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Expanding the Genetic and Phenotypic Spectrum of Popliteal Pterygium Disorders

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Abstract

The popliteal pterygia syndromes are a distinct subset of the hundreds of Mendelian orofacial clefting syndromes. Popliteal pterygia syndromes have considerable variability in severity and in the associated phenotypic features but are all characterized by cutaneous webbing across one or more major joints, cleft lip and/or palate, syndactyly, and genital malformations. Heterozygous mutations in *IRF6* cause popliteal pterygium syndrome (PPS) while homozygous mutations in *RIPK4* or *CHUK (IKKA)* cause the more severe Bartsocas-Papas syndrome (BPS) and Cocoon syndrome, respectively. In this study we report mutations in six pedigrees with children affected with PPS or BPS. Using a combination of Sanger and exome sequencing, we report the first case of an autosomal recessive popliteal pterygium syndrome caused by homozygous mutation of *IRF6* and the first case of uniparental disomy of chromosome 21 leading to a recessive disorder. We also demonstrate that mutations in *RIPK4* can cause features with a range of severity along the PPS-BPS spectrum and that mutations in *IKKA* can cause a range of features along the BPS-Cocoon spectrum. Our findings have clinical implications for genetic counseling of families with pterygia syndromes and further implicate *IRF6*, *RIPK4*, and *CHUK* (*IKKA*) in potentially interconnected pathways governing epidermal and craniofacial development.

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Keywords

orofacial cleft; popliteal pterygia; exome; uniparental disomy; recessive

Introduction

Orofacial clefts are a component of a group of heterogeneous disorders that are collectively the most common craniofacial malformation. The majority of orofacial clefts arises without additional clinical features and is classified as nonsyndromic but approximately 30% consist of a diverse assortment of syndromes in which the cleft occurs with additional structural or cognitive abnormalities. Over 500 Mendelian syndromes in which orofacial clefts are a defining feature have been described, a subset of which have a known genetic cause [Dixon et al. 2011]. The clefting syndromes including limb pterygia form one distinct group. These syndromes are variably characterized by cutaneous webbing across one or more major joints, cleft lip and/or palate, syndactyly, and genital malformations.

The least severe of the pterygia syndromes is popliteal pterygium syndrome (PPS; MIM 119500), an autosomal dominant disorder caused by mutations in the transcription factor *interferon regulatory factor 6 (IRF6)* [Kondo et al. 2002]. The clinical presentation of PPS can mimic the allelic disorder Van der Woude syndrome (VWS) in mild cases [Matsuzawa et al. 2010] or can include multiple pterygia, ankyloblepharon, syndactyly, and syngnathia among other malformations in more severely affected patients. It is estimated that 97% of PPS cases are explained by *IRF6* mutations and that this disorder is highly associated with mutations affecting residues of the DNA-binding domain predicted to contact DNA (e.g. Arg84Cys, Arg84His) [de Lima et al. 2009].

The more severe cases of PPS may be indistinguishable from patients diagnosed with Bartsocas-Papas syndrome (BPS; MIM 263650), a recessive disorder caused by mutations in the receptor-interacting serine/threonine kinase *RIPK4* [Kalay et al. 2012; Mitchell et al. 2012]. The substantial clinical overlap between PPS and BPS is due to the presence of popliteal pterygia, syndactyly, ankyloblepharon, and genital malformations. *IRF6* mutations have not been reported in patients with BPS [Shanske et al. 2004; Zaki et al. 2012]. The phenotypic similarities in human patients are paralleled by the phenotypic similarities in mice. *Ripk4*$^{-/-}$and *Irf6*$^{-/-}$mice both have fused oral cavities and epithelial defects [Holland et al. 2002; Ingraham et al. 2006]. Furthermore, *RIPK4* is a direct transcriptional target of ΔNp63α [McDade et al. 2012; Mitchell et al. 2012], the latter also activating IRF6 in a feedback loop mechanism [Moretti et al. 2010; Thomason et al. 2010].

The most extreme end of this spectrum of disorders may be the lethal condition known as Cocoon syndrome (MIM 613630). This disorder, which has been described as an extreme form of Bartsocas-Papas syndrome, is caused by mutationsin *CHUK* (also known as inhibitor of nuclear factor kappa-B kinase subunit alpha or IKKA) [Lahtela et al. 2010]. In this article, we investigated six families with pterygia syndromes and report novel mutations, inheritance mechanisms, and expanded phenotypic spectrum for these families with popliteal pterygium and Bartsocas-Papas syndromes.

