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Lack of Mutation-histopathology Correlation in a Patient with Proteus Syndrome

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Abstract

Proteus syndrome (PS) is characterized by progressive, disproportionate, segmental overgrowth and tumor susceptibility caused by a somatic mosaic *AKT1* activating mutation. Each individual has unique manifestations making this disorder extremely heterogeneous. We correlated three variables in 38 tissue samples from a patient who died with PS: the gross affection status, the microscopic affection status, and the mutation level. The *AKT1* mutation was measured using a PCR-based RFLP assay. Thirteen samples were grossly normal; six had detectable mutation (2-29%) although four of these six were histopathologically normal. Of the seven grossly normal samples that had no mutation, only four were histologically normal. The mutation level in the grossly abnormal samples was 3-35% and all but the right and left kidneys, skull, and left knee bone, with mutation levels of 19%, 15%, 26%, and 17% respectively, had abnormal histopathology. The highest mutation level was in a toe bone sample while the lowest levels were in the soft tissue surrounding that toe, and an omental fat nodule. We also show here that PS overgrowth can be caused by cellular proliferation or by extracellular matrix expansion. Additionally, papillary thyroid carcinoma was identified, a tumor not previously associated with PS. We conclude that gross pathology and histopathology correlate poorly with mutation levels in PS, that overgrowth can be mediated by cellular proliferation or extracellular matrix expansion, and that papillary thyroid carcinoma is part of the tumor susceptibility of PS. New methods need to be developed to facilitate genotype-phenotype correlation in mosaic disorders.

Keywords

Proteus syndrome; *AKT1* mutations; somatic mutation; histopathology

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INTRODUCTION

Proteus syndrome is a mosaic postnatal overgrowth disorder that occurs sporadically, with an estimated prevalence of <1 patient per 1 million [Biesecker, 2006]. It is characterized by progressive, segmental, disproportionate overgrowth. Skin, bone, and adipose tissues are the most commonly involved, though any tissue may be affected. The lesions seen in Proteus syndrome typically present within the first year or two of life [Biesecker, 2001; Twede et al., 2005]. Proteus syndrome is caused by a post-zygotic somatic activating mutation in *AKT1*, in the AKT-PI3K cellular growth signaling pathway [Lindhurst et al., 2011], supporting the somatic mosaicism hypothesis of segmental overgrowth [Happle, 1987].

Individuals with somatic mosaicism have two (or more) genotypically distinct populations of cells that may be contained in different parts of the body [Biesecker and Spinner, 2013]. Molecular detection of mosaicism is challenging for several reasons. First, mosaicism levels can vary widely and can be difficult to detect with molecular assays. The levels of mutations in a tissue can range from 50% (non-mosaic heterozygous level) down to, and sometimes below, the level of even the most sensitive assay. Second, mosaicism may be tissue-specific or tissue-limited. Sampling from multiple tissues within an individual may be necessary for detection of mosaicism, which can severely constrain testing due to the necessary and appropriate limitations of sampling, and in some cases, ethical considerations. In the case of Proteus syndrome, the *AKT1* somatic mutation is rarely present in blood [Lindhurst et al., 2011], and a skin biopsy or other tissue sampling is necessary for molecular detection.

Genotype-phenotype correlations for non-mosaic disorders are carried out by comparing the genes (for disorders with locus heterogeneity) and the gene variants (for disorders with allelic heterogeneity) that are mutated in affected individuals. In mosaic disorders, the challenge is to adapt our genotype-phenotype correlation approaches to add the complexity of within-subject variation in tissue mutational level. Proteus syndrome is an excellent candidate for this challenge because it has, to date, exhibited neither locus nor allelic heterogeneity – to date every patient has the same heterozygous mutation in the same gene. The mutational load of the c.49G>A *AKT1* variant in each tissue is hypothesized to be the primary source of variation.

We set out to test the hypothesis of correlation of mutation levels to tissue phenotype due to the opportunity provided by the tragic and untimely death of a young woman with this disorder and the generosity of her next of kin. The Proteus syndrome pathological-mutational analysis presented here is an unprecedented evaluation of somatic mosaicism in tissues that are normally unfeasible to sample.

