

Clinical and Pathological Features of Pachyonychia Congenita

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Pachyonychia congenita (PC) is a rare genodermatosis affecting the nails, skin, oral mucosae, larynx, hair, and teeth. Pathogenic mutations in keratins K6a or K16 are associated with the PC-1 phenotype whereas K6b and K17 mutations are associated with the PC-2 phenotype. Analysis of clinical, pathological, and genetic data from the literature and two research registries reveal that >97% of PC cases exhibit fingernail and toenail thickening, and painful plantar keratoderma. Prospective evaluation of 57 PC patients from 41 families revealed variable clinical findings: hyperhidrosis (79%), oral leukokeratosis (75%), follicular keratosis (65%), palmar keratoderma (60%), cutaneous cysts (35%), hoarseness or laryngeal involvement (16%), coarse or twisted hair (26%), early primary tooth loss (14%), and presence of natal or prenatal teeth (2%). Stratification of these data by keratin mutation confirmed the increased incidence of cyst formation and natal teeth among PC-2 patients, although cysts were more commonly seen in PC-1 than previously reported (25%–33%). Previously unreported clinical features of PC include development of painful oral and nipple lesions during breastfeeding, copious production of waxy material in ears, and inability to walk without an ambulatory aid (50%). Possible pathogenic mechanisms are discussed with respect to the clinicopathologic and genetic correlations observed.

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Pachyonychia congenita (PC) is a rare, autosomal dominant keratin disorder that typically affects the nails and palmo-plantar skin, and often the oral mucosa, tongue, larynx, teeth, and hair. Because of its rarity, the condition has been difficult to characterize or investigate, and no controlled prospective clinical trials have been published using this patient population. Our understanding of the clinical and pathological features of the disorder has been based upon case reports, a few case series, and some excellent reviews that have attempted to unify the case report literature (Kumer, 1935; Moldenhauer and Ernst, 1968; Schonfeld, 1980; Franzot *et al*, 1981; Stieglitz and Centerwall, 1983; Sivasundram *et al*, 1985; Feinstein *et al*, 1988; Su *et al*, 1990; Paller *et al*, 1991; Dahl *et al*, 1995). A comprehensive bibliography of PC, complete with translations of non-English articles, is available at <http://www.pachyonychia.org>. Unfortunately, many publications include photographs of dramatic or unusual manifestations of the condition and there is little discussion about the spectrum of clinical or

pathological phenotypes that may be observed. This bias makes it extremely difficult for the average practitioner, who may only see one or two cases in an entire career, to be assured that they have made the correct diagnosis, especially in an individual without classic symptomatology.

History and classification of PC. The term *pachyonychia congenita* (Greek: thick nails from birth) was coined by Jadassohn and Lewandowski in 1906 and this case report is frequently quoted as the original description of the condition (Jadassohn and Lewandowski, 1906). But based on the descriptions and photographs of cases reported by Müller in 1904 and Wilson in 1905, it is likely that they were also describing PC (Müller, 1904; Wilson, 1905). Jan Bondeson has performed a comprehensive review of the medical history of PC (Bondeson, 1993). This paper provides compelling evidence that St George Ash described an Irish case of PC as early as 1685, and that the first reported case of PC-tarda was by the philosopher John Locke in 1695 (Ash, 1685; Locke, 1695). A doctoral dissertation in 1716 by Carl Musaeus on “monstrous nails” was apparently the first to postulate that the constellation of symptoms seen in PC represented a systemic disease called “morbus corneus” (Musaeus, 1716). The form of PC with widespread pilosebaceous cysts was first described in the recent literature by Jackson and Lawler (1951).

Abbreviations: H&E, hematoxylin and eosin; HIM, helix initiation motif; HTM, helix termination motif; IPCRR, International Pachyonychia Congenita Research Registry; NRIIRD, National Registry for Ichthyosis and Related Disorders; PC, pachyonychia congenita

Prior to the discovery of the genetic basis of PC, several clinical classification schemes were proposed (Kumer, 1935; Schonfeld, 1980; Sivasundram *et al*, 1985; Feinstein *et al*, 1988; Dahl *et al*, 1995). A review of the modern literature by Feinstein *et al* (1988) summarized the majority of PC reports in the worldwide literature and discussed each of the major classification schemes proposed up to 1985. Based on these 168 cases of PC reported in the literature, Feinstein *et al* (1988) proposed a classification of PC into four overlapping subtypes which are frequently used in case reports. The literature was again reviewed in 1995 (Dahl *et al*, 1995), and simplified criteria were proposed in which the diagnosis of PC could be made when the characteristic nail changes (major criteria) occurred in association with at least one minor criterion (autosomal dominant inheritance, palmoplantar keratoderma, leukokeratosis oris, follicular keratosis, bullae on palms or soles, or laryngeal leukokeratosis). Unfortunately, classification of PC by purely clinical criteria was hindered by significant variability of the phenotype, even within affected members of the same family, a common feature of most or all keratin disorders (Irvine and McLean, 1999).

Clinical genetics of PC. A major advance in the capacity to classify PC came in 1995 when the genes encoding keratins K16 and K17 were identified as harboring the first genetic mutations underlying PC (McLean *et al*, 1995), following genetic linkage data showing a probable keratin defect (Munro *et al*, 1994). These findings were followed by identification of mutations in keratins K6a and K6b (the polymerization partners of K16 and K17) in additional PC families (Bowden *et al*, 1995; Smith *et al*, 1998). To date, over 82 mutations in these four keratins have been identified in independently ascertained families (Fig 1 and Smith *et al*, this issue). Nearly all the reported mutations occur at either the start or the end of the central keratin rod domain. These regions are known as the helix boundary motifs of the keratin polypeptide, or, individually, the helix initiation motif (in the 1A domain) and the helix termination motif (in the 2B domain). As discussed elsewhere in this issue (Smith *et al*, McLean *et al*), these regions are critically important for end-to-end association of protein subunits in the assembly of keratin filaments and, furthermore, represent mutation hot-spots in all keratin genes so far associated with human disease phenotypes.

In the genetic studies described above, the phenotypic classification of PC families into two major subtypes emerged, termed as PC-1 (or the Jadassohn–Lewandowski type) and PC-2 (or the Jackson–Lawler type) based on early clinical descriptions (Jadassohn and Lewandowski, 1906; Jackson and Lawler, 1951). The most prominent clinical feature of both PC subtypes is hypertrophic nail dystrophy. Specifically, the nail changes in PC consist of three abnormal findings: hyperkeratosis of the nail bed; thickening of the nail plate; and distortion or curvature of the nail plate. Importantly, both PC types also show variable degrees of a focal palmoplantar keratoderma with accentuation in weight-bearing or traumatized areas. Both PC-1 and PC-2 patients sometimes develop follicular keratoses of the elbows, knees, and hips. The clinical discrimination between PC-1 and PC-2 usually depends on more prominent oral leukokeratosis in PC-1, or, conversely, in findings of

steatocystomas/pilosebaceous cysts, vellus hair cysts, hair abnormalities (alopecia, *pili torti* (twisted hair)), and natal teeth in PC-2. The occurrence of natal teeth appears to be diagnostic of PC-2, but, unfortunately, this feature is not fully penetrant, i.e., not all PC-2 patients present with natal teeth. The PC-1 clinical phenotype is associated with mutations in K6a and K16 whereas the PC-2 phenotype is associated with mutations in K6b and K17. The major phenotypic differences between the two types are clearly correlated with known differences in the expression ranges of these two pairs of keratins (Lane, 1993). A delayed onset, or tarda subtype of both PC-1 and PC-2 has also been described and keratin mutations associated with PC-tarda have been found outside the helix boundary motif regions of the K16 and K17 proteins (Connors *et al*, 2001; Xiao *et al*, 2004). It is tempting to speculate that the tarda phenotype occurs because these mutations are not so disruptive to keratin assembly (see McLean *et al*, this issue), but additional cases will be necessary to confirm this hypothesis. Despite the incomplete information available regarding the mechanistic details, practitioners can now confirm clinical suspicion of PC by mutation testing.

