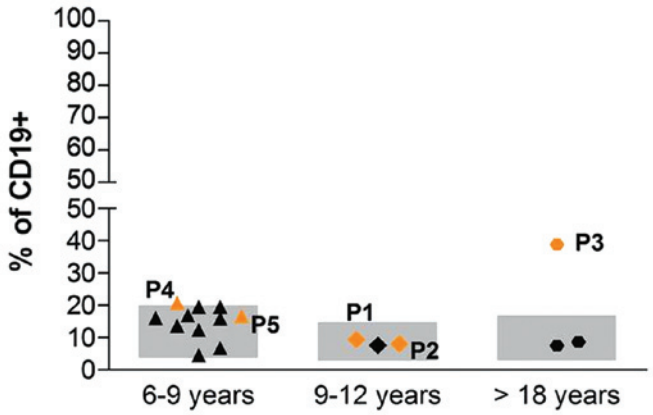

Figure 1. Percentage of B-switched memory cells which had class-switched to CD19⁺ CD27⁺ IgD/IgM. Class switched memory B cells are identified as CD19 and CD27 positive with the absence of surface IgD and IgM. Patients (orange dots) and control individuals (black dots) are divided into three age groups. The grey bars represent the range values. P1-5, patients 1-5.

the patients and *MVK* mutations, even taking into account the mutations, which change amino acids located close to each other in the protein sequence, for example, p.V377I and p.G336S, and that were considered to affect the same MK protein domain. Of considerable clinical importance is the p.G336S mutation which, unlike other mutations, determines inhibition of the metabolic pathway, causing high UMA levels, which also occur outside of fever attacks (5).

With the aim of identifying novel potential modifier genes/variations associated with MKD phenotype variability, the present study analyzed the entire exome of the five patients with MKD, hypothesizing that MKD may be a polygenic disease. Considering the heterogeneity of the clinical phenotypes, the present study investigated the possible presence of modifier genes with potential to affect the phenotype of the patients, in any way.

The variants obtained from a first analysis, which filtered variants with an MAF <0.05 did not explain the wide range of MKD phenotypes observed in the patients

When the analysis was performed with the second filtering criteria, two putative genes were identified, which potentially explained the clinical phenotype of two MKD patients (P2 and P5).

Patient P2 was identified with a mutation in the *PEX11γ* gene, which is a member of the PEX11 family, whose predominant function is associated with the tabulation, enlargement and clustering of peroxisomes, thus being important in human metabolism (34). Peroxisomal gene defects are also known to be associated with severe disorders (35-38).

In mammals, there are three PEX11-associated genes, *PEX11-α*, *PEX11-β* and *PEX11-γ*. Several previous studies analyzing expression have been performed on *PEX11-α*, and a few studies using animal models have been performed examining the β proteins (35,39). The γ protein has been reported to interact with α and β, and, by being present at the crossroads of PEX11, activates the peroxisome proliferation pathways, possibly being involved in homotypic interactions. In addition, PEX11-γ overexpression has been suggested to have the ability to induce early formation of juxtaposed elongated peroxisomes, suggesting that the γ protein either acts upstream of PEX11-α and PEX11-β, or that is their limiting factor. PEX11-γ appears to recruit the other PEX11 proteins to facilitate aggregation, and is required for the elongation of the peroxisome membrane (40).

Previously, a case of a patient carrying a homozygous nonsense mutation in the *PEX11-β* gene has been described. Using immunofluorescence microscopical analyses in over-expression experiments at 37˚C/40˚C, it was suggested that the occurrence of the enlarged catalase-containing peroxisomes is associated with the *PEX11-γ* gene (38).

The PEX11γ gene and the c.C646T; p.L216F variant have received limited attention in previous studies. The present study hypothesized that this variant, rarely detected in the general population (MAF, ~0.024), affected and modified the phenotype of P2. Patient P2 presented with elevated UMA values due to mutations in the *MVK* gene. However, in addition to showing all the typical signs of a MKD phenotype, the patient presented with visual blurring, not associated with cataracts (37).

