Allelic heterogeneity of Proteus syndrome

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Abstract Proteus syndrome is a mosaic disorder that can cause progressive postnatal overgrowth of nearly any organ or tissue. To date, Proteus syndrome has been exclusively associated with the mosaic c.49G > A p.(Glu17Lys) pathogenic variant in *AKT1*, a variant that is also present in many cancers. Here we describe an individual with severe Proteus syndrome who died at 7.5 yr of age from combined parenchymal and restrictive pulmonary disease. Remarkably, this individual was found to harbor a mosaic c.49_50delinsAG p.(Glu17Arg) variant in *AKT1* at a variant allele fraction that ranged from <0.01 to 0.46 in fibroblasts established from an overgrown digit. This variant was demonstrated to be constitutively activating by phosphorylation of AKT(S473). These data document allelic heterogeneity for Proteus syndrome. We recommend that individuals with a potential clinical diagnosis of Proteus syndrome who are negative for the p.(Glu17Lys) variant be tested for other variants in *AKT1*.

[Supplemental material is available for this article.]

INTRODUCTION

Until this report, a somatic variant in the *AKT1* gene—c.49G > A, p.(Glu17Lys)—was the only reported cause of Proteus syndrome (Lindhurst et al. 2011). The AKT1 protein is a serine-threonine kinase that signals in the AKT/PI3K/mTOR pathway. The pathophysiology of Proteus syndrome is attributed to constitutive activation of AKT1 (Carpten et al. 2007). This activation limits apoptosis and promotes growth, among other effects (Carpten et al. 2007). The clinical manifestations are dominated by skeletal overgrowth, but the disorder is highly pleiotropic including central nervous system (CNS) overgrowth and neuronal migration defects, vascular anomalies, overgrowth of many other organs and tissues, and bullous or cystic disease of the lungs. Here we report an individual with severe manifestations of Proteus syndrome who has constitutive AKT activation because of a distinct variant in *AKT1*.

RESULTS

Clinical Presentation and Family History

The proband was born by emergent cesarean delivery at 37 wk of gestation to a G1P1 21-yr-old mother because of fetal decelerations. He underwent surgical repair of an umbilical

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government.

Ontology terms: contractures of the large joints; fibrocystic lung disease; increased adipose tissue; lower limb asymmetry; overgrowth; upper limb asymmetry; venous malformation

Published by Cold Spring Harbor
Laboratory Press

doi:10.1101/mcs.a005181

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hernia after birth and was fed via a nasogastric tube because of poor oral intake for the first 13 d. He developed infantile seizures that were treated with an antiepileptic. At birth, he was noted to have slight asymmetric overgrowth of the left leg and arm and vascular markings on both legs, diagnosed as cutis marmorata telangiectasia.

By 4 mo of age, the seizures resolved, and the left leg measured 1.5 cm longer than the right. Linear verrucous epidermal nevi streaked his left arm, left trunk, and left leg. His left sole had a cobblestone appearance (which we now term “pre- CCTN,” the precursor lesion of the cerebriform connective tissue nevus (Keppler-Noreuil et al. 2019)). At 5 mo, a lipoma on the left lower back was noted, and at 7 mo, macrodactyly and divergence of the third and fourth fingers of the left hand were noted. He was hospitalized for pneumonia at 14 mo. At 15 mo, his leg length discrepancy had progressed to 5 cm with a left knee valgus deformity of 30°.

Magnetic resonance imaging at 17 mo showed pleural effusions of the lungs, irregularly shaped vertebral bodies, and an enlarged spleen and portal vein. A CCTN was noted on the left fourth finger and the progressive CCTN on the left sole was associated with deviation of the left great toe into an ~30° extension at rest. Cutaneous capillary malformations were present on both calves.

At 18 mo, he underwent hemiepiphysiostasis surgery with tension band plates placement at the left distal medial femur (Crenshaw et al. 2018). At 21 mo, he had a left orchiectomy for removal of a serous borderline cystadenoma, and at 22 mo, he had an adenoidectomy. At 2 yr 2 mo, he had a second orthopedic surgery replacing the tension band plates on his left femur and adding additional plates to his left proximal tibia. By 2 yr 5 mo of age, his left knee contracture was 90°, and he had regressed to crawling. Subsequently, an above-the-knee amputation of the left leg was performed. Also, at this time, increased lipomatous lesions on his lower left back, left chest, left abdomen, and bilateral axillae were present. His left foot measured 3 cm longer than the right, and digits 1–4 in his left hand were overgrown. He experienced daily emesis, which was attributed to gastroesophageal reflux disease. An esophagoduodenoscopy reported the duodenum was thickened and pale.