As genetic testing of PC patients is becoming more common, the complexity of the relationship between the keratin mutation status and the clinical phenotype also becomes more obvious (Munro, 2001). Mutations within the same functional domain of the keratins produce variable clinical manifestations, and even patients with the same mutation sometimes display different levels of severity and different clinical spectra. For example, the same mutation in K17 gives rise to the full PC-2 phenotype in some families and to steatocystoma multiplex without nail changes in others (Smith *et al*, 1997; Covello *et al*, 1998). Similarly, mutations in the helix initiation motif of K16 can produce a full-blown PC-1 presentation (Smith *et al*, 1999a, b; 2000) or just focal non-epidermolytic palmoplantar keratoderma in other cases (Shamsher *et al*, 1995). The variability of phenotype and incomplete penetrance seen in PC strongly suggests that genetic and/or environmental modifier effects are modulating the genotype–phenotype relationship. Comprehensive investigation of these genotype–phenotype–environment interactions in PC may ultimately provide clinically relevant clues to the pathogenesis and optimal treatment of the disease. Thus, the goals of this paper are to provide the medical community with a comprehensive review of the clinical and pathological spectrum seen in PC, report new prospective data on a cohort of 57 PC participants, correlate the clinical findings of PC patients with their genetic mutation status, and provide clinicians with a user-friendly summary of genetic test results seen in PC.

Results

Literature review and analysis of PC cases Three sources of cases were evaluated: cases in the literature, cases from the International Pachyonychia Congenita Research Registry (IPCRR), and cases from the National Registry for Ichthyosis and Related Disorders (NRIRD). A comprehensive literature review revealed a total of 198 articles containing 457 independent cases of PC from 214 different

Keratins Mutated in Pachyonychia Congenita

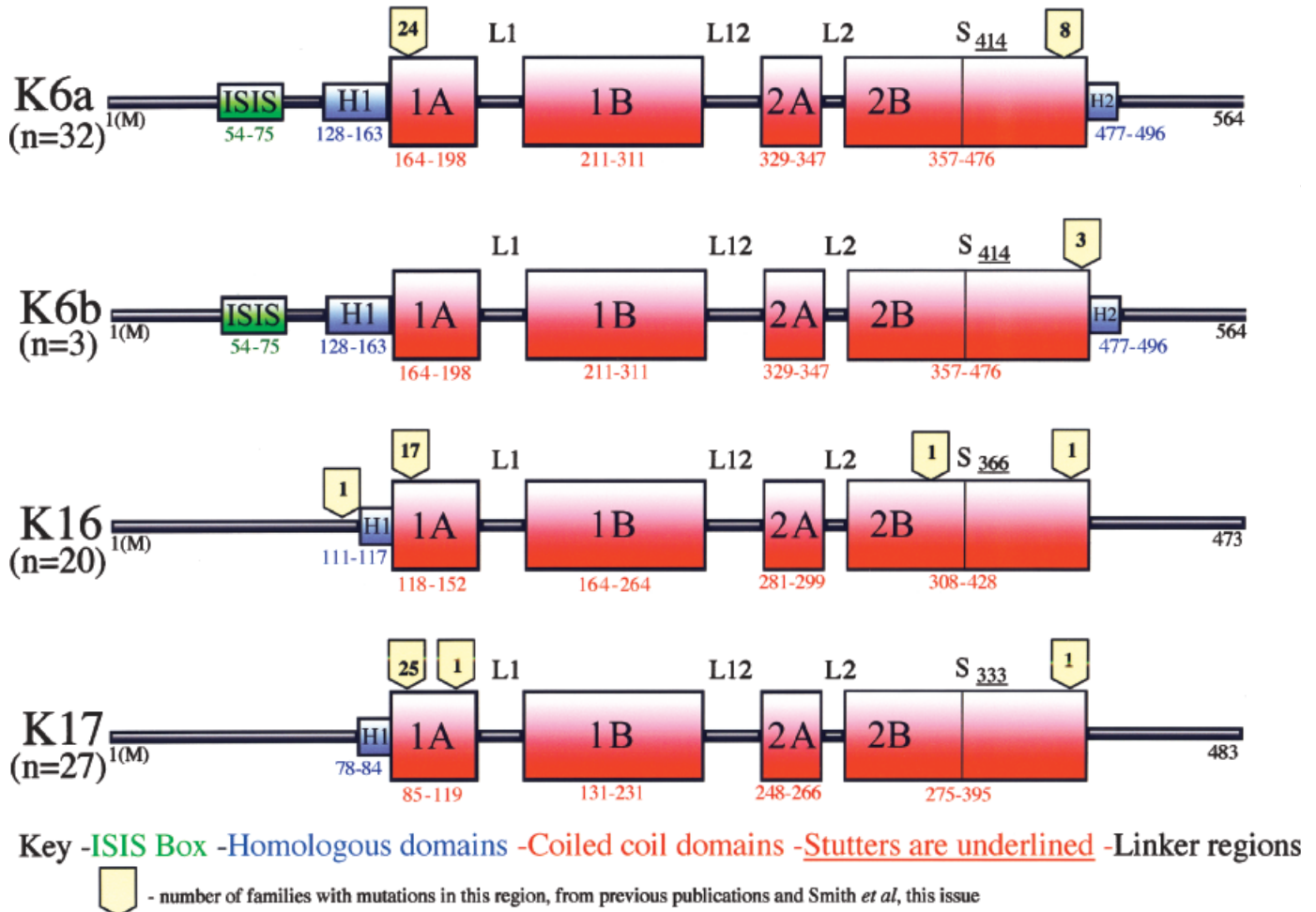


Figure 1

Keratin mutations in pachyonychia congenita (PC). A schematic representation of the protein domain organization of each of the four keratins associated with PC is shown (K6a, K6b, K16, and K17). A total of 82 mutations have been published to date including those reported in this issue (Smith *et al*). The approximate location of mutations is indicated by numbered boxes. The numbers within the boxes indicate the number of families reported with mutations in this region, i.e., the number of independent occurrences of mutations. Note that the vast majority of mutations are found in or near the helix initiation motif in the 1A domain or the helix termination motif at the end of the 2B domain. The numbers below the schematic represent the amino acid residue number with the protein. The domains shown include the ISIS box (green), and homology subdomains H1 and H2 (blue), and the coiled coil domains 1A, 1B, 2A, and 2B (red), separated by non-helical linkers L1, L12, and L2 (black). Stutter sequences (S) are underlined.

pedigrees. A complete, searchable bibliography containing these papers was developed and can be found at <http://www.pachyonychia.org>. All cases from these reports were utilized for the analysis of clinical phenotype. A total of 55 of these cases have a reported keratin mutation. For a complete listing showing the references used and corresponding data extracted, please see supplemental online information accompanying this paper (website to be included by JID). In addition to the cases from the literature, 57 participants from 41 families were prospectively enrolled in IPCRR and results of keratin mutation tests were completed for 33 participants. The 57 IPCRR participants reside in 10 different countries around the world. A total of 11 participants with PC were identified within the NRIRD and the mutational status of these participants was unknown.

