Screening of PRKAR1A and PDE4D in a Large Italian Series of Patients Clinically Diagnosed with Albright Hereditary Osteodystrophy and/or Pseudohypoparathyroidism

Screening of *PRKAR1A* and *PDE4D* in a large Italian series of patients clinically diagnosed with Albright hereditary osteodystrophy and/or Pseudohypoparathyroidism

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Supporting grant: This work was supported by a grant from the Italian Ministry of Health to G.M. (GR-2009-1608394) and by Ricerca Corrente Funds to Fondazione IRCCS Ca' Granda Policlinico. Moreover, the authors are members and acknowledge the Euro-Pseudohypoparathyroidism network (EuroPHP) and the EUCID.net (COST action BM1208 on imprinting disorders; www.imprinting-disorders.eu).

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Supplemental data: 4 Supplemental Tables.

Disclosure statement: The authors have nothing to disclose

Abstract

The cyclic adenosine monophosphate (cAMP) intracellular signaling pathway mediates the physiological effects of several hormones and neurotransmitters, acting by the activation of G-protein coupled receptors (GPCRs) and several downstream intracellular effectors, including the heterotrimeric stimulatory G-protein (Gs), the cAMP-dependent protein kinase A (PKA) and cAMP-specific phosphodiesterases (PDEs). Defective G protein-mediated signaling has been associated to an increasing number of disorders, including Albright hereditary osteodistrophy (AHO) and pseudohypoparathyroidism (PHP), a heterogeneous group of rare genetic metabolic disorders due to molecular defects at the GNAS locus. Moreover, mutations in *PRKAR1A* and *PDE4D* genes have been recently detected in patients with acrodysostosis (ACRDYS), showing a skeletal and endocrinological phenotype partially overlapping with AHO/PHP.

Despite the high detection rate of molecular defects by currently available molecular approaches, about 30% of AHO/PHP patients still lack a molecular diagnosis, hence the need to screen patients negative for GNAS epi/genetic defects also for chromosomal regions and genes associated to diseases that undergo differential diagnosis with PHP.

According to the growing knowledge on Gsα-cAMP signaling-linked disorders, we investigated our series of patients (n=81) with a clinical diagnosis of PHP/AHO but negative for GNAS anomalies for the presence of novel genetic variants at *PRKAR1A* and *PDE4D* genes. Our work allowed the detection of 8 novel missense variants affecting genes so far associated to ACRDYS in 9 patients. Our data further confirm the molecular and clinical overlap among these disorders and we present the data collected from a large series of patients and a brief review of the literature, in order to compare our findings with already published data, to look for *PRKAR1A/PDE4D* mutation spectrum, recurrent mutations and mutation hot spots, and to identify specific clinical features associated to ACRDYS, that deserve surveillance during follow-up.

Key words (5): GNAS; AHO; PRKAR1A; PDE4D; Acrodysostosis.

Introduction

The cyclic adenosine monophosphate (cAMP) intracellular signaling pathway mediates the physiological effects of several hormones and neurotransmitters, including the parathyroid hormone (PTH), acting by the activation of G-protein coupled receptors (GPCRs). This signaling cascade relies on the transient activation of the heterotrimeric stimulatory G-protein (Gs), adenylyl cyclase (AC) and cAMP-dependent protein kinase A (PKA), resulting in the phosphorylation of effectors and the generation of cellular responses. Defective G protein-mediated signaling has been associated to an increasing number of retinal, endocrine, metabolic, and developmental disorders. [1-5]

Inactivating mutations in the α-stimulatory subunit of the Gs protein (Gsα), encoded by the *GNAS* gene, cause Albright hereditary osteodystrophy (AHO), a syndrome with characteristic skeletal and developmental abnormalities (short stature, brachydactyly, subcutaneous ossifications, centripetal obesity, rounded facies, and mental and/or developmental deficits). Due to the tissue-specific imprinted nature of *GNAS*, patients who inherit Gsα mutations from their mother, in addition to the AHO phenotype, also develop resistance to various hormones (mainly PTH and TSH), a condition referred to as Pseudohypoparathyroidism type 1A (PHP1A, MIM103580). In contrast, paternal inheritance of the same defects is associated with the AHO phenotype only, also called Pseudopseudohypoparathyroidism (PPHP, MIM612463). [6-7] Moreover, sporadic or maternally-inherited GNAS epigenetic defects lead to Pseudohypoparathyroidism type 1B (PHP1B, MIM603233), that may be also occasionally associated with signs of AHO. [8]