Patients and Methods

Subjects

Informed consent was obtained for all participants and protocols were approved by Institutional Review Boards at the University of Iowa, Vanderbilt University, and Seattle Children's Hospital; or the UK National Research Ethics Service. Six families with orofacial clefts and popliteal pterygium were included in this study. Based on the clinical descriptions, three families were diagnosed with popliteal pterygium syndrome and three were diagnosed with Bartsocas-Papas syndrome.

Sequencing

Sanger sequencing of all exons of *IRF6* and *RIPK4* was performed using oligonucleotide primers, the sequences of which have been published previously [Kondo et al. 2002], [Mitchell et al. 2012]. The effects of missense variants were predicted using the Ensembl Variant Effect Predictor software and variants were compared against 1000 Genomes, NHLBI Exome Variant Server, and previously identified mutations in *IRF6* [Leslie et al. 2013b] or *RIPK4* [Gripp et al. 2013; Kalay et al. 2012; Mitchell et al. 2012]. For exome sequencing, targeted enrichment and sequencing were performed on 3 μg of DNA extracted from peripheral blood of the affected child. Enrichment was performed using the SureSelect Human All Exon 50 MB v4 Kit (Agilent, Santa Clara, CA, USA) for the Illumina system. Sequencing was carried out on a HiSeq 2500 sequencer (Illumina Inc, San Diego, CA, USA), following the manufacturer's protocols. Sequence data were mapped using BWA software with the hg19 human genome as a reference. Approximately 7.60 gigabases of sequence mapped to the human genome with 93.8% of the targeted exome covered at twenty-fold or higher. Variants were called using GATK v2.4.7 software then filtered for those SNPs with ≤5× coverage. SNPs were initially annotated using Ensembl v72. Using Ensembl's defined consequence hierarchically system the highest impacting consequence for a variant in a gene was retained. Variants were filtered out if they were non-functional, in dbSNP138 (unless seen in the Human Genetic Mutation Database (HGMD)), and in our in-house variant database. At the time of the comparison, the latter database consisted of 275 exomes. For the recessive mutation model, homozygous variants were filtered, with a further filtering at novel allele depth of twenty-fold.

Array CGH and Genotyping

Array comparative genomic hybridization (aCGH) was performed using a Human CGH 2.1M Whole-Genome Tiling v2.0D array (Roche Nimblegen, Inc., Madison, WI). 1ug of patient or control DNA was labeled with Cy3 or Cy5. 34ug of each labeled DNA was cohybridized to the array according to manufacturer protocols. Paternity was assessed by genotyping 16 Taqman SNP Genotyping Assays on the ABI 7900HT (Life Technologies Corp., Carlsbad, CA). Whole genome genotyping was performed at The Genome Institute at Washington University using the HumanOmni1_Quad array (Illumina, Inc., San Diego, CA).

Results

Six families with one or more affected children were included in this study. All affected individuals had popliteal pterygium syndromes and received a clinical diagnosis of either popliteal pterygium syndrome (PPS) or Bartsocas-Papas syndrome (BPS). The characteristic features of PPS and BPS are listed in Table I along with a summary of the clinical features of the three families diagnosed with PPS (PPS1, PPS2, and PPS3) and the three families diagnosed with BPS (BPS1, BPS2, BPS3). Below we describe the clinical presentations for each of these families.

PPS1—The proband was a nine day old neonate born as a result of a third degree consanguineous marriage. Multiple congenital malformations were noted including adhesions between left eyelids (ankyloblepharon), bilateral cleft lip, cleft of the hard palate, lower lip pit, a band of soft tissue connecting lower lip and palate, pterygia of popliteal fossae (more severe on the left than the right), bilateral club foot, and syndactyly of all four toes on both feet (Figure IA). Clinical examination of the mother and sister showed a short frenulum but the father was normal. No lip pits, hypodontia, or palatal defects were noted in the parents. Other family members were not available for examination.