General clinical characteristics Among PC cases identified from the literature, IPCRR, or NRIRD, all but three cases

in two unrelated families (Chong-Hai and Rajagopalan, 1977; Haber and Rose, 1986) are consistent with an autosomal dominant pattern of inheritance. One of these two reports bases the claim of recessive inheritance upon parental consanguinity plus the fact that no other family members exhibited the disease (Chong-Hai and Rajagopalan, 1977); however, it is more likely that this case represented a spontaneous dominant mutation rather than recessive inheritance. In the other possible recessive family, there were two affected offspring born to consanguineous parents (Haber and Rose, 1986); however, this could also be explained by one parent carrying a dominant mutation as a gonadal mosaic. In fact, the rate of spontaneous mutation appears to be relatively high, with 85 of 294 cases (29%) from the literature reporting a negative family history. Similarly in the IPCRR, 26 of the 57 participants enrolled (46%) have a negative family history. Age of onset was typically reported in the literature as "by the first year of life," "during

childhood,” or “in adulthood.” In the literature, 216 of 260 (83%) cases developed within the first year of life, 36 of 260 (14%) developed during childhood, and eight of 260 (3%) developed during adulthood. In the IPCRR, the ages when plantar keratoderma appeared were defined as 0–1, >1–19, and ≥ 20 y. Data from the IPCRR were 11 of 57 (19%) before 1 y of age, 44 of 57 (77%) after the first year but before age 20, and one of 57 (2%) after age 20. The male:female ratio from the literature was 1:1 (192 male:191 female), demonstrating no significant gender bias in development of the disease; however, of note, two families within IPCRR have reported gender-based differences in severity of the disease within the family. Unfortunately, these data are too limited to draw definitive conclusions. Reports of worsening of the painful plantar keratoderma with weight gain and pregnancy, or improvement with weight loss or bed rest, were common in IPCRR participants. Systematic tracking of these parameters is now underway.

Frequency of occurrence of clinical features in PC The most common clinical features of PC and the frequency of occurrence of these manifestations as reported in the literature, the IPCRR, and the NRIRD are summarized in Table I. Of note, in the cases reported in the literature, not every report commented on each clinical finding. If no mention of the feature was made, that report was not included in the overall assessment of frequency. This is likely to lead to an overestimation of the frequency. The frequency of clinical findings from the literature was, however, very similar in most instances to that of the registry data, which were prospectively collected. The most common of all the findings is onychodystrophy (90%–98%) (Fig 2), followed by plantar pain (91%–96%), and plantar keratoderma (91%–96%) (Fig 3). Notable discrepancies between the IPCRR and literature-based data are in the rate of cyst formation (35% in IPCRR vs 72% in the literature), laryngeal involvement or hoarseness (16% in IPCRR vs 70% in the literature), hair involvement (26% in IPCRR vs 53% in the literature),

and natal or prenatal teeth (2% in IPCRR vs 50% in the literature). It should be noted that the IPCRR data are strongly skewed toward PC-1 (K6a/K16) cases (Table II). Specifically, of 55 case studies with known genotypes in the literature, 26 had K6a/K16 mutations compared with 29 with K6b/K17 defects, i.e., a PC-1:PC-2 ratio of 1:1.1. In contrast, of 33 genotyped case studies collected by the IPCRR, 30 had K6a/K16 mutations compared with three with K6b/K17 mutations, giving a PC-1:PC-2 ratio of 10:1. It is not clear why this apparent skewing has occurred, but nevertheless the discrepancies between the IPCRR and literature-based data, notably the percentage of cyst formation, may be because of this PC-1/PC-2 bias.

Onychodystrophy Onychodystrophy is the most consistent physical finding observed in PC patients (Table I); however, the character of the nail changes and the severity of the dystrophy are variable from patient to patient (Fig 2). Clinically, all 20 nails are usually (but not always) hard and thickened and sometimes discolored. The nail findings are one of the earliest manifestations of the disease and are frequently affected at or soon after birth. There is a prominent thickening of the nail bed, often with progressive distal elevation. The surface of the nail can be rough or smooth and some nails develop a “pincer” or “omega” pattern whereas other nails taper off prematurely before reaching the distal fingertip. The fingernails of these patients develop an apparent recession of the nail plate that leaves the distal fingertip with a slightly bulging appearance. Constant grooming of the nails is required to prevent overgrowth and excessive trauma. The nail dystrophy often results in difficulty with fine motor skills such as opening drink cans or bottles. Nail loss and re-growth is frequently associated with infection and/or trauma.

Palmoplantar keratoderma The palmoplantar keratoderma seen in PC is usually more prominent on the plantar than the palmar surface (Figs 3 and 4). It is a hard, non-ery-

Table I. Percent of patients with phenotypic symptoms

Phenotype	Case studies (N = 481)	IPCRR (N = 57)	NRIRD (N = 11)
Toenails	426 of 435 (98%) ^a	55 of 57 (98%)	Nine of Ten (90%)
Fingernails	424 of 434 (97%)	56 of 57 (98%)	Ten of 11 (91%)
Plantar pain	71 of 74 (96%)	54 of 57 (95%)	Ten of 11 (91%)
Plantar keratoderma	288 of 312 (92%)	55 of 57 (96%)	Ten of 11 (91%)
Oral leukokeratosis	216 of 309 (95%)	43 of 57 (75%)	NR
Palmar keratoderma	171 of 215 (80%)	35 of 57 (60%)	Seven of 11 (63%)
Follicular keratoses	149 of 189 (79%)	37 of 57 (65%)	NR
Cysts (any type)	72 of 100 (72%)	20 of 57 (35%)	NR
Larynx (hoarseness)	31 of 44 (70%)	Nine of 57 (16%)	NR
Hyperhidrosis	111 of 169 (66%)	42 of 53 (79%)	4 of 11 (36%)
Hair abnormalities	64 of 120 (53%)	15 of 57 (26%)	0 of 11 (0%)
Natal/prenatal teeth	54 of 108 (50%)	One of 53 (2%)	0 of 11 (0%)

IPCRR, International Pachyonychia Congenita Research Registry; NRIRD, The National Registry for Ichthyosis and Related Disorders; NR not reported.

^aPercentage of reported.



Figure 2

Features of onychodystrophy in pachyonychia congenita (PC) patients. The clinical appearance of nail dystrophy is shown in PC patients with defined keratin mutations (shown below each panel). Variation in nail thickening and subungual hyperkeratosis is apparent in all images and illustrated especially well by comparison of K6a L170F and K6a L469P. Distal hyperpigmentation of the nails is seen in patients with the K6a L469P, K6a I462N, and K16 N125D mutations. Premature tapering of the nails (nail recession) is seen in individuals carrying mutations K6a N171K, K6a N171del, and K6b E472K. Sparing of the proximal nail is seen in some patients (K6a L469P, K6b E472K, K6a L170K, K6a I462N). "Pincer" nails or "Omega" sign is apparent in some cases when examined end-on (K6a I462N). One example of onycholysis is shown (mutation K6a N171K).

thematous, focal keratoderma that is accentuated in pressure points of the feet or in areas of chronic use on the hands (e.g., on the thenar eminences where crutches are held, or in finger creases). Specialized footwear or insoles that modify the pressure distribution on the feet can also alter the pattern of the plantar keratoderma over time. There is no extension of the keratoderma onto the dorsal aspects of the hands or feet, unless there has been friction or trauma in that area. Development of the plantar keratoderma is later than the nail and oral findings, typically developing at the time a child begins ambulating and bearing weight. Blistering often precedes the hyperkeratosis, and blisters can often be seen at the periphery of the hyperkeratosis or underlying the callosities. Although some degree of keratoderma is seen in most PC patients, the severity is highly variable (Fig 3). The plantar keratoderma is usually exquisitely painful and the level of discomfort experienced is often out of proportion to the degree of hyperkeratosis present. Severe cases frequently develop fissuring with secondary infection and routine paring of the hyperkeratotic areas is required to control pain.

In our histology specimens, areas of plantar hyperkeratosis from patients carrying K6a N171K and L469P mutations show prominent ortho- and parakeratosis surmounting an acanthotic epidermis (Fig 5). This pattern is compatible with rapid keratinocyte proliferation and differentiation.

There is no cytologic atypia associated with plantar lesions, although transformation to squamous cell carcinoma has been reported in a chronic plantar lesion (Su *et al*, 1990). In addition to immunostaining of K14 (expected), K6, K16, K17 are seen in the basal cell layer. K6, K16, K17, and K14 staining persists and K10 staining appears in the suprabasal epidermis (Su *et al*, 1990; Wollina *et al*, 1991).

