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Original article

Development of simple and effective PCR based assay to detect *PCCA* mutation (c.425G > A) among Saudi carriers and functional study of the homozygous *PCCA* mutations

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ABSTRACT

The aim of this study is to develop a rapid and effective method to screen for Saudi carriers of one of the most common propionic acidemia mutations (c.425G > A) and to study the functional impact of this mutation. Using allele-specific primers, we have developed a qPCR assay that clearly distinguishes heterozygotes from mutated and wild type homozygotes that overcome the dependence on labor-intensive gene sequencing. We show here that (*i*) qPCR rapid test has strong accuracy in detecting (c.425G > A) mutation in heterozygotes and homozygotes individuals and that the Ct-value cut-offs were estimated to be and 23.37 ± 0.04 (CV-6 %, 95 %CI-7.25) for homozygote, 25.06 ± 0.02 (CV-3.5 %, 95 %CI-7.85) for heterozygote *PCCA* c.425G > A mutation and 29.55 ± 0.002 (CV-11 %, 95 %CI-1.41) for *PCCA* wild type; (*ii*) the incidence of PA heterozygotes/carriers in Saudi population is about 550/100,000; (*iii*) skin fibroblast assays show that homozygote c.425G > A mutation induced propionyl-CoA carboxylase activity abrogation, (*iv*) PA patients showed an increased level of propionyl carnitine C3 in blood and 3-hydroxy propionic acid and methyl citrate in urine. *Conclusion*: qPCR represent an effective strategy to assess for *PCCA* mutation carriers in the Saudi population and we believe that will help in preventing homozygosity in the population after been implemented in pre-marriage screening program.

1. Introduction

Propionic Acidemia (PA: MIM #606054) is a rare hereditary disorder caused by a deficiency in the mitochondrial enzyme Propionyl CoA Carboxylase (PCC) leading to an accumulation of propionic acid and other metabolites. The disease may present within the first days of neonatal life with repeated episodes of metabolic acidosis including hyperammonemia initiated by period of vomiting, lethargy, viral infection, and resulting in bone marrow suppression with neutropenia and thrombocytopenia that may be lethal (Kalloghlian et al., 1992). These symptoms sometimes progress to more serious medical problems, including heart abnormalities, seizures, coma, and possibly death. In some instances, some patients show milder symptoms and long survival rate associated with chronic late-onset form (Pérez-Cerdá et al., 2000).

Abbreviations: Ct, Cycle Threshold; PA, Propionic Acidemia; *PCC*, Propionyl-CoA Carboxylase; PCR, Polymerase Chain Reaction.

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PA affects about 1 in 2,000 to 1 in 5,000 individuals according to the tribe in Saudi Arabia, 1 in 100,000 people in the United States and the condition appears to be more common in several populations worldwide, including the Inuit population of Greenland and some Amish communities (Pena et al., 2012). Saudi PA patients present with a severe phenotype and acute intercurrent infections are unusually frequent (80 %) (Al Essa et al., 1998) making early diagnoses essential. The diagnoses of PA in the neonatal period is suspected with elevated propionylcarnitine (C3) by tandem MS/MS followed by biochemical testing that includes plasma acylcarnitine and amino acid profile, urine organic acid for elevated 3-hydroxypropionate, and confirmatory enzymatic assays for PCC or *PCCA*/*PCCB* mutational analyses.

A number of pathogenic, likely pathogenic, variants of uncertain significance (VUS), likely benign and benign *PCCA*/*PCCB* variants in ClinVar and HGMD that may serve as confirmatory diagnostic tests have been reported in PA. For example, the variant c.425G > A (p.Gly142Asp; NM_000282.3) is reported to be the most common and a founder variant causing PA in Saudi Arabian population. The deletion c.111_130del20 has been recently reported in three families but functional data to support the deleterious nature of this deletion has to be shown (Al-Hamed et al., 2019). We, therefore, used PCC enzyme activity assay (Baumgartner et al., 2014) in cultured skin fibroblasts as a valuable functional assay to assess the effect of the novel deletion detected in one of our PA patients compared to the known pathogenic variant (c.425G > A). A combination of PCC enzymatic assay and molecular genetic testing of *PCCA*/*PCCB* gene mutations that may include single gene testing, multigene panels or genomic (whole exome and whole genome) approaches may be necessary in the confirmation of PA diagnosis. This will particularly hold true in the case of novel mutations detected in the process of diagnostic work-up of PA patients or by whole genome approaches. Despite advances in molecular testing and enzymatic assays for the diagnoses of PA, rapid and efficient molecular testing methodology based on PCR is lacking. We, therefore, designed a qPCR CybrGreen-based study (n = 2000 individuals) using allele-specific primers to detect the most common and a founder variant causing PA in Saudi Arabian population, c.425G > A (homozygous) and carriers (heterozygous).