A mutation in the *UNG* gene encoding an important DNA repair enzyme, was also found in P2.

The deletions in the *UNG* gene (c.391_393delC, c.426_429delAT c.568_571delTA) and the missense mutations (c.752T>C p.F251S) have been previously described to be associated with hyper IgM immunodeficiency type 4 (40,41). Hyper-IgM syndrome is characterized by normal or increased levels of IgM, and is associated with low or absent serum levels of IgG, IgA and IgE, indicating a defect in the class-switch recombination process (42). In 2003, Imai *et al* (43) reported on three patients with HIGM5, in which deleterious mutations within the catalytic domain of the UNG protein were identified. Functional investigations of immunoprecipitation and immunoblot assays confirmed the absence of expression in the three patients, supporting the hypothesis of a correlation between the identified mutation and protein instability.

In the present study, the p.R88C missense mutation in the *UNG* gene identified in P5, was previously described by Torseth *et al* (41), but only in a heterozygotic state. Despite the fact that this mutation is rare, its presence in homozygosis in the patient in the present study had no effect on the levels of IgG, IgA and IgE (Fig. 1). In addition, the patient was found to have a normal percentage of B-switched memory lymphocytes, indicating normal immunity, with regards to the B-cell compartment. Furthermore, patient four (P4) who was not a carrier of mutations in the *UNG* gene, had high levels of IgA, as previously reported described in several patients with MKD (44,45) but with normal levels of IgG and IgM. Taken together, these results suggested that investigation of the polymorphism found on the *UNG* gene requires caution, as it does not correlate directly with any specific disease phenotype.

The primary aim of the present study was to identify novel potential modifier genes for MKD disease. A number of preliminary results have shown that the clinical profile of heterogeneous phenotypes of patients with MKD may be associated with novel genes involved in modulating the MKD clinical picture (15,18).

In the present study, following WES in five patients with MKD, it was found that variations in genes encoding proteins of the cholesterol pathway were not associated with the modulation of MKD clinical phenotypes.

A missense variation, namely the c.C646T; p.L216F (NM_080662) in exon 5 of the *PEX11γ* gene was observed in homozygosis in P2, possibly correlating with visual blurring.

A rare *UNG* gene variant, namely (c.C262T; p.R88C), was detected in homozygosis in P5, however this did not correlate with a specific clinical phenotype. A number of other variants were found in the five MKD patients analyzed, however, no correlations with phenotype were observed. The lack of a direct correlation between genetic variations and phenotype suggest a possible role of post-transcriptional mechanisms, which may affect protein expression or function.

In the patients examined in the present study, no mutation was identified, but a a synonymous variation on NM_003956:exon1:c.C657T:p.N219 N in the gene coding for the *CH25H* gene was identified in the P2, P4 and P5 patients.

The patients in the present study were also analyzed using direct sequencing to verify the presence of the intronic polymorphism, identified by Moura *et al* (46). The intronic NM_001510.3:c.89-32007 A>G polymorphism (rs1450500) of the human glutamate receptor δ-2 (*GRID-2*) gene was not identified in any of the patient. Therefore, it was concluded that, at least in these patients, and in patients with more severe features, *GRID2* does not appear to be significantly involved.

Taking into account the primary limitation of the present study lacking patients with MKD exhibiting different clinical phenotypes, but sharing the same *MVK* mutations, the use of WES, despite being an attractive approach, only provided evidence of an association between one genetic variant, in the *PEX11γ* gene, and one clinical characteristic, visual blurring, in one of five patients. Therefore, further analysis required, using NGS approaches on a larger sample size of patients with MKD sharing the same *MVK* mutations and, ideally, exhibiting extreme clinical phenotypes, in order to identify genes and variants that correlate with clinical features. Considering the fact that MKD is a rare, orphan disease, this approach may be possible providing there are a sufficient number of patients with the above-mentioned characteristics. The identification of modifier genes specific for HIDS and MA may assist in the diagnoses of these two forms of the same disease at an earlier stage.

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