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ORIGINAL ARTICLE

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Genetic analysis of adult Slovenian patients with combined pituitary hormone deficiency

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Abstract

Purpose Among genetic causes of combined pituitary hormone deficiency (CPHD), mutations of genes coding for transcription factors involved in pituitary development have been implicated. Congenital CPHD is a rare disease; therefore, it is important to expand the knowledge about incidence and regional distribution of specific mutations. The aim of this paper is to report results of genetic analyses of adult Slovenian patients with CPHD.

Methods Twenty-three adult Slovenian patients with early childhood onset CPHD were included in the study. Blood samples were collected through the GENHYPOPIT network to assess possible mutations of six genes (*PROP1/HESX1/LHX4/LHX3/POU1F1*) involved in the pituitary development following an established algorithm.

Results In seven out of 23 patients (30%) a specific mutation in genes encoding pituitary transcription factors was discovered. In five patients, two different mutations of the *PROP1* gene (c.150delA and c.301-302delAG) were identified. One patient was heterozygous for a missense variant in the *LHX4* gene. Additionally, one patient was positive for a mutation in the gene coding for prokineticin receptor-2.

Conclusions Our study confirms that the two most common mutations of the *PROP1* gene globally are also the most frequent mutations in the cohort of adult Slovenian patients with CHPD. Other mutations of pituitary transcription factor genes are extremely rare.

Keywords Pituitary · Hypopituitarism · CPHD · *PROP1* · *LHX4*

Introduction

Congenital hypopituitarism encompasses a group of different disorders and may manifest as an isolated hormone deficiency, or alternatively several pituitary axes may be defective resulting in combined pituitary hormone deficiency (CPHD) [1].

Among the genetic causes of CPHD, mutations of genes coding for specific or non-specific transcription factors involved in the pituitary ontogenetic development have been implicated, which can result in either non-syndromic CPHD (e.g. mutations of *PROP1*, *POU1F1*) or syndromic CPHD in association with ocular defects, midline brain abnormalities or other features (e.g. mutations of *HESX1*, *LHX4*) [2, 3]. Almost

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all mutations of the genes reported to date as being involved in CPHD have been discovered via murine models with consequent extrapolation on human phenotypes and followed empirically, and in many cases the phenotypes are variable and overlapping [2–5]. Nevertheless, the timing and combination of pituitary hormone deficiencies, neuroimaging and associated features may guide the diagnosis. Based on available data on phenotype–genotype correlations in the genes coding for transcription factors involved in the pituitary development, an algorithm was developed by the GENHYPOPIT network to guide the plan of genetic analysis in CPHD patients [2]. However, the aetiology of most cases of CPHD remains unexplained since the overall incidence of mutations in known transcription factors is low, indicating that many genes or non-genetic causes remain to be identified [1–3].

Since congenital CPHD is a very rare disease, it is extremely important to expand the knowledge about incidence and regional distribution of mutations by reporting results of genetic analyses. This study was designed to investigate a possible genetic aetiology of CPHD in adult Slovenian patients.

Methods

Twenty-three patients from 22 families assessed and treated for CPHD at the Department of Endocrinology, Diabetes and Metabolic Diseases of the University Medical Centre Ljubljana (until December 2017), which is a referral centre for ~2 million inhabitants of the whole country of Slovenia, were included in this study. We included all patients with CPHD who had been transferred to us from pediatric endocrinologists, one patient (No. 3) who had been treated by endocrinologists in Croatia in childhood and one patient (No. 6) who came to our outpatient clinic when he moved from Switzerland back to Slovenia on retirement. After transition, the patients' documentation was carefully reviewed and in any dubious cases, hormone testing repeated. In their histories, they all had a very early appearance of growth retardation and/or symptoms of other pituitary hormone deficiencies. There were two siblings in the cohort (patients No. 1 and 4). Except for a mother of patient No. 2 who complained of tiredness and fatigue but had normal height, none of the parents of other patients were affected with short stature or symptoms of other hormone deficiencies and were therefore not endocrinologically tested. Adrenocorticotropin (ACTH) deficiency was diagnosed by an inadequate response to a standard short Synacthen test. Thyrotropin (TSH) deficiency was diagnosed by low free thyroxine and either low or inappropriately normal TSH levels. Gonadotrophin deficiency was diagnosed on the basis of biochemical (basal gonadotrophin, testosterone/oestradiol levels and gonadotrophin-releasing hormone stimulation test) and clinical parameters —none of the patients entered a spontaneous puberty. The standard workup for suspected growth hormone deficiency at the Department of Paediatric Endocrinology in the eighties and nineties included three stimulation tests (insulin tolerance test, L-dopa and glucagon test) before patients were put on growth hormone (GH) replacement therapy. GH deficiency in adulthood was assessed by inappropriately low insulin-like growth factor 1 levels for age and sex as they all had had multiple pituitary hormone deficiencies. In dubious cases, GH and ACTH deficiency were confirmed by an insulin tolerance test. Prolactin levels were assessed by measuring basal levels. Magnetic resonance images (MRI) of the patients were investigated to define the appearance of the pituitary, visibility of the pituitary stalk, possible ectopia of the posterior pituitary and other associated features such as optic nerve hypoplasia or midline forebrain abnormalities. None of the patients had an obvious cause of acquired hypopituitarism, such as pituitary adenoma, cranial irradiation or trauma. The diagnosis of septo-optic dysplasia (SOD) was based on any two out of the three features: (1) midline forebrain defects (e.g. absent septum pellucidum, agenesis of the corpus callosum); (2) optic nerve hypoplasia or (3) anterior pituitary hypoplasia and/or pituitary hormone deficiencies [6]. Other outstanding clinical features were recorded if present.

