Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Lindhurst MJ, Sapp JC, Teer JK, et al. A mosaic activating mutation in *AKT1* associated with the Proteus syndrome. N Engl J Med 2011;365:611-9. DOI: 10.1056/NEJMoa1104017.

Supplementary Appendix *Lindhurst et al.*

Table of contents

Table 1 Clinical features table of individuals analyzed in the study **2**

Exome sequencing methods **3**

Custom PCR restriction enzyme assay methods & validation **4**

Custom PCR restriction enzyme assay validation results figure **5**

Cell culture methods **6**

Table 2 Sample characteristics and mutation status **7**

Supplementary Table 1. ***Clinical features table of individuals analyzed in the study***

	GenI	A	B1(LVEN)	B2(Bones)	B3(Tumors)	C1(Fat)	C2(Vasc)	C3(Lung)	C4(Face)
PS5	Y	Y	Y	Y	N	Y	Y	U	Y
PS14	Y	Y	N	Y	N	Y	N	Y	N
PS23	Y	Y	Y	Y	Y	N	Y	Y	N
PS29	Y	Y	Y	Y	N	Y	Y	Y	Y
PS34	Y	Y	Y	Y	N	N	Y	N	N
PS38	Y	Y	Y	Y	N	Y	Y	Y	Y
PS43	Y	Y	Y	Y	N	Y	Y	Y	Y
PS46	Y	Y	Y	Y	N	Y	Y	N	N
PS52	Y	Y	Y	Y	N	Y	N	N	N
PS53	Y	Y	N	Y	N	Y	Y	Y	N
PS54	Y	Y	Y	Y	N	Y	Y	N	N
PS57	Y	Y	Y	Y	N	Y	N	N	N
PS58	Y	Y	N	Y	N	N	N	N	N
PS59	Y	Y	Y	Y	N	Y	Y	N	N
PS60	Y	Y	Y	Y	N	Y	Y	N	N
PS64	Y	Y	Y	Y	Y	Y	Y	Y	N
PS69	Y	N	Y	Y	N	Y	Y	N	Y
PS73	Y	Y	Y	Y	N	Y	Y	N	N
PS74	Y	U	Y	Y	N	N	N	N	Y
PS75	Y	Y	N	Y	N	Y	Y	N	N
PS77	Y	Y	Y	Y	N	Y	N	N	N
PS78	Y	Y	Y	Y	N	Y	Y	N	Y
PS82	Y	Y	Y	Y	N	Y	Y	Y	Y
PS83	Y	Y	Y	Y	N	U	U	N	N
PS84	Y	Y	Y	Y	N	Y	Y	U	Y
PS87	Y	Y	Y	Y	N	Y	Y	N	N
PS91	Y	Y	N	Y	N	N	N	N	N
PS93	Y	Y	Y	Y	N	Y	N	N	N
PS95	Y	Y	Y	Y	N	Y	Y	U	N

The columns indicate which of the published diagnostic criteria[3] each individual manifested. GenI; the three general criteria of sporadic, mosaic, and progressive. Criterion A is the cerebriform connective tissue nevus, B1 is the linear verrucous epidermal nevus, B2 is distorting skeletal overgrowth, B3 is bilateral ovarian cystadenomas or monomorphic adenomas of the parotid gland, C1 is abnormal fat distribution, C2 is cutaneous vascular anomalies, C3 is pulmonary bullae, C4 is the characteristic facial phenotype. See reference 1 for details.

Exome Sequencing Methods

To prepare sequencing libraries, 3-5 μg of sample was fragmented using sonication (Covaris Inc), end-repaired, and ligated to paired-end sequencing adapters (Illumina, Inc., SureSelect Human All Exon Kit and SureSelect Human All Exon 50Mb kit, Agilent, Inc) as described[8]. Samples were gel purified and size selected to 200-300 bp and then amplified. The amplified library was purified on MinElute columns (Qiagen Inc), and eluted in 10 μL Buffer EB. 500 ng of this library was mixed with the SureSelect hybridization buffer and denatured at 95 ^{o}C for 5 min. The biotinylated RNA baits (SureSelect kit) were added, and the reaction incubated 24 hours at 65 ^{o}C. The hybridized DNA-bait duplexes were captured on streptavidin beads, washed, and eluted. Following desalting, samples were PCR-amplified 18 cycles (Herculase II Fusion DNA polymerase, Agilent Technologies, Stratagene Products), purified using AMPure beads (Beckman Coulter Genomics), quantified, and then used for cluster generation. Sequencing was performed as described by manufacturer's protocols (Illumina Inc.) Samples were quantified for cluster generation using quantitative-PCR to improve cluster number targeting. An exome capture was sequenced in one or more lanes of an Illumina GAIIx sequencer and considered successful if >85% of the CCDS+3 regions were covered with a high quality (MPG ≥10) genotype. If one lane did not pass this threshold, the same sample was run on a second lane.