CLINICAL REPORT

The patient, a 21-year-old Caucasian female, was admitted to the primary hospital with a chief complaint of constipation, abdominal pain, and severe, acute rectal bleeding. The history was significant for a clinical diagnosis of Proteus syndrome requiring multiple surgeries, including suboccipital decompression with laminectomy and shunting of an Arnold-Chiari type I malformation, several bilateral external auditory canaloplasties with

subsequent bone regrowth, and a right bone assistance hearing aid placement. She had been bedridden for seven years before expiration and suffered from multiple pressure ulcers. She was anemic and hypothermic on admission. An abdominal CT scan showed a large lobular mass of the rectum and anus compatible with a malignancy with suspected metastatic disease in the retroperitoneum and the spleen. An MRI showed multiple perirectal lesions and bowel wall thickening. Subsequent rectal biopsy showed only acute inflammatory changes and was negative for malignancy. Shortly after the patient's arrival to the referral facility, she began to vomit brown emesis and became unresponsive. She was resuscitated and intubated. An esophagogastroduodenoscopy demonstrated multiple gastric ulcers. The patient's condition deteriorated, she became hypotensive and acidotic, and experienced worsening pulmonary function. She expired eight days after admission. The primary cause of death per autopsy was "Proteus syndrome complicated by rectal bleed (with ulceration and extensive internal rectal hemorrhoids) and acute respiratory failure with pulmonary microemboli and focal acute lung injury."

MATERIALS AND METHODS

Autopsy

The autopsy was performed using the Letulle method. Samples of grossly normal and abnormal tissue (if present) from each organ were placed in 10% neutral-buffered formalin in addition to being snap frozen for molecular studies. After a 48-hour formalin fixation the samples were embedded in paraffin and stained with hematoxylin and eosin for histopathologic examination. The brain was fixed in formalin for an additional 12 days.

Tissue Preparation and DNA Isolation

From the initial tissue samples obtained by autopsy, we dissected between one and four pieces of approximately 1 cm³ for DNA isolation to evaluate mutation level across the sample. Tissue was digested using 125 µg of proteinase K for 24 hours. Genomic DNA was subsequently isolated by phenol/chloroform extraction and ethanol precipitation. Bone samples were digested overnight in proteinase K and the supernatant was removed and processed following the procedure for soft tissue. The remaining bone fragments were partially powdered using a TissueRupter (Qiagen, Valencia, CA) and re-digested overnight with proteinase K. DNA was extracted from the lysate as described above.

AKT1 Mutation Analysis

The *AKT1* mutation level in a DNA sample was determined using a custom restriction fragment length polymorphism (RFLP) assay as described [Lindhurst et al., 2011]. The DNA was first amplified in a polymerase chain reaction using a FAM-labeled forward primer and a modified reverse primer that encompasses the Proteus syndrome *AKT1* mutation (c.49G>A) to create an MboII restriction site in the presence of the mutation. Subsequent MboII digestion produces a 122 bp fragment from cut mutant alleles, whereas the uncut wildtype allele is 141 bp.

Fragments were detected on the ABI 3130 using several injection times for optimal peak height. The mutation level was calculated as a ratio of the area under the mutant peak

divided by the combined areas under the mutant and wildtype peaks. This assay has a lower limit sensitivity of 0.5%. Individual DNA preparations were tested in duplicate and with the same reagent mix to improve accuracy. Mutation levels from individual DNA preparations from soft tissues were averaged to obtain the final mutation level for each sample. For bone samples, the mutation levels in the soft tissue and bone were reported separately.

This study is not considered human subjects research under US Federal regulations (45CFR46).

RESULTS

Figures that show images for the systems described in each section are identified in the section headings. All tissue samples and mutation levels referred to in the results section are identified and described in Table I.

Autopsy Findings

The decedent was a Caucasian female with a weight of 112 lbs, length of 188 cm, and BMI of 14.4, appearing consistent with the stated age of 21.

Musculoskeletal and Skin (Figures 1A, 1B, 2, 3, Supplementary Figure 1)

The head was dolichocephalic and deformed, measuring 45.5 cm in circumference. The palpebral fissures were narrow and down-slanted. The nose had a low bridge with widened nares. The mouth was open with the teeth in good state of repair. There was a large sagittal bony prominence of the skull measuring 5 cm in thickness. The diploic space of the skull bone was grossly widened and the cortex was grossly thickened. There was also a 1.5×0.8 cm bony intracranial tumor near the right auditory meatus. The ears were low set. The external auditory canals were nearly occluded by bony overgrowth. There was a bone-anchored hearing aid on the right temporal region.

There was extreme kyphoscoliosis with marked deformity of the rib cage shifted to the left. The extremities were asymmetric. The long bones of the arms and legs were markedly elongated and thin. The knees were grossly enlarged and deformed (circumferences of 47.5 cm left and 41 cm right). The toes were grossly deformed bilaterally. There were multiple, large cerebriform masses on the soles of the feet covering over half of the soles that were >5 cm thick. There was a large violaceous linear vascular lesion from the trochanter to mid calf on the lateral left leg. The right index and middle fingers were enlarged at the proximal and distal interphalangeal joints and were deformed with ulnar deviation of the middle finger.