We believed that due to the high rate of consanguineous marriages that such disease is high in Saudi Arabia compared to other countries. Prevention is the golden practice to overcome such problem. In the current study, we sought to establish carrier screening for the most common PA mutation seen in the Kingdom of Saudi Arabia to enhance prevention strategies for propionic acidemia, by developing a rapid and cost effective CybrGreen-based qPCR assay that clearly distinguishes heterozygotes from wild type homozygotes to detect *PCCA* mutation (c.425G > A) among carriers in Saudi Arabia, and thereby overcome labor and time-consuming gene sequencing and the dependence on invasive skin biopsy for enzymatic assay. To our knowledge, this is the first such study in the literature reported thus far and would have potential implications globally in using real-time PCR based screening for PA.

2. Materials and methods

2.1. Sample selection and DNA extraction

All patients, Male and Female adult participants with no known previous Propionic Acidemia history and from different region of the country were enrolled at King Fahad Medical City Tertiary Hospital under approved Institutional Review Board protocol (IRB# 13-025). The participants have signed an informed consent prior to inclusion. Three ml EDTA blood samples were collected, and DNA were extracted using Viral NA large volume isolation kit DNA Blood LV 3.1 protocol, according to the manufacturer's instructions (Roche Applied Science, Indianapolis, USA). Extracted DNA samples were stored at −80 °C for further analysis.

2.2. Real-time PCR assay development

Predicted amino acid numbering is based on cDNA with the 'ATG' as the start codon using transcript ID NM_000282.3; NP_000273.2, and the entire exon 6 nucleotide sequence for the *PCCA* gene with the corresponding amino acid as well as mutation position highlighted in bold (Fig. 1**A**). We have developed a CybrGreen-based PCR assay using allele specific primers (Fig. 1**B**) that clearly distinguishes heterozygotes from wild type (WT) or mutant homozygotes (Fig. 1**C**). The assay has been validated using WT DNA spiked with template mimicking either WT or mutant, diluted to a level that gives the same cycle threshold as genomic DNA. Cycle threshold (Ct) ranged from 26.85 to 27.43 for heterozygous mutant and 30.84 to 31.16 for wild type (Fig. 1**C**), clearly distinguishing genotypes with no overlap in seven replicate assays.

Briefly, the assay has been designed to discriminate between the normal and mutated alleles of the c.425A > G in the *PCCA* gene using alleles specific primers (Fig. 1**B**) designed using the NCBI Primer Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/primertool.cgi?ctg_time=1654634711&job_key=XFaDoHx2cd5-W5OHh7IHF05aa1OG7ic_8ug). DNA was amplified by real time PCR using SYBR Green Mix: 10 µl; Forward Primer: 1 µl (10 mM), Reverse Primer: 1 µl (10 mM), DNA: 2 µl (15 ng/µl) and H2O: 6 µl and the following cycling conditions: initial denaturation of 600 s at 95 °C followed by 45 cycles of 3-steps amplification (95 °C for 20 s, 52 °C for 30 s-single acquisition mode, 72 °C for 30 s) and terminated by melting (95 °C for 10 s, 65 °C for 60 s and 97 °C for 1 s, continuous acquisition mode).

2.3. Propionyl-CoA carboxylase activity

The PCC carboxylase activity was analyzed on skin fibroblasts at the Center for Inherited Disorders of Energy Metabolism (CIDEM, Cleveland, USA). Briefly, Fibroblasts were cultured from skin biopsies as described by Atkin et al. (1979). The procedure of the propionyl-CoA carboxylase activity involved fixation of $^{14}CO_2$, dependent on pyruvate in the presence of ATP plus acetyl-CoA and citrate synthase; or dependent on IDP (Inosine-5′-diphosphate) in the presence of phosphopyruvate, NADH, and malate dehydrogenase for phosphoenolpyruvate Carboxykinase (PEPCK) (Atkin et al., 1979; Kerr et al., 1987; Marsac et al., 1982).

2.4. Tandem MS

Propionyl carnitine C3 level (C3/C1 ratio) in blood, 3-hydroxy propionic acid and methyl citrate levels in urine were tested using Tandem/MS and GC/MS, respectively according to the established protocol at Biochemical Genetics and Toxicology laboratory of PCLMA, KFMC (CAP accredited laboratory).