Follicular keratoses Follicular, keratotic spikes of variable severity occur primarily in areas of friction such as the waistband, elbows, and knees (Fig 4). The thickening and follicular keratoses are more accentuated in individuals who frequently crawl on their knees to avoid plantar pain. The primary lesions are reminiscent of severe keratosis pilaris. Development of the follicular keratoses is usually in childhood and they often improve or resolve as the patient ages. Histologically, the follicular keratoses in our specimens demonstrate a parakeratotic horn surmounting a broadly papillomatous and acanthotic epidermis with a pronounced granular layer (Figs 6 and 7). A few pyknotic keratinocytes are seen within the upper layers of the epidermis and are reminiscent of those seen in coronoid lamellae of porokeratosis (100 ×). The horn can also be orthokeratotic (Perrot *et al*, 1973). A normal pattern of expression of epidermal keratins is seen, with suprabasal expression of K6, K16, and K10, with full-thickness staining with K14 (Fig 7).



Figure 3

Plantar keratoderma in pachyonychia congenita. The pressure point distribution of plantar keratoderma is highly variable (all images). Note the variation of keratoderma among individuals with same mutation (K16 L132P, *bottom right*). There is evidence of blistering preceding formation of hyperkeratosis in some patients (K6a N171del, *top: second from right*) and fissuring (K6a N171del, *top right*).

Cysts PC patients are susceptible to developing cysts (Fig 8), including epidermal inclusion cysts, and pilosebaceous cysts such as steatocystomas and vellus hair cysts (Feinstein *et al*, 1988; Su *et al*, 1990; Moon *et al*, 1994). The epidermal inclusion cysts are extremely variable in size and severity. Some patients have only an occasional lesion that is less than 1 cm, whereas others have numerous lesions greater than 5 cm in diameter. These cysts have a punctum, contain a cheesy keratinous material, and frequently become painful, inflamed, and infected. The pilosebaceous type of cysts, including steatocystoma and vellus hair cysts, are typically round to oval, flesh-colored to yellow dermal papules without a visible punctum located on the face and upper trunk and arms. Despite previous reports to the contrary, PC-1 patients develop typical epidermal inclusion cysts in an intertriginous distribution which can occasionally be severe enough to resemble hidradenitis suppurativa (Fig 8). PC-1 patients do not appear to develop steatocystomas or vellus hair cysts. Conversely, PC-2 patients develop all types of cysts, including epidermal inclusion cysts, pilosebaceous cysts, steatocystomas, and vellus hair cysts. Although steatocyst and vellus hair cyst formation appears to be a reliable discriminator between PC-1 and PC-2 subtypes, the delay in onset until the time of puberty makes it

less helpful. In Fig 7, a small epidermal inclusion cyst is shown, which is lined by stratified squamous epithelium with primitive hair follicle differentiation ($100\times$) derived from a carrier of the N171K mutation in K6a. Suprabasal keratinocytes are immunoreactive with K6, K16, and K10, whereas K14 stains the full thickness of the epithelium and hair follicle (Fig 7).

Oral manifestations Oral leukokeratosis of the tongue and buccal surfaces of the mouth are frequently seen in PC (Fig 9). The gingival surfaces are rarely involved. These oral findings often present soon after birth and may be the earliest sign of PC. The leukokeratosis can be worsened by and interfere with breastfeeding in infancy. The tongue develops a white-to-yellow thickening that can mimic oral candidiasis, white sponge nevus, or hairy tongue. The clinical appearance of the buccal leukokeratosis resembles pre-malignant leukoplakia, but is often accentuated along the bite line. Another common, intermittent oral manifestation is angular cheilitis. The cheilitis frequently demonstrates secondary bacterial and yeast infection. Histologically, no cytological atypia is observed. The mucosal lesions show acanthosis and marked parakeratosis in the absence of a granular layer (Fig 7). The pathology is similar to that seen

Table II. Phenotypic features by genotype

Phenotype	Case studies with known genotype (N = 55)				IPCRR with known genotype (N = 33)			
	K6a (N = 16)	K16 (N = 16)	K6b (N = 2)	K17 (N = 27)	K6a (N = 20)	K16 (N = 10)	K6b (N = 2)	K17 (N = 1)
Toenails	16 of 16 (100) ^a	Eight of nine (89)	Two of two (100)	Two of two (100)	20 of 20 (100)	Ten of ten (100)	Two of two (100)	One of one (100)
Fingernails	16 of 16 (100)	Eight of nine (89)	One of one (100)	22 of 22 (100)	20 of 20 (100)	Ten of ten (100)	One of two (50)	One of one (100)
Plantar pain	NR	One of one (100)	NR	Two of two (100)	19 of 20 (95)	Ten of ten (100)	Two of two (100)	One of one (100)
Plantar keratoderma	Nine of 13 (69)	Six of seven (86)	Two of two (100)	15 of 16 (94)	20 of 20 (100)	Ten of ten (100)	Two of two (100)	One of one (100)
Oral leukokeratosis	Nine of ten (90)	Six of seven (86)	One of two (50)	One of five (20)	18 of 20 (93)	Six of ten (60)	One of two (50)	One of one (100)
Palmar keratoderma	Six of ten (60)	Three of five (60)	One of two (50)	Six of seven (86)	11 of 20 (55)	Nine of ten (90)	Two of two (100)	One of one (100)
Follicular keratosis	Six of nine (33)	Three of five (60)	NR	Five of nine (56)	16 of 20 (80)	One of ten (10)	Two of two (100)	One of one (100)
Cysts (any type)	One of nine (11)	NR	Two of two (100)	20 of 20 (100)	Five of 20 (25)	Three of ten (33)	One of two (50)	One of one (100)
Larynx (hoarseness)	Zero of one (0)	NR	NR	Two of two (100)	Eight of 20 (40)	Zero of ten (0)	One of one (100)	Zero of two (0)
Hyperhidrosis	NR	NR	NR	One of one (100)	16 of 20 (80)	Seven of eight (88)	Two of two (100)	One of one (100)
Hair abnormalities	One of one (100)	NR	Zero of one (0)	Four of 10 (40)	Two of 20 (10)	Five of ten (50)	Zero of two (0)	One of one (100)
Natal/prenatal teeth	Zero of ten (0)	NR	One of one (100)	Nine of 16 (56)	Zero of 20 (0)	Zero of ten (0)	Zero of two (0)	Zero of one (0)

IPCRP, International Pachyonychia Congenita Research Registry; NR not reported.

^aPercentage of reported.

with white sponge nevus caused by keratin K4 or K13 mutations (Richard *et al*, 1995; Rugg *et al*, 1995), but there is no dyskeratosis in PC.

Laryngeal lesions Laryngeal involvement usually presents as hoarseness and occasionally as a life-threatening respiratory stridor requiring emergent tracheotomy. Assessment of this finding is subjective because of the invasive nature of the requisite diagnostic laryngoscopy. In cases in which laryngoscopy and biopsy have been performed, however, the larynx demonstrates a white-to-pink thickening or an exophytic mass formation (Cohn *et al*, 1976; Benjamin *et al*, 1987; Wudy *et al*, 1995). Histologically, there is a hyperplastic squamous epithelium with focal intracellular vacuolization that spares the basal layer. Cellular atypia is not present. Several IPCRR participants report that overuse or speaking in a loud voice seems to precipitate hoarseness, but because laryngoscopy has not been performed in these cases, the nature of the hoarseness is not known. Most trauma-induced hoarseness, however, resolves over 1–2 d. One confirmed death because of respiratory compromise from laryngeal involvement has been reported to the IPCRR, and practitioners need to be aware of this severe complication (see Smith *et al*, this issue).

