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Gene Expression Profiling in Pachyonychia Congenita Skin

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Abstract

Background—Pachyonychia congenita (PC) is a skin disorder resulting from mutations in keratin (K) proteins including K6a, K6b, K16, and K17. One of the major symptoms is painful plantar keratoderma. The pathogenic sequelae resulting from the keratin mutations remain unclear.

Objective—To better understand PC pathogenesis.

Methods—RNA profiling was performed on biopsies taken from PC-involved and uninvolved plantar skin of seven genotyped PC patients (two K6a, one K6b, three K16, and one K17) as well as from control volunteers. Protein profiling was generated from tape-stripping samples.

Results—A comparison of PC-involved skin biopsies to adjacent uninvolved plantar skin identified 112 differentially-expressed mRNAs common to patient groups harboring K6 (*i.e.*, both K6a and K6b) and K16 mutations. Among these mRNAs, 25 encode structural proteins including keratins, small proline-rich and late cornified envelope proteins, 20 are related to metabolism and 16 encode proteases, peptidases, and their inhibitors including kallikrein-related peptidases (*KLKs*), and serine protease inhibitors (*SERPINS*). mRNAs were also identified to be differentially

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Conflict of Interest

None.

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expressed only in K6 (81) or K16 (141) patient samples. Furthermore, 13 mRNAs were identified that may be involved in pain including nociception and neuropathy. Protein profiling, comparing three K6a plantar tape-stripping samples to non-PC controls, showed changes in the PC corneocytes similar, but not identical, to the mRNA analysis.

Conclusion—Many differentially-expressed genes identified in PC-involved skin encode components critical for skin barrier homeostasis including keratinocyte proliferation, differentiation, cornification, and desquamation. The profiling data provide a foundation for unraveling the pathogenesis of PC and identifying targets for developing effective PC therapeutics.

Introduction

Pachyonychia congenita (PC) is a rare keratinization disorder resulting from autosomal dominant-negative mutations in keratin genes that are expressed in epithelial tissues [1–3]. Symptoms include thickened dystrophic nails, palmoplantar keratoderma, leukokeratosis, and follicular hyperkeratosis [4–6]. The major patient complaint is painful plantar keratoderma (possibly due to fissuring, secondary infections, increased pressure on nerve endings, underlying blistering and/or cutaneous thromboses [7–10]), where intense pain negatively impacts quality of life and often necessitates ambulatory aides and pain medication.

Keratins heterodimerize and further associate to form intermediate filaments, which provide mechanical strength and resilience to epithelial cells and tissues [11, 12]. Mutant keratins negatively impact skin structure and function, at least in part, by destabilizing the existing intermediate filament networks within cells [13–15], resulting in faulty intermediate filaments and cell fragility, which can lead to skin disorders including epidermolytic ichthyosis (also known as epidermolytic hyperkeratosis, caused by keratin (*KRT*) 1/*KRT10* gene mutations) [16, 17], epidermolysis bullosa simplex (*KRT5/KRT14*) [18, 19], and PC (*KRT6A/6B/16/17*) [1, 2, 5]. The majority of PC mutations are heterozygous missense or small insertion/deletion mutations that disrupt cytoskeletal function, presumably by preventing proper protein/protein interactions [20, 21]. In PC, this manifests as hyperplasia and hyperkeratosis in the subset of differentiated epithelial tissues in which mutant keratins including K6a, K6b, K16 and K17 are predominantly expressed [5], specifically in the palmoplantar epidermis, nail bed, oral mucosa, and the pilosebaceous unit. Surprisingly, *Krt16* knockout mice exhibit palmoplantar keratoderma (PPK)-like lesions, one of the hallmark features of PC [9, 22], in the absence of keratin mutations, suggesting that the pathogenesis of PC is more complex than previously appreciated.

In this study, we performed gene expression microarray analysis of RNA extracted from PC patient biopsies to examine changes in mRNA expression in PC-involved versus uninvolved and non-PC plantar skin to better understand the pathogenesis of PC at the molecular level, including the intense idiopathic pain associated with plantar keratoderma. Proteomic profiling of stratum corneum supported the findings herein.

Materials and Methods

Subjects

Seven patients from the International PC Research Registry (IPCRR) were identified by the Pachyonychia Congentia Project (www.pachyonychia.org) for evaluation in this study. Specific mutations in all seven IPCRR patients were previously identified, and the genotyping results were confirmed by a Clinical Laboratory Improvement Amendments (CLIA)-certified laboratory. The mutations of the participating PC patients are shown in Table I. Using local anesthesia, a matched pair of 3 or 4 mm full-thickness plantar skin punch biopsies, one from PC-involved skin and the other from adjacent uninvolved skin (typically within 1–2 cm but as far as 5 cm), was collected from each patient listed in Table I. The biopsy sites for one of the PC patients (IPCRR #1009, harbors a K16 R127C mutation) are shown in Fig. 1. Plantar biopsies from two healthy, non-PC volunteers (controls) were also collected from locations corresponding to the involved and uninvolved sites in PC patients. Biopsies were obtained using standard surgical techniques with patient consent under W-IRB #2004/0468/1057496.

RNA extraction and quantitative RT-PCR

Biopsied skin samples were collected and mechanically disrupted in a FASTprep24 (MPBio, Santa Ana, CA) with Lysing Matrix D as previously described [22, 23]. Total RNA was isolated using the RNeasy Mini Plus kit (Qiagen), according to manufacturer's instructions. Total RNA (up to 1 µg) isolated from skin samples was reverse transcribed with random hexamer primers using the Superscript III First-Strand Synthesis System (Life Technologies, Grand Island, NY) following the manufacturer's instructions.