2.5. Sanger sequencing

Genomic DNA from PBMC was used to amplify the twenty-four coding exons and corresponding flanking sequences of *PCCA* gene. The PCR products were analyzed by sequencing in both the forward and reverse directions using an established protocol.

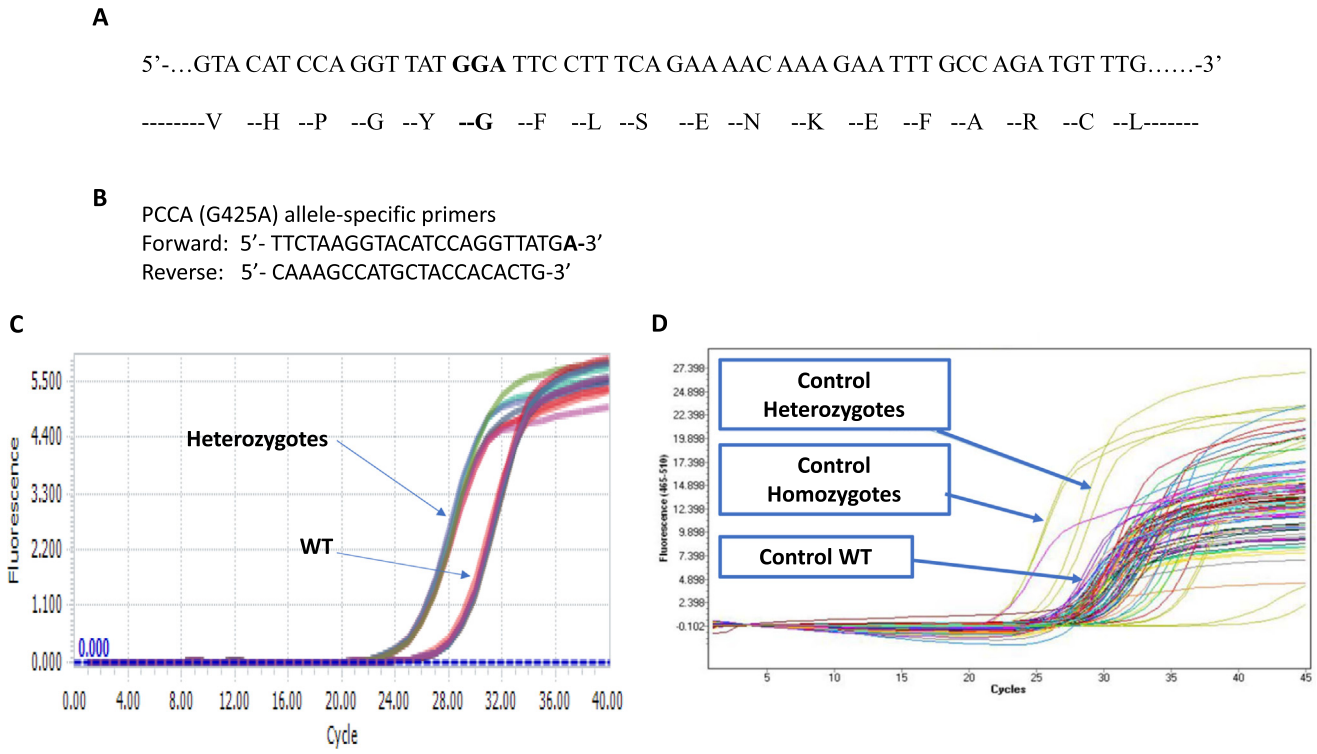

Fig. 1. qPCR method for the detection of PCCA mutation (c.425G > A). A) The design of alleles specific primers was based on the PCCA sequence showing the mutation position on the DNA, B) Sequence of alleles specific primers and C) Detection of mutated allele c.425A > G of the PCCA gene in heterozygote samples compared to wild type samples by Real-time PCR using LightCycler 480 (Roche Applied Science, Indianapolis, USA as mentioned in the Materials and Methods section. D) Screening of Saudis for PCCA mutation (c.425G > A). Mutated allele c.425A > G in the PCCA gene. The result is an illustration of amplification curves from the qPCR showing clear amplification of Homozygote, heterozygote and wildtype controls and 40 samples per run in duplicate.

