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► To cite this version:

Arnaud Molin, Marie Nowoczyn, Nadia Coudray, Céline Ballandonne, Geneviève Abeguilé, et al.. Molecular characterization of a recurrent 10.9 kb CYP24A1 deletion in Idiopathic Infantile Hypercalcemia. *European Journal of Medical Genetics*, 2018, 62 (11), pp.103577. 10.1016/j.ejmg.2018.11.011 . hal-02272241

HAL Id: hal-02272241

<https://hal.science/hal-02272241>

Submitted on 20 Jul 2022

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Molecular characterization of a recurrent 10.9 kb *CYP24A1* deletion in Idiopathic Infantile Hypercalcemia.

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Running title: *CYP24A1* deletion in Idiopathic Infantile Hypercalcemia

Conflict of interest

The authors declare no conflict of interest.

Abstract

Loss-of-function mutations in *CYP24A1* (MIM 126065 20q13.2), the gene encoding the 24-hydroxylase responsible for 25-OH-D and 1,25-(OH)₂D degradation, are identified in about 20% of patients presenting Idiopathic Infantile Hypercalcemia (IIH) (MIM 143880). Common features of this autosomal recessive condition included hypercalcemia with hypercalciuria, suppressed PTH and a high 25-OH-D₃:24,25-(OH)₂D₃ ratio. Medical care mainly relies on sun protection and life-long contraindication of vitamin D to avoid complications such as early nephrocalcinosis and renal failure.

Molecular diagnosis therefore keeps a crucial place in the diagnosis of IIH, and genetic counseling should be systematically recommended to prevent vitamin D administration in affected siblings.

In this report is described the molecular characterization of a *CYP24A1* deletion identified in two unrelated families. This highlights the potential role of *CYP24A1* copy number variations (CNV) in IIH. Considering the presence of CNV affecting *CYP24A1* in public databases, CNV analysis should be systematically added to the sequencing studies in IIH. Targeted Massively Parallel Sequencing (MPS) study coupled with a CNV detection tool called CovCop is a powerful method to detect genic rearrangement and improve genetic analysis.

Key words: nephrocalcinosis, vitamin D, hypercalcemia

Introduction

Loss-of-function mutations in *CYP24A1* (MIM 126065 20q13.2) are identified in about 20 % of patients presenting Idiopathic Infantile Hypercalcemia (IIH) (MIM 143880) (Molin et al. 2015). This gene encodes the mitochondrial enzyme 25-hydroxyvitamin D

24-hydroxylase which catabolizes the vitamin D metabolites. High levels of active vitamin D cause increased calcium intestinal absorption, leading to high serum and urine calcium with suppressed parathyroid hormone (PTH). A high 25-OH-D3:24,25-(OH)₂D3 ratio (substrate: product ratio), which directly reflects the enzymatic defect, has been specifically associated with this condition and proposed as a screening tool (ref). In children, common features of this condition include acute symptomatic hypercalcemia, renal stones and nephrocalcinosis (Schlingmann et al. 2011). Medical care mainly includes sun protection and life-long contraindication of vitamin D from childhood, to avoid acute complications of hypercalcemia and chronic hypercalciuria and subsequent renal failure.

Molecular diagnosis therefore has a crucial place in the diagnosis of IIH, and genetic counseling should be systematically recommended to prevent vitamin D administration in affected siblings. Since the first publication (Schlingmann et al. 2011), the phenotypic spectrum has been extended to adults with renal disease (Castanet, Mallet, and Kottler 2013; Colussi et al. 2014; Dauber et al. 2012; Dinour et al. 2013; Fencel et al. 2013; Figueres et al. 2015; Jacobs et al. 2014; Meusburger et al. 2013; Molin et al. 2015; Nesterova et al. 2013; Tebben et al. 2012). The vast majority of observations suggested a recessive autosomal inheritance, but some authors have suggested that there might also be a dominant inheritance pattern (Tebben et al. 2012).

Most *CYP24A1* mutations previously reported are point variations, essentially missense, with at least 2 recurrent mutations (Molin et al. 2015). They were detected using targeted sequencing or whole exome sequencing (WES) (Dauber et al. 2012).

This report highlights the requirement of Copy Number Variations (CNV) detection procedures during *CYP24A1* analysis for molecular diagnosis of IIH to search for CNV which could go undetected with conventional Sanger sequencing.

Patients (Figure 1)

Family 1 has previously been reported by Castanet *et al.* (Castanet, Mallet, and Kottler 2013). The proband (F1-II1) is the first child of a nonconsanguineous healthy couple, who received vitamin D (1900 UI/day) until the diagnosis of severe hypercalcemia with hypercalciuria at the age of 6 months. PTH level was low and nephrocalcinosis was detected on ultrasonography. Molecular diagnosis allowed precocious identification of an affected sibling (F1-II3) and prevent vitamin D administration and complications of the disease.