Quantitative RT-PCR was performed on cDNAs (diluted 25-fold) from PC-involved and uninvolved biopsies of four patients (IPCRR #8, 233, 1009, and 1015). *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase), *KLK10*, *RGS20*, small proline-rich protein 1a (*SPRR1a*), *ADAM23*, *FABP5*, *RND3*, and *IFRD1* inventoried TaqMan gene expression assays were obtained from Applied Biosystems (ABI, Foster City, CA). Samples were analyzed using the ABI 7500 Fast Real-Time PCR System under standard conditions. The data were analyzed with the Applied Biosystem's Sequence Detection software (version 1.4) and reported as relative quantitation using *GAPDH* mRNA as the reference gene. All data points reported for individual patients are the mean of three replicate assays and changes in mRNA expression levels in PC-involved versus uninvolved are displayed as mean \pm standard deviation (n=4).

RNA profiling

RNA profiling was performed on RNA samples from all 7 patients listed in Table I as described in Supplemental Materials and Methods Briefly, total RNA (100 to 200 µl of 50 ng/µl per sample) was processed following Agilent's (Santa Clara, CA) Two-Color Microarray-Based Gene Expression Analysis Protocol (Version 5.7). The Cy3- and Cy5-labeled and amplified cRNA were quantified using a NanoDrop 1000 UV-Vis spectrophotometer (Thermo Scientific, Waltham, MA). In some cases the above procedure was repeated on a different day and then respective samples were pooled and quantified

before hybridization (see Table SI). 627 to 825 ng of each cRNA sample was hybridized to Agilent 4×44K Human Whole Genome Gene Expression microarrays (part # G4112F). Data were extracted from the scanned images using Agilent Feature Extraction and imported into Rosetta (Kirkland, WA) Resolver (v7.2.2.0). For the differential expression analysis, fold changes and p-values were computed for each two-channel experiment using Rosetta's standard ratio experiment pipeline. Rosetta performs a two-sided, two-sample t-test on the means of the probe intensities corresponding to a single gene in each channel. The null hypothesis is that there is no fold change between the genes in the two channels. The p-values were corrected using Benjamini-Hochberg Multiple Test Correction [24] (for more information, see Whole Genome Microarray Processing and Table SI in Supplementary Materials and Methods). The full RNA profiling dataset has been deposited in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) site (www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE63326). An Excel file containing the subset of these data used for this study is provided in Supplemental Material.

Differentially-expressed genes were identified via two rounds of selection. For the first round, all genes with p-values and absolute fold changes satisfying the criteria of statistical significance (p-value < 0.1 and absolute fold change > 3.5 compared to matched PC-uninvolved skin) in at least one of three patients in either the K6 or K16 phenotypic group were selected. For the second round of selection, fold changes of individual genes that were selected in the first round were averaged within their respective K6 or K16 patient group using Student's t-test. The average fold changes were then filtered, limiting the set to those showing changes > 3.5-fold. This method of selection was chosen based on the limited number of PC skin samples available for the study. Due to the small sample size, patient-to-patient variability and the limiting amount of RNA extractable from 3–4 mm punch biopsies (maximum recommended to minimize scarring and other complications to patients), a more rigorous statistical analysis was not possible. Differentially-expressed genes common to K6 and K16 patients or unique to either K6 or K16 patients were sorted using Microsoft Office Excel. To identify differentially-expressed genes related to pain and neuropathy, a screening limit of 2-fold change in transcript levels was used, as described in the footnote for Tables SIII and SV.

Immunohistochemistry

Biopsied skin, embedded and frozen in optimal cutting temperature (O.C.T.) medium, was cryosectioned to 10 µm. Frozen skin sections were fixed (20 min in acetone at –20°C) and then incubated overnight at 4°C with SPRR1a primary antibody (rabbit polyclonal, affinity purified for amino acids 40-89, Abcam, Cambridge, MA) diluted 1:300 to a concentration of 3 µg/mL in PBS containing 0.025% Triton-X 100 (PBST). Rinsed sections were incubated for 3 h with Alexa Fluor-546 goat anti-rabbit secondary antibody (Life Technologies) in PBST. The sections were rinsed and mounted with Hydromount containing 4',6-diamidino-2-phenylindole (DAPI, 1 µg/mL; Sigma-Aldrich, St. Louis, MO) to facilitate visualization of nuclei. Sections were imaged using a Zeiss Axio Observer.A1 inverted fluorescence microscope equipped with FITC, Cy3 and DAPI filter sets.

Proteomic profiling

Using five successive 2.2 cm tape circles (D-Squame Pro Kits, CuDerm Corp, Dallas, TX), samples of plantar epidermis were collected from the heel and arch (analyzed separately) of three K6a N171K genotyped patients and from three healthy non-PC control subjects. Corneocytes were eluted from the tapes by immersion overnight in 2% sodium dodecyl sulfate, 0.1 M sodium phosphate pH 7.8, rinsed twice in the same solution, reduced and alkylated with iodoacetamide, precipitated with ethanol and digested with stabilized bovine trypsin [25] in 0.1 M ammonium bicarbonate/10% acetonitrile. Peptides were analyzed using a Thermo-Finnigan LTQ iontrap mass spectrometer, essentially as previously described [26]. X! Tandem was set up to search a March 11, 2013 Uniprot human database (91,573 proteins), appended to an identical but reversed database for calculating false discovery rates. Scaffold (version Scaffold_4.2.1, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Protein identifications were accepted if they could be established at greater than 95% probability (protein decoy false discovery rate 4%; peptide decoy false discovery rate 0.6%). Proteins sharing significant peptide evidence were grouped into clusters, where spectral counts were adjusted for shared peptides using the weighted peptide option in Scaffold. This option appropriates a percentage of each count divided among the protein groups that share that peptide. The formulation for that percentage for peptide(i) assigned to protein(j) is $PPS(j)/\text{sum}(PPS(1\dots n))$, where $PPS(j)$ is the sum of the peptides(1...m) for protein(j) and (1...n) is the set of proteins that contain peptide(i). The weighted spectral count is the sum of those percentages for each protein group. This is similar to method 3-a in Zhang et al [27], but Scaffold uses summed probabilities as normalizers instead of summed exclusive counts. The R statistical computing environment (v3.0.1) [28] was used to median normalize weighted spectral counts and to compare differences in protein abundance between subject groups using independent sample t-tests on shifted logarithm transformed data or quasi-poisson models without transformation. The two comparison approaches gave equivalent results, and spectral count differences for individual proteins were considered significant when $p < 0.1$. Illustrated are proteins for which significant changes were detected and where an average of at least 5 weighted spectral counts after normalization were detected in the most prevalent condition.