3. Results

3.1. Detection of c.425A > G variant using cybergreen-based realtime PCR

In order to establish a quick and robust molecular screening method such as a PCR to detect homozygous and/or heterozygous carriers of *PCCA* (c.425G > A) mutation, we have designed a point mutation specific sense and anti-sense primers based on *PCCA* c.425G > A mutation detected by sanger sequencing as described in the methods and (Fig. 1**A**). The data show that the developed qPCR clearly distinguishes between *PCCA* mutation (c.425G > A) heterozygote and wild type (Fig. 1**C**). Based on the homozygote, heterozygote and WT controls tested in each run (Fig. 1**D**), we established the Ct-value cut-off for *PCCA* homozygotes was 23.37 ± 0.04 (CV-6 %, 95 %CI-7.25), *PCCA* heterozygote was 25.06 ± 0.02 (CV-3.5 %, 95 %CI-7.85) and for wild-type was 29.55 ± 0.002 (CV-11 %, 95 %CI-1.41) (Table 1).

3.2. Prevalence of PCCA mutation (c.425G > A) heterozygote in Saudi carriers

A total of 2,000 consented individuals were tested for *PCCA* mutation (c.425G > A) using real time PCR assays as shown in (Fig. 1**D**). Based on the heterozygote and homozygote controls tested in each run, the Ct-value cut-off were established and any sample below this cut-off with an appropriate RGP PCR curve was considered as positive for the *PCCA* mutation. Of the total specimens analyzed, 11 samples out of 2,000 showed *PCCA* heterozygosity giving an overall incidence of 550/100,000 individuals.

3.3. A functional study of pathogenic mutations in the PCCA gene

We describe here the clinical characteristics and functional analysis of pathogenic *PCCA* mutation (c.425G > A) and the novel homozygous frameshift deletion (c.111_130del20) in 14 cases of PA patients consulting at the Department of Pediatrics, Medical Genetics Section, King Fahad Medical City, Riyadh, Saudi Arabiabetween 2012 and 2020 (Table 2). 13 patients are homozygotes for (c.425G > A) *PCCA* mutation in which PA disease was detected early in life with maximum age survival of 14 years. They all showed poor feeding, lethargy and metabolic acidosis. 4 patients died and 10 still alive with mild to severe mental and developmental retardation, seizure and failure to drive (Table 2). The screening for inherited metabolic diseases markers showed a high level of propionylcarnitine (C3) and high excretion in Urine of 3-hydroxypropionic acid and methylcitrate in all the patients thereby confirming the diagnosis of PA as revealed by Tandem-

Table 1
Ct-value determination of PPCA (c.425G > A) mutation for homozygote, heterozygote, and wild-type.

	Mean Ct	SD	SE	CV	1.96*SE ~ 95 %CI
HOMOZYGOTE N = 38	**23.37**	1.4076072	0.03704229	6.0232922	7.253557887
HETEROZYGOTE N = 38	**25.06**	0.86353469	0.0227246	3.44550966	7.847437792
Wild type N = 1924	**29.55**	3.2148735	0.00176157	10.8796159	1.412802617

Table 2
Clinical characteristics of PA patients.

Patient #	*Genotype*[*]	*Age (year)*	*Alive*	*Onset Neonatal*	Propionylcarnitine (μmol/L)[a]	Urine organic acid Secretion[b]	Current clinical outcomes
P1	A	7	Yes	Yes	19	High	Mild developmental delay, normal Growth
P2	B	6	Yes	Yes	18.3	High	Severe mental retardation, seizure disorder, GT feeding,
P3	B	8	Yes	Yes	26	High	severe mental retardation, seizure disoder, GT feeding, spatic quadriplagia
P4	B	10	Yes	Yes	9	High	Moderate mental retardation, failure to thrive, GT feeding
P5	B	10	Yes	Yes	14	High	Severe menetal retardation, seizure disoder, failure to thrive, GT feeding
P6	B	10	Yes	Yes	26	High	Severe mental retardation, seizure disoder, GT feeding, spatic quadriplagia
P7	B	10	Yes	Yes	23	High	Severe mental retardation, seizure disoder, failure to thrive, GT feeding
P8	B	11	Yes	Yes	8.1	High	Moderate developmental delay, Post-liver Tx.
P9	B	10	Yes	Yes	22	High	Severe mental retardation, failure to thrive, GT feeding
P10	B	4	Yes	Yes	40	High	Moderate to severe mental retardation
P11	B	14	No	Yes	16	High	
P12	B	14	No	Yes	21	High	
P13	B	14	No	Yes	25	High	
P14	B	14	No	Yes	27	High	

[*] Genotype A: PCCA: c.111_130del20; p.C44LfsX6 and Genotype B: PCCA: c.425G > A; p.G142D.
[a] Cut-off value < 6.86 μmol/L.
[b] 3-hydroxypropionic acid, propionylglycine, methylcitrate and ketone bodies.