PPS2—The proband of PPS2 was a female infant born at 40 weeks gestation to a 20-year-old G2P2 mother. Pregnancy was complicated by treated maternal hypothyroidism. The mother reported a distant paternal cousin with cleft lip and palate requiring surgical repair. There was no evidence of parental consanguinity (Figure IB). At birth this child was noted to have multiple dysmorphic features including ankyloblepharon, skin webbing behind the knees, bilateral cleft lip and palate with attenuated lower lip pits (Figure IB), underdeveloped external genitalia, and a sacral dimple. A renal ultrasound showed mild left hydronephrosis and a head CT was normal. There was an intraoral fibrous band connecting maxillary and mandibular alveolar ridges. No other ectodermal changes or folliculitis was noted. An echocardiogram shortly after birth showed a patent foramen ovale, patent ductusarteriosus, and mild left ventricular hypertrophy.

PPS3—In addition to the phenotypic characteristics summarized in Table I, examination of the feet revealed a dramatic malformation of the toes, which was symmetric on all digits and on both feet (Figure IC). The hypoplastic nails erupted from within the toe rather than on the surface. On close examination, the dorsal aspect of the distal foot bilaterally had increased subcutaneous tissue, reminiscent of a hypoplastic sole of the foot on the dorsal surface. He also had ectodermal dysplasia with sparse, dry slow growing hair, dry, peeling and cracked skin on his soles and palms. We note that he was born from a consanguineous relationship between first degree relatives and, as a result, some of the features may be due to homozygosity at other loci.

BPS1—The proband of BPS1, who was born as the result of a consanguineous union, presented with typical features of Bartsocas-Papas syndrome (Table I; Figure IIA).

BPS2—The proband of BPS2, who was born to healthy, consanguineous parents, presented with a milder phenotype that included bilateral cleft lip/palate, lower lip pits,

ankyloblepharon, bands between the jaws, bilateral club feet, popliteal pterygia, absent eyelashes and eyebrows and sparse scalp hair, brittle nails, absent/small toenails, III-IV-V syndactyly of the right foot, and triangular overgrowth of skin over the nail of the first right toe (Figure IIB).

BPS3—This female infant was born by Caesarean section at term to a 26 years old Saudi primigravida. The parents were first degree cousins and were reportedly healthy with no family history of birth defects and there were no exposures to recognized human teratogens (Figure IIC). The child was found to have alopecia totalis with absent eyebrows and eyelashes. The cranial suture and anterior fontanel were wide and the occiput was prominent with low set ears with over folded helices. She had bilateral microophthalmia, ankyloblepharon, a skin tag on the right eyelid, and cloudy corneas. She had bilateral cleft lip and palate and the nose was distorted with absent alaenasi. Micrognathia and intraoral bands limited mouth opening and movement of the small tongue. She had hypoplastic nipples and a short sternum and the abdominal examination showed no organomegaly, a highly positioned umbilical stump and umbilical cord fused to the abdominal wall with skin tag on it. The upper extremities were short with bilateral cubital webs and hands were small with complete syndactyly bilaterally. The lower extremities were very short and had popliteal webs extending from the upper thigh to the feet. There were multiple webs that include axillae, cubital, inguinal, and popliteal areas. Skin bands were noted between the fused forefeet and genital area. The examination of genitalia showed skin tag and hypoplasia of labia majora, labia minora, and clitoris. Overall there were four skin tags: on the scalp, on right eye lid, on the umbilical cord, and on the vagina. A skeletal survey showed the presence of only 3 metacarpal bones, hypoplasia of the proximal phalanges, and bilateral aplasia of distal phalanges; absence of left foot bones except the talus; absence of the calcaneus, tarsal bones, and hypoplasia of the phalanges of the right foot.

An *IRF6* recessive mutation in PPS

We sequenced the protein coding exons of *IRF6* in the probands of three families diagnosed with PPS (PPS1, PPS2, and PPS3), as one of the recommended strategies for genetic testing in PPS [Schutte et al. 1993]. Sequencing of *IRF6* was negative for the Arg84Cys and Arg84Hismutations in each case; however a novel, homozygous missense mutationin exon 9, p.Leu439Pro (NM_006147.3:c.1316T>C), was identified in PPS1 that was carried by both parents (Figure IA). Although not reported previously, several lines of evidence support this variant as causative. First, bioinformatic predictions using Polyphen2 and SIFT indicate this variant is probably damaging. Second, this variant was absent from 6500 exomes of the NHLBI Exome Sequencing Project and from 1091 genomes from the 1000 Genomes Project. Finally, the leucine at position 439, located in the protein-binding domain of IRF6, is highly conserved across species and this position is evolutionarily constrained as measured by GERP scores [Cooper et al. 2005].