Despite the high detection rate of *GNAS* molecular defects, about 30% of patients with a clinical suspect of PHP/AHO still lack a confirming molecular diagnosis, hence the need to screen patients negative for *GNAS* defects also for alterations within chromosomal regions and genes associated to diseases clinically similar to AHO. Notably, it is sometimes difficult to distinguish between AHO and other phenotypically related skeletal disorders only on the basis of clinical and radiological findings. [9] Mutations in genes encoding proteins crucial for cAMP-mediated signaling have been

recently detected in a small subset of patients negative for *GNAS* defects, showing a phenotypic overlap between PHP and Acrodysostosis. [10-20]

The term Acrodysostosis (ACRDYS) describes a group of rare skeletal disorders characterized by severe brachydactyly, nasal and/or midfacial hypoplasia and variable intellectual/developmental/behavioural disabilities; resistance to multiple hormones that bind to GPCRs (including PTH and TSH), progressive growth failure with short stature, advanced bone age and obesity are frequently observed features. [21] Genetic defects affecting *PRKAR1A* (cAMP-dependent protein kinase type I-α regulatory subunit) and *PDE4D* (cAMP-specific phosphodiesterase 4D), both crucial for cAMP signaling pathway, were associated to ACRDYS in 2011 and 2012 by different research groups. [10-12]

According to the growing knowledge on Gsα-cAMP signaling-linked disorders, we investigated our series of patients with a clinical diagnosis of PHP1A/AHO but negative for *GNAS* defects for the presence of novel genetic variants at *PRKAR1/PDE4D* genes. In particular, in the present work we present the data collected from 9 mutated of 81 investigated cases and a brief review of the literature, in order to compare our findings with already published data, to look for *PRKAR1A/PDE4D* mutation spectrum, recurrent mutations and mutation hot spots, and to identify poorly investigated clinical features associated to ACRDYS, that deserve surveillance during follow-up.

Materials and Methods

Patients

The present series involved 28 patients with a clinical diagnosis of PHP1A (14 females and 14 males) and 53 with apparently isolated AHO (36 females and 17 males). The inclusion criteria were the presence of at least two of AHO manifestations: brachydactyly (shortening of fourth and/or fifth metacarpals), ectopic ossifications, short stature (height below the 3th percentile for chronological age), rounded facies (broad face, depressed nasal bridge, hypertelorism), and

intellectual disabilities and/or behavioural problems (milt-to-moderate mental retardation, behavioral disorders and/or developmental disabilities). Among required AHO signs, skeletal abnormalities had to be present in order to be included the study. The diagnosis of PHP1A was based upon the associated detection of at least PTH resistance (i.e. hypocalcemia, hyperphosphatemia and raised serum PTH levels). The Ellsworth-Howard test was performed only in one *PRKAR1A*-mutated patient (pt PHP4), showing a blunted cAMP and phosphaturic urinary response, as previously described by Linglart and co-workers[10]. Twenty four of the patients also showed an elevated TSH, documented by raised serum TSH levels, absence of anti-thyroid antibodies and presence of normal thyroid scan. Clinical details of mutated patients and the whole investigated series are resumed in Table 1 and Supplemental Table 1, respectively.

The presence of genetic/epigenetic defects affecting GNAS locus had been previously excluded in all samples by Sanger sequencing of Gsα coding exons 1-13 and Methylation Specific-Multiplex Ligand-dependent Probe Amplification (MS-MLPA) of STX16 and GNAS loci, both methods previously described. [22, 23]

Informed consent was obtained from all patients (or legal guardians for minors) and relatives included in the present study.

Mutational analysis of PRKAR1A and PDE4D genes

Genomic DNA was extracted from peripheral blood leukocytes by Nucleon BACC2 genomic DNA purification kit (GE Healthcare, Piscataway, NJ, USA) according to the manufacturer's instructions. Obtained DNA was amplified by PCR for *PRKAR1A* and *PDE4D* exons and flanking intronic sequences (*PRKAR1A* ENSG00000108946; *PDE4D* ENSG00000113448), using specific primers resumed in the Supplemental Table 2. Direct sequencing was performed with the AmpliTaq BigDye Terminator kit and the 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA), as previously described. [22] The mutation nomenclature follows the guidelines indicated by Human Genome Variation Society (HGVS - available at http://www.hgvs.org/mutnomen/). Nucleotide

numbering is based on the *PRKAR1A* transcript ENST00000392711/NM_002734, while for the *PDE4D* gene on the LRG sequence (available also at www.lovd.nl/PDE4D), corresponding to the *PDE4D* transcript ENST00000502484/NM_001165899.