Neural (Supplementary Figure 1)

The right eye was proptotic. The right sclera was blood-injected and the left sclera was white. The brain weighed 1,325 g (fixed) with multiple surface indentations. There were several 1–1.5 cm intracranial bosselated calvarial soft tissue tumors in the posterior fossa, and along the greater wing of the right sphenoid bone.

Cardiothoracic (Supplementary Figure 2)

The chest was asymmetric with an increase in anteroposterior diameter and measured 100.5 cm in circumference. The sternum was displaced to the left. The thoracic organs were in their normal anatomic position. The mediastinum was shifted due to the severe scoliosis. The heart weighed 140 g (expected for weight: 166–356 g). The coronary arteries had mild atherosclerotic changes. The left and right cardiac chambers were not dilated. The foramen ovale was sealed. The endocardium was transparent with no mural thrombi. The myocardium was red-brown and uniform without focal lesions. The right ventricle was 0.2 cm thick and the left ventricle was 0.7 cm thick. Cut sections of papillary muscles were unremarkable. The lungs weighed 450 g on the right and 110 g on the left (expected 450 and 375 g, respectively). The small left lung was associated with the severe scoliosis and secondary skeletal restriction. The right lung was grossly normal.

Alimentary tract (Figure 1C, Supplementary Figure 3)

The abdominal organs were in their normal anatomic position. The omental and mesenteric fat was abundant and overgrown. The omentum had nodular areas ranging in size from 1.8 – 4.0 cm in greatest dimension. The colon contained melanotic stool. The mucosa was intact. Multiple large diverticuli were identified. The rectum had a 1 × 0.5 cm mucosal ulcer and internal hemorrhoids forming rectal masses. The liver (660 g, expected 1,650 g) had a sharp anterior margin. The capsule was smooth and glistening. On cut section, the parenchyma was red-brown and firm. No focal lesions were seen. The pancreas was small and yellow with preserved architecture.

Genitourinary (Figure 1D, Supplementary Figure 4)

The kidneys had capsules that stripped with difficulty to show a smooth surface with several small simple cysts filled with yellow fluid and larger hemorrhagic cysts present at the inferior pole of the right kidney. There were bilateral perirenal hemorrhagic masses composed of cysts filled with blood. The vaginal mucosa was intact. The uterus was 14 × 5.5 × 4 cm and was remarkable for a 1.5 × 1.5 cm myometrial nodule located at the dome. The rest of the myometrium was unremarkable. There were two periuterine multicystic hemorrhagic masses: one measuring 6 × 3 × 3 cm and the other measuring 1.5 × 1 cm. The ovaries were bilaterally pale and enlarged measuring 8 × 3.5 cm on the right and 8 × 4.5 cm on the left. The stroma was homogenous with numerous dilated follicular cysts on cut sections. No focal masses were identified. The breasts were grossly unremarkable.

Endocrine (Figure 1E, Supplementary Figure 5)

The thyroid weighed 14 g and was red-brown with a 1.5 × 0.5 cm white-tan pale nodule in the upper right lobe.

Lymphohematopoietic (Supplementary Figure 5)

The spleen weighed 160 g (expected for age: 155 g) and had an irregular nodular surface. Upon sectioning the splenic parenchyma contained numerous well-circumscribed microcystic brown to black nodules ranging from 0.5 to 3 cm in greatest dimension.

Microscopic Evaluation and Mutation Levels

Musculoskeletal and Skin (Figures 1A, 1B, 2, 3, Supplementary Figure 1)

Microscopic sections of the sagittal bony prominence demonstrated viable and unremarkable mature bone. The soft tissue surrounding the bone (sample T2) demonstrated a mutation level of 26%. The bony intracranial tumor (sample V2) was microscopically consistent with an osteoma demonstrating thickened bony trabeculae and a mutational level of 26% with the surrounding soft tissue demonstrating a level of 21%. Multiple sections of grossly normal skin from the feet (samples A1, B1, E1) demonstrated preservation of the superficial and deep dermis with scattered eccrine glands. Focal areas of the deep dermis appeared to have thickened bands of collagen embedded in loose connective tissue. The underlying skeletal muscle was atrophic. The epidermis showed mild keratosis and preserved rete pegs. Molecular testing demonstrated mutational levels of 0–4%. Sections of abnormal skin (samples A2, B2, D2, E2, F2, G2, J2, K2) demonstrated attenuation of the epidermis with loss of rete pegs and surface hyperkeratosis. The superficial dermis was completely replaced by diffuse bands of collagen, while deeper in the dermis the collagen formed nodules. Scattered fibroblasts were seen in the hypertrophic collagen bundles. Deep collagenous nodules were separated by loose connective tissue containing scattered eccrine glands. No hair follicles were identified in sections examined. Deep nerve trunks were surrounded by bands of collagen extending from adjacent nodules. These findings are consistent with a diagnosis of cerebriiform connective tissue nevi (CCTN) of the feet. Molecular testing demonstrated mutational levels of 8–14% for these samples. Multiple sections of grossly normal (samples C1 and D1) and abnormal (samples C2 and H2) bone demonstrated osteopenic viable bone with normal cortices and fatty marrow. Molecular testing demonstrated mutational levels of 17% for bone from D1 with the surrounding soft tissue demonstrating 29%, 35% for bone from C2 with the surrounding soft tissue demonstrating 3%, and 21% for bone from H2 with the surrounding soft tissue demonstrating 16%. Bone from the right medial malleolus (sample C1) was negative for mutation. Bone from the left lateral knee (sample I2) demonstrated a fibrocollagenous cup with no significant histopathologic findings. Molecular testing demonstrated mutational levels of 24% with the surrounding soft tissue demonstrating 17%.