Results

Differentially-expressed genes in PC skin biopsies are clustered on chromosomal loci

Using a microarray approach, 18,771 unique genes were surveyed, and differentially-expressed genes were identified (by fold change) in PC-involved vs. uninvolved biopsied skin. Fold changes in differentially-expressed transcripts were averaged according to genotype and plotted against chromosomal location (Fig. 2). This analysis did not take p-values into consideration due to the small sample sizes (seven total PC-involved vs. uninvolved pairs consisting of three K6 patients (two K6a and one K6b), three K16 and one K17, Table I). Differentially-expressed genes in common with the non-PC volunteer control samples (potentially false positives) were removed from the analysis. Heterogeneity in gene expression levels was observed among all patients including those within a given genotype (*e.g.*, within K6 or K16 patient groups). Compared to changes in healthy non-PC volunteer

control matched biopsies (corresponding to the general location of PC-involved and uninvolved skin biopsies), there were several obvious hotspots on the chromosomes where gene expression levels were markedly altered, the most dramatic being the epidermal differentiation complex (EDC) on chromosome 1 (1q21), where 11 genes in K6 patients and 5 genes in K16 patients were upregulated by greater than 8-fold, respectively. These include *S100A*, *SPRR*, and late cornified envelope (*LCE*) protein genes. Other hotspots include the type I keratin cluster on chromosome 17 (17q12-q21) and the type II keratin cluster on chromosome 12 (12q11-q13). *KRT6A*, *KRT6B*, *KRT6C* and *KRT75* were among the most upregulated type II keratin genes in PC patients, while various type I keratin genes were differentially expressed among the patient groups (Fig. 2).

Classification of differentially-expressed genes

Using the cut-off criteria of statistical significance of p-value ≤ 0.1 and an average fold change of ≥ 3.5 when compared to matched PC-uninvolved skin biopsies for a given patient, a total of 193 genes were identified in the K6 patient group and 253 genes in the K16 group. Of these genes, 112 are common to both K6 and K16 patient groups, 81 genes are differentially expressed in the K6 genotype alone and 141 genes are found only in K16 patient samples (Fig. 3).

The 112 differentially-regulated genes shared by genotypes K6 and K16 were classified into nine categories based on gene ontology (Fig. 3). Detailed classification information, including chromosomal location and primary function, on all 112 genes is listed by category in Table SII and genes with an absolute fold change greater than 10 are summarized in Table II. As only one biopsy for a K17 patient was available, the dataset from this patient was excluded from the analysis. The hotspots of differentially-expressed genes (shown in Fig. 2) were confirmed using the cut-off criteria of p-value ≤ 0.1 and average fold change ≥ 3.5 , with 11 highly differentially-expressed genes clustering on the 1q21 locus and encoding either antimicrobial and inflammatory (*S100A8*, *S100A9*, *S100A7*, and *S100A7LI*) or structural (*LCE3A*, *LCE3D*, *Involucrin (IVL)*, *SPRR3*, *SPRR1A*, *SPRR1B*, and *SPRR2C*) proteins (Table SII). Among the 10 cytokeratin genes on either the type I or II cluster, transcripts of *KRT6A*, *KRT6B*, and *KRT6C* increased by 26-, 50-, and 30-fold, respectively. Other keratins such as *KRT16* and *KRT17*, *KRT75*, *KRT79*, *KRT80*, and *KRT85* are also upregulated significantly, whereas *KRT2* is downregulated and *KRT1* and *KRT10* are unchanged.

In the category of proteases, peptidases, and their inhibitors, two kallikrein-related peptidases (*KLK10* and *KLK5*; 5.7- and 5.0-fold changes, respectively), one chymotrypsin (*CTRB2*, 3.8-fold) and one metallopeptidase (*ADAM23*, 5.8-fold) are upregulated in PC-involved skin. Cathepsins, a class of proteases, are largely unchanged or slightly decreased (data not shown). Strikingly, many genes encoding protease inhibitors are upregulated including secretory leukocyte peptidase inhibitor (*SLPI*, 6.9-fold), skin-derived peptidase inhibitor 3 (*PI3*, also called elafin, 29-fold), cystatin A (*CSTA* or stefin A, 4.5-fold), serine peptidase inhibitor, Kazal type 6 (*SPINK6*, 20-fold), and three members from the SERPIN family (*SERPINS* B3, B4, and B13; 12-, 11-, and 5.8-fold, respectively). Transcripts of genes responsible for cell-cell adhesion such as corneodesmosin (*CDSN*), desmocollin 2

(*DSC2*), and gap junction beta 2 protein (*GJB2* also known as connexin 26) are also increased significantly. Several of the differentially-expressed genes are related to either lipid biogenesis or hydrolysis such as lipoprotein lipase (*LPL*), perilipin (*PLIN*), elongation of long chain fatty acids family member 7 (*ELOVL7*), and *KIAA1881* (Table SII).

Genes that are differentially expressed exclusively in either K6 or K16 patients were similarly classified and information on these genes is listed in Tables SIII and SIV (compare with Table SII). Notably, *KRT9* is dramatically downregulated by 22-fold in the K6 patient group (n=3) while unchanged in K16 patients (our related finding of a reciprocal correlation between *KRT6* and *KRT9* expression will be published elsewhere, Kaspar *et al.*, submitted). On the other hand, *KRT15* (7.6-fold), *KRT18* (4.7-fold) and *KRT19* (16-fold) are dramatically downregulated in K16 patients compared to minor changes (3.5-fold) in K6a patients (n=3), and therefore do not meet the selection cut-off criterion (3.5). Unexpectedly, several immunoglobulin genes (*IGHG4*, *IGLV2-14*, *IGHA2*, *IGLL1*, *IGJ*, and *IGKV1-5*) are upregulated in only K6 patients while only one, *IGH@*, is upregulated in both K6 and K16 patients (Tables SII and SIII).