Recessive *RIPK4* mutations in PPS and BPS

We hypothesized that mutations in *RIPK4* could explain a portion of the cases of PPS in which an *IRF6* mutation had not been identified. To test this hypothesis, we sequenced the probands of pedigrees PPS2 and PPS3 and three additional individuals who had been

diagnosed with BPS (BPS1, BPS2, and BPS3). We identified novel *RIPK4* mutations in 4 of these 5 individuals.

In the PPS2 proband, we identified an apparently homozygous *RIPK4* mutation (p.Arg618His, NM_020639.2:c.1853G>A) (Figure IB). However, sequencing of the parents showed that only the mother carried the mutation. We genotyped 16 SNPs distributed across the genome, confirming paternity. Because the father was homozygous for 8 of the 11 variants we detected in *RIPK4*, we used array CGH to rule out the possibility that the father carried a deletion of *RIPK4*. No deletion was detected in either the father or proband (data not shown). However, genotyping using a SNP micro array demonstrated copy-neutral loss of heterozygosity on chromosome 21 (Figure ID). Only one maternal allele was inherited by the proband indicating maternal isodisomy of chromosome 21.

The proband of PPS3 carried a homozygous *RIPK4* mutation (p.Ala448Pro, NM_020639.2:c.1342G>C) (Figure 1C). In BPS1, we identified the homozygous transition c.566C>T in exon 3 of *RIPK4* which resulted in the novel missense variant p.Pro189Leu, locatedin the serine/threonine kinase domain of the protein (Figure IIA). Both parents were heterozygous for this variant. Finally, we identified a homozygous missense mutation,p.Arg618His, located in the serine/threonine kinase domain of *RIPK4* in BPS2 (NM_020639.2:c.1853G>A) (Figure IIB).

Exome sequencing reveals *CHUK* (*IKKA*) mutation in BPS3

In the third family with BPS (BPS3), *RIPK4* sequencing was negative and the girl had a normal 46, XX karyotype; therefore, whole exome sequencing was performedto identify the causative mutation(s). Among the 53 unique homozygous sequence variants identified across the exome of the affected child, we identified a homozygous mutation in the exon 10 splice acceptor site of *CHUK* (IKKA) (NM_001278.3:c.934-2A>G) (Figure IIC). Sanger sequencing confirmed that the parents were heterozygous for the mutation.

Discussion

In summary, by using a combination of Sanger sequencing and exome sequencing to identify the genetic causes of a set of patients with popliteal pterygia and orofacial clefts, we have identified the first case of recessive PPS caused by homozygous mutation of *IRF6*, demonstrated that mutations in *RIPK4* can cause a range of severity along the PPS-BPS spectrum, and expanded the phenotypic spectrum of disorders caused by mutation of *CHUK*.

The majority of *IRF6* mutations reported in PPS patients reside in the DNA-binding domain and are thought to act in a dominant-negative manner (Figure IID) [Leslie et al. 2013b]. As no homozygous mutations in *IRF6* have been reported previously, it is possible that complete loss of IRF6 is lethal as it is in the mouse knockout [Ingraham et al. 2006; Richardson et al. 2006]. We hypothesize that the p.Leu439Pro variant is a hypomorphic mutation. Thus, inheriting two copies of the mutant allele results could result in less than the equivalent of one functional *IRF6* allele (as in VWS), resulting in a more severe phenotype in the proband but no discernable phenotype in the parents.

We also showed that uniparental disomy (UPD) led to BPS in one of our families. UPD is estimated to occur in 1 in 3,500 live births [Robinson 2000] and can result in a phenotype when it occurs in an imprinted region (as in Prader Willi or Angelman syndromes) or when the inherited chromosome carries a recessive mutation. Henderson et al. [1994] reported maternal isodisomy of chromosome 21 in a case of early embryonic demise but at least six other publications have reported UPD(21) in phenotypically normal individuals [Liehr 2010], indicating that chromosome 21 is not imprinted. Examples of recessive disorders caused by isodisomy are relatively rare (≈50 patients) [Engel 2006] and there have been no published examples for chromosome 21. Thus, we report the first case of matUPD(21) leading to a recessive disorder (in this case, BPS).