Neural (Supplementary Figure 1)

Representative sections of brain were unremarkable microscopically. The intracranial soft tissue tumors demonstrated whorls of spindle cells and psammoma bodies and were consistent with meningiomas. A section of one of the meningiomas (sample U2) demonstrated a mutation level of 27% (+/– 3%).

Cardiothoracic (Supplementary Figure 2)

Both the left and right ventricles in addition to the left and right coronary arteries were microscopically unremarkable (Supplementary Figure 4). Both lobes of the left lung microscopically demonstrated atelectasis, fibrous thickening of the interlobular septa with prominent enlarged muscular pulmonary veins in the septa and focal dystrophic calcifications. The right lung was grossly unremarkable but microscopically demonstrated

intraalveolar hemorrhage, focal atelectasis, congestion, and thickened alveolar septa. Additionally, the right upper and lower lobes demonstrated regional acute lung injury with hyaline membranes and superimposed acute pneumonitis. Both lungs demonstrated multiple pulmonary thromboemboli with focal early organization and focal marked fibrous intimal hyperplasia (muscular pulmonary arteries), consistent with hypertensive arteriopathy. Both the right and left lung (samples K1 and L1) demonstrated no detectable *AKT1* mutation.

Alimentary tract (Figure 1C, Supplementary Figure 3)

The omental nodules (samples L2 and M2) demonstrated hemorrhagic fat necrosis with mutation levels of 6% (+/- 1%) and 3% (+/- 0.3%). Normal omental fat (sample G1) demonstrated mature adipose tissue with a mutation level of 13% (+/- 2%). Microscopically, the rectal ulcer demonstrated denuded mucosa and granulation tissue. Rectal varices (samples R1 and S1) demonstrated large, ectatic vessels filled with blood and organizing thrombi. The liver (sample J1) demonstrated sinusoidal congestion and bile stasis and had no detectable mutation levels. The pancreas (sample U1) was microscopically unremarkable.

Genitourinary (Figure 1D, Supplementary Figure 4)

Both kidneys on microscopic examination demonstrated focal interstitial nephritis and occasional glomerulosclerosis. A right renal cyst (sample AA2) demonstrated a fibrous lining with hemosiderin. The right kidney (sample X2) demonstrated a mutation level of 19% (+/- 3%) while the left kidney (sample Y2) demonstrated a mutation level of 15% (+/- 1.0%). The perirenal hemorrhagic cysts microscopically represented vascular malformations. The vaginal mucosa (sample H1) was intact with congestion on microscopy and demonstrated a mutation level of 17% (+/- 5%). The myometrial nodule (sample S2) located at the dome of the uterus confirmed microscopically to represent a hyalinized leiomyoma, demonstrated a mutation level of 26% (+/- 5%). Both of the periuterine multicystic masses (samples O2 and P2) microscopically represented vascular malformations and had mutation levels of 12% (+/- 3%) and 16%. Microscopic sections of the ovaries showed extensive fibrosis and hyalinization of the outer cortex forming a band-like collagenous surface layer containing fibroblasts/myofibroblasts, but no primary follicles. The underlying ovarian stroma, though morphologically normal, was significantly attenuated and contained scattered primary follicles, some of which contained ova. There were a few subcortical, cystically dilated follicles lined by granulosa cells and a bilayer of theca interna and externa cells. None of these follicles were larger than 1 cm. No corpora lutea were identified in any of the sections examined. There were few atretic follicles present. There was extensive hilar fibrosis containing intermixed dense and loose patterns of fibrous tissue. The ovaries (samples Q2 and R2) demonstrated a mutation level of 26% (+/- 1%) and 25% (+/- 2%).