In silico analysis of novel genetic variants of PRKAR1A and PDE4D genes

In order to predict the possible *in vivo* effect of novel *PRKAR1A* and *PDE4D* genetic defects detected in our series, we performed an extensive review of published data and an *in silico* analysis using different computer generated algorithms. [10-20] *In silico* analyses aren't a substitute for functional studies in determining the pathogenicity of a genetic variant, but allow to evaluate the impact of mutations on the protein product, based on combined analysis of protein multiple sequence alignment and protein structural and functional attributes. In particular, we used the following algorithms: Polymorphism phenotyping program 2. Polyphen2 (available at http://genetics.bwh.harvard.edu/pph2/), Sort Intolerant From Tolerant human Protein, SIFT human Protein program (available at http://sift.jcvi.org/www/SIFT_enst_submit.html) and Mutation Taster (available at http://www.mutationtaster.org/). [22]

Results

In our cohort of 81 patients with a clinical diagnosis of PHP1A or AHO but negative for *GNAS* genetic and/or epigenetic defects, in 9 patients we identified 8 novel genetic variants affecting the 2 genes so far associated to ACRDYS: these were all missense alterations leading to changes in the amino acidic sequence, as reported in Table 1. In particular, we detected 4 mutations in the *PRKAR1A* coding sequence and 4 in the *PDE4D* gene, one of which demonstrated to co-segregate with the disorder in the only familial case (pts PHP1 & PHP2) (Table 1, Figure 1 & 2). Sequencing analysis of healthy control individuals (n=50) did not reveal any of the variants found in our cases. Moreover, these genetic alterations were also absent in online databases of polymorphisms (dbSNP, available at http://www.ncbi.nlm.nih.gov/SNP/) and mutations previously associated to

159 Carney Complex (*CNC1* – MIM160980), a multiple neoplasia syndrome characterized by cardiac,
160 endocrine, cutaneous, and neural myxomatous tumors, and ACRDYS (*PRKAR1A* Mutation
161 Database, available at http://prkar1a.nichd.nih.gov/hmdb/mutations.html; LOVD *PDE4D*,
162 available at http://www.LOVD.nl/PDE4D).
163
164 *PRKAR1A genetic defects in our series*
165 The *PRKAR1A* gene encodes for the most abundantly expressed regulatory subunit of PKA, the
166 type 1α regulatory subunit (RIα), that consists of a dimerization domain (DD), an inhibitory site
167 (IS) and two cyclic nucleotide-binding domains (NBD-A and NBD-B). [3]
168 Sanger sequencing analysis of coding exons 2-11 in our patients unraveled 4 previously
169 undescribed heterozygous missense variants (c.524A>G, c.625A>G, c.806A>G and c.879C>G)
170 affecting exons 6, 7 and 9 (Table 1, Figure 1). The variant c.524A>G (pt AHO25) determines the
171 amino acid change in position 175 from tyrosine to cysteine. The c.625A>G transition (pt PHP26)
172 determines the substitution of the polar uncharged hydrophilic threonine in position 209 with a
173 nonpolar and hydrophobic alanine residue. This amino acid is localized in the NBD-A, and is part
174 of the most conserved feature of this functional domain, the phosphate-binding cassette-A (PBC-A).
175 The other two novel defects affect the NBD-B and, in particular, the c.806A>G transition (pt PHP4)
176 changes the negatively charged aspartic acid 269 located in the conserved N3A motif, into a
177 glycine, while the c.879C>G transversion (pt PHP3) changes the aromatic hydrophobic
178 phenylalanine 293 with an aliphatic hydrophobic leucine residue. *In silico* analysis predicted a
179 pathological effect for such defects, as they cause the substitution of highly conserved amino acid
180 residues located within cAMP-binding domains, and more likely affect the ability of these domains
181 to sequentially bind cAMP molecules, first to NBD-B and then to NBD-A, and to trigger the
182 activation of PKA catalytic subunits.
183
184 *PRKAR1A mutation spectrum*

The updated *PRKAR1A* mutational spectrum comprises 18 variants (14 known and 4 novel), of which 16 missense and 2 nonsense (Table 2, Figure 1). [10-20] Considering the distribution of the mutations, exon 11 is the most affected site (52.9%), followed by exon 9 (23.5%), exon 7 (17.6%) and exon 8 (5.9%). No mutations were observed in other exons, acceptor-donor splice sites and introns.