Reads were aligned to a human reference sequence (UCSC assembly hg18, NCBI build 36) using "efficient large-scale alignment of nucleotide databases" as part of the standard Pipeline Analysis v1.3.4 and v1.4.0 (ELAND, Illumina Inc). Reads that failed the Illumina chastity filter were omitted. Reads that aligned uniquely were grouped into genomic sequence intervals of about 100 kb, and reads that failed to align were binned with their paired-end mates. Reads in each bin were subjected to a Smith-Waterman-based local alignment algorithm, *cross_match* using the parameters –minscore 21 and –masklevel 0 to their respective 100 kb genomic sequence (http://www.phrap.org). Genotypes were called at all positions with high-quality sequence bases (Phred-like Q20 or greater) using a Bayesian algorithm (Most Probable Genotype – MPG[8]). Genotypes with a MPG score of 10 or greater showed >99.89% concordance with SNP Chip data. The targeted regions included the Consensus Coding Sequence regions plus three bases on each side (CCDS+3): 28,793,846 bases of the genome. The baited regions included 37,640,396 bases (SureSelect All Exon) or 51,646,629 bases (SureSelect All Exon 50MB). Our annotation of cSNVs (coding single nucleotide variants) was based on UCSC 'known genes'. Missense variants were sorted by the degree of severity of functional disruption prediction using CDPred[32].

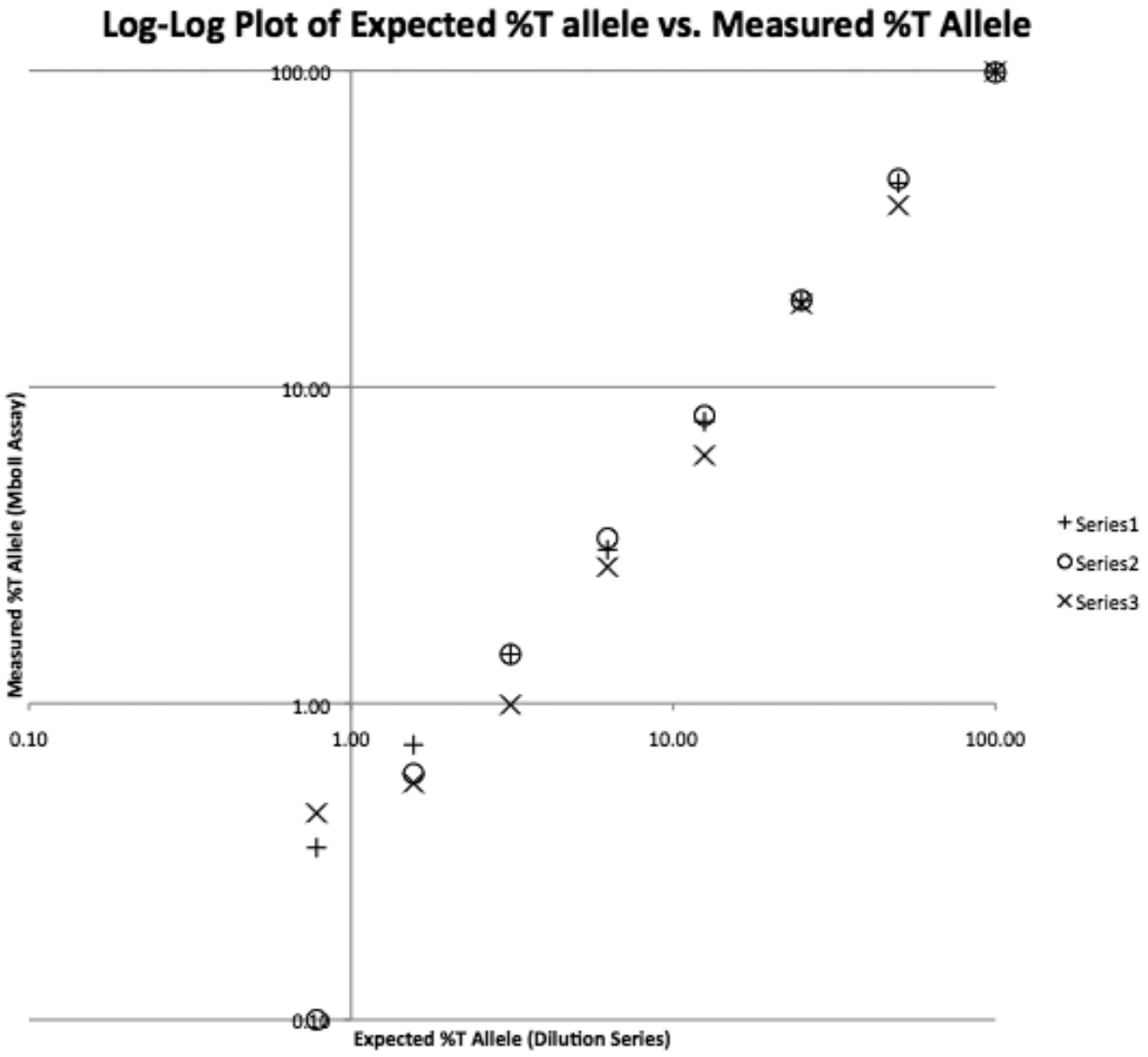
Custom PCR restriction enzyme assay for the c.49G>A mutation in *AKT1*