Microscopic sections of the breast showed diffuse and extensive fibrous expansion of the stroma with numerous atrophic terminal duct lobular units. There was no adipose tissue identified. Many lobules appeared hyalinized with a thickened basement membrane. The bilayer of epithelial and myoepithelial cells was preserved. The intralobular stroma in many

areas blended with a dense and hyalinized extralobular stroma containing scattered myofibroblasts. Right breast tissue (sample F1) had a mutation level of 12% (+/- 3%).

Endocrine (Figure 1E, Supplementary Figure 5)

Sections of the thyroid showed simple goiter. The thyroid nodule sections showed a diffuse sclerosing variant of papillary thyroid carcinoma with numerous psammoma bodies. The tumor focally retained its nodular appearance and showed mixed papillary and follicular differentiation. The background stroma appeared dense and focally hyalinized with prominent myofibroblasts and scattered chronic inflammatory cells. No extrathyroidal extension or lymphovascular invasion was identified. Normal-appearing thyroid (sample M1) demonstrated an undetectable mutation level (sample M1) while the thyroid nodule (sample Z2) had a mutation level of 19% (+/-15%).

Lymphohematopoietic (Supplementary Figure 5)

Microscopic sections showed a background of normal spleen containing numerous vascular malformations. The proliferating vessels showed mixed cavernous and microcystic thin-walled patterns focally separated by bands of collagenous stroma. Focally, the stroma contained crystalloid deposits of calcium mixed with iron, reminiscent of Gamna-Gandy bodies. Normal-appearing spleen (sample I1) demonstrated an undetectable mutation level while the vascular malformations (sample W2) demonstrated a mutation level of 7% (+/- 1%).

DISCUSSION

The proliferation of mosaic genetic disorders demands that we develop methods to perform genotype-phenotype associations, now at the tissue level. We describe here a broad mutation-histopathology correlation of a patient with severe manifestations of Proteus syndrome. Proteus syndrome is universally caused by a heterozygous, mosaic gain of function mutation in *AKT1* [Lindhurst et al., 2011] and it has been proposed that the distribution and level of the mutation in tissues is what determines the severity and extent of the disorder in affected patients. Because of sampling limitations, it has never before been possible to determine the level of the *AKT1* mutation and histology in multiple tissues from a single patient (Figure 4). Our primary questions were whether there was a correlation of abnormal gross appearance/histopathology with the level of mutation in tissues and what the distribution of mutation positive tissues was in the body.

In this patient, all grossly abnormal tissues were mutation positive, though four demonstrated no significant histopathologic findings (I2, T2, X2, Y2) and were considered microscopically normal. The mutation level in the grossly abnormal samples ranged from 3-35%. Six of 13 grossly normal tissues were mutation positive (range 2-29%). Five of the grossly normal tissues had significant histopathologic findings (C1, D1, F1, K1, L1) and were considered microscopically abnormal; however, only two of these were mutation positive (D1 and F1). Eight of the grossly normal tissues were considered microscopically normal; four of these were mutation positive (B1, E1, G1, H1). Of the seven grossly normal,

mutation negative tissues, three had a notable histopathologic finding (C1, K1, L1) and were considered microscopically abnormal.

Twenty-four of 38 tissues from the autopsy of this patient were judged to be grossly and/or microscopically abnormal with evidence of neoplasia, hyperplasia, extracellular matrix expansion, or gross overgrowth. Of these abnormal tissues, all were found to harbor the c. 49G>A *AKT1* mutation. Six other samples were determined to be grossly or microscopically abnormal without evidence of overgrowth and of these, three were mutation negative (C1, K1, L1). Overall, 30 of 38 tissues were determined to be grossly and/or microscopically abnormal and of these 30, only three were mutation negative. In contrast, of the eight tissues judged to be grossly and histologically normal, four were identified to have detectable levels of the mutation. From these data, we conclude that mutation level does not correlate well with gross and microscopic abnormality; grossly and microscopically normal tissue may still harbor a mutation load, though grossly affected tissues demonstrate an increased, but wide range of mutation levels (3-35%). In addition, the mutation positive tissues were apparently randomly distributed throughout the body. Interestingly, the highest mutation level was in a bone sample from the second digit of the right foot while the lowest levels were found in the soft tissue surrounding that bone, as well as in an omental fat nodule.

Multiple tissue samples dissected from the same organ harbored distinct mutation levels. For example, grossly normal splenic tissue was negative for mutation while the splenic vascular malformation taken from an adjacent region was positive. Even when specimens were subdivided, they exhibited distinct mutation levels as was shown by the widely varying standard deviations from several tissues (Table I). Nearly all of the specimens, including those that were mutation negative, were divided into two to four pieces that were processed and assayed for the *AKT1* mutation separately. The greatest variation was found in the specimen from the thyroid tumor where the individual pieces had mutation levels of 8% and 30%. This, in addition to the phenotypic findings, exemplifies the mosaic state of this syndrome.