Alteration of pain-relevant gene expression in PC-involved skin

Since persistent severe pain is the most problematic symptom for PC patients, genes with putative roles in nociception and neuropathy pathways were specifically investigated. Upregulation of 12 pain-related genes was observed in PC-involved skin compared to the uninvolved samples (Table SV). Of particular note, *SPRR1A* (11-fold, n=6), *KLK10* (5.7-fold, n=6), and regulator of G-protein signaling 20 (*RGS20*) (5-fold, n=6) are common to both K6 and K16 patients (Table SV), while *KLK12* (31-fold, n=3) appears to be specific to K6 patients (Tables SIII and SV).

To confirm the microarray results, RT-qPCR was performed on seven genes identified as differentially expressed by the microarray analysis that are potentially involved in nociception and neuropathy pathways. The RT-qPCR results of *KLK10*, *RGS20*, *SPRR1A*, *ADAM23*, *FABP5*, *RND3*, and *IFRD1* revealed similar fold changes compared to microarray analysis (Fig. 4A). *SPRR1A*, a gene encoding a structural protein but also related to neuropathy [29], was further subjected to immunohistochemical staining and exhibited increased protein levels in PC-involved skin sections compared to uninvolved (Fig. 4B & D). These results are in agreement with the increased RNA levels observed by RNA profiling and RT-qPCR analysis.

Proteomic analysis

To investigate expression differences at the protein level, samples of plantar callus from three PC patients with K6a N171K mutations were compared to normal control subjects by shotgun proteomics. The 37 proteins with significantly different expression present in at least moderate amounts (5 peptides or more in the most prominent condition) are listed in Table SVI and a subset graphically presented in Fig. 5. The most striking change was the evident suppression of K9 in the PC state. This was accompanied by suppression of the much less prominent desmocollin-1 and arachidonate 12-lipoxygenase (12R type). By contrast, keratins expressed at higher levels in the PC samples included K5, K6a, K14 and

K1. In addition, transglutaminases 1 and 3, S100A9 (as well as A7 and A8), SPRR2G (as well as 2F and 2D) are also substantially higher in the normal epidermis; also higher are the keratinocyte proteins gasdermin-A (10.7-fold), LCE 1B (11.5-fold), skin specific protein 2F (2.2-fold), filaggrin (4.6-fold), histidine ammonia lyase (17-fold), SERPINB3 (53.4-fold) and desmoglein-1 (1.8-fold) (Table SVI). Of the 37 proteins with significant changes (p-value<0.1), mRNAs for nine change in the same direction as observed in the DNA microarray experiments, where the rest were not observed with one exception. Unlike at the protein level, cornifin mRNA is nearly 4-fold higher in PC samples. Several prominent keratins (K6b, K16, K17) are seen to change in the same direction as their mRNAs but the p-values are 0.11–0.13 due to variability in the peptide counts and are therefore not included.

Discussion

PC plantar keratoderma is extremely painful, compromises patient mobility, and is the most problematic feature according to patients [4–6]. Unfortunately, the complex pathological mechanisms of disease progression are only now being elucidated, and no effective approved therapies exist that target the underlying disease mechanism. Several potential PC mouse models have been generated by altering the expression of genes associated with PC, including *Krt6a*, *Krt6b*, *Krt16* and *Krt17* [9, 14, 22, 30–34]. Surprisingly, among these models, only the *Krt16*-null mice exhibit palmoplantar keratoderma (PPK) with a possible decrease in overall mobility, potentially due to discomfort from painful keratoderma [9, 22].

In the current study, global gene expression profiling was performed on RNA extracted from PC patient skin biopsies. Differentially-expressed mRNA transcripts were identified in PC-involved versus uninvolved skin and classified into several major functional categories (specific to either the K6 or K16 genotype or common to both), including structural proteins, metabolism- or immune response-related proteins, proteases and peptidases and their inhibitors. Interestingly, these genes overlap extensively with those differentially expressed in the *Krt16*-null mouse model that exhibits paw keratoderma [9] and include genes involved in maintaining the mechanical integrity of the skin and desquamation [22]. Protein profiling on tape-stripping samples of PC patient plantar scale compared to non-PC control subjects revealed complementary, but not identical, results. The differences in the RNA profiling and proteomics analyses could be due to several reasons including: 1. the proteomic samples were obtained from tape-stripping of the stratum corneum whereas RNA profiling used full-thickness skin biopsies; 2. the proteomic samples were only from patients harboring the K6a N171K mutation and clear differences in RNA profiling are observed between patient groups (see Tables SIII and SIV); 3. the RNA profiling study was designed to compare intra-patient PC-involved to adjacent PC-uninvolved skin biopsies, whereas the proteomics compared PC skin samples to non-PC control subjects; and 4. the level of sensitivity of the proteomics analysis is lower than for the RNA profiling analysis.

Keratins form filamentous fibers that provide strength and stability to keratinocytes and the epidermis. Unlike the majority of keratins, K6, K16, and K17 are “inducible” and their expression can be rapidly switched on after stress or injury. In PC-involved skin biopsies, marked increases are observed in the transcript levels of the *KRT6A*, *KRT6B*, *KRT16*, and

KRT17 genes, mutations in which are known to cause PC [35]. It is plausible that mechanical forces acting on the skin (e.g., walking or friction from shoes) lead to upregulation of both wild-type and mutant forms of these keratins and that faulty intermediate filament formation due to mutant keratin incorporation into the fibers and/or the presence of free unincorporated mutant keratin protein may trigger the molecular pathogenesis leading to the PC phenotype [14, 15]. The structurally weakened PC keratinocytes and correspondingly compromised skin tissue are likely more prone to injury, thus increasing susceptibility to further insult and subsequent expression of additional mutant keratin as part of the wound healing response, resulting in a destructive downward cycle.