The study was approved by the Republic of Slovenia National Medical Ethics Committee (No. 0120-184/2017/8). Blood samples were collected through the GENHYPOPIT network for the study of genetic determinants of hypopituitarism. A written informed consent was obtained from the patients before the genetic analysis. The analyses were performed in the Laboratory of Molecular Biology of the Conception Hospital in Marseille, France. DNA was extracted from blood lymphocytes. Genomic DNA was PCR-amplified from all index cases using sets of flanking intronic primers for direct sequencing of all coding exons of *PROP1, HESX1, LHX4, LHX3* and *POU1F1* (primer sequences available as supplementary material). Amplification was carried out using the Hot Start Taq polymerase kit protocol (Qiagen GmbH, Hilden, Germany). Sequencing was performed with a 3130 XL Genetic analyser (LifeTechnologies, New-York, USA). Sequences were analysed with the Variant Reporter software (LifeTechnologies, New-York, USA). The sequencing of genes (*PROP1/HESX1/LHX4/LHX3/POU1F1*) was guided by a previously described algorithm [2]. An exception is the patient No. 7, whose DNA had been analysed in another laboratory as part of a collaborative study with results published previously (referred to as pt. no. II in that study) [7].

Results

Seven patients out of 23 were found to have mutations in the genes involved in the pituitary development (30%). The

results of genetic analyses and clinical characteristics of the affected patients are summarized in Table 1. Table 1 reports also the final height of the patients; however, all patients except patient No. 6 had been treated with growth hormone (GH) replacement at some time in their childhood and adolescence due to short stature. Since the patients had been transferred from paediatric endocrinologists at their transition period or later, we do not have exact data on their height before GH replacement had been introduced in their childhood as this had happened decades ago (none of the patients included in the study was born after the year 1988). In adulthood, GH replacement was re-introduced at different ages of individual patients but soon after they were referred to the Department of Endocrinology (for adult patients).

In five patients (22%), mutations in the *PROP1* gene were identified. Patients No. 1 and No. 4, who were siblings, were homozygotes for the mutation c.150delA (NM_006261.4: c.150delA; NP_006252.3: p.(Arg53Aspfs*112), minor allele frequency (MAF) 0.013%); patient No. 3 was homozygous for the same mutation. Patient No. 2 was homozygous for another *PROP1* mutation p.(Leu102fs) (NM_006261.4: c.301_302delAG; NP_006252.3: p.(Leu102Cysfs*8), MAF 0.014%); furthermore, this patient's mother was tested and was found to be heterozygous for the same mutation but had normal pituitary function. Patient No. 6, who was the oldest in the study, was compound heterozygous for two above defined *PROP1* mutations (c.150delA and c.301_302delAG).

Patient No. 5 was found to be heterozygous for a missense variant in the *LHX4* gene p.(Gln346Arg) (NM_033343.3: c.1037 A > G; NP_203129.1: p.(Gln346Arg)) [8]. Polymorphism Phenotyping v2 tool (Polyphen-2 (http://genetics.bwh.harvard.edu/pph2/)) predicted p.(Gln346Arg) variant to be possibly damaging (HumVar score 0.459) and the Mutation Taster tool (http://www.mutationtaster.org/) predicted the variant to be disease causing (probability 0.97).

Patient No. 7 was found positive for the heterozygous rare variant p.(Arg85Leu) (MAF 0,06%) in the *PROKR2* gene coding for prokineticin receptor-2 (NM_144773.3: c.254G>T; NP_658986.1:p.(Arg85Leu)), already reported to be likely pathogenic [7].

No mutations in the *LHX3* and *HESX1* genes were found.

Two patients in the study fulfilled criteria for SOD besides CHPD. However, no mutations in the encoding transcription factors or other genes were identified in these two patients.