Proteus syndrome is considered to be a tumor predisposition and overgrowth syndrome [Cohen et al., 2002; Gordon et al., 1995]. There are a number of overgrowth syndromes that have a numerically large relative risk of one or two tumors (e.g., Wilms tumor and hepatoblastoma in Beckwith-Wiedemann syndrome). In contrast, patients with Proteus syndrome seem to be at risk for a wide range of tumors, each of which are numerically low risks. For example, a single patient has been reported to have a mesothelioma of the tunica vaginalis [Malamitsi-Puchner et al., 1990] and ductal carcinoma *in situ* of the breast [Iqbal et al., 2006]. The patient described here had a papillary thyroid carcinoma (PTC), which has not to our knowledge been reported with Proteus syndrome, though it is the most common type of thyroid cancer and accounts for about 74–80% of all thyroid cancers.

The histopathologic patterns identified in this patient can be categorized as cellular overgrowth and extracellular matrix expansion (Figure 1). Of the total of 38 specimens that were tested, ten demonstrated cellular overgrowth (G1, L2, M2, O2, P2, S2, U2, V2, W2, Z2). These findings can be explained by the anti-apoptotic and pro-proliferative nature of

activated AKT1 [Osaki et al., 2004]. However, the majority of the specimens manifested extracellular matrix expansion (12 of 38) in the skin, breast, ovaries and bone. We hypothesize that the specific type of overgrowth may be due to which cell types harbor the mutation. If fibroblasts harbor the mutation, the resultant phenotype is extracellular matrix expansion. If other cell types are mutation positive, the resultant phenotype is cellular overgrowth, which can manifest as nodules, tumors and cysts or simply as increased tissue mass as was seen in the increased amount of omental fat. Previous studies on superficial skin scrapings from epidermal nevi, which are characterized by epidermal hyperplasia, demonstrated high levels of mutant allele in the keratinocytes [Wieland et al., 2013]. Our previous work showed that in epidermal nevi both the keratinocytes and fibroblasts are mutation positive, and in CCTN where there is massive deposition of extracellular matrix, only the fibroblasts harbor the PS mutation [Lindhurst et al., 2014]. The data from this patient corroborate this finding in additional tissues and demonstrate that PS overgrowth can be due to either or both cellular overgrowth and extracellular matrix expansion.

These data show not only that the cellular mechanism of overgrowth in Proteus syndrome can be heterogeneous, but more importantly show that the overgrowth is poorly correlated with bulk tissue mutation levels from specimens obtained at autopsy. Indeed, if we cannot identify genotype-phenotype correlations from dozens of samples at autopsy, there is little reason to believe that we can predict phenotype from mutation determination through sampling of limited tissues in a living patient. That these data do not show a correlation of phenotype to bulk mutational load in these tissues leads us to hypothesize that the distribution of mutant cells within specific cellular subtypes determines the mechanism of overgrowth. New methods to identify mutation positive cells *in situ* will be necessary to test this hypothesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Representative Examples of Overgrowth

A) The left knee was grossly overgrown but microscopically (I2) demonstrated no significant histopathologic diagnoses and showed neither cellular proliferation nor extracellular matrix (ECM) expansion. The mutational level for the bone and surrounding soft tissue was 24% (I2B) and 17% (I2ST), respectively. B) The left foot grossly showed a cerebriform connective tissue nevus on the sole. Microscopic sections of the lateral sole (J2) and medial sole (K2) demonstrated attenuation of the epidermis, diffuse bands and nodules of dense collagen in the dermis and expansion of the ECM. The specimens demonstrated mutational levels of 11% (+/- 1) and 10% (+/- 1), respectively. C) The abdominal cavity was significant for omental overgrowth but was grossly normal. Microscopically, the omentum (G1) demonstrated mature adipose tissue with no histopathologic abnormalities and a mutational level of 13% (+/- 2). D) The right breast was grossly normal but demonstrated fibrous stromal overgrowth with atrophic terminal duct lobular units and lack of adipose tissue microscopically (F1). The mutational level was 12% (+/-2). E) The right thyroid lobe was significant for a firm white-tan nodule that demonstrated papillary thyroid carcinoma microscopically (Z2) with a mutational level of 19% (+/-15). The red area of the pie charts labeling the microscopic sections represent the *AKT1* c.49G>A mutation level in the corresponding sample used for molecular testing.