The second proband (F2-II3) (patient 17 in (Molin et al. 2015)) is a 10-year-old girl, the third child of a healthy nonconsanguineous couple. At the age of 14 months, receiving 1200 UI/day of vitamin D, she presented polydipsia and vomiting in a context of postnatal growth failure, hypercalcemia and hypercalciuria with suppressed PTH. Renal ultrasound showed medullary nephrocalcinosis grade 2 (mild increase of echogenicity at whole pyramid). Vitamin D supplementation was ceased.

Biochemical features are included in Supplemental material table 1.

Written consent was obtained from the patients and/or their parents for the collection of clinical and laboratory data and for genetic investigation. The molecular analysis of relatives was suggested through genetic counseling after the identification of a mutation in the probands.

Methods

Genomic DNA was extracted from whole blood samples with routine procedures.

Two sequencing methodologies were used, i. e. Sanger and targeted Massively Parallel Sequencing (MPS) of genes involved in vitamin D metabolism including *CYP24A1*.

Reference sequence (ENST00000216862/NM_000782.4) was obtained from ensembl database (<http://www.ensembl.org>).

Sanger: The 11 coding exons of *CYP24A1* and their flanking regions were amplified by PCR using specific primers as previously described (Castanet, Mallet, and Kottler 2013; Molin et al. 2015).

MPS: The exons 1 to 12 were sequenced using a custom Ampliseq library and the Ion Torrent Personal Genome Machine (PGM) (ThermoFisher Scientific, Waltham, Massachusetts, USA). Variants were annotated using IonSuit (ThermoFisher Scientific, Waltham, Massachusetts, USA) and Nextgene (SoftGenetics, State College, Pennsylvania, USA). A read depth (RD)-based approach was used to detect CNV using the CovCop (v1.2 - <https://omictools.com/cov-cop-tool>) tool (Derouault et al. 2017).

This procedure also identified single nucleotide polymorphisms (SNP) which were used to determine pathogenic haplotype at *CYP24A1* locus.

Quantitative PCR: Copy number of exons 9 and 11 was assessed using specific TaqMan probes (Hs00815832_cn targeting the junction between intron 8 and exon 9 and Hs01863481 targeting the junction between intron 10 and exon 11) (ThermoFisher Scientific, Waltham, Massachusetts, USA).

Breakpoints determination: Sixteen primers were designed: a forward primer in exon 9, and 15 reverse primers (R1 to R15) from intron 10 each placed about 800 pb away from other ([Supplemental material 1](#)). Amplification was performed following standard routine protocol and PCR products were sequenced.

Results

In the proband F1-III1 (family 1) (Castanet, Mallet, and Kottler 2013), the exon 9 to 11 were not amplifiable. Thus, a deletion which included at least these exons in a homozygous state was suspected. This was coherent with quantitative PCR analysis; the deletion was inherited from both heterozygous parents.

In Family 2, sequencing analysis in the proband F2-II3 found the recurrent exon 9 mutation p.Leu409Ser in an apparently homozygous state. However parental analyses revealed that it was in a heterozygous state in the maternal DNA while no variation was inherited from the father. Quantitative PCR suggested a paternally inherited deletion. Targeted MPS with CovCop (Derouault et al. 2017) detected the deletion in every patient, either in a homozygous (patient F1-III1) or in a heterozygous state (F1-II1, F1-II2, F2-II1).

The SNP study showed that pathogenic haplotype at the *CYP24A1* locus was the same between the two families (Figure 1).

Breakpoints study evidenced the same 10,924-bp long deletion in both families, from the end of exon 9 to the *CYP24A1* downstream region (hg19 chr20:52763705_52774628del) (Figure 2).

DISCUSSION

In two unrelated families with IIH, a large *CYP24A1* deletion was characterized which might go undetected without adequate molecular diagnosis procedure.

The examination of the Database of Genomic Variants (DGV, <http://dgv.tcag.ca/dgv/app/home>) revealed several deletions at *CYP24A1* locus, ranging from 0.6 to 28 kb, with an estimate of the frequency of 0.01% (3/29,084) (14). Two similar deletions (nsv1063981 and esv2762085) were detected using SNP-array in an American patient with developmental delay and in a Swiss healthy man (Figure 2) (Coe et al. 2014; Vogler et al. 2010). Their length (11,296 bp) was defined according to the array's probes' localization, which may explain the difference with our deletion's length. Unfortunately, no DNA sample from these patients was available to test this hypothesis. Allelic frequency of this particular deletion is currently unknown. It was identified with the same haplotype in the 3 unrelated heterozygous parents (F1-I1, F1-I2, F2-I1), which may suggest a founder effect (as both families shares common geographic origins in Normandy) and a significant frequency in general population. Thus, a special attention should be paid to CNV analysis during molecular diagnosis of IIH, as it might represent a significant part of *CYP24A1* molecular defects.

Intragenic deletion can easily remain undetected by Sanger sequencing when at least one primer targets a deleted sequence, leading to misdiagnosis, wrong interpretation of genetic data and wrong genetic counseling. In patients harboring the mutation in a homozygous state, the lack of PCR amplification draws attention, as illustrated in family 1. Conversely, in the case of a deletion in a heterozygous state, only one allele is amplified and sequenced, which leads to an incorrect diagnosis of homozygosity, as illustrated in family 2. Indeed, the lack of amplification of both allele of exon 9 explains why the p.Leu409Ser mutation appeared in a homozygous state in F2-II3.