We confirm that *PRKAR1A* genetic defects are mainly private mutations. Up to now, the only mutational hot spot is the c.1102C>T nonsense mutation, p.(Arg368X), that, since its first description in 2011 by Linglart and colleagues, has been identified in 15 unrelated patients (Supplemental Table 3). [10, 11, 14, 16, 18] Only another mutation, c.866G>A, has been observed in two unrelated subjects (Supplemental Table 3). [14, 19] In addition, our analysis highlighted the presence of 2 amino acid residues, arginine 335 and tyrosine 373, that may be considered as putative mutational hot spots, being mutated in 5 unrelated patients through 5 different genetic variations (c.1003C>T, c.1004G>C, c.1004G>T, c.1117T>C and c.1118A>G) changing Arg to Cys/Pro/Leu and Tyr to His/Cys (Table 2). [11, 14, 16] These amino acids are located in highly conserved positions of the RIα subunit (Arg335 in the PBC-B and Tyr373, subject to phosphorylation, in an interaction site with PKA), thus playing a key functional role. As the association between *PRKAR1A* defects and ACRDYS represents a recent discovery, the number of recurrent mutations will likely increase as some mutations, affecting key amino acids, showed to recur in unrelated subjects. [11, 14, 16, 19]

Although most of known *PRKAR1A* mutations alter the NBD-B (76.5%), our findings support the previous observation that also mutations affecting the NBD-A (23.5%) may be associated to ACRDYS. [13, 16, pt A1]

We also cross-referenced mutations associated to ACRDYS with those associated to Carney Complex and we did not find any molecular overlap, suggesting that different substitutions lead to different and opposite effects on protein function, with consequent different clinical phenotypes. [24]

PDE4D genetic defects in our series

The *PDE4D* gene codifies a class IV cAMP-specific phosphodiesterase that hydrolyzes cAMP, important to control specificity and temporal/spatial compartmentalization of cAMP-induced PKA signalling. [25, 26]

We sequenced all coding exons, from 2 to 17, of the long isoform, thus containing both Upstream Conserved Regions (UCRs), and we discovered 4 heterozygous missense variants (c.1279A>C, c.1600A>C, c.1666C>T and c.2047G>T), all novel to the literature and localized in the region encoding the catalytic unit (Table 1, Figure 2). Both c.1279A>C and c.1600A>C transversions determine the substitution of a polar uncharged hydrophilic threonine with a proline (p.(Thr427Pro) in pt AHO7 and p.(Thr534Pro) in pt AHO3). Proline is unique in that it is the only amino acid where the side chain is connected to the protein backbone twice, forming a five-membered nitrogen-containing ring, so that it is unable to occupy many of the main chain conformations easily adopted by all other amino acids. The c.1666C>T transition detected in pt AHO5 changes the polar histidine 556 into an aromatic, partially hydrophobic tyrosine. Finally, the c.2047G>T transversion causes the change of glycine 683 with a cysteine residue (pts PHP1 and PHP2). *In silico* analysis predicted a pathological effect for these *PDE4D* variants, as they affect highly conserved amino acid residues located in the catalytic domain. Moreover, in 5 patients (3 unrelated and 1 kindred), we found 4 new, inherited and probably benign, intronic variants (c.464+26_464+27del, c.501+17G>C, c.575+13T>C and c.626-24C>T) (Table 1). When available, we investigated patients' RNA without finding any abnormal splicing products, and parent's genotype, all reported to be clinically unaffected, determining the inheritance pattern of such previously unreported noncoding variants.

PDE4D mutation spectrum

The *PDE4D* mutational spectrum now includes 25 missense variants (21 already published and 4 found in our series) (Table 3, Figure 2). [11, 12, 14, 15, 17, 18, 20] Considering the distribution of these

mutations, exon 5 is the most affected site (36%), followed by exon 15 (16%), exons 8 and 17 (12% each), exon 9 (8%) and exons 4, 6, 13 and 16 (4% each). No mutation has been observed to date in other exons, acceptor-donor splice sites and introns.

According to the division into encoded protein domains, mutations are spread all along the three main functional domains, (44% in the UCR1, 20% in the UCR2 and 36% in the catalytic domain), although the region spanning the amino acid stretch 163-169, and in particular Pro164 and Phe165, seems to be a key functional site and a mutational hot spot, as 10 different nucleotide changes affect these 7 resides of the UCR1 (Table 3). [11, 12, 14, 15, 17, 18, 20] To note that also threonine in position 526, localized in the catalytic domain, needs a careful evaluation, being affected in two ACRDYS patients. [11, 18]

Most of *PDE4D* mutations described here and elsewhere are private mutations confined to one patient or kindred, with the exception of 4 variants affecting the catalytic unit (c.803T>C, c.1586A>C, c.1835G>A and c.1850T>C), which recurred in more than one unrelated case, thus suggesting an underlying common molecular mechanism of formation, rather than a founder effect (Supplemental Table 4). [12, 14, 15, 17, 18]