MS. In order to confirm molecularly the diagnoses of PA, we performed mutational analyses of the *PCCA* gene using PCR and Sanger sequencing of the coding regions and splice junctions in the *PCCA* gene that identified two copies of a sequence variant, c.425G > A (Fig. 2). Both the forward and reverse primers gave a homozygous missense variant as shown (Fig. 2**A and 2B, upper and lower panel-patient #2 and #3**). This variant c.425G > A predicts a missense mutation of glycine (G) to aspartate (D) at codon 142 of the carboxylase protein (p.G142D) and is a known pathogenic mutation in *PCCA* causing PA. These results indicate that these 13 patients were affected with PA due to presence of a missense variant c.425G > A in exon 6 of the *PCCA* gene.

In order to measure the impact of this mutation on the PCC enzyme activity, we used the gold standard skin fibroblast assay from 2 patients having c.425G > A mutation (P2 & P3). The results of skin fibroblasts in these 2 patients were well below the range of controls (Table 3). The activity of Pyruvate Carboxylase, another biotin dependent carboxylase was observed to be relatively high

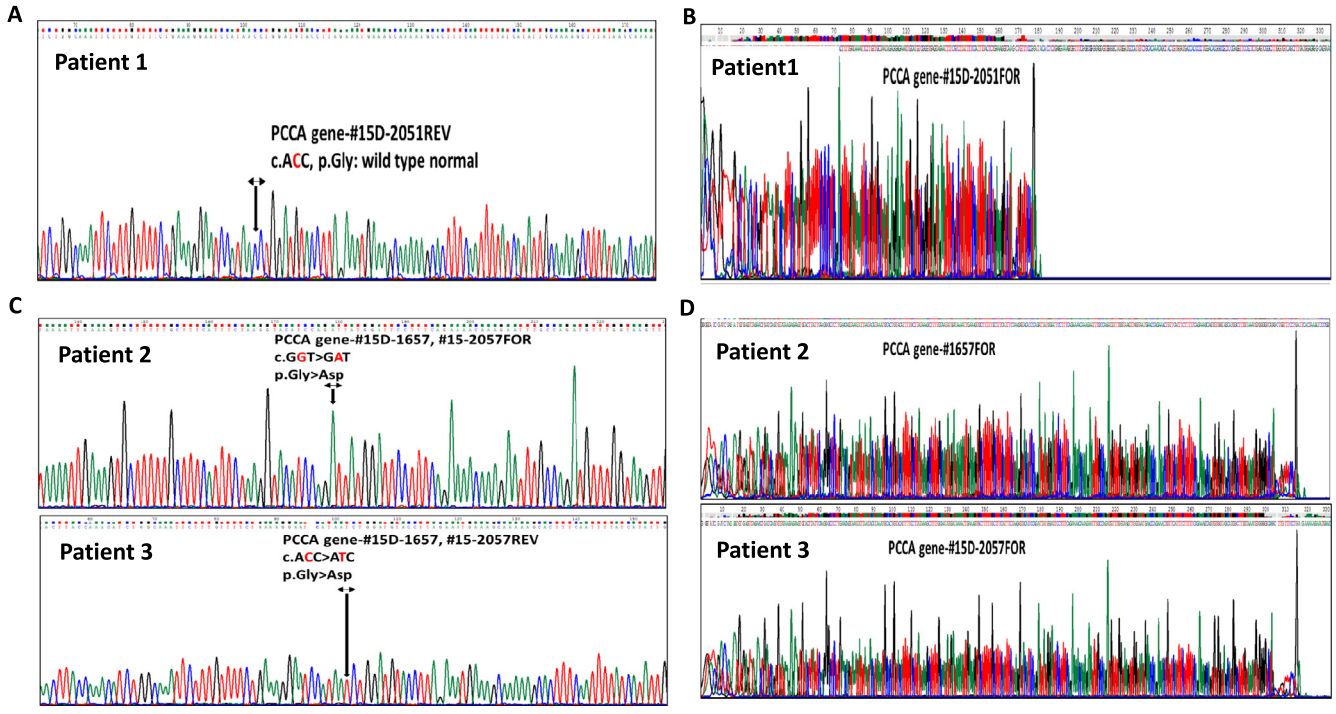

Fig. 2. Genomic DNA sequencing using amplified coding exons and corresponding flanking sequences of the PCCA gene. While the normal wild type sequencing result was obtained with the reverse primer used (A), a clear deletion was seen with the forward primer used (B). Both the forward and reverse primers gave a homozygous missense variant as shown in (C and D), upper and lower panel-patient #2 and #3. Compressed electrophoretograms of Patient P1, P2 and P3 to distinguish the deletion in P1 from a single nucleotide variation in P2 and P3.