Figure 2. Skeletal deformities

A) The bilateral lower extremities were elongated and thin with grossly enlarged knees bilaterally. The skin of the left lateral leg showed a large violaceous linear vascular lesion. The left lateral femoral head (D1) and the right medial knee (H2) both demonstrated osteopenic viable bone with normal cortices and fatty marrow. Mutation levels for the left lateral femoral head bone (D1B) and the surrounding soft tissue (D1ST) was 29% and 17%, respectively. The mutational levels for the right medial knee bone (H2B) and surrounding soft tissue (H2ST) were 21% and 16%, respectively. B) The index and middle fingers of the right hand were enlarged at the proximal and distal interphalangeal joints and were deformed with ulnar deviation of the middle finger. The left hand was grossly normal. C) The spine demonstrated extreme kyphoscoliosis with marked deformity of the rib cage shifted to the left. The red area of the pie charts labeling the microscopic sections represent the *AKT1* c.49G>A mutation level in the corresponding sample used for molecular testing.

Figure 3. Foot deformities

A) The right foot (medial view) showed a large cerebriiform connective tissue nevus on the sole of the foot in addition to deformation of the digits. Grossly normal skin from the medial sole (A1) and the dorsum (B1) microscopically demonstrated mild epidermal keratosis and preserved rete pegs with preservation of the superficial and deep dermis with scattered eccrine glands. The mutational levels were 0 and 4% (+/-1), respectively. Grossly abnormal skin from the right great toe (A2), medial sole (E2), central sole (F2), and medial heel (G2) microscopically demonstrated attenuation of the epidermis with loss of rete pegs and surface hyperkeratosis in addition to diffuse bands and nodules of dense dermal collagen. Mutational levels were 13% (+/-6), 12% (+/-4), 14 (+/-2), and 7% (+/-0.2), respectively. Bone from the medial malleolus (C1) demonstrated osteopenic viable bone with normal cortices and fatty marrow. The bone (C1B) and the surrounding soft tissue (C1ST) were both negative for mutation. B) A lateroplantar view of the right foot further shows the extent of the cerebriiform nevus. Bone from the second right toe (C2) microscopically demonstrated osteopenic viable bone with normal cortices and fatty marrow. The mutational level was 35% for the bone (C2B) and 3% for the surrounding soft tissue (C2ST). Grossly abnormal skin from the second right toe (B2) and fifth right toe (D2) microscopically demonstrated attenuation of the epidermis with loss of rete pegs and surface hyperkeratosis in addition to diffuse bands and nodules of dense dermal collagen. Mutational levels were 13% (+/-2) and 8%, respectively. C) The left foot (dorsal view) was grossly similar to the right foot. Grossly normal skin from the dorsum (E1) microscopically demonstrated mild epidermal keratosis and preserved rete pegs with preservation of the superficial and deep dermis with scattered eccrine glands. The mutational level was 2% (+/-3). The red area of the pie charts labeling

the microscopic sections represent the *AKT1* c.49G>A mutation level in the corresponding sample used for molecular testing.

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Figure 4. Diagram indicating locations and level of *AKT1* c.49G>A mutation of specimens collected for molecular testing

Square = grossly normal specimens, Triangle = grossly abnormal specimens, Blue = 0% mutational level, Dark purple = 1–5% mutational level, Light purple = 5–10% mutational level, Light pink = 10–15% mutational level, Dark pink = 15–20% mutational level, Red = >20% mutational level

Table 1

Descriptions and mutational levels of samples assayed for the *AKT1* c.49G>A mutation.