The modified PCR/restriction endonuclease assay amplified a 141 bp segment (chr14:105,246,521-105,246,661 hg19) of genomic DNA using AmpliTaq Gold® (Applied Biosystems), a FAM labeled forward primer, and an unlabeled modified reverse primer (Figure 2A). The reverse primer was modified at chr14:105,246,548, creating an MboII restriction site in the presence of c.49G>A. MboII digestion of a mutant allele (MT) gave a 122 bp fragment and uncut wild type (WT) alleles gave a 141 bp fragment. Fragments were detected on the ABI3130 (Figure 2B) using a minimum signal threshold of 100 relative florescence units (rfu); peaks below this were assigned a value of zero. When no MT peak was evident, injection times were increased until the WT peak was nearly off scale to increase sensitivity. Areas under peaks were used to calculate the proportion of mutant PCR product (MT area/(MT area + WT area)). Injection times were altered for optimal peak height. The assay results correlated well (r^2=0.9993) with a series of two-fold diluted mutant DNA samples

Custom PCR restriction enzyme assay *validation methods*

PCR products encompassing the AKT1 c.49G>A point mutation were cloned into the TA cloning vector as per the manufacturer's instructions (Invitrogen Corp.) Clones were sequenced and a pair of wild type and mutant clones were selected. DNA from these clones was quantitated and either the undiluted mutant clone or a series of two-fold dilutions were subjected to the MboII assay in triplicate.

Custom PCR restriction enzyme assay validation results figure



Supplementary figure. Results of the above-described Custom PCR restriction enzyme assay validation experiment. Plot of expected vs. measured %T allele frequency from a dilution series. The correlation of the average of the three values with the predicted values was r^2=0.9993

Cell culture methods

Cultured cells from patients with Proteus syndrome and controls were grown from surgically collected explants and maintained in DMEM or αMEM medium with 10-20% fetal bovine serum, penicillin/streptomycin +/- fungizone. For AKT western blots, cells were grown overnight in serum-containing medium and washed once with phosphate buffered saline (PBS). The medium was replaced with either serum-containing or serum-free medium and the cells were grown for eight hours. Cells were lysed in PBS containing 1% Triton X-100, 0.1% SDS, protein and phosphatase inhibitors, using a 25 gauge needle. Solubilized protein was collected following centrifugation and used for immunoblotting. Antibodies included p-AKT(S473), p-AKT(T308), pan-AKT (#4060, #2965, #4691, Cell Signaling Technology) and β-actin (A5441, Sigma-Aldrich).