Dentition PC-2 patients sometimes present with teeth at birth (prenatal or natal) or within the first 30 d of life (neonatal). These teeth are typically located in the frontal position, are soft and friable, are prone to caries, and are usually lost within the first few months of life (Clementi *et al*, 1986; Feinstein *et al*, 1988; Munro *et al*, 1994; Munro, 2001). Natal teeth may cause lacerations of the infant's tongue or mother's breast during breastfeeding and can be an aspiration risk. In some cases, second primary teeth will develop in addition to the natal teeth but are ultimately replaced by normal permanent teeth during childhood. In addition to natal teeth, some IPCRR participants have reported early multiple tooth development at 4–5 mo of age as well as one case of early tooth loss without immediate permanent tooth replacement. Histologically, the dental papillae show mucosal hyperplasia and irregular rete ridge proliferation. Cytoplasmic vacuolization and edema are seen in the upper and spinous layers of the mucosa. Irregular osteodentine-like structures with cell inclusions and interglobular dentine are seen (Anneroth *et al*, 1975). The finding of natal teeth is not consistently present, even within the same PC-2 family. Thus, the existence of natal or prenatal teeth is highly suggestive of a PC-2 phenotype and a probable K6b/K17 mutation, but absence of this finding does not suggest a PC-1 phenotype.

Hair manifestations Hair manifestations are most commonly seen in PC-2 patients, although PC-1 patients in the IPCRR have reported hair abnormalities (Irvine and McLean, 1999; Munro, 2001). Hair is thickened or coarse and sometimes brittle or curly. *Pili torti* (twisted hair) has been reported in association with PC-2 (Irvine and McLean, 1999; Munro, 2001). K17 has been shown to be expressed not only in the sebaceous gland and infundibulum of the hair follicle, but also in the hair shaft itself (McGowan and Coulombe, 2000). Mice in which the K17 gene has been ablated



Figure 4
Keratotic follicular spikes. Examples of follicular spikes occurring in areas of friction such as the elbow (K6a N171del), waistband (K6a N171K), knees (K6a L468P), and palms (K6a N171K and K16 N125D) are shown.

develop age- and strain-dependent alopecia, again consistent with a hair phenotype associated with PC-2 (McGowan *et al*, 2002), although the null mutations in these mice are obviously different from those commonly seen in PC-2. Hair findings become apparent when the hair grows in during early childhood. The hair findings are not consistently present, even within PC-2 patients and are variably penetrant within a given family. If hair abnormalities are seen in conjunction with pilosebaceous cysts or in a patient who had natal or prenatal teeth, it is virtually diagnostic of a PC-2 phenotype. Hair findings associated only with onychodystrophy and/or palmoplantar keratoderma, however, are not good predictors of PC-1 (K6a/K16) *versus* PC-2 (K6b/K17) phenotypes.

Other clinical features in PC Additional features of PC have been reported, but are extremely rare and not clearly related to PC. These features include deafness, mental disability, cataract formation, skeletal changes, delayed development of genitalia, and corneal dyskeratosis (Moldenhauer and Ernst, 1968). In one reported family, autosomal dominant cataract formation was shown to be an unrelated coincidental finding in some members affected by PC-1 and carrying a K16 mutation (Smith *et al*, 1999a). The IPCRR has also identified additional findings that may be associated with PC but for which there is still limited data. These features include excessive production of a waxy material in the ears (29 of 57), corneal abnormalities (two of 57), and abnormal cytology on pap smears (two of 57).

Genotype–phenotype relationships in PC An evaluation was also performed of all the IPCRR registry participants and of case reports in whom a mutation has been reported (Table II). Patients with identical mutational status demonstrated a variable phenotype (Table II; Figs 2–5). As

described above, the phenotypic features of PC can be subdivided into a PC-1 (K6a or K16) and PC-2 (K6b or K17) subtype based on a few specific clinical features. The phenotypic features reported in the literature and through IPCRR were subdivided by the type of keratin mutation carried by the patient (Table II). Most features demonstrated no association with a specific keratin mutation (K6a, K6b, K16, or K17). The data from the literature and from the IPCRR do support increased hair involvement, natal tooth development, and cyst formation in PC-2 relative to PC-1, but it again should be noted that most IPCRR-identified cases carried K6a/K16 mutations (30 of 33). The phenotypic features were also divided based on which functional domain of the keratin contained the mutation: the helix initiation motif (HIM), or helix termination motif (HTM), as shown in Table III. Although the data are limited, it is possible that mutations in the HTM may be more pathogenic in the larynx (eight of 11 cases in the HTM vs four of 22 cases in the HIM) and that mutations in the HIM may be more pathogenic in the hair (seven of 22 cases in the HIM vs two of 11 cases in the HTM). Furthermore, the non-HIM/HTM mutations may be associated with a late-onset (*tarda*) presentation (Connors *et al*, 2001; Xiao *et al*, 2004), as discussed elsewhere in this issue (Smith *et al*, McLean *et al*). Overall, numbers are still too small to make firm genotype–phenotype conclusions.

Quality-of-life issues in PC Few quality-of-life questions were included in the IPCRR questionnaire; however, the participants were asked about the time required for grooming and concerns about the cosmetic appearance of their skin and nails. Of 57 participants, 38 reported that time for skin and nail care negatively impacted their quality of life and 45 reported cosmetic concerns that negatively impacted their quality of life. In addition, 29 of 57 participants re-