Other techniques for CNV detection such as quantitative PCR, Multiplex Ligation Probe Analysis (MLPA) (targeted approaches), arrayCGH or SNP-array (pangenomic approaches) have been used to further genetic analysis in case of highly specific phenotype with negative sequencing, in various diseases (Ellingford et al. 2018). Accuracy of these techniques greatly depends on the number and localization of probes. More recently, specific algorithms to detect CNV among MPS data (whole genome sequencing (WGS), WES or targeted MPS) are being developed to improve the sensitivity of genetic analysis (Ellingford et al. 2018; Zhao et al. 2013). Our targeted MPS with CNV detection method based on CovCop (Derouault et al. 2017), a user friendly tool made to identify CNV among amplicons sequencing data, was able to detect the deletion in every patient including heterozygous patient.

In conclusion, instead of illustrating one new mutation, this report **confirms** the role of *CYP24A1* CNV in IIH. Physicians must keep in mind that a non-contributory *CYP24A1*

coding sequence analysis (e.i. no mutation or a mutation in a heterozygous state) in a IIH patient may have two different meanings, either a truly negative result, which should lead to consider another mechanism of IIH (*SLC34A1* mutation), or a CNV which may be detected with an appropriate method. In that perspective, evaluation of 24,25-dihydroxyvitamin D (Kaufmann et al. 2014) and familial study are critical for a relevant interpretation of genetic data and to point patients who may harbor intragenic rearrangement.

Acknowledgements

The authors would like to thank the patients and their families, Anne-Sophie Lia and the CovCop team, Christian Vogler and Candy Lee.

Conflict of interest

The authors declare no conflict of interest.

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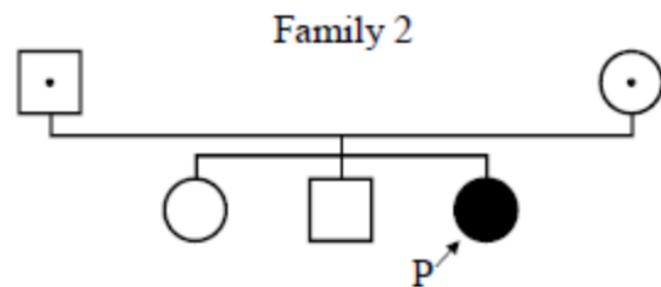
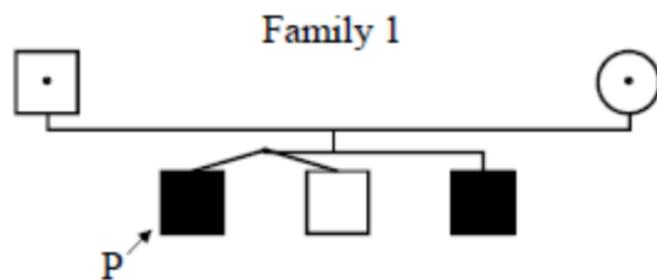
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Legend:

Figure 1 Pedigree trees and haplotypes study. Nine single nucleotide polymorphisms (SNP) and one *CYP24A1* mutation c.1226T>C / p.Leu409Ser (rs6068812) were used to determine a common pathogenic haplotype. Grey boxes symbolize exonic SNP.

Figure 1 (A) Schematic representation of the *CYP24A1* rearrangements indexed in Database of Genomic Variants (DGV) (<http://dgv.tcag.ca/dgv/app/home>). Blue lines are duplications, red lines are deletion. (B) Schematic representation of the *CYP24A1* deletion characterized through Sanger sequencing of the 595-pb fragment obtained from PCR with R14. Black arrows represent Taqman assays.



c.450-583C>T (rs11699278)	TC	CC	TC	CC	CC	CC	--	--	CC	CC
c.543+814T>C (rs13038432)	TC	CC	TT	CC	TC	CT	--	--	CC	TC
c.641-1280C>T (rs2762941)	CT	TT	CC	TT	CT	TT	--	--	TT	TT
c.641-706T>A (no rs)	TA	AA	TT	AA	TA	AT	--	--	AT	TT
c.641-319G>T (rs3787555)	GT	TT	GG	TT	GT	TG	--	--	TT	TT
c.641-66A>C (rs4809958)	CC	CC	CA	CC	AC	CA	--	--	CC	CC
c.733-162A>G (rs6013905)	AG	GG	AA	GG	AG	GA	--	--	GG	GG
c.744G>A / p.Thr248Thr (rs6068816)	GA	AA	GG	AA	GA	AG	--	--	AA	AA
c.845-792T>C (rs3886163)	CT	TT	CC	TT	CT	TC	--	--	TT	TT
c.1226T>C / p.Leu409Ser (rs6068812)	TT	TT	TT	TT	TT	TT	--	--	TC	TC

A



B