At 3.5 yr, the left fourth finger was amputated because of progressive bony overgrowth. Respiratory issues starting at 4 yr of age necessitated a chest CT, which showed severe bullous pulmonary disease and cervical vertebrae elongation. He was placed on pediatric hospice care at 6 yr. Imaging studies at 6 yr 8 mo showed the bullae and fibrosis had progressed, with an estimate of 40% normal lung tissue remaining (Fig. 1C). Moreover, severe thoracolumbar scoliosis contributed a restrictive component to his lung disease. Pulmonary function tests showed elevated residual volume (211%) and reduced total lung capacity (80%). His oxygen saturations on room air ranged from 70% to 88%, and, thus, he used supplement oxygen at night and as needed during the day. Further imaging showed a coxa valga deformity in the left femoral head, shortening of the left ulna, and myocardial fat infiltration in his right ventricular septum. A vascular lesion in the left optic nerve region was also reported.

At 6 yr 11 mo, he began taking the AKT inhibitor, miransertib (ARQ 092), at a dose of 5 mg/m² through a compassionate use protocol. However, because of the patient’s worsening condition, the study drug was stopped after 3 mo. The patient died at 7 yr 6 mo of age from respiratory complications secondary to advanced bullous lung disease. This individual’s manifestations exceeded the 2006 criteria needed to support a clinical diagnosis of Proteus syndrome (Table 1; Biesecker 2006).

Molecular Analyses

Molecular testing of DNA isolated from five fibroblast cultures from the finger amputation at 3.5 yr using a custom restriction fragment length polymorphism (RFLP) assay (Lindhurst et al.

Figure 1. Patient images showing (A) lipoma of left lower back at 22 mo; (B) cervical vertebrae elongation and fusion of the posterior elements of C2–C3 and C4–C5 at 6 yr 8 mo; (C) bilateral pulmonary bullae via chest CT at 6 yr 10 mo; (D) cerebriiform connective tissue nevus (CCTN) of right sole; (E) linear verrucous epidermal nevi streaking upper left arm and trunk; and (F,G) bony and soft tissue overgrowth of first–third digit, post-fourth finger ray amputation. A CCTN is noted on lower palm (F). Images D–G were obtained at 6 yr 8 mo of age.

Table 1. Proteus syndrome diagnostic criteria in the proband

Proteus syndrome diagnostic criteria ^a	Proband
Mosaic distribution of lesions	Yes
Sporadic occurrence	Yes
Progressive course	Yes, severe
(A) Cerebriiform connective tissue nevus	Yes
(B) Linear epidermal nevus	Yes
(B) Asymmetric, disproportionate overgrowth	Yes
(B) Bilateral ovarian cystadenoma or parotid monomorphic adenoma before second decade	No
(C) Dysregulated adipose tissue	Yes
(C) Vascular malformations	Yes
(C) Bullous lung disease	Yes
(C) Facial phenotype	No

^aDiagnosis requires all three general criteria AND either one from (A), two from (B), or three from (C).

2011) for the expected *AKT1* c.49G > A p.(Glu17Lys) variant was negative. Additional testing using custom RFLP assays for the *PIK3CA* “hotspot” variants (c.3140A > G p.(His1047Arg), c.3140A > T p.(His1047Leu), c.1624G > A p.(Glu542Lys), and c.1633G > A p.(Glu545Lys) (Lindhurst et al. 2012; Keppler-Noreuil et al. 2014) was negative. We next interrogated DNA from two cultures using a custom capture next-generation sequencing (NGS) panel containing 153 PI3K/AKT pathway genes. Examination of the *AKT1* sequence in one of the samples (FB1) identified 58/515 (0.113) alternate reads for a c.49_50delinsAG variant. Visualization of the data using the Integrative Genomics Viewer showed that both substitutions always occurred on the same read (Fig. 2A). The second DNA interrogated by custom capture/NGS (FB4) had 3/474 (0.006) alternate reads for the c.49_50delinsAG variant. Sanger sequencing confirmed the presence of both base changes in DNA directly isolated from a CLIA cell line established from the amputated finger (Fig. 2B). The RFLP assay for the

Figure 2. Sequencing results at Chr 14:105,246,550–105,246,551 from the proband. (A) Snapshot of the next-generation sequencing reads in sample FB1 visualized using the Integrative Genomics Viewer (Robinson et al. 2011) showed that reads either had both alternate bases or had reference calls at these positions, indicating they were in *cis*. The color of a row denotes direction of read; note that alternate bases occurred on reads from both directions. (B) Sanger sequencing showed two peaks at each position (arrows) in both the forward and reverse direction that were present in the proband and absent in control DNA.