Discussion

In this study, we report the results of the genetic testing of a cohort of adult Slovenian patients with early childhood onset CHPD. A mutation in genes encoding pituitary transcription factors was identified in seven (30%) patients, which is rather high. The mutation rate was reported to vary considerably among the different geographical areas: while Western-European, US, Australian and Japanese cohorts presented a low mutation prevalence, the Eastern-European and Russian cohorts showed a much higher frequency reaching 64.8% in the Lithuanian population [9–16]. Some other rare genetic disorders, autoimmune polyglandular syndrome 1 and thyroid dyshormonogenesis were also reported in Slovenian population with a higher than usual incidence [17, 18].

Five patients (22%) were positive for two different *PROP1* mutations (c.150delA and c.301_302delAG). The *PROP1* mutation c.150delA, found in patients 1 and 4 (siblings) and in patients 3 and 6, is the variant that was originally described in patients with dwarfism from two adjacent villages on the island of Krk in northern Croatia, close to Slovenia (where since 1864, 25 related dwarfs have been described) [19]. Interestingly, four out of our five patients with the *PROP1* mutation come from the south-eastern part of Slovenia (Dolenjska, Bela Krajina), which is the closest to the island of Krk. The same type of mutation was also reported in two sisters from Serbia with CHPD [20]. This mutation and also the other identified mutation of the *PROP1* gene, c.301_302delAG, are the two variants that were reported to be the most common genetic causes of congenital CHPD in a majority of European patients and are most probably founder variants, as the ancestral origin of the variant c.301_302delAG was demonstrated to arise ~101 generations ago and the variant c.150delA was estimated to appear 44 generations ago [21]. The recently published study of Slovenian paediatric patients with CPHD reported two different *PROP1* mutations (c.301_302delAG and c.362G > C (p.(Arg121Thr)) and one *HESX1* mutation in four patients (22 analysed patients from 20 families). However, no c.150delA mutation of the *PROP1* was found in this paediatric cohort [22].

Interestingly, our subject No. 6 was a compound heterozygote for two most frequent mutations in *PROP1* gene (c.150delA and c.301_302delAG), which were likely responsible for the phenotype. Although he is now in his seventies and is tested yearly, he did not develop ACTH deficiency up to the present. Corticotroph deficiency is reported to be present in 50% of cases of CPHD with *PROP1* mutation and usually develops in adulthood [2].

Patient No. 5 was heterozygous for a variant in the *LHX4* gene, p.(Gln346Arg), that is reported in another patient with a similar phenotype [8] and in only 1/246246 participants in the genome aggregation database (gnomAD). In silico tools predict the variant to be possibly damaging. Nevertheless, as reported previously, in vitro functional study failed to confirm the pathogenicity of this variant, which is located C-terminal to the recognized functional domains of the LHX4 transcription factor [8]. As the American College of Medical Genetics and

Table 1 Clinical data and hormonal characteristics of seven mutation carriers out of 23 Slovenian patients with CHPD

Patient no.	Sex	Year of birth	Height (cm)	Pituitary deficiency (year of diagnosis if known)	MRI finding	Genetic testing results[a]	Other outstanding clinical features
1	F	1983	160	GH (1984) + TSH (1984) + ACTH (2007) + LH/FSH (1996)	Hypoplasia of anterior pituitary, posterior pituitary in place, pituitary stalk visible	*PROP1* Exon 2; c.150delA; p. (Arg53Aspfs*112) homozygote	/
2	F	1982	170	GH (1987) + TSH (1986) + ACTH (2012) + LH/FSH + PRL	Not known	*PROP1* Exon 2; c.301_302delAG p. (Leu102Cysfs*8) homozygote	/
3	F	1965	156,5	GH (1967) + TSH (1971) + ACTH (2011) + LH/FSH (1982) + PRL	Hypoplasia of anterior pituitary, ectopic posterior pituitary	*PROP1* Exon 2; c.150delA p. (Arg53Aspfs*112); homozygote	Hypercholesterolemia
4	M	1987	182,5	GH (1988) + TSH (1988) + ACTH (2010) + LH/FSH + PRL	Not known	*PROP1* Exon 2: c.150delA; p. (Arg53Aspfs*112) homozygote	Depression
5	M	1983	176	GH (1993) + TSH (1993) + ACTH (2001) + LH/FSH (1999)	Hypoplasia of anterior pituitary, ectopic posterior pituitary, pituitary stalk not visible	*LHX4* Exon 6; c.1037A > G p. (Gln346Arg) heterozygote	Coeliac disease Hypertriglyceridemia
6	M	1944	165	GH + TSH + LH/FSH + PRL	Hypoplasia of anterior pituitary, posterior pituitary in place, pituitary stalk visible	*PROP1* Exon 2; c.150delA; p. (Arg53Aspfs*112) heterozygote + Exon 2; c.301_302delAG p. (Leu102Cysfs*8) heterozygote	/
7	M	1965	165	GH (1971) + TSH (1971) + ACTH (1991) + LH/FSH (1991)	Aplasia of anterior pituitary, ectopic posterior pituitary, pituitary stalk not visible	*PROKR2* c.254G > T p. (Arg85Leu) heterozygote	/