Non-PC-related keratins were also identified to be differentially expressed in involved vs. uninvolved skin. Unexpectedly, the hair follicle-associated keratins *KRT75* (formerly known as *KRT6hf*) and *KRT85* (also known as *KRTHB5*) are markedly increased in PC-involved skin. K9, a keratin that is abundantly and specifically expressed in terminally differentiating keratinocytes of palmoplantar epidermis, is dramatically reduced in the involved skin of K6, but not K16 or K17 patients (see also Kaspar et al., submitted for publication, for determination of absolute K9 and other keratin mRNA levels in PC skin). This observation was further confirmed by proteomic analysis in which a >60-fold K9 decrease was demonstrated in scale from K6a patients (see Fig. 5). In addition, downregulation of the basal cell keratins including *KRT15*, *KRT18* and *KRT19* is observed in K16 patients and unchanged or minimally altered in K6 patients. Genetic mouse models [34, 36] and the identification of causative gene mutations in human skin diseases [37–40] suggest that, individually or collectively, some of these keratins may be involved in PC pathogenesis.

Keratins have been implicated in the modulation of Akt and mTOR signaling pathways through direct physical interaction with the adapter protein 14-3-3 σ stratifin [41], AMP-activated protein kinase (AMPK) [42] and Akt/PKB [43, 44], or via transcriptional regulation of the effector genes in the pathway [14, 45]. Previous studies on the regulation of nociceptive mechanisms show that sensory axonal protein synthesis also occurs under the control of the mTOR signaling pathway [47–49]. It would be intriguing to investigate the activation status of the mTOR pathway in PC-involved skin and whether it may contribute to the persistent pain experienced by PC patients.

Three clear clusters of induced gene expression are observed in PC-involved skin (see Fig. 2). In addition to the Type I and Type II keratin clusters, the EDC cluster (on chromosome 1q21) clearly stands out. The EDC comprises a large number of non-keratin genes involved in the maturation of the human epidermis [51, 52], including the formation of the cornified cell envelope. Many of the overexpressed EDC genes encode key structural players in stratum corneum formation including involucrin, loricrin, SPRRs, elafin, CSTA, LCEs and desmosomal proteins [53, 54], whose abnormal synthesis can result in inherited keratodermas [54–56]. These results are in general agreement with the increased protein levels observed in proteomic analysis of tape stripping samples obtained from PC scale as well as in other human skin disorders where the barrier is breached [57, 58]. The mechanism of coordinated upregulation of these clustered EDC genes during keratinocyte differentiation

or following barrier breach remains to be elucidated but epigenetic factors, such as DNA methylation, may be involved [58].

In normal human skin, homeostasis exists as terminally-differentiated corneocytes are shed (desquamation) and replaced by underlying differentiating keratinocytes, resulting in a relatively constant thickness of the epidermis [59, 60]. Desquamation is mediated by desmosomes as well as various proteases and protease inhibitors, many of which are upregulated in PC-involved skin. *DSC2* and *CDSN*, two major components of desmosome junctions, are significantly upregulated and may hinder desquamation. Furthermore, the proteases *KLK5* (serves as a primary regulator of the stratum corneum kallikrein cascade to cleave components of desmosomes and initiate desquamation [59, 61, 62]), *KLK10*, chymotrypsinogen B2 (*CTRB2*), and metallopeptidase domain 23 (*ADAM23*) are also upregulated. Some proteases, however, such as cathepsins remained unchanged or are slightly decreased (data not shown).

Protease activity can be controlled by various inhibitors, many of which, including *SPINK6*, SERPINs (*B3*, *B4*, and *B13*), elafin, and SLPI, are markedly upregulated. These protease inhibitors have distinct localization patterns, e.g., SERPINs are intracellular inhibitors that may have a role in protecting tissue from proteolysis [63, 64], while SPINKs are secreted and act extracellularly [65, 66]. Similar proteomics findings were observed from tape-stripping PC-involved skin compared to control skin. In addition, *CSTA*, a cysteine protease inhibitor present in skin and sweat that impedes corneodesmosome breakdown [67], is also upregulated in PC-involved skin biopsies.

Abnormal expression of these proteases and/or their inhibitors has the potential to dramatically change the state of desquamation, altering the equilibrium and the thickness of the stratum corneum. For example, mutations in the *SPINK5* gene cause Netherton syndrome, a severe disorder characterized by accelerated desquamation and dramatic skin peeling, defective keratinization, hair shaft defects, and recurrent infections [68, 69]. Accelerated corneocyte desquamation is also observed in palmoplantar hypokeratosis resulting from decreased expression of *SPINK5* and corneodesmosin, along with an increased expression of *KLK5* [70]. Conversely, delayed desquamation and palmoplantar keratoderma are observed with mutations in genes encoding the desquamation-related protease cathepsin C [71] or adhesion protein *GJB6* (also known as connexin-30) [72, 73]. Although it is difficult to assess the net activity of the proteases such as *KLK5* in the stratum corneum, it is reasonable to speculate that protease activity is suppressed and desquamation impaired in PC-involved skin, given the broad spectrum of these induced protease inhibitors (SERPINs, *SPINK6*, elafin, SLPI, and *CSTA*), the extent of this upregulation, and the increased thickness of PC-involved skin.