Table 2. Variant levels in fibroblasts from the reported individual

Sample	VAF (NGS ^a)	VAF (RFLP ^b)
FB1	0.113	0.02
FB2	NA	0.46
FB3	NA	0.05
FB4	0.006	0.004
FB5	NA	0.31

(VAF) Variant allele fraction, (NGS) next-generation sequencing, (RFLP) restriction fragment length polymorphism.

^aFraction of alternate reads at Chr 14:105,246,550–105,246,551 identified by next-generation sequencing.

^bVariant allele fraction detected by custom restriction fragment length polymorphism assay for the c.50A > G substitution.

Proteus c.49G > A variant was negative because the additional base change destroys the MbolI restriction site used in the assay. We were unable to develop a RFLP assay that detected the dinucleotide change but instead developed one that detects the c.50A > G substitution component of the 2-bp insertion-deletion (it is not sensitive to the change at nucleotide 49). We tested the fibroblast DNAs and found that all had measurable levels of this change with variant allele fractions (VAFs) ranging from <0.01 to 0.46 (Table 2). This further supports that the substitutions are in *cis*, because only the c.50A > G and not the c.49G > A assay was positive. The discordance in VAF between the NGS and RFLP results for FB1 was due to the use of DNA isolated from aliquots of the culture that had different admixtures of positive and negative cells. Admixture variation between aliquots is not uncommon for mosaic cell lines (M.J. Lindhurst, unpubl. obs.). We conclude that this individual had a somatic mosaic AKT1 c.49_50delinsAG p.(Glu17Arg) 2-bp variant associated with Proteus syndrome (Table 3).

To test if the p.(Glu17Arg) variant confers constitutive activation, we measured the phosphorylation level of AKT in multiple fibroblast lines from this individual grown in serum-free medium and compared it to levels in control fibroblasts without AKT1 variants and to levels in fibroblast single cell clones from an affected individual with and without the typical p.(Glu17Lys) variant (Fig. 3). Lysates from p.(Glu17Arg)-containing fibroblasts showed a two-fold relative activation (FB1 cell line), three- to fourfold relative activation (FB3), a three- to 11-fold (FB4), and a 23- to 25-fold (FB2) increase compared to the averaged levels in the variant-negative lysates. FB2 had a VAF of 0.46, which is quite similar to the heterozygous affected c.49G > A cloned fibroblasts (VAF 0.5, by definition), the latter showing a 17- to 19-fold increase activity compared to control cells. As expected, these data show a proportionality of VAF to S(473) activation in the various cell lines. We cannot, however, conclude that these levels of VAF or S(473) phosphorylation are relevant to the degree of disease effect in the source tissue as they could instead simply be a local sampling artifact. Given that the S(473) activation in the VAF 0.46 p.(Glu17Arg) sample is as high or higher than that of a cloned p.(Glu17Lys) variant, we conclude that the c.49_50delinsAG p.(Glu17Arg) variant is

Table 3. Variant table

Gene	Chromosome	HGVS DNA reference	HGVS protein reference	Variant type	Predicted effect	dbSNP/dbVar ID	Genotype
AKT1	Chr 14: g.105,246,550_105,246,551 delinsCT (GRCh37)	c.49_50delinsAG	p.(Glu17Arg)	Dinucleotide substitution	Missense	VCV000800567.1	Mosaic (VAF 0.004–0.46)

Figure 3. AKT remains active in variant-positive fibroblasts in serum-free medium. Duplicate plates of four fibroblast cultures established from the amputated finger of the proband (blue bars), variant-positive (red bars), and variant-negative single cell fibroblast clones from an individual with a *AKT1* c.49G > A; p.Glu17Lys mutation, and fibroblasts from a control individual (green bars) were grown without serum for 24 h prior to lysis and western analysis. The histogram shows the ratio of the infrared signal from the phospho- and total AKT antibodies. The variant allele fraction (VAF) for each culture is indicated. Cultures with high levels of variant-positive cells (FB2 and SCC1) had high levels of pAKT compared to variant-negative fibroblasts when grown in the absence of growth factors.

at least as strongly activating as, and may have a stronger activating potential than, the more common c.49G > A p.(Glu17Lys) Proteus variant.