Anatomic Location	External Description	Organ/Structure	Gross Findings	Sample ID	Sample Description	Gross Description	Microscopic Description	Mutation Level (%)	Cellular Proliferation vs. ECM Expansion	Microscopic Findings
Head/Neck	Large, thickened sagittal bony prominence of skull bone; facies consistent with features of Proteus syndrome	Brain	Multiple indentations; compression	NT	NT	Abnormal	Normal	NT	None	NSHA
		Intracranial soft tissue	Multiple intracranial soft tissue tumors	U2	Intracranial soft tissue tumor	Abnormal	Abnormal	27 (±3)	Cellular Proliferation	Meningioma
		Skull	Sagittal bony prominence; intracranial bone tumor	T2	Bone from skull	Abnormal	Normal	Soft tissue - 26	ECM Expansion	NSHA
				V2	Intracranial bone tumor	Abnormal	Abnormal	Bone - NA Soft tissue - 21 Bone - 26	Cellular Proliferation	Osteoma
Trunk	Abdominal distension, sternal and rib deformities; severely decreased left thoracic cavity; severe spinal deformity to the left	Breast	Normal	F1	Right breast	Normal	Abnormal	12 (±3)	ECM Expansion	Fibrous stromal overgrowth
		Kidney	Large hemorrhagic cyst of right kidney	X2	Right kidney	Abnormal	Normal	19 (±3)	None	NSHA
				Y2	Left kidney	Abnormal	Normal	15 (±1)	None	NSHA
		Liver	Normal	J1	Liver	Normal	Normal	0	None	Congestion, bile stasis; NSHA
		Lungs	Left lung severely compressed and small; right lung normal	K1	Right lung	Normal	Abnormal	0	None	Atelectasis
				L1	Left lung	Normal	Abnormal	0	None	Pulmonary thromboemboli
		Omentum	Overgrowth	G1	Omental fat	Normal	Normal	13 (±2)	Cellular Proliferation	NSHA
				L2	Omental nodule 1	Abnormal	Abnormal	6 (±1)	Cellular Proliferation	Fat necrosis
				M2	Omental nodule 2	Abnormal	Abnormal	3 (±0.3)	Cellular Proliferation	Fat necrosis
		Ovaries	Bilateral enlargement; follicular cysts	Q2	Right ovary	Abnormal	Abnormal	26 (±1)	ECM Expansion	Fibrous stromal overgrowth
				R2	Left ovary	Abnormal	Abnormal	25 (±2)	ECM Expansion	Fibrous stromal overgrowth
		Spleen	Multiple cystic and solid nodules	I1	Spleen	Normal	Normal	0	None	NSHA
		Thyroid	Right lobe nodule	W2	Splenic tumor	Abnormal	Abnormal	7 (±1)	Cellular Proliferation	Vascular malformation
				M1	Thyroid, left lobe	Normal	Normal	0	None	Simple goiter; NSHA
		Uterus	Uterine dome nodule; perituterine hemorrhagic cysts	Z2	Thyroid tumor	Abnormal	Abnormal	19 (±15)	Cellular Proliferation	Papillary thyroid carcinoma
				O2	Perituterine hemorrhagic cyst 1	Abnormal	Abnormal	12 (±3)	Cellular Proliferation	Vascular malformation
				P2	Perituterine hemorrhagic cyst 2	Abnormal	Abnormal	16	Cellular Proliferation	Vascular malformation
		Vagina	Normal	S2	Uterine dome nodule	Abnormal	Abnormal	26 (±5)	Cellular Proliferation	Leiomyoma
				H1	Vagina	Normal	Normal	17 (±5)	None	Congestion; NSHA

Anatomic Location	External Description	Organ/Structure	Gross Findings	Sample ID	Sample Description	Gross Description	Microscopic Description	Mutation Level (%)	Cellular Proliferation vs. ECM Expansion	Microscopic Findings
Lower Extremities		Right leg	Knee enlargement	H2	Bone - right medial knee	Abnormal	Abnormal	Soft tissue - 16 Bone - 21.4	None	Osteopenic bone with fatty marrow
				C1	Bone - right medial malleolus	Normal	Abnormal	Soft tissue - 0 Bone - 0	None	Osteopenic bone with fatty marrow
		Right foot	Severely deformed; CCTN	A1	Skin - medial sole of right foot	Normal	Normal	0	None	Thickened collagen; NSHA
				B1	Skin - dorsum of right foot	Normal	Normal	4 (±1)	None	Thickened collagen; NSHA
				E2	Skin - medial sole of right foot	Abnormal	Abnormal	12 (±4)	ECM Expansion	CCTN
				F2	Skin - central sole of right foot	Abnormal	Abnormal	14 (±2)	ECM Expansion	CCTN
				G2	Skin - medial heel of right foot	Abnormal	Abnormal	7 (±0.2)	ECM Expansion	CCTN
				A2	Skin - right great toe	Abnormal	Abnormal	13 (±6)	ECM Expansion	CCTN
				B2	Skin - second right toe	Abnormal	Abnormal	13 (±2)	ECM Expansion	CCTN
				C2	Bone - second right toe	Abnormal	Abnormal	Soft tissue - 3 Bone - 35	None	Osteopenic bone with fatty marrow
		Left leg	Knee enlargement; cutaneous vascular malformation	D2	Skin - fifth right toe	Abnormal	Abnormal	8	ECM Expansion	CCTN
				D1	Bone - left lateral femoral head	Normal	Abnormal	Soft tissue - 17 Bone - 29	None	Osteopenic bone with fatty marrow
		Left foot	Severely deformed; CCTN	I2	Bone - left lateral knee	Abnormal	Normal	Soft tissue - 17 Bone - 24	None	Fibrocollagenous cup; NSHA
				E1	Skin - dorsum of left foot	Normal	Normal	2 (±3)	None	Thickened collagen; NSHA
		Left foot		J2	Skin - lateral sole of left foot	Abnormal	Abnormal	11 (±1)	ECM Expansion	CCTN
				K2	Skin - medial sole of left foot	Abnormal	Abnormal	10 (±1)	ECM Expansion	CCTN

ECM, extracellular matrix; NT, not tested; NSHA - no significant histopathologic abnormalities; CCTN, cerebriform connective tissue nevus