The intense pain associated with plantar keratoderma is the most problematic symptom for PC patients. Surprisingly, only a few genes potentially related to nociception and neuropathy appear to be differentially-expressed in PC-involved skin (similar results were also obtained in less stringent screens of the data). Among the upregulated “pain-related” genes identified, *KLK10* catalyzes the production of bradykinin, which initiates pain sensation after binding its receptor on nerve fiber endings [74]. Furthermore, *SPRR1a*, one of the important

structural proteins expressed in keratinocytes, is also expressed by neurons and promotes axonal outgrowth, guidance, and branching [75]. It is unclear, however, whether increased *SPRR1A* expression, along with other neuropathy-related genes, directly affects plantar pain [10]. In addition, the increased expression of antimicrobial peptides such as defensins (*DEFB4* and *DEFB103A*), 2'-5'-oligoadenylate synthetase-like (*OASL*), S100 proteins (A7, A8 and A9), and the immunoglobulin genes suggest the presence of infection (fungal and/or bacteria colonization) [10] and inflammation. In the recently described *Krt16*-null mouse model [9, 22], inflammation and increased numbers of infiltrating immune cells were observed in the hindpaws along with reduced mobility and possibly pain. These data suggest that inflammation, which might potentiate pain sensation from mechanical stress on the impaired skin barrier, may be a part of PC pathogenesis. To investigate the role of inflammation in PC, preliminary immunohistochemical staining has been performed on a small number of PC patient samples. Although some patients appear to have immune infiltrates, the small sample size and high patient-to-patient variability preclude firm conclusions and additional studies with greater numbers of PC and control biopsies are required.

Gene profiling studies have been performed in other skin disorders, notably psoriasis [76, 77]. There is some overlap in the upregulated genes that are common to both psoriatic and PC skin including *S100A7* (psoriasin) [52, 78], *S100A9*, *OASL*, *SERPINB3/B4*, *SPRR2C* [77], *IVL* and *CSTA* [76]. All of these known psoriatic markers are involved in atypical epidermal cellular organization and differentiation [76]. Down-regulation of *KRT18* and *KRT19* are also observed in psoriatic lesional skin [77]. Notably, in contrast to psoriatic skin in which inflammatory cytokines, interleukins, and other immunological components are highly upregulated [77], changes in immune-relevant gene expression are less evident in PC-involved skin with the exception of a few immunoglobulin genes (particularly in K6 patients) and the pro-inflammatory alarmins *S100A7/A8/A9* [79–82]. These striking gene expression similarities and differences in psoriatic and PC skin warrant further investigation.

In summary, this study identified dramatic changes in expression of a multitude of genes potentially related to PC pathogenesis. Many of these genes encode structural proteins, proteases, and protease inhibitors that are critical for cornification or desquamation, an imbalance in which likely leads to PC palmoplantar keratoderma. Fig. 6 schematically depicts how PC-relevant keratin mutations may ultimately lead to the observed keratoderma and pain, incorporating many of the molecular players identified in this study together with other published and unpublished observations. It is important to note that the sample size was small and the samples were collected in different batches; therefore, minimal statistics were utilized. The data generated from this study should form a framework for understanding the sequelae resulting from mutant keratin expression in PC skin and may lead to a rational approach for identifying appropriate therapies for PC patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Both mRNA and protein profiling of PC-involved skin biopsies to uninvolved plantar skin identified differentially-expressed genes.
- The differentially-expressed genes cluster into 9 different classes according to their primary functions.
- A model of the molecular pathways involved in PC pathogenesis is proposed.



Fig. 1. Physical locations of plantar biopsy sites for one of the participating patients (K16-R127C mutation)

Sites (involved and uninvolved) where biopsies were obtained (as described in Methods and Materials) are circled. Similar biopsy pairs were collected from all PC participants and non-PC volunteers (controls).

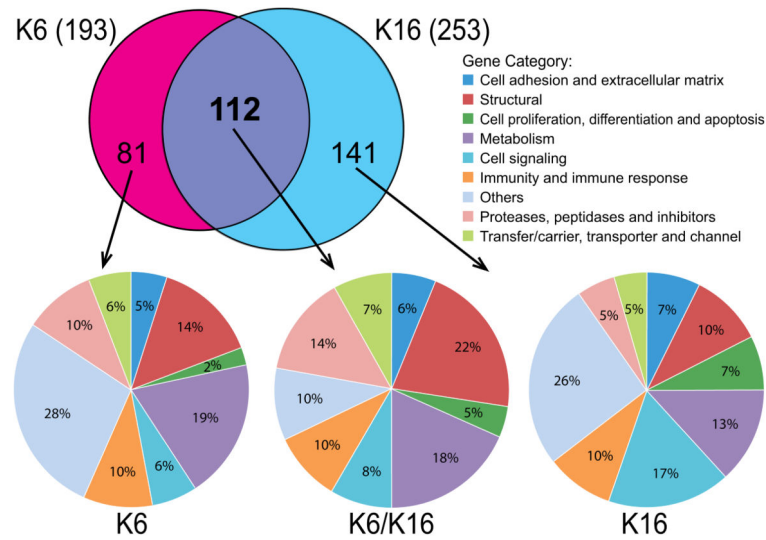


Fig. 3. Venn diagram and classification of differentially-expressed genes by genotype mRNA profiling results were divided into K6 and K16 groups according to patient genotype. Genes satisfying the criteria of fold change ≥ 3.5 and p-value ≤ 0.1 (when compared to matched PC-uninvolved skin biopsies) were identified as differentially expressed (see Materials and Methods). Of the resulting 193 genes in K6 patients (n=3) and 253 genes in K16 patients (n=3), 112 are common to both K6 and K16 patient groups, while 81 genes are differentially expressed only in K6 patients and 141 genes only in K16 patients. The identified genes were classified into different categories with color coding (applies to pie chart) according to function.