DISCUSSION

Proteus syndrome is a very rare syndrome (prevalence < 1/1,000,000) and was first described in the modern medical literature in 1976 (Temtamy and Rogers 1976) and as Proteus syndrome several years later (Wiedemann et al. 1983). The manifestations of Proteus syndrome are associated with an activation of the AKT1 oncogene product (Lindhurst et al. 2011). *AKT1* was first recognized to be important for human health when it was found to be mutated in a number of human cancers (COSMIC database; <https://cancer.sanger.ac.uk/cosmic>) (Tate et al. 2019). Importantly, in most cancers, the variant is the same c.49G > A variant as is found in the mosaic state in individuals with Proteus syndrome. The pathogenesis of Proteus syndrome and the *AKT1*-mutated cancers is a ligand-independent activation of the AKT1 signal, which is downstream from a number of tyrosine kinase growth factor receptors in the AKT/PIK3CA/mTOR pathway (Carpten et al. 2007).

Whereas nearly all variants in the COSMIC database are the c.49G > A p.(Glu17Lys) variant, there are a growing number of other activating variants in some less common tumors (Tate et al. 2019). Recent studies have shown heterogeneous *AKT1* variants, including in-frame duplications in sclerosing pneumocytoma—remarkably, 93% and 100% of two cohorts of affected individuals had *AKT1* variants (Yeh et al. 2020). An atypical *AKT1* p.(Arg23Gln) variant was observed in two neuroendocrine tumors, although causality was not established

(Park et al. 2019). Finally, one individual with a histopathologically indeterminate epithelioid neoplasm was found to harbor a novel fusion gene comprising the *LAMTOR1* and *AKT1* genes (Slotkin et al. 2019). From these publications it is clear that allelic heterogeneity is relevant to tumorigenesis and this report is the first to demonstrate this in Proteus syndrome.

This individual unambiguously met the 2006 clinical criteria for Proteus syndrome—he has nearly all manifestations of the disorder (Biesecker 2006). Recently, we have updated these simple clinical criteria to incorporate an integrated clinical-molecular scoring system (Sapp et al. 2019). In this scheme, clinical manifestations are scored on a point scale from –10 to +28 points, with positive points for attributes of Proteus syndrome and negative points for attributes associated with *PIK3CA*-related overgrowth spectrum. This individual scored 24 points, missing only the criteria for deep vein thrombosis and the facial phenotype. Note that in those updated clinical diagnostic criteria, we specified that diagnosis was based on a dyad of phenotype and genotype and that the latter was a pathogenic variant in *AKT1*, which we conclude to be the case here. Therefore, we conclude that this individual has *AKT1*-related Proteus syndrome, even though his variant is different from all other affected individuals to date.

The natural history of the disease in this individual was severe. Although it is known that the mortality of Proteus syndrome is considerable (Sapp et al. 2017), he is one of the younger individuals to expire with this disease. The cause of death was respiratory insufficiency secondary to pulmonary disease, a known manifestation of Proteus syndrome (Lim et al. 2011). The diffuse nature of his bullous disease was such that lobectomy was not felt to be beneficial and he was evaluated as a poor lung transplant candidate because of the advanced nature of the disease and his other medical issues. It is critical to note that his severe parenchymal lung disease was compounded by the restrictive lung disease because of vertebral and chest wall overgrowth and deformation. This combination of respiratory disease is difficult to treat and may primarily be a manifestation that can best be avoided through primary drug treatment (Keppler-Noreuil et al. 2019; Leoni et al. 2019), rather than postsymptomatically.

We conclude that this variant is pathogenic for Proteus syndrome using an adaptation of the ACMG/AMP criteria, which were designed for constitutional variants. We would cite a modification of the PS2 (de novo) criterion, reasoning that a mosaic alteration of this VAF that so strongly matches the phenotype is as strong a piece of evidence as is a constitutional de novo variant. We cite criterion PS3 (functional) in that the AKT phosphorylation assay is a well-established assay that is proximate to the actual pathophysiology of this disease (Carpten et al. 2007). We would cite PM2_Sup (absence in controls) as there is only a single occurrence of this variant in gnomAD, though it is in fact a variant from a tumor sample erroneously included in the database (A O'Donnell, Broad Institute, pers. comm.). We would cite PM5 as codon 17 is known to be mutated in the p.(Glu17Lys) variant of COSMIC and Proteus syndrome (these data could arguably be used instead for the hotspot criterion PM1). We would cite PP2 as multiple in silico programs predict this variant to be deleterious. We would score PP4_Strong as we believe that the phenotype in this patient is highly specific for individuals with variants in this gene; we are aware of no individual with this degree of Proteus syndrome severity and specificity who has a variant in another gene. These total three Strong, one Moderate, and two Supporting, which yields a Pathogenic assertion in (Richards et al. 2015) (their Table 5) and a posterior probability of 0.99993 in the Bayes calculator of (Tavtigian et al. 2018), two orders of magnitude above the Pathogenic threshold.