Clinical presentation of ACRDYS patients in our series

Clinical features of our mutated patients at diagnosis (6 male and 3 females, age ranging from 7 to 47 years) are resumed in Table 1. All patients but one showed variable degrees of mental retardation, some behavior disorders and/or mild developmental delay, and all of them presented severe obesity. More than half of patients had resistance to hormones acting through GPCR-Gsα-cAMP-PKA signaling pathway, in particular PTH and TSH values higher than the standard. Typical clinical and/or x-ray features of brachydactyly and severe short stature were reported in 6 subjects. Facial dysmorphisms such as rounded face were observed in 5 mutated patients. It is to note that after obtaining the molecular results, these patients were re-evaluated and a slight flattening of nasal bridge was recorded. As additional features, 2 patients showed IUGR and neonatal hypoglycemia,

while other clinical characteristics associated only to single patients are resumed in Table 1. Of note, patient PHP26 also manifested subcutaneous ossifications at her right leg soon after diagnosis at 8 years of age. At clinical examination, before molecular analysis, no apparent differences in the skeletal phenotype were noted that could help differentiate patients carrying *PRKAR1A* and *PDE4D* mutations.

Considering our series according to the affected gene, PTH resistance was diagnosed in 3 of 4 *PRKAR1A*-mutated patients (pts PHP3, PHP4 and PHP26) and 3 of them (pts PHP4, PHP26 and AHO25) displayed also TSH resistance. Patients PHP3, PHP4 and AHO26 also showed short stature and mild mental retardation/developmental delay, while patients PHP4, PHP26 and AHO25 had brachydactyly and obesity.

As for *PDE4D*-mutated patients, only siblings PHP1 and PHP2 showed elevated PTH and TSH levels due to hormone resistance, while patient AHO7 had resistance to TSH. All subjects presented with variable degrees of mental retardation and developmental delay, and were obese. Only 3 of 5 demonstrated short stature. To note that they were affected by severe brachydactyly, with the exception of patients PHP1 and PHP2, while only patients AHO3 and AHO5 showed a typical facial dysostosis.

Clinical features associated to ACRDYS

We next considered the clinical presentation of all ACRDYS patients, both our cases and those previously reported in the literature, with the aim of pointing out differences between phenotypes associated with *PRKAR1A* and *PDE4D* mutations, and to identify specific clinical features associated to ACRDYS deserving surveillance during follow-up (Supplemental Tables 3 and 4).

Our investigation confirmed that the phenotype resulting from *PRKAR1A* defects is frequently associated with multihormone resistance (rPTH= 76%, rTSH= 73% and rPTH+TSH= 64%), while in case of *PDE4D* mutation resistance to PTH or TSH is present only in a small subset of patients (rPTH= 27%, rTSH= 8% and rPTH+TSH= 5%). An altered response to FSH was reported in about

18% of *PRKAR1A*-mutated patients, and cryptorchidism and/or lack of pubertal spurt, possibly secondary to hormone resistance, were described in about 19% of patients with *PDE4D* mutations. Overall, the most frequent clinical features were brachydactyly (*PRKAR1A*= 97% and *PDE4D*= 92%) and dysmorphic facies (*PRKAR1A*= 75% and *PDE4D*= 90%). Obesity and advanced bone age were reported in less than half of the cases, with no apparent differences between the two ACRDYS subtypes. Phenotypic characteristics which appeared to have different frequencies according to the mutated gene were short stature (*PRKAR1A*= 94% and *PDE4D*= 57%), cone-shaped epiphyses (*PRKAR1A*=72% and *PDE4D*=16%) and mental/behavioural defects (*PRKAR1A*=48% and *PDE4D*=95%). These values could be underestimated, as different research groups focused their attention on different clinical aspects, suggesting the need to harmonize clinical protocols and to deeply evaluate the endocrine status in all patients, independently of the mutated gene.

Finally, in a subset of patients we recorded additional recurring comorbidities that deserve further investigation in larger cohorts in order to define their possible relationship with ACRDYS. In particular, 6% of patients with *PRKAR1A* defects showed hearing loss and 15% IUGR, while about 8% of patients with *PDE4D* defects were affected by hearing loss, recurrent otitis media, intracranial hypertension, shypo-deformity of knees and shoulders and atopy/rhinitis/eczema.

For both genes, we did not observe significant gender difference (% mutated females vs males: *PRKAR1A*=55% vs 45%, *PDE4D*= 41% vs 59%), not even in the presentation of specific signs, including PTH and/or multihormone resistance, facial dysmorphic features, brachydactyly, short stature, obesity and the age at diagnosis. Notably, male patients bearing *PRKAR1A* mutations (11 of 15 reported cases) showed an increased frequency of intellectual disabilities and/or behavioural problems respect to mutated females (5 of 18 cases). However, due to the recent discovery of ACRDYS genetic defects, these data should not be considered as conclusive, deriving from the analysis of a small series (*PRKAR1A* n=33 and *PDE4D* n=37).