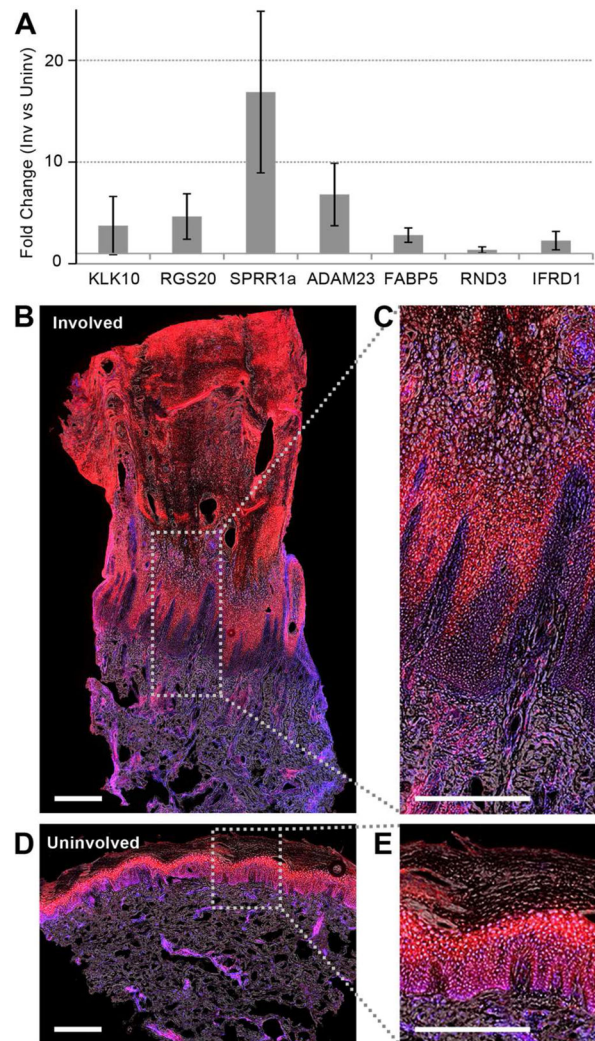


Fig. 4. Validation of mRNA profiling results by RT-qPCR and immunohistochemistry
 Selected differentially-expressed genes identified by mRNA profiling that are potentially related to nociception and neuropathy were further analyzed by RT-qPCR to confirm the mRNA profiling findings. For each gene analyzed, RT-qPCR was performed (> three replicates) and the average fold change of expression levels (involved vs. uninvolved) was plotted as mean \pm standard deviation (n=4) (A). SPRR1A was selected for further investigation by immunohistochemistry of frozen skin sections (10 μ m). Increased protein expression of SPRR1a (red) is observed in PC-involved skin biopsies (B) compared to uninvolved (D). Enlarged images of areas marked by squares are also shown (C and E). Nuclei were counterstained with DAPI (blue) Scale bar = 500 μ m.

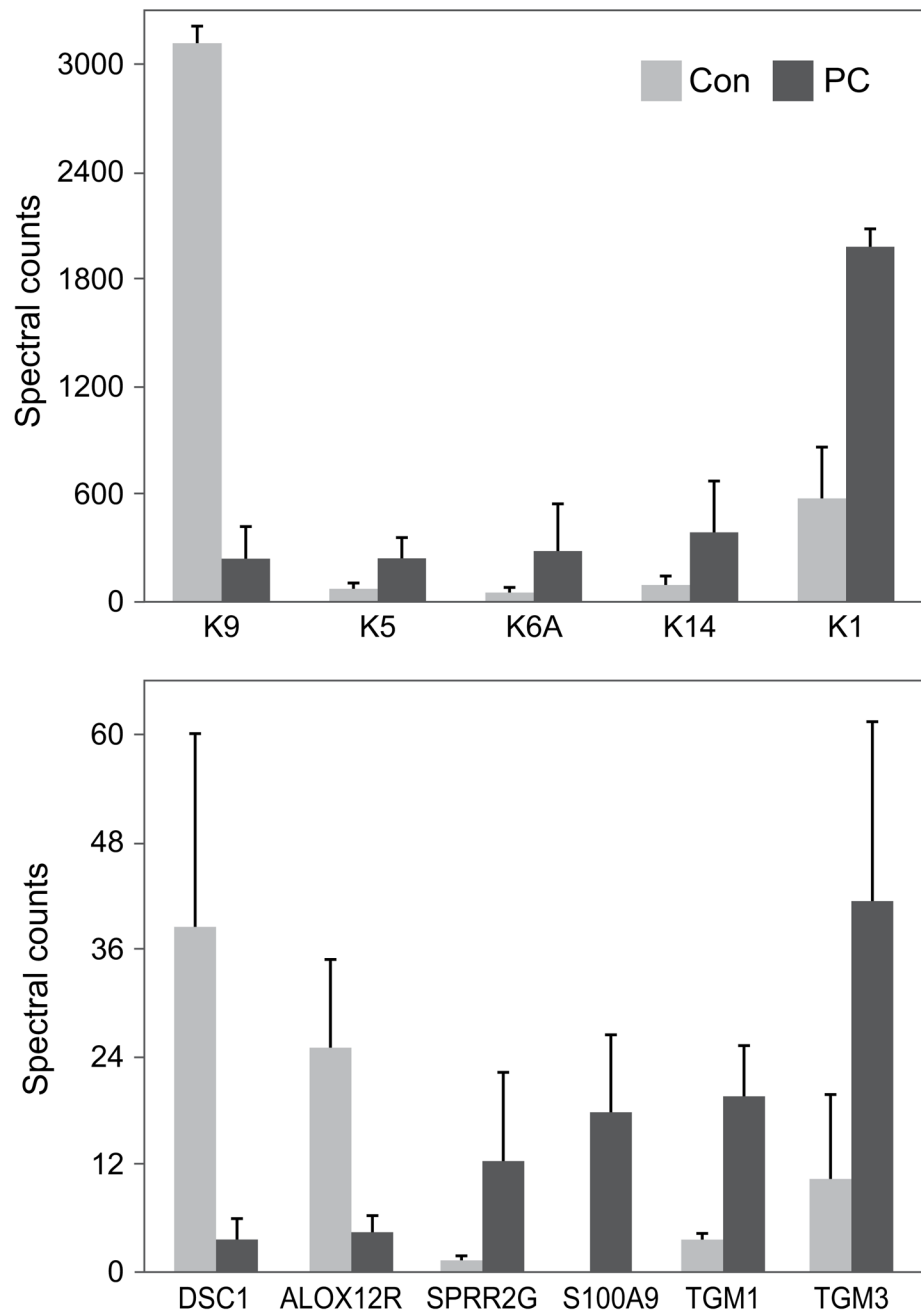


Fig. 5. Differences in protein profile between PC and normal epidermis

Illustrated are the normalized weighted spectral counts of keratins (top) and other prominent keratinocyte proteins (bottom) from three subjects, each harboring the K6a N171K mutation.