Table 3
PCC enzyme activity in cultured skin fibroblasts.

	P1	**Concurrent Controls**	**Skin fibroblasts prior controls**			
LAB#	*61,596*	*60,011*	*Mean ± SD*	*Range*	*n=*	*% mean*
PC, pyruvate carboxylase	2.58	2.29	1.42 ± 0.79	0.56–3.22	338	182 %-High
Propionyl CoA carboxylase	**0.41**	**1.85**	**1.42 ± 0.27**	**0.96–2.54**	**9**	**29 %- Low**
CS, citrate synthase	56.8	57.8	37.9 ± 10.8	22.0–57.9	254	150 %-High
PC/CS ratio	4.54	3.96	4.4 ± 2.31	1.56–8.74	195	103 %
Protein ug/T75	2090	1527	1562 ± 925	444–3358	198	134 %
	P2					
LAB#	*61,220*	*60,011*	*Mean ± SD*	*Range*	*n=*	*% mean*
PC, pyruvate carboxylase	3.93	2.59	1.42 ± 0.79	0.56–3.22	338	277 %-High
Propionyl CoA carboxylase	**0.03**	**2.07**	**1.42 ± 0.27**	**0.96–2.54**	**9**	**2 %- Low**
CS, citrate synthase	66.9	66.5	37.9 ± 10.8	22.0–57.9	254	177 %-High
PC/CS ratio	5.87	3.89	4.4 ± 2.31	1.56–8.74	195	134 %
Protein ug/T75	1616	1458	1562 ± 925	444–3358	198	103 %
	P3					
LAB#	*61,219*	*60,011*	*Mean ± SD*	*Range*	*n=*	*% mean*
PC, pyruvate carboxylase	5.23	2.59	1.42 ± 0.79	0.56–3.22	338	368 %-High
Propionyl CoA carboxylase	**0.06**	**2.07**	**1.42 ± 0.27**	**0.96–2.54**	**9**	**4 %- Low**
CS, citrate synthase	60.3	66.5	37.9 ± 10.8	22.0–57.9	254	159 %-High
PC/CS ratio	8.67	3.89	4.4 ± 2.31	1.56–8.74	195	197 %
Protein ug/T75	1419	1458	1562 ± 925	444–3358	198	91 %

(P2 = 277 %, P3 = 368 % of prior mean). Similarly, Citrate Synthase, a mitochondrial reference enzyme activity was elevated relative to controls (P2 = 177 %; P3=, 159 % of mean).

Patient (P1) presented with fever, metabolic acidosis and vomiting with no skin rash, non-palpable spleen and with no dysmorphic features. He was from a smooth pregnancy, had no craniofacial, no limb/skeletal abnormalities and had normal tone. Imaging studies showed that the heart is not enlarged, and lungs show bilateral perihilar linear opacities. No major atelectasis or consolidation and no pleural effusion or pneumothorax was observed. Newborn screening result on dried blood spots for acylcarnitine came positive with elevated propionylcarnitine (C3), elevated C3/C2, elevated C3/C16 and work-up for PA diagnosis was started. Screening for inherited metabolic diseases showed high level of propionylcarnitine (C3) at 19 μmol/L (cut-off value at <6.86 μmol/L, C3/C1 ratio was elevated and observed to be 0.8 (cut-off <0.32) and the level of plasma glycine was 1933 μmol/L (range 107–460 μmol/L) (Table 2**)**. These results suggested either PA or methylmalonic acidemia, resolved by urine organic acid analyses using GCMS that showed high excretion of 3-hydroxypropionic acid and methylcitrate thereby confirming the diagnosis of PA as revealed by Tandem-MS.