The detection of a novel variant causing activation of *AKT1* in this patient is the first to our knowledge in Proteus syndrome. We have carefully evaluated the COSMIC database and do not see evidence of this variant among the nearly 250 tumors that have *AKT1* variants, nor is it reported among the variants detected in sclerosing pneumocytoma (Yeh et al. 2020). We saw no evidence of the c.49_50delinsAG p.(Glu17Arg) variant in COSMIC, although it is possible that that data set may be artifactually depleted for dinucleotide variants for

methodologic reasons (Tate et al. 2019). We conclude that this variant is a cause of Proteus syndrome, that functional evidence and the severity of the patient could be consistent with a more strongly activating mutational effect (relative to p.Glu17Lys), and that cancer mutation-al surveys and diagnostic testing for Proteus syndrome should check for other variants in *AKT1* if the typical c.49G > A p.(Glu17Lys) variant is not detected.

METHODS

Fibroblast cultures were established from five separate samples of tissue from the left fourth finger amputation. DNA was isolated using the Gentra Puregene Blood kit (QIAGEN). Genome libraries with ~325 base inserts were prepared for each sample from 100 ng of genomic DNA using the Accel-NGS 2S Plus DNA Library Kit (Swift Biosciences). Libraries were barcoded and pooled in an equimolar ratio for capture enrichment. The custom capture panel targeted 0.69 Mb of sequence covering 153 PI3K/AKT pathway genes. Capture was performed according to the SeqCap EZ Library SR User's Guide (Roche Nimblegen). The captured exome pool was sequenced on a HiSeq 2500 using version 3 chemistry (Illumina). At least six million paired-end 126 base reads were obtained for each sample. Data were processed using RTA ver. 1.18.64 and CASAVA 1.8.2. The target coverages were calculated using Picard CollectHSMetrics (Picard; <http://broadinstitute.github.io/picard>) with default parameters (Base Quality [BQ], Mapping Quality [MQ] = 20). The target BED file was converted to the Picard interval file using Picard BedToIntervalList (Supplemental Table S1). The custom RFLP assay used to detect the *AKT1* c.50A > G substitution was modified from the c.49G > A assay described in Lindhurst et al. (2011) by using the following primers: (6FAM)-CAGGCATCCCAGGCACATCTGTCC and 5'-AGTAGCGTGGCCGCCAGGTCTTGAGGTAC followed by digestion with KpnI.

Functional studies were conducted on fibroblasts grown with or without serum for 20–24 h before cell lysis as described (Lindhurst et al. 2011). Western analyses were performed as described (Lindhurst et al. 2012, 2015) using antibodies to pAKT(S473) and pan-AKT (products #4060 and #2920, Cell Signaling Technologies).

ADDITIONAL INFORMATION

Data Deposition and Access

The sequencing data is available in the National Center for Biotechnology Information (NCBI) database of Genotypes and Phenotypes (dbGaP; <http://www.ncbi.nlm.nih.gov/gap>) under the accession number phs002006.v1.p1. The variant has been submitted to ClinVar (<https://www.ncbi.nlm.nih.gov/clinvar/>) under the accession number VCV000800567.1.

Ethics Statement

This study was reviewed and approved by the National Human Genome Research Institute (NHGRI) Institutional Review Board, protocol 94-HG-0132. Written informed consent by the mother was provided on behalf of this minor. The mother of the affected individual has reviewed this manuscript and approves publication of the report.

Acknowledgments

The authors thank the individual's family and the home medical team for their devoted care and advocacy for this young man. The authors thank Celine Hong for completing the dbGap submission and for compiling the sequencing coverage table. The authors thank the

Competing Interest Statement

L.G.B. received in-kind research support from ArQule, Inc. and is a Deputy Editor of this Journal.

Referees

Marcella Zollino
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Received January 21, 2020;
accepted in revised form
February 19, 2020.

National Institutes of Health (NIH) Intramural Sequencing Center for performing the NGS capture and sequencing.

Author Contributions

A.B. and L.G.B. wrote the manuscript; all authors edited it. J.C.S. coordinated and organized clinical data acquisition. K.M.K.-N. clinically evaluated the individual. M.J.L. performed the molecular and functional analyses. H.C.K. and M.R.Y. performed molecular analyses.

Funding

This study was supported by the intramural research program of the NHGRI, HG200328 and HG200388. Miransertib was provided by ArQule, Inc.

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Cold Spring Harb Mol Case Stud 2020, **6**: a005181 originally published online April 23, 2020
Access the most recent version at doi:[10.1101/mcs.a005181](https://doi.org/10.1101/mcs.a005181)

**Supplementary
Material**

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