[a]The reference mRNA and protein sequences used to annotate variants were the following: PROP1: NM_006261.4, NP_006252.3; LHX4: NM_033343.3, NP_203129.1; PROKR2: NM_144773.3, NP_658986.1

Genomics (ACMG) criteria [23] for benign and pathogenic effect of p.(Gln346Arg) are contradictory, this variant is considered to be of unknown significance for the phenotype of CHPD. Considering the fact that the same variant was seen in two unrelated patients with the CPHD phenotype and was on the other hand exceptionally rare in controls, the question remains whether its functional consequences on the pituitary development are driven through other mechanisms not tested by the functional studies. Of note, a homozygous mutation of *LHX4* was also reported recently, which was lethal after 3 weeks of life despite optimal therapy for hypopituitarism; the affected two children also had mid-facial hypoplasia and lung anomalies [24]. Surprisingly, functional studies in this case did not reveal any difference from the wild-type *LHX4* in the activation of target promoters.

We have not identified any *HESX1* mutation in the two patients with SOD. This is in agreement with data reported by Avbelj Stefanija et al. for Slovenia and also by studies from other countries [9, 22, 25]. In 20 patients with SOD investigated by Dattani et al., only one *HESX1* mutation in two siblings was found [26]. In their more recent evaluation, the *PROKR2* variations were present in ~2% of their cohort with SOD; thus *PROKR2* variations occur more frequently than any other genetic abnormalities identified in association with SOD [7, 27]. Therefore, our findings are in agreement with their conclusion that *HESX1* mutations are an uncommon cause of SOD and hypopituitarism [27]. Moreover, Rainbow et al. reported a significantly younger maternal age at birth in SOD patients compared with the CHPD patients suggesting that in the pathogenesis of SOD, environmental factors rather than genetic factors are important [9]. In most cases, SOD occurs sporadically but has also been reported to occur with exposure to several teratogens including alcohol [28].

The genetic testing in our study was based on a previously developed and described algorithm using phenotypes previously confirmed (in the literature) to be associated with mutations [2]. However, recent data suggest that some genes initially thought to be related to a specific phenotype can be associated with a wider range of phenotypes [2, 3]. Availability of next-generation sequencing (NGS) analysis allows to analyse concomitantly all the genes proved to be implicated in CPHD phenotype, either in a dedicated panel or in an exome analysis, which is likely to correct some of the limitations of such algorithms.

We also report a variant in the *PROKR2* gene (p.(Arg85Leu)) in heterozygous state in the patient No. 7 that had been discovered as part of another study before we joined the GENHYPOPIT network [7]. In this study, a cohort of patients with complex forms of congenital hypopituitarism ($n = 422$) were screened for mutations in *PROK2* and *PROKR2* and five *PROKR2* variants in 11 patients with SOD and/or congenital hypopituitarism were detected: novel p.(Gln371Arg) and previously reported p.(Ala51Thr), p.(Arg85Leu), p.(Leu173Arg), and p.(Arg268Cys); known as functionally deleterious variants [7]. The p.(Arg85Leu) variant in heterozygous state was previously reported also in a patient with Kallmann's syndrome in combination with additional heterozygous *FGFR1* gene variant [29]. The p.(Arg85Leu) variant harboured by our patient presumably affects G protein-coupling of the *PROKR2* [30, 31]; however, based on the classification of ACMG, this variant is considered likely pathogenic in homozygous state and likely benign when heterozygous [31]. No potentially pathogenic variants were identified in the second allele of *PROKR2* gene or in *PROK2* gene in this patient [8]. The patient's parents were deceased but were reported to be of normal stature by the patient. The patient's brother was of a normal stature and did not have any clinical problems and refused testing.

The strength of this study is that the cohort of included patients was retrieved from a single-centre of well-characterised adult patients with CPHD, which is a referral centre for approx. 2 million inhabitants of the whole country of Slovenia and that genetic testing followed a well-defined algorithm. However, the data to determine the type and timing of occurrence of hormonal deficiencies were collected retrospectively from the patients' files and in some cases reflect the time of diagnosis and not precisely the exact time of occurrence of a hormone defect.

Conclusion

Determination of the genetic basis of CPHD and SOD has prognostic implications for the development of hormone deficiencies, timing of initiation of replacement therapy and genetic counselling of an individual patient. We discovered genetic causes of early childhood onset CPHD in 30% of patients. Our study confirms that the two most common mutations of the *PROP1* gene globally are also the most frequent mutations in the cohort of adult Slovenian patients with CPHD.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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