Discussion

Gsα-cAMP signaling-linked disorders demonstrated a substantial overlap from the clinical point of view, and it is still difficult to make a conclusive diagnosis without a molecular confirmation of the underlying genetic defect. This paper presents our data obtained through the screening of genetic variants at *PRKAR1A/PDE4D* genes in a series of patients with an initial clinical diagnosis of PHP1A/AHO but negative for *GNAS* defects.

Although we did not perform functional studies to confirm the pathological effect of *PRKAR1A* and *PDE4D* genetic variants found in our cases, conservation and *in silico* analysis prompt their causative role in the development of the clinical phenotype. Our findings are further supported by previous studies demonstrating that *PRKAR1A* mutations discovered in patients cause a defect in PKA activation by cAMP, associated with a decreased responsiveness of PKA to cAMP, and their dominant negative effect on PKA function. [10, 13] Recently, Kaname and colleagues performed functional studies to analyze *PDE4D* mutants and generated Pde4d knockout rats, demonstrating that *PDE4D* loss results in the skeletal dysplasia phenotype observed in Acrodysostosis. [18] Meanwhile, the functional consequences of the *PDE4D* coding changes was also confirmed in zebrafish, suggesting a dominant negative effect. [17] Moreover, 10 patients belonging to 4 families with 4 different *PDE4D* mutations (including the kindred described in the present paper) have been reported, confirming the co-segregation of these molecular defects with the disorder (Supplemental Table 4). [15, 18, pts P1/P2] Interestingly, structural variants of chromosome 5q12.1 determining haploinsufficiency of *PDE4D* resulted in a novel intellectual disability syndrome, but several opposing features compared with Acrodysostosis (characteristic faces with prominent nasal bridge and maxillary hyperplasia, low BMI, long extremities and fingers). [17]

The review of published mutations associated to ACRDYS (summarized in Tables 2 and 3) demonstrated that *PRKAR1A*/*PDE4D* genetic variants may affect different functional domains and are mainly private mutations. Only few variants recurred in more than one unrelated case but, since

ACRDYS-associated genes have been recently discovered, the number of recurrent mutations is likely to increase.

Previous reports documented the phenotypic similarities and differences associated with *PRKAR1A* and *PDE4D* mutations causing ACRDYS, and identified two subtypes of this entity: type 1 (ACRDYS1 - MIM101800), with hormonal resistance and resulting from *PRKAR1A* defects, and type 2 (ACRDYS2 - MIM614613), resulting from *PDE4D* defects. [21] Typical skeletal and facial dysmorphisms characterizing these subtypes are quite similar, possibly more severe when the *PDE4D* gene is affected, and comprise: broad face, widely spaced eyes, maxillonasal hypoplasia, small hands/feet affected by brachydactyly type E (BDE), severe short stature, cone-shaped epiphyses with early epiphyseal fusion and advanced bone age. Bone growth is regulated by the PTHrp/PTH receptor type 1 (PTH1R) activation that stimulates slow chondrocyte differentiation into hypertrophic cells, thus it was proposed that skeletal abnormalities derive from a general impairment of the cAMP/PKA pathway. [14] Contrarily, other clinical features seem to be more frequently associated with a specific subgroup, like intrauterine growth restriction and hormonal resistance in *PRKAR1A* mutated patients and mental retardation in patients with *PDE4D* defects. Although both genes are involved in the GPCR-Gsα-cAMP-PKA pathway, *PRKAR1A* ubiquitous expression compared to *PDE4D* isoforms tissue-specific distribution may account for these observed phenotypic differences.

Clinical data collected in our cohort of ACRDYS patients confirmed an elevated phenotypic heterogeneity, both in *PRKAR1A* and *PDE4D* mutated patients, and no apparent differences in skeletal phenotype that could help distinguish patients before genotyping were noted (Table 1). Afterwards, we analysed all ACRDYS patients, both our cases and previously reported in the literature, to find phenotypic differences between *PRKAR1A* and *PDE4D* mutated patients and specific clinical features associated to ACRDYS, deserving a careful surveillance during follow-up (Supplemental Tables 3 and 4).