In order to confirm the diagnoses of PA, we performed mutational analyses of the *PCCA* gene using Sanger sequencing. We identified two copies of 20 nucleotide deletion, c.1111_130del20 in the coding region of the *PCCA* gene (Fig. 2). While the normal wild type sequencing result was obtained with the reverse primer used (Fig. 2**C**), a clear deletion was seen with the forward primer used (Fig. 2**D**). This deletion predicts a sequence frameshift due to loss of six codons and subsequent juxtaposition of the second nucleotide of codon 44 for cysteine to the second nucleotide of codon 37 for valine, inserting 5 amino acids and a premature termination codon (X) in exon 1 of the carboxylase protein (p-C44_fsX6). Additionally, two other sequence variants and polymorphisms identified in this patient were c.136A > G; p.M46V (Homozygous) and c.627A > G; p.A209A (homozygous).

The results of the skin fibroblast assay revealed PCC enzymatic activity to be well below the range of controls, corresponding to 29 % of the mean of prior controls (Table 3**)**. The activity of Pyruvate Carboxylase, another biotin dependent carboxylase was observed to be relatively high (182 % of prior mean). Similarly, Citrate Synthase, a mitochondrial reference enzyme activity was elevated relative to controls (150 % of mean). These results demonstrate that the novel mutation is in fact able to abrogate the PCC enzyme activity in skin fibroblasts, suggesting its causative nature in PA.

4. Discussion

PA is a rare autosomal recessive metabolic disorder that presents with nonspecific symptoms and high frequency of infections, particularly in Saudi Arabia (Al Essa et al., 1998) making the clinical diagnosis not only difficult but suggesting an underlying immune deficiency. Moreover, since metabolic decompensation plays a major role in the neurologic problems and sequel observed in patients with PA (Fenichel, 1996; Hoffmann et al., 1994; Nyhan et al., 1999) it is therefore, very important to recognize early signs of dehydration, poor feeding, seizures, and respiratory distress, and educating the patient's family to improve patient outcome.

Molecular characterization of PA is not only important in diagnosing the disease and establishing genotype to phenotype relationship but also important in community screening and developing preventive strategies including prenatal and preimplantation genetic diagnosis. The hallmark of this study is first, the functional study of a novel homozygous frameshift deletion in *PCCA* gene that has functional consequences on the PCC enzyme activity in cultured skin fibroblasts, and second, successful establishment and use of real-time PCR based detection of a specific most common and founder *PCCA* mutation.

The 15 cases of PA described in this manuscript presented with a high level of propionylcarnitine C3 and hyperammonemia and excess glycine in serum and urine suggestive of PA. Soriano et al (Soriano et al., 1967) and Childs et al (CHILDS et al., 1961) described hyperammonemia and ketoacidosis as a generalized defect in utilization of amino acids resulting in excessive deamination of certain amino acids in muscle, in addition to glycine encephalopathy in patients with PA. Surtees et al (Surtees et al., 1992) divided patients with PA into 2 subgroups: those with early onset disease presenting in the first week of life and those with late-onset disease presenting after age 6 weeks. The early onset group was characterized by mental retardation and early death, with the median survival period being 3 years. Those who didn't die in the neonatal period rarely survived beyond the first decade. The late-onset group was characterized by severe movement disorders and dystonias. Patients with late-onset disease usually have permanent neurologic damage.

Elevated propionylcarnitine C3, 3-hydroxypropionate, glycine with increased secondary markers including C3/C2 and C3/C16 ratios in all our PA patients is in conformity with diagnostic accuracy proposed by Couce et al. (2011). In the diagnostic work-up of PA, detection of biallelic pathogenic variants in the *PCCA/PCCB* gene alone or in combination with PCC enzymatic activity assay has a confirmatory role with the later as a valuable functional readout. 13 PA patients showed a known pathogenic *PCCA* mutation c.425G > A (p.G142D) while one patient revealed a novel homozygous 20 nucleotide deletion, c.1111_130del20 in the coding region. Approximately 50 % of PA patients carry pathogenic *PCCA* mutations and 18 % as copy number variants although most are described to occur in a single family (Kraus et al., 2012). While many reported homozygous missense pathogenic variants in *PCCA* such as p.Ala138Thr, p.Ile164Thr, p.Arg288Gly are associated with a less severe phenotype however, loss-of-function null variants such as p.Arg313Ter, p.Ser562Ter and frameshift small deletions/insertions are associated with a more severe form of PA (Desviat et al., 2006, 2004). Kaya et al. (2008) reported a 72,967 bp deletion in *PCCA* gene in the family with surprisingly only mild–severe observed PA phenotype in the face of such a large deletion. In line with our results, the reasons for this phenotypic variability are attributable to: a) the heterogeneity observed in the mutations of *PCCA* compared with limited number of mutations in the *PCCB*, b) the variable stability of the mutant *PCCA* protein and c) the time of onset of specialized clinical management (Clavero et al., 2004; Yorifuji et al., 2002).