Table 2. Sample characteristics and mutation status

Patient	Anatomical Source[1]	Tissue Affection Status[2]	DNA Source Type[3]	Mutant allele (%)[4]	Exome (mutant/ total)[5]
PS5	Skin, LVEN - right chest	+	C	28	10/28
	Skin, normal - right chest	-	C	3	2/55
PS14	Skin, hyperplastic lip	+	C	3	0/48
	Lipoma - groin	+	C	0	
	Groin tumor (primarily lipoma) A	+	C	0	
	Skin, normal	-	C	0	0/31
PS23	Skin, hypertrophic - right foot sole	+	T	9	
			C	0	
PS29	Skin, CCTN - right paranasal mass	+	C	35	7/34
	Skin, CCTN –left medial canthus	+	C	39	
	Skin, CCTN - right hand middle finger	+	C	39	
	Skin, CCTN - right hand ring finger	+	C	47	32/63
PS34	Skin, atypical epidermal nevus - left anterior axilla	+	C	0	
	Skin, cutaneous capillary malformation - right abdomen	+	C	0	
	Skin, normal - right upper arm	-	C	0	
PS38	Skin, CCTN - side of left foot	+	C	0	
	Bone - right second toe	+	C	3	
	Skin, abnormal	+	C	12	
	Skin, normal - left upper arm	-	C	0	
	Skin, normal - top of left foot	-	C	0	
PS43	Bone, growth plate - head of right distal femur	+	T	9	
PS46	Skin, abnormal -overlying right knee	+	C	0	
PS52	Skin, normal - right upper arm	-	C	0	
	Skin overlying excess fat - left palm	?	C	0	
PS53	Skin, CCTN - ball of right foot	+	T	8	
			C	14	
	Skin, CCTN - heel of right foot	+	T	6	
			C	9	
	Skin, CCTN - right foot arch	+	T	7	
			C	17	
	Skin, CCTN - right foot outside edge	+	T	5	
			C	22	
	Skin, CCTN - ball of left foot	+	C	34	
	Skin, CCTN - heel of left foot		C	29	
	Skin overlying second toe left foot	+	C	23	
	Skin overlying fifth toe left foot	+	C	13	
	Skin, normal - top of right foot over fifth metatarsal	-	T	0	
			C	3	
	Skin, normal - top of right foot over first metatarsal	-	T	2	
			C	2	
	Skin, normal - right mid calf	-	T	0	

			C	0	
	Skin, normal skin - right mid shin	-	T	2	
			C	5	
	Skin, normal - top of foot near left ankle	-	C	4	
	Skin, normal - over left knee	-	C	0	
	Skin, border of CCTN – lateral right foot	?	T	0	
			C	2	
	Skin, border of CCTN - medial right foot	?	T	4	
			C	3	
	Skin, normal - over abnormal right patella	?	T	3	
			C	12	
PS54	Capillary vascular malformation - right outer knee	+	C	3	
	Skin, normal– medial aspect left thigh	-	C	7	
PS57	Skin, CCTN - right foot instep, inferior	+	C	0	
	Bone - right tibia	+	C	0	
	Skin, normal - right foot instep, superior	-	C	0	
	Bone - right fibula	-	C	0	
	Iliotibial band - right	?	T	2	
			C	0	
	Bone, growth plate - right tibia proximal epiphysis	?	T	18	
PS58	Skin, CCTN - left great toe	+	T1	14	
			T2	0	
			T3	5	
			T4	11	
			T5	5	
PS59	Skin, nevus - right superior antecubital fossa	+	C	0	
	Bone, osteoma - left ear canal	+	T	12	
	Bone, contents of left mastoid	+	T	0	
	Skin, normal - right upper inner arm	-	C	0	
PS60	Skin, LVEN - right shoulder	+	T	6	
			C	6	
	Skin, CCTN - right foot near fifth toe	+	C	0	4/72
	Skin, normal - left foot near fifth toe	-	C	7	9/35
PS64	Skin, CCTN - left lateral foot	+	C	4	
	Bone - distal femur core biopsy	+	C	0	
	Bone - proximal tibia core biopsy	+	C	0	
	Skin, normal - right iliac crest	-	C	0	
PS69	Bone, large nodule - floor of right ear canal	+	C	36	
	Bone, small nodule - lateral right ear canal	+	C	18	
	Skin, normal - behind right ear, piece A	-	C	6	
	Skin, normal - behind right ear, piece B	-	C	0	
	Skin over large nodule - right ear canal	?	T	13	
			C	34	
PS73	Skin, abnormal - left medial knee	+	T	17	
			C	34	
	Skin, mildly abnormal - left distal thigh	+	T	5	