Considering our subjects affected by *PRKAR1A* mutations, 3 of them were initially diagnosed as having PHP1A because of the presence of PTH resistance and AHO signs, while the other one as possibly affected by AHO. The detection of *PRKAR1A* mutations in these latter patients further supports the hypothesis that also defects in the NBA-A domain can impair PKA RIα activity. [13, 16] As for *PDE4D*-mutated patients, multihormone resistance was observed only in the kindred PHP1&2 (elevated PTH and TSH levels), while 2 patients showed a resistance limited to TSH. So, as expected, resistance to PTH and/or TSH is mainly related to *PRKAR1A* defects, but endocrine disorders signaling cannot be completely excluded in the presence of *PDE4D* defects so that patients should be carefully screened also for an altered response to hormones acting through GPCRs. Moreover, since 16% of all *PDE4D*-mutated patients were affected by cryptorchidism and/or lack of pubertal spurt, it would be worthwhile investigating whether these signs are secondary to hormone deficiency or resistance. Finally, there is no evident explanation as to why *PRAKAR1A/PDE4D* mutated patients exhibit hormone resistances similar to those observed in *GNAS*-mutated subjects, despite no tissue-specific imprinting has been demonstrated for ACRDYS-related genes.

No apparent differences between ACRDYS subtypes were found in terms of frequent clinical features, such as brachydactyly, obesity and advanced bone age. Other phenotypic characteristics (short stature, cone-shaped epiphyses, mental/behavioural defects) seemed to have different frequencies according to the mutated gene, and a subset of patients presented additional comorbidities (hearing loss, IUGR, recurrent otitis media, intracranial hypertension and atopy). Further studies, involving larger series of patients and aimed to investigate specific clinical features, are needed to obtain conclusive data.

In conclusion, the present study reports 8 novel mutations in *PRKAR1A* and *PDE4D* coding exons associated with ACRDYS, discovered in 9 patients who were previously diagnosed as having PHP/AHO, further expanding the spectrum of mutations and underlining the importance of identifying such genetic alterations for both diagnostic and research purposes.

Furthermore, thanks to the review of all published mutations associated to ACRDYS, the present work provides an updated compilation of mutational and phenotypic data. Overall, the molecular and clinical overlap among these Gsα-cAMP signaling-linked disorders indicates the need for different classification models and for a deeper investigation of the mechanisms through which defects of the cAMP signaling cascade cause either common or specific clinical phenotypes, in order to elaborate patient-specific algorithms.

Author's roles: Study design: GM, FME. Study conduct: FME, PB, VB. Data collection: GM, FME, FG, LdS, EV, MS. Data analysis: GM, FME, FG, PB, VE. Data interpretation: GM, AS, FME. Drafting manuscript: GM, FME. Revising manuscript content: GM, AS, LdS, FME, MS. Approving final version of manuscript: GM, AS, FME, LD, PB, VB, EV, MS. GM and FME take responsibility for the integrity of the data analysis.

References

1. Spiegel AM, Shenker A, Weinstein LS. Receptor-effector coupling by G proteins: implications for normal and abnormal signal transduction. Endocr Rev. 1992; 13(3): 536-65.
2. Spiegel AM, Weinstein LS. Inherited diseases involving g proteins and g protein-coupled receptors. Annu Rev Med. 2004; 55: 27-39.
3. Taskén K, Skålhegg BS, Taskén KA et al. Structure, function, and regulation of human cAMP-dependent protein kinases. Adv Second Messenger Phosphoprotein Res. 1997; 31: 191-204.
4. Taylor SS, Buechler JA, Yonemoto W. cAMP-dependent protein kinase: framework for a diverse family of regulatory enzymes. Annu Rev Biochem. 1990; 59: 971-1005.
5. Lania AG, Mantovani G, Spada A. Mechanisms of disease: Mutations of G proteins and G-protein-coupled receptors in endocrine diseases. Nat Clin Pract Endocrinol Metab. 2006; 2(12):681-93.