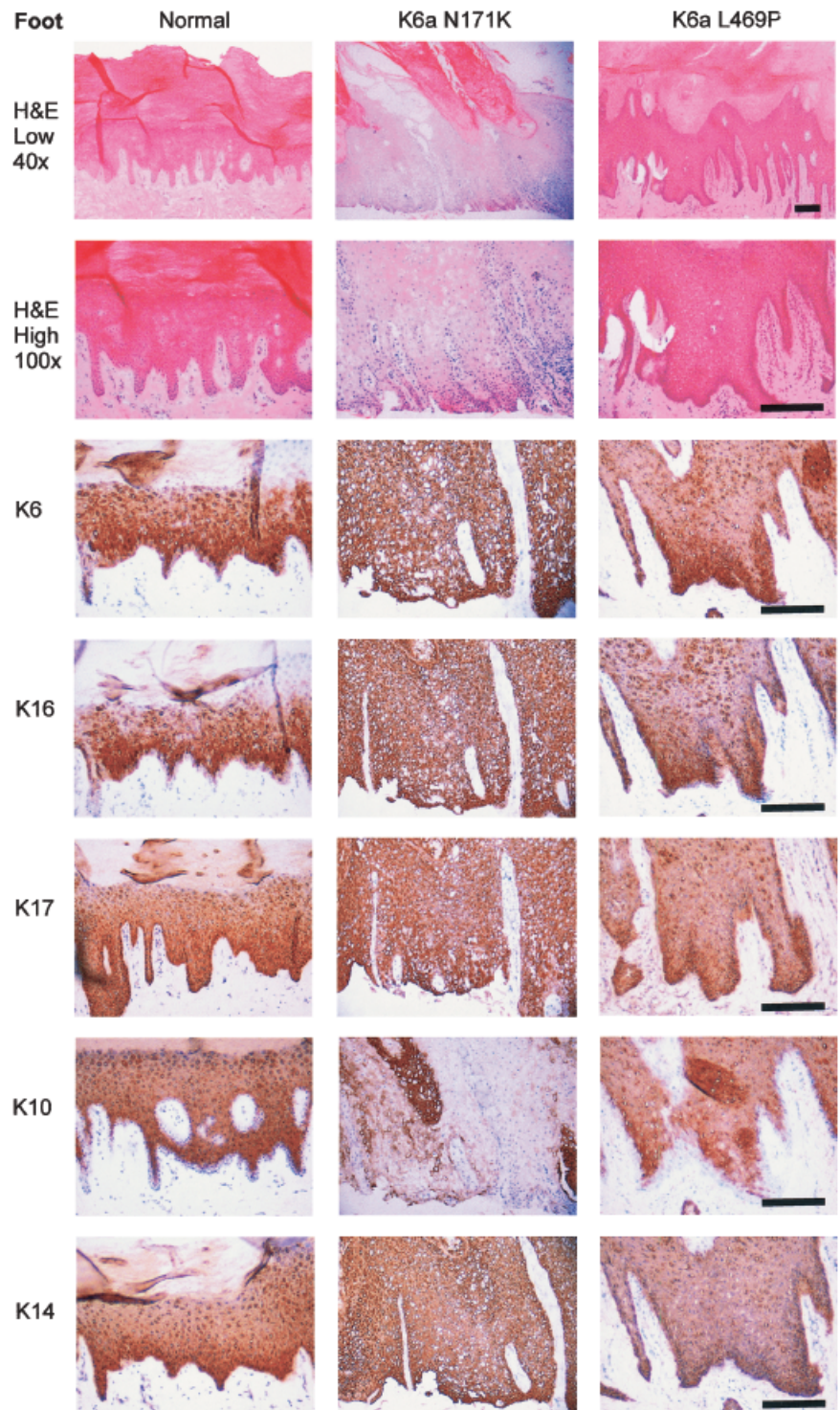


Figure 5
Hematoxylin and eosin (H&E) staining and anti-keratin immunostaining of plantar biopsy material. Plantar biopsy material from two pachyonychia congenita (PC) patients with defined mutations and a normal unrelated control were analyzed by H&E staining and immunohistochemical staining with keratin antibodies. In normal skin, there was a similar suprabasal keratin immunostaining pattern seen for K6, K16, K10, whereas K17 and K14 showed diffuse epidermal staining that included the basal cell layer. In specimens from the patients carrying the K6a N171K and K6a L469P mutations, there is a prominent orthokeratosis and parakeratosis surmounting an acanthotic epidermis. Keratin immunostaining showed diffuse epidermal staining, including the basal cell layer, for K6, K16, K17, and K14. The K6a N171K sample shows markedly decreased K10 expression. All scale bars = 100 μ m.

ported an inability to ambulate without a walking aid (crutches, wheelchair, etc.) and 12 of 57 reported that they are unable to work or attend school because of the severity of their condition. The plantar pain is reported as the most disabling feature (54 of 57 cases), something which has been underrecognized until now. Several IPCRR participants have elected not to have children because of the fear of having a child with PC; however, first-trimester prenatal diagnosis is now possible for individuals with characterized mutations (Smith *et al*, 1999b).

Discussion

PC is a rare, but well-characterized genetic disorder of the nails, skin, hair, and mucosa that exhibits significant phenotypic variability across genotypic groups and families. In order to evaluate which features are most consistently seen in the disorder as well as evaluate the spectrum of presentation, we have analyzed case reports from the literature and prospectively collected data from the IPCRR and NRIRD. We have added 313 additional cases to the 168

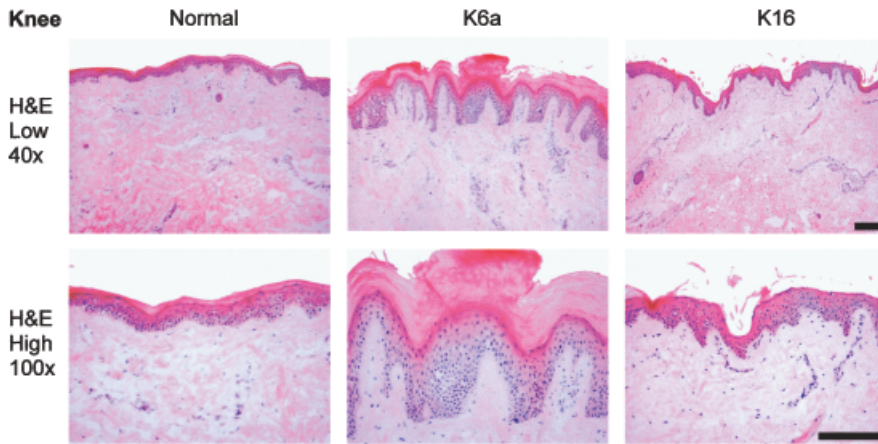


Figure 6
Follicular keratoses from the knee in pachyonychia congenita (PC). Epidermal biopsy material from the knees of two PC patients with defined mutations and a normal unrelated control were analyzed by hematoxylin and eosin (H&E) staining. Hyperkeratosis and epidermal papillomatosis was observed in the K6a patient as compared with the normal control and K16 subject. All scale bars = 100 μ m.

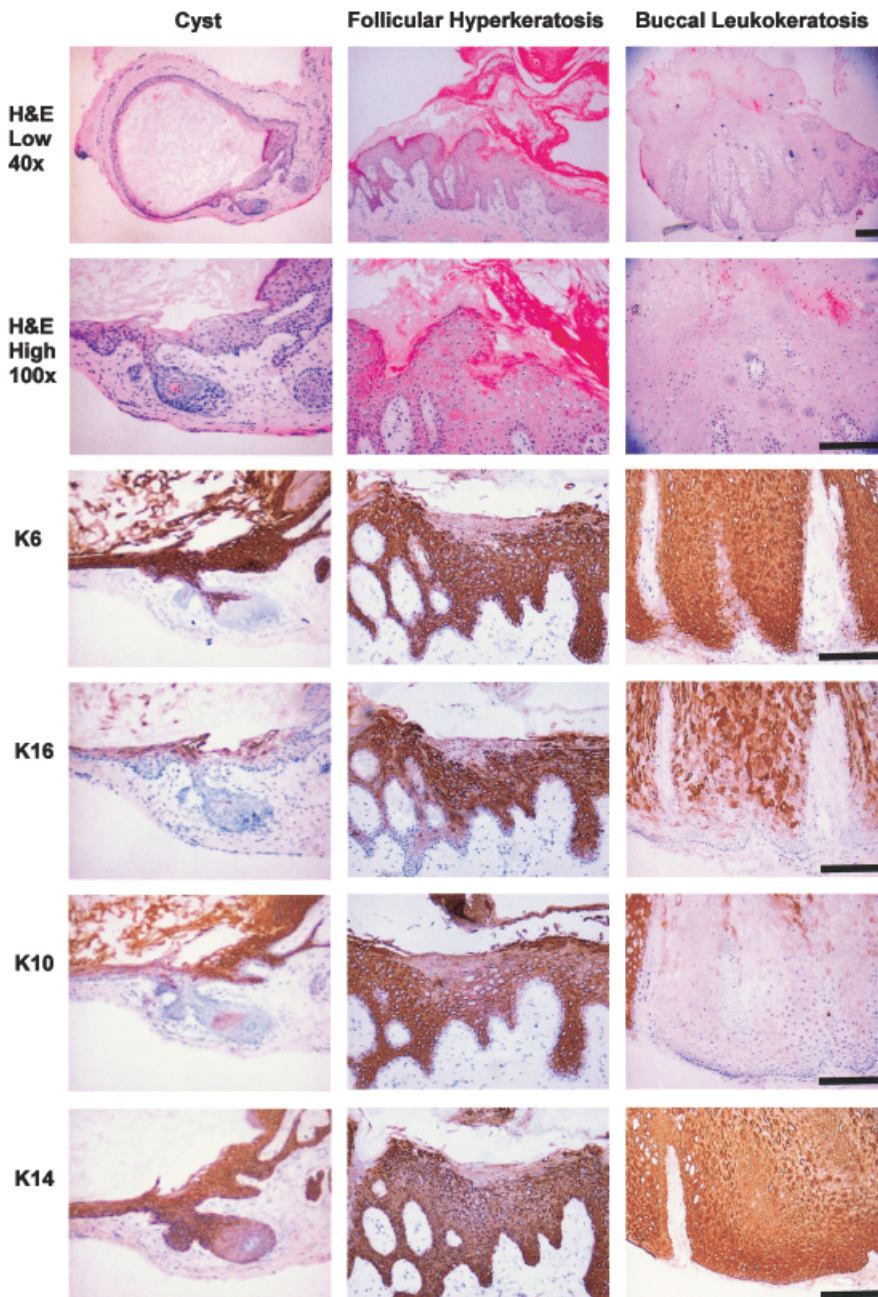


Figure 7
Comparative histology and immunostaining on biopsies from a K6a N171K mutation carrier. Dermal cyst: Cysts were seen to be lined by stratified squamous epithelium with primitive hair follicle differentiation (100 \times). The cyst was immunoreactive with K6, K16, and K10, whereas K14 stains the full thickness of the epithelium and hair follicle. Follicular hyperkeratosis: A few pyknotic keratinocytes were seen within the upper layers of the epidermis and are reminiscent of those seen in coronoid lamellae of porokeratosis (100 \times). The normal pattern of expression of epidermal keratins was seen, with suprabasal expression of K6, K16, and K10, with full-thickness staining with K14. Oral leukokeratosis: hematoxylin and eosin (H&E) staining revealed acanthotic squamous mucosa surmounted by parakeratosis in the absence of keratinocyte atypia. Similar suprabasal immunoreactivity with K6 and K16 was seen, although K10 staining is patchy and is present mostly within the superficial keratinocyte layers. Strong K14 immunoreactivity was observed over the full-thickness of the squamous mucosa. All scale bars = 100 μ m.