Ikka, *Irf6*, and *Ripk4* mutant mice share a thick, tightened epidermis and cleft palate or fused oral cavity. Both *IRF6* and *RIPK4* are transcriptional targets of p63 [McDade et al. 2012; Mitchell et al. 2012], mutation of which is known to cause several Mendelian syndromes including Hay-Wells (AEC) and ectrodactyly-ectodermal dysplasia-clefting (EEC) syndromes. As with other IRF family members, IRF6 is phosphorylated [Bailey et al. 2005], although thekinase responsible has not been identified. It is possible that either IKKA or RIPK4 acts as a kinase for IRF6. The majority of the mutations in *RIPK4* involve residues of the kinase domain (Figure IID). In 2010, Rountree and colleagues [Rountree et al. 2010] crossed a transgenic mouse expressing RIPK4 under control of the human keratin-14 promoter with the $Ikka^{-/-}$ mouse which did not rescue the epidermal differentiation defects of the $Ikka^{-/-}$ mouse. These experiments indicate that RIPK4 acts upstream of IKKA or that these genes function in separate molecular pathways. Experiments such as these, and development of additional murine models, will help to further explore the relationships between *p63*, *IKKA*, *RIPK4*, and *IRF6*.

Lower lip pits are part of the diagnostic criteria for VWS and PPS, but until now had not been documented in patients with BPS. However, Lees and colleagues [Lees et al. 1999] reported a family linked to the IRF6 locus segregating a dominant PPS phenotype, although none of the affected individuals was reported to have lower lip pits. Others have reported a *RIPK4* mutation in a patient with features more consistent with a diagnosis of Hay-Wells syndrome than BPS [Gripp et al. 2013]. We identified the same mutation in a patient with BPS (BPS2) in a second patient (PPS2) with the milder PPS phenotype. Thus although mutations in *RIPK4* have been associated with the more severe pterygia disorder BPS, it appears that *RIPK4* mutations can result in a range of severity with overlapping phenotypes including orofacial clefts, lip pits, and popliteal pterygia.

Phenotypic heterogeneity, reduced penetrance, and variable expressivity are hallmarks of Mendelian disorders. While the molecular basis of most phenotypic heterogeneity is not well understood, it may be due to locus or allelic heterogeneity, modifier genes, epigenetics, or environmental covariates. Although a recent candidate gene study failed to identify common modifier alleles in VWS [Leslie et al. 2013a], future studies utilizing genome-scale approaches may successfully identify other variants contributing to these multifaceted syndromic phenotypes.

Our findings have important implications for genetic counseling and recurrence risk for families diagnosed with these disorders. PPS is an incompletely penetrant syndrome with variable expression and recurrence risks ranging from <1% (due to *de novo* mutations) to 50% (inherited, dominant mutations). In the examples of PPS1 and PPS3, both diagnosed as having a classic PPS phenotype but no identified mutation, the recurrence risk has dropped from a maximum risk of 50% (for autosomal dominant PPS) to 25% (PPS1, recessive) and to <1% (PPS3, uniparental disomy). On the other hand, due to the nearly identical phenotypes in some patients, it is possible that patients diagnosed with BPS could have dominant mutations in *IRF6*, which could similarly result in an increase in recurrence risk. In addition there may be other genes that are involved such related disorders with overlapping features as demonstrated in our reported family with BPS and mutation in *CHUK*. Thus, in small families with a single affected individual, it is difficult to accurately predict recurrence risk without a molecular diagnosis. Although there is currently only a select group of genes contributing to PPS, BPS, and other related syndromes (VWS, AEC, and EEC), not all patients with these disorders have a molecular diagnosis. Exome sequencing may prove to be the most efficient approach for genetic testing in patients presenting with cleft lip and palate, popliteal pterygia, and other congenital anomalies.