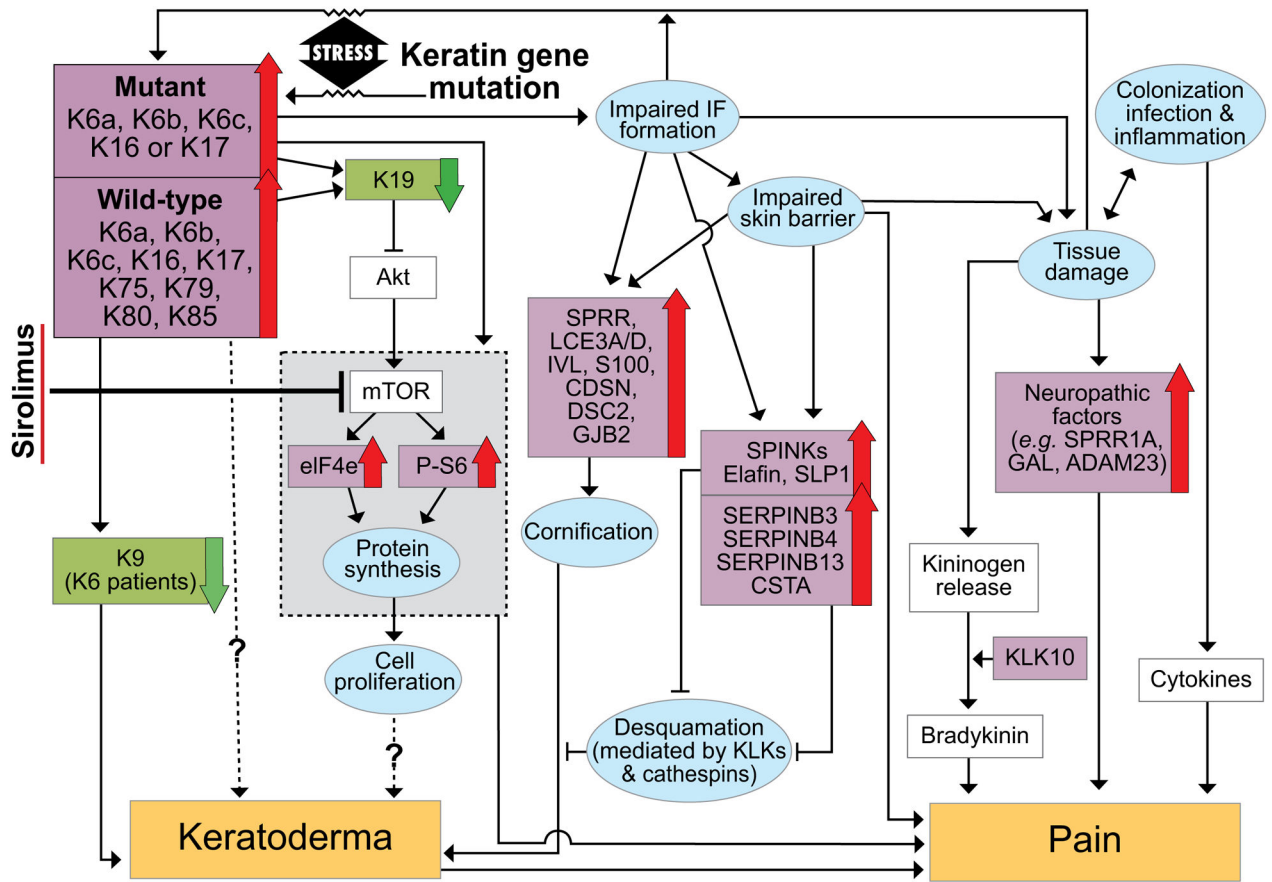


Fig. 6. Proposed model of molecular pathways involved in PC pathogenesis

In healthy skin, keratinocyte cornification and desquamation exist in equilibrium to maintain the appropriate epithelium thickness. In PC, this process is imbalanced as exemplified by acanthosis associated with increased levels of structural (keratins, SPRRs, IVL, and late cornified envelope) and adhesion (CDSN, DSC2, and GJB2) proteins in the SC.

Desquamation is putatively delayed by inhibition of key desquamation enzymes such as KLK5, KLK7, and cathepsins, likely through increased production of protease inhibitors including SPINK6, SPINK5, elafin, SLPI, and SERPINS. This imbalance likely leads to the observed keratoderma. Mechanical stress acting on the altered skin barrier may trigger pain, potentiated by mTOR pathway activation, increased KLK10 and bradykinin levels and/or tissue damage caused by microbial colonization and infection in the skin.

Table I

IPCRR numbers and corresponding mutations of PC patients

IPCRR #	Mutation
8	K6a N171K
10	K6a N171K
661	K6b E472K
233	K16 R127G
1009	K16 R127C
1015	K16 R127C
394	K17 N92S

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Table II

List of differentially-expressed genes identified in RNA profiling study common to K6 and K16 patient groups with absolute fold change* at least 10 and p-value less than 0.1

Gene Category	Genes
Cell proliferation, differentiation and apoptosis	GoS2
Immunity and immune response	S100A7, S100A7L1, S100A8, S100A9, DEFB4, DEFB103A, OASL, IGH@
Proteases, peptidases and inhibitors	PI3, SPINK6, SERPINB3, SERPINB4
Structural	ACTG2, KRT6A, KRT6B, KRT6C, K16, KRT75, LCE3A, LCE3D, SPRR1A, SPRR1B, SPRR2C, SPRR3
Transfer/carrier, transporter and channel	RHCG

* For absolute mean fold change of individual genes as well as a more comprehensive list of differentially-expressed genes, see Supplementary Table SII.