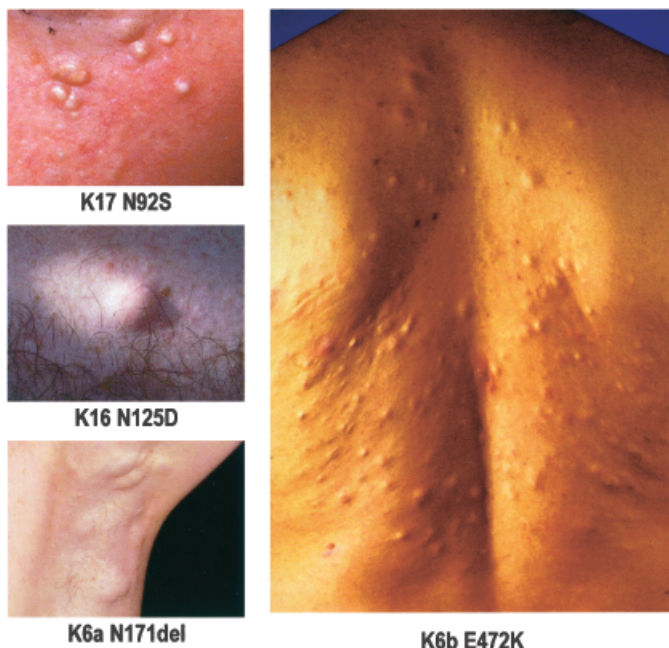


Figure 8

Cysts associated with pachyonychia congenita (PC). Steatocysts (or pilosebaceous cysts) are commonly found on the face (K17 N92S) and are widespread across the trunk (K6b E472K) in post-pubescent PC-2 individuals. Inclusion cysts are usually found on the trunk (K16 N125D) and axilla (K6a N171del) in PC-1 cases.

cases included in the metaanalysis of Feinstein *et al* in 1988. In addition, 57 participants enrolled in the IPCRR and 11 participants enrolled in the NRIRD were reported here. The combination of these data sets permits us to draw more informed conclusions regarding the clinical and pathological features of PC and the genotype–phenotype relationships.

Literature-based metaanalyses, however, have potential sources of bias and misinterpretation. In particular, cross-study comparisons are difficult to perform because of reporting inconsistencies. Retrospectively, it is frequently unclear whether lack of discussion of a particular symptom is because of absence of the finding, or a failure to report an unrecognized component of the phenotype. Therefore, we calculated our percentage of cases having a given clinical finding from the total number of cases in which the finding was reported rather than the total number of case reports. Although better than using the total overall number of cas-

es, this method leads to an overestimation of the incidence of any given finding because of the tendency of authors to report positive findings. In addition, reports of unusual presentations of rare disorders are preferentially published, also leading to an overestimation of the number of cases with unusual findings. Thus, it is unlikely that a rare finding such as corneal dyskeratosis is actually found in 7.8% of PC cases as reported previously (Feinstein *et al*, 1988).

Another source of bias in reporting includes findings that cannot be visually confirmed, such as laryngeal thickening. Although prominent hoarseness may be an indication of laryngeal involvement, the number of PC cases with laryngeal involvement is probably underestimated because laryngoscopy is not a routine part of the examination and assessment of hoarseness is subjective. Additionally, historical reports of findings such as mental retardation in association with PC are often difficult to interpret because of lack of clarity of the degree or type of mental impairment, lack of comment regarding co-segregation with PC in the family, and lack of comparison of rate of occurrence relative to the general population. Thus, although defining PC subtypes based on summaries of clinical case reports was a necessary exercise in order to begin to investigate and understand the syndrome, this method of classification is also fraught with many potential sources of confusion. Because of these multiple sources of bias, we have categorized all the less commonly associated features as “other clinical features” rather than attempting to estimate incidence rates.

A superior alternative to collecting cases from the worldwide literature is to collect cases prospectively through a registry in which information can be consistently obtained. But in disorders as rare as PC, collecting adequate numbers is difficult. For example, our set of 57 IPCRR participants represents the largest prospectively enrolled group of PC patients described to date, but is still small, especially if subset analysis is attempted. Also, Internet access to IPCRR contact information is a likely source of bias toward a more affluent and better-educated population. Therefore, rather than attempting to draw definitive conclusions about genetic subsets of our IPCRR population, we present the raw data for the reader to evaluate (Tables II and III).

Despite the considerable difficulties in evaluating the clinical and pathological phenotypes of PC, our data collectively support the conclusion that there are two primary

Figure 9

Oral leukokeratosis in pachyonychia congenita (PC). Oral leukokeratosis appears to be more pronounced in K6a mutation carriers. Note this is similar in clinical appearance to oral candidiasis. Angular cheilitis can also be observed in PC patients (K6a L468P shown here).

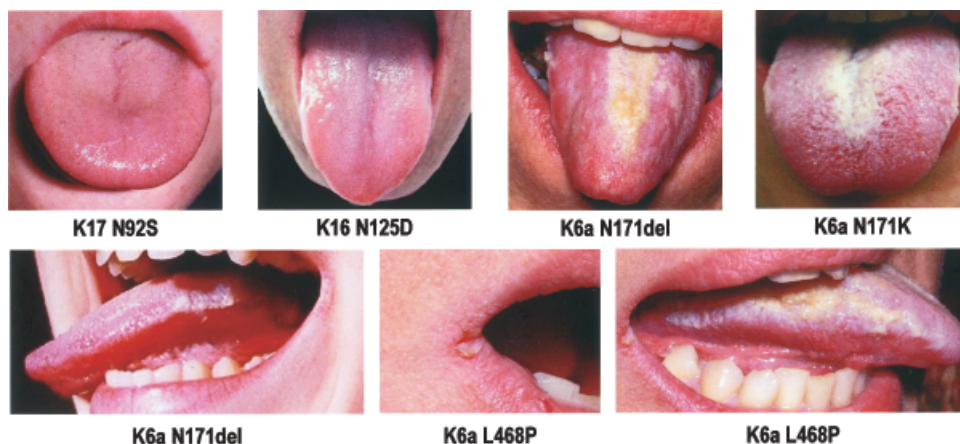


Table III. Clinical features of PC based on location of keratin mutation

Phenotype	Case studies with known genotype (N = 48)			IPCRR with known genotype (N = 33)	
	1AHIM ^a (N = 45)	All other domains (N = 1)	2BHTM ^b (N = 2)	1AHIM ^b (N = 22)	2BHTM ^b (N = 11)
Toenails	43 of 44 (98%) ^c	One of one (100%)	Two of two (100%)	21 of 22 (95%)	11 of 11 (100%)
Fingernails	42 of 44 (95%)	One of one (100%)	Two of two (100%)	22 of 22 (100%)	Ten of 11 (91%)
Plantar pain	Four of four (100%)	NR	NR	22 of 22 (100%)	11 of 11 (100%)
Plantar keratoderma	28 of 33 (85%)	Zero of one (0%)	Two of two (100%)	22 of 22 (100%)	11 of 11 (100%)
Oral leukokeratosis	14 of 31 (45%)	NR	Two of two (100%)	18 of 22 (82%)	Eight of 11 (73%)
Palmar keratoderma	13 of 20 (65%)	Zero of one (0%)	Two of two (100%)	14 of 22 (64%)	Ten of 11 (91%)
Follicular keratoses	Ten of 20 (50%)	One of one (100%)	Zero of two (0%)	13 of 22 (59%)	Seven of 11 (64%)
Cysts (any type)	21 of 30 (70%)	Zero of one (0%)	Zero of two (0%)	Seven of 22 (32%)	Four of 11 (36%)
Larynx (hoarseness)	Zero of one (0%)	NR	NR	Four of 22 (18%)	Eight of 11 (72%)
Hyperhidrosis	One of one (100%)	Zero of one (0%)	NR	15 of 21 (71%)	Ten of ten (100%)
Hair abnormalities	Zero of one (0%)	NR	NR	Seven of 22 (32%)	Two of 11 (18%)
Natal/prenatal teeth	Six of 26 (23%)	Zero of one (0%)	Zero of two (0%)	Zero of 22 (0%)	Zero of 11 (0%)

PC, Pachyonychia Congenita; IPCRR, International Pachyonychia Congenita Research Registry; HIM, helix initiation motif; HTM, helix termination motif; NR not reported.