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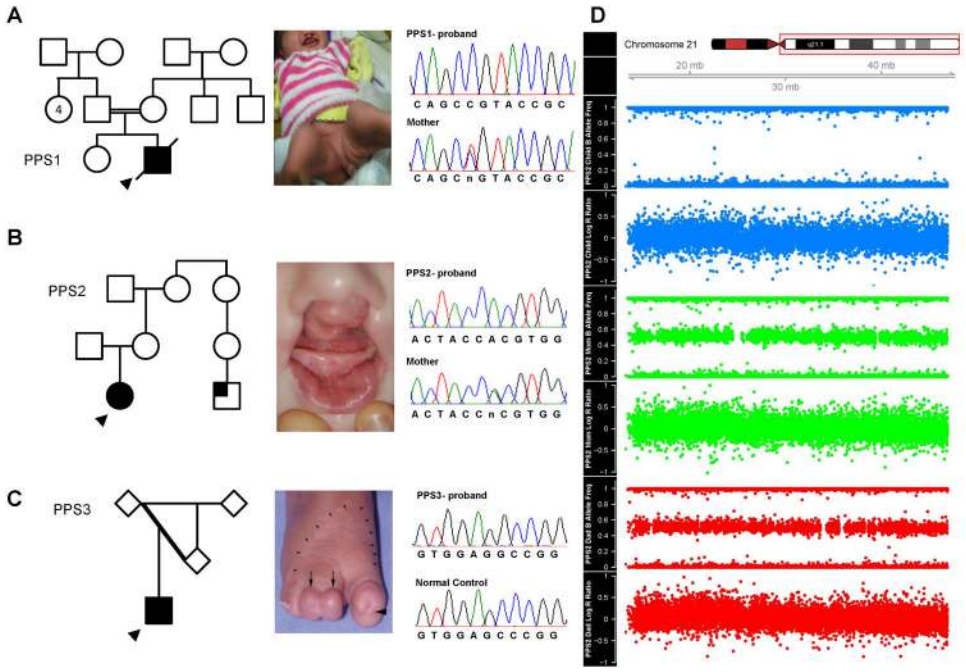

Figure I. Phenotypic and molecular characterization of families clinically diagnosed with popliteal pterygium syndrome

(A) Pedigree PPS1 and clinical picture of the affected child. Genital hypoplasia and popliteal pterygia are apparent. The sequence chromatograms show the homozygous *IRF6* mutation (NM_006147.3:c.1316T>C) resulting in the missense change p.Leu439Pro, which was inherited from heterozygous parents.

(B) Partial pedigree of PPS2 and clinical picture of the affected child with bilateral cleft lip and palate and the attenuated lower lip pit. The sequence chromatograms show the homozygous RIPK4 mutation (NM_020639.2:c.1853G>A) resulting in the missense change p.Arg618His.

(C) Pedigree of PPS3 and clinical picture of the affected child showing the malformation of the toes (large arrows) and increased subcutaneous tissue on the dorsal surface of the foot (small arrows). Sequence chromatograms show the homozygous *RIPK4* mutation NM_020639.2:c.1342G>C, which resulted in the missense change p.Ala448Pro.

(D) Chromosome 21 Log R ratios and B allele frequencies from SNP array data for members of pedigree PPS2 indicate uniparental disomy. Two chromosomes are present in the affected child (top plots in blue), as indicated by the normal logR ratio, but there is no heterozygosity, as shown by the B allele frequencies. The plots for the parental data (mother in middle in green; father at bottom in red) are normal.