6. Mantovani G. Clinical review: Pseudohypoparathyroidism: diagnosis and treatment. J Clin Endocrinol Metab. 2011; 96(10): 3020-30.
7. Mantovani G, Elli FM. Albright Hereditary Osteodystrophy and Pseudohypoparathyroidism type I. In: CLINICAL GENOMICS : Practical considerations for adult patient care. Eds. MCGRAW–HILL 2013; 327-330.
8. Elli FM, de Sanctis L, Bollati V et al. Quantitative Analysis of Methylation Defects and Correlation With Clinical Characteristics in Patients With Pseudohypoparathyroidism Type I and GNAS Epigenetic Alterations. J Clin Endocrinol Metab. 2014; 99(3): E508–E517.
9. Ablow RC, Hsia YE, Brandt IK. Acrodysostosis coinciding with pseudohypoparathyroidism and pseudo-pseudohypoparathyroidism. AJR Am J Roentgenol. 1977; 128(1): 95-99.
10. Linglart A, Menguy C, Couvineau A et al. Recurrent PRKAR1A mutation in acrodysostosis with hormone resistance. N Engl J Med. 2011; 364(23): 2218–2226.
11. Michot C, Le Goff C, Goldenberg A et al. Exome sequencing identifies PDE4D mutations as another cause of acrodysostosis. Am J Hum Genet. 2012; 90(4):740-745.
12. Lee H, Graham JM Jr, Rimoin DL et al. Exome sequencing identifies PDE4D mutations in acrodysostosis. Am J Hum Genet. 2012; 90: 746-751.
13. Nagasaki K, Iida T, Sato H et al. PRKAR1A mutation affecting cAMP-mediated G protein-coupled receptor signaling in a patient with acrodysostosis and hormone resistance. Clin Endocrinol Metab. 2012; 97(9): E1808-1813.
14. Linglart A, Fryssira H, Hiort O et al. PRKAR1A and PDE4D mutations cause acrodysostosis but two distinct syndromes with or without GPCR-signaling hormone resistance. J Clin Endocrinol Metab. 2012; 97(12):E2328-2338.
15. Lynch DC, Dyment DA, Huang L et al. Identification of novel mutations confirms PDE4D as a major gene causing acrodysostosis. Hum Mutat. 2013; 34(1): 97-102.
16. Muhn F, Klopocki E, Graul-Neumann L et al. Novel mutations of the PRKAR1A gene in patients with acrodysostosis. Clin Genet. 2013; 84(6): 531-538.

17. Lindstrand A, Grigelioniene G, Nilsson D et al. Different mutations in PDE4D associated with developmental disorders with mirror phenotypes. J Med Genet. 2014; 51(1) :45-54.
18. Kaname T, Ki CS, Niikawa N et al. Heterozygous mutations in cyclic AMP phosphodiesterase-4D (PDE4D) and protein kinase A (PKA) provide new insights into the molecular pathology of acrodysostosis. Cell Signal. 2014; 26(11): 2446-2459.
19. Li N, Nie M, Li M et al. The first mutation identified in a Chinese acrodysostosis patient confirms a p.G289E variation of PRKAR1A causes acrodysostosis. Int J Mol Sci. 2014; 15(8): 13267-13274.
20. Mitsui T, Kim OH, Hall CM et al. Acroscyphodysplasia as a phenotypic variation of pseudohypoparathyroidism and acrodysostosis type 2. Am J Med Genet A. 2014; 164(10): 2529-2534.
21. Silve C, Le-Stunff C, Motte E, Gunes Y, Linglart, A Clauser. Acrodysostosis syndromes. BoneKEy Reports. 2012; 1, 225: 1-7.
22. Elli FM, deSanctis L, Ceoloni B et al. Pseudohypoparathyroidism type Ia and pseudo-pseudohypoparathyroidism: the growing spectrum of GNAS inactivating mutations. Hum Mutat. 2013; 34(3): 411-416.
23. Elli FM, de Sanctis L, Bollati V et al. Quantitative analysis of methylation defects and correlation with clinical characteristics in patients with pseudohypoparathyroidism type I and GNAS epigenetic alterations. J Clin Endocrinol Metab. 2014; 99(3): E508-517.
24. Horvath A, Bertherat J, Groussin L et al. Mutations and polymorphisms in the gene encoding regulatory subunit type 1-alpha of protein kinase A (PRKAR1A): an update. Hum Mutat. 2010; 31(4): 369-379.
25. Bolger GB, Erdogan S, Jones RE et al. Characterization of five different proteins produced by alternatively spliced mRNAs from the human cAMP-specific phosphodiesterase PDE4D gene. Biochem J. 1997; 328(Pt 2): 539-548.

26. Richter W, Jin SL, Conti M. Splice variants of the cyclic nucleotide phosphodiesterase PDE4D are differentially expressed and regulated in rat tissue. Biochem J. 2005; 388(Pt 3): 803-811.

Figure Legends

Figure 1: Novel *PRKAR1A* mutations associated to ACRDYS. The upper part shows the *PRKAR1A* structure (protein domains and gDNA) and the genomic location of previously detected mutations in ACRDYS patients (dots over exons). At the bottom, the electropherograms of missense mutations found in our series are compared with wild-type reference sequences.

Figure 2: Novel *PDE4D* mutations associated to ACRDYS. The upper part shows the *PDE4D* structure (protein domains and gDNA) and the genomic location of previously detected mutations in ACRDYS patients (dots over exons). At the bottom, the electropherograms of missense mutations found in our series are compared with wild-type reference sequences.

Supplemental Data

Supplemental Table 1 Clinical data of patients enrolled in this study.

Supplemental Table 2 *PRKAR1A* and *PDE4D* primer sequences.

Supplemental Table 3 Clinical data of *PRKAR1A* mutated patients reported in the literature.

Supplemental Table 4 Clinical data of *PDE4D* mutated patients reported in the literature.