	Skin, abnormal - left second toe	+	T1	13
			T2	11
	Subcutaneous fat over iliotibial band	+	T	7
	Iliotibial band - left	+	T	2
	Skin, normal - right upper inner arm	-	T	0
			C	3
PS74	Skin, LVEN - right neck, culture A	+	C	0
	Skin, LVEN - right neck, culture B	+	C	3
PS75	Skin, CCTN - medial left great toe	+	T	3
			C1	5
			C2	0
	Skin, CCTN - left second toe	+	C	11
	Bone/Cartilage - left distal tibia	+	T	5
			C	0
	Bone, growth plate - proximal phalanx of left great toe	+	T	5
	Bone, growth plate - distal phalanx of left great toe	+	T	13
	Bone, periosteum, and patellar tendon - left leg	+	T	14
	Bone, heterotopic - left patella	+	C1	37
			C2	28
	Skin, normal - right calf	-	T	0
			C1	0
			C2	0
	Skin, normal - left upper forearm	-	C	0
	Skin, normal - right lateral leg	-	C	0
	Bone, possibly abnormal - left patella	?	C	35
	Skin, scar - right lateral leg	?	C	0
	Iliotibial band	?	T	0
PS77	Skin - left nasal mass	+	T1	3
			T2	6
	Skin, CCTN - left foot		C	4
PS78	Skin, abnormal - left medial knee	+	T	4
			C	5
	Skin, CCTN - left foot sole, outer heel	+	C	1
	Skin, normal - left lateral mid-thigh	-	C	0
	Skin, normal - right upper arm	-	C	0
	Skin, overgrown - left knee	?	C	0
PS82	Lung	+	T1	9
			T2	6
			C	17
	Skin, back	?	C	0
PS83	Skin, CCTN - subcutaneous tissue - right second toe	+	T	16
			C1	37
			C2	27
	Bone; tissue on bone surface - right second toe	+	T	10
			C	13
	Soft tissue - right second toe	+	C1	14
			C2	5

	Bone - right second toe	+	C	1	
	Bone; growth plate & cartilage - right tibia	+	C1	0	
			C2	0	
			C3	0	
	Skin, abnormal - right second toe	+	C	0	
	Skin, normal - left iliac crest	-	C1	0	
			C2	0	
	Bone, periosteum - right lateral proximal tibia	?	C1	0	
			C2	0	
PS84	Skin, CCTN - left nares	+	C	16	
	Soft tissue mass - frontal skull	+	C1	20	
			C2	11	
	Skin, normal - right supraclavicular region	-	C	10	
PS87	Skin, CCTN - outside of left foot near arch	+	C	20	
	Skin, normal - left upper inner arm	-	C	0	
	Skin - overlying left femur	?	C	4	
PS91	Skin, CCTN - right fourth toe	+	C	4	0/49
	Skin, normal - right upper inner arm	-	C	0	
PS93	Skin, CCTN - arch of right foot	+	C	3	3/20
	Skin, normal - left upper inner arm	-	C	2	0/20
PS95	Skin, CCTN - left third toe	+	T	5	
			C	2	
	Skin, normal - right abdominal flank	-	T	0	
			C	4	

[1]Source tissue for sample, LVEN, linear verrucous epidermal nevus; CCTN, cerebriform connective tissue nevus

[2]Affection status of sample, +, affected; -, unaffected; ?, unknown

[3]Type of sample used for DNA isolation; C, cultured cells, T, fresh or frozen uncultured tissue. For some larger specimens, multiple cultures were established or DNA was isolated from separate pieces of the tissue.

[4]Percent c.49G>A mutant allele determined by MboII assay

[5]Number of c.49G>A mutant sequences found by massively parallel sequencing/total reads at that position for each exome.