Since the novel homozygous 20 nucleotide deletion, c.1111_130del20 in one of our PA patients is a frameshift mutation, we presumed it to be causative and thus have a functional consequence. This was evidenced by ACMG classification criteria-based pathogenicity of the mutation and functional enzymatic assay that clearly demonstrated reduced PCC activity well below the range of controls. In concordance with the enzymatic activity in cultured fibroblasts, this novel deletion predicts a sequence frameshift that is expected to result in reduction of the nonfunctional carboxylase protein due to either nonsense mediated mRNA decay and/or rapidly degraded truncated protein. Our results would suggest deficient propionate carboxylation in cultured fibroblast as the basic defect in PA albeit with differences in the molecular signature that could give them variable enzymatic activities. This is in concordance with the results reported by Hsia et al. (1971) who demonstrated deficient propionate carboxylation in cultured fibroblats as the basic defect in ketotic hyperglycinemia, which was later reported to be the same as PA. In a male Pakistani offspring of first-cousin parents, Gompertz et al. (1975) and Hommes et al. (1968) described acidosis and ketosis in PA, leading to death at 8 days of age due to a defect involving mitochondrial PCC. Although there was no relationship between the high level of C3 and the activity of PCC determined in cultured fibroblasts however, the activities were observed to be related to the presence of different molecular mutations in our patient.

Given the correlation between various pathogenic variants and the severity of PA phenotype, it would not be an exaggeration to state early diagnoses (before birth or soon after birth) becomes increasingly essential in order to have the best prognosis through the use of a quick and robust qPCR-based method to detect PCCA mutations. We, therefore, established a qPCR-based protocol with c.425G > A as a model and proof-of principle concept. The high incidence of heterozygote/carriers: ~550 per 100.000 is not surprising given high rates of consanguineous marriages in Saudi population especially first cousin marriages (El-Mouzan et al., 2007). Alfadhel et al. (2017) suggest that because of this high consanguinity rate, the prevalence of autosomal recessive disorders in general and inborn errors of metabolism (IEM), PA included, in specific is exceedingly high causing great social impact and heavy economic burden. In Saudi Arabia, PA is included in the National Newborn Screening Program and has the highest incidence rate of 1:14,000 among top 16 inborn errors of metabolism. Wiley et al. (1999) have reported the overall incidence of IEM in Saudi Arabia at 1:1043, which is higher than USA, Europe and Australia (1:1792 and 1:4500 correspondingly) (Marquardt et al., 2012; Wiley et al., 1999). These data are comparable with the data reported by other countries of Gulf Cooperation Council like Qatar and United Arab Emirates which were reported as 1:1,327 and 1:1,047 correspondingly (Al Hosani et al., 2014; Lindner et al., 2007).

In summary our data could pave the way for community screening and developing preventive strategies including prenatal and preimplantation genetic diagnosis using simple and robust qPCR-based methodologies. Our results will have great implications in this direction in Saudi Arabia and globally.

What is known

- Propionic acidemia mutation (c.425G>A) is one of the most common mutations in Saudi population (PA affects about 1/2,000 to 1/5,000).
- The diagnostic of propionic acidemia mutations require a labor and time-consuming gene sequencing and invasive skin biopsy for PCC enzymatic activity testing.

What is new

- We have development a novel PCR assay that efficiently detect *PCCA* mutation (c.425G>A) and clearly distinguishes heterozygotes from homozygotes and wild type. The assay shows that the incidence of *PCCA* heterozygotes/carriers in Saudi population is about 550/100,000.
- Skin fibroblast assays shows that homozygote c.425G>A mutation induced propionyl-CoA carboxylase activity abrogation and PA patients shows an increased level of propionyl carnitine C3 in blood and 3-hydroxy propionic acid and methyl citrate in urine.

Declarations

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Authors' contributions: Chentoufi AA, is principal Co-investigator of the *PCCA* project, designed the theoretical and technical aspect of the project, supervised data collection, writing and editing the manuscript.

Al-Asmari AM, is the principal investigator of the project, involved in the clinical management of the patient, performed all the clinical investigations and involved in writing of the clinical part of the manuscript.

Peer-Zada AA, performed molecular data analyses, involved in conceptual design, writing and editing of the manuscript.

AlDehaimi A, is principal co-investigator of the project and performed Tandem MS and provide the related data.

Polychronakos C, designed the primers and validated the PCR assay.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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