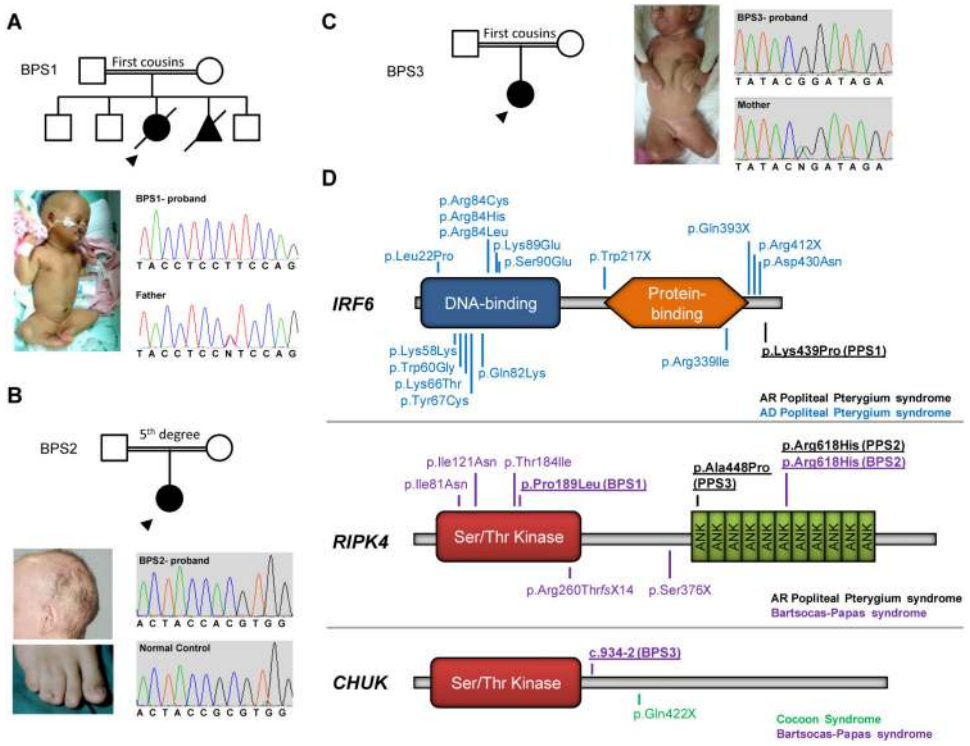

Figure II. Phenotypic and molecular characterization of families clinically diagnosed with Bartsocas-Papas syndrome

(A) Partial pedigree of BPS1 and clinical picture of the affected proband with typical features of Bartsocas-Papas syndrome. The sequence chromatograms show the homozygous *RIPK4* mutation (NM_020639.2:c.566C>T) resulting in the missense change p.Pro189Leu, which was inherited from heterozygous parents.

(B) Partial pedigree of BPS2 and clinical picture of the affected proband showing alopecia and III-IV-V syndactyly of the right foot. The sequence chromatograms show the homozygous *RIPK4* mutation (NM_020639.2:c.566C>T) resulting in the missense change p.Arg618His.

(C) Pedigree of BPS3 and clinical picture of the affected child who underwent exome sequencing. Sequence chromatograms show the homozygous *CHUK* mutation NM_001278.3:c.934-2A>G, which occurs at a splice acceptor site.

(D) Schematics of proteins with reported mutations. Mutations reported in this study are bolded and underlined. The *IRF6* mutations reported in autosomal dominant (AD) popliteal pterygium syndrome are clustered in the DNA binding domain. The mutation in PPS1 (p.Lys439Pro) is located at the 3′ end of the gene. *RIPK4* mutations in Bartsocas-Papas syndrome cluster in the kinase domain, with mutations causing milder phenotypes occurred in the ankyrin domains. Mutations in CHUK in Bartsocas-Papas syndrome and Cocoon syndrome are predicted to result in a protein truncated after the kinase domain.

Table I

Phenotypic characteristics of Bartsocas-Papas syndrome, popliteal pterygium syndrome and the six patients sequenced in this study

	Bartsocas-Papas Syndrome	Popliteal Pterygium Syndrome	PPS1	PPS2	PPS3	BPS1	BPS2	BPS3
			IRF6	*RIPK4*				*CHUK (IKKA)*
			p.Lys439Pro	p.Arg618His	p.Ala448Pro	p.Pro189Leu	p.Arg618His	c.934-2 splice acceptor
Homozygous?	+	–	+	maternal UPD	Presumed	+	Presumed	+
Orofacialclefting	+	+	+	+	+	+	+	+
Pterygia	+	+	+	+	+	+	+	+
Ankyloblepharon	+	+	+	+	–	+	+	+
Oral synechia	+	+	+	+	+	+	+	+
Syndactyly	+	+	+	–	+	+	+	+
Genital hypoplasia	+	+	+	+	+	+	+	+
Sparse Hair	+	–	–	+	+	+	+	+
Lip Pits	–	+	+	+	–	–	+	–
Nails	+	+	N/A	N/A	Dysplastic	Hypoplastic	Hypoplastic	N/A
Dentition	Oligodontia (1 report)	Hypodontia	N/A	N/A	Hypodontia	N/A	N/A	N/A