^aN terminus

^bC terminus

^cPercentage of total reported for location of specific keratin mutation

clinical categories of PC (PC-1 and PC-2) and that they correlate well with mutations in K6a/K16 and K6b/K17, respectively. The best discriminating clinical features include findings of natal teeth, pilosebaceous cysts, or hair abnormalities in K6b/K17 mutation carriers, but incomplete penetrance and overlap of these findings make clinical examination a poor discriminator. It is also clear that dramatic phenotypic diversity exists, both in terms of the exact features that are present and in the severity of the finding. For example, PC without nail findings has been reported and steatocystoma multiplex with natal teeth has been reported as a "new syndrome" (King and Lee, 1987), but molecular analysis of similar cases has revealed K17 mutations (Smith *et al*, 1997; Covello *et al*, 1998). Thus, at least some cases of steatocystoma multiplex are allelic variants of PC-2. Similarly, some families carrying mutations in K16 present with focal non-epidermolytic palmoplantar keratoderma with very trivial or no nail changes (Shamsher *et al*, 1995; Smith *et al*, 2000), showing that this condition is allelic with PC-1 (see also Smith *et al*, this issue). Even within a single family, considerable clinical variability may be observed, suggesting that environmental or co-inherited genetic modifiers play an important role in disease manifestation. This feature of PC may ultimately be of benefit to patients because once the modifier effects are identified they may be utilized in a therapeutic role. Unfortunately, identifying genetic modifiers is difficult or impossible in humans using genetic linkage approaches because of limitations on pedigree size; however, this is more tractable using mouse genetics.

Based on the questionnaire data from the IPCRR, it appears that the quality of life of PC patients is significantly compromised, and further investigation of this finding is warranted. The most common debilitating feature is not the

most obvious (nails), but rather the plantar pain. In addition, laryngeal involvement may be more common and more serious than originally believed. As respiratory compromise may be preceded by a hoarse cry, all parents of infants with PC should be educated to listen for hoarseness and watch for signs of respiratory compromise to permit corrective surgery when necessary. Because of the rarity of PC, surgical pathologists need to be advised of the underlying condition at the time of biopsy in order to facilitate clinicopathologic correlation and correct diagnosis.

Clinicopathologic correlations reveal that the underlying defect in PC is probably similar to that of other keratin disorders such as epidermolysis bullosa simplex or epidermolytic hyperkeratosis, namely skin fragility. Histologically, vacuolization of suprabasal keratinocytes is seen in most affected areas. Blister formation is common on the plantar surface and blisters are easily induced on the feet of PC patients by the pencil-eraser friction method (S. A. Leachman, unpublished observation). The plantar keratoderma becomes worse when stress forces are increased: by increasing weight bearing, hydrating the stratum corneum, or walking long distances. Buccal mucosal involvement is accentuated along the bite line. Palmar involvement is distributed in areas of chronic use. Similarly, follicular keratoses also develop in areas of friction. These findings point to keratinocyte fragility as a prominent pathomechanism in PC. Of further consequence is the fact, in addition to constitutive expression in the tissues of involvement, that expression of the keratin pairs associated with PC, K6a/K16, and K6b/K17 is induced by wound healing and trauma. This creates a cycle in which keratinocyte fragility leads to increased damage or trauma, which leads to increased expression of deficient keratins, which leads to increased fragility, and so on. We hypothesize that the

plantar keratoderma in PC is an accentuation of "normal" callusing that occurs following trauma. Just as a normal individual develops blisters and subsequent calluses following a day of hard work, the PC patient develops blisters and compensatory callus formation. But unlike the normal individual, the callus is not transient or protective, but rather further increases fragility by increasing shear forces and by having elevated levels of the pathogenic, trauma-induced keratins.

We were unable to identify any histological or immunohistochemical features that are pathognomonic for PC or for a specific keratin mutation. Thus, at the present time, the most objective definition of PC subtypes comes from mutation analysis of the keratin genes: mutations in K6a or K16 result in PC-1 whereas defects in K6b or K17 cause PC-2. The discovery of the molecular basis of PC gives physicians the capacity to classify the disease based upon the mutational status rather than relying on variable and subjective physical examination. Given the number of genetic tests that have now been performed for PC patients (Fig 1), it is now relatively straightforward to provide useful and accurate genetic counseling information to PC patients. In addition, the comparison of a variety of patients with similar mutations will ultimately allow physicians to better appreciate the full spectrum of the disease. Indeed, it is likely that comparison of patients with the same mutation may lead to the recognition of other genetic or environmental modifier effects and help elucidate fundamental mechanisms of pathogenesis.

Conclusions

In summary, mutations in K6a/K16 or K6b/K17 are associated with clinical phenotypes PC-1 and PC-2, respectively. Mutation screening of these keratin genes in patients with the PC phenotype will allow confirmation of the clinical diagnosis, classification of the PC subtype based on genotype, and provide the option for prenatal genetic diagnosis. In addition, the phenotypically and genotypically defined cohort of the IPCRR will be crucial for future clinical trials when testing therapeutic agents under development in laboratories around the world.

Materials and Methods

Literature searches and analysis of reported PC cases A comprehensive literature review was performed which included a PubMed/MEDLINE search for primary references, a collection and review of all relevant secondary references from these original sources, and translation of most non-English language articles. The search string used was: [pachyonychia OR Jadassohn OR Jackson-Lawler OR keratoderma OR onychogryphosis OR keratin 6a OR K6a OR keratin 6b OR K6b OR keratin 16 OR K16 OR keratin 17 OR K17 OR steatocystoma OR follicular keratosis OR leukoplakia OR leukokeratosis]. This search revealed 8868 primary articles, many unrelated to PC. The titles and abstracts of these articles were reviewed and 282 papers relevant to PC (or with the potential of having a case report of PC) were identified and obtained. Each of the primary papers was reviewed and an additional 246 references were selected for a total bibliography of 582 articles. Of these references, more than 80 non-English language articles were translated (31 German, 19 French, two Italian, two

Japanese, 13 Russian, nine Spanish, two Dutch, one Latin, and one Hungarian). A total of 41 references were not available for review. For analysis of case reports, each reference was reviewed and each clinical feature of interest was assigned a status of "affected," "unaffected," or "unreported." The percentage of cases affected was calculated by utilizing the number of affected cases divided by the total number reported (i.e., affected plus unaffected). The undocumented cases were not included in the calculation.

IPCRR The IPCRR is a research registry resource approved by an Institutional Review Board (IRB) that complies with all principles of the Helsinki Accord (Western IRB #1057496). Participant recruitment is primarily by self-referral to the web-based registry (<http://www.pachyonychia.org/Registry.html>), although direct physician referral occurs as well. Participants in the registry have the option to complete an epidemiologic, genetic, and medical history questionnaire, provide clinical photographs, receive mutation testing (K6a, K6b, K16, K17), and donate blood or tissue samples for research purposes. Each participant that completes a questionnaire and provides a series of standard photographs (or receives a physical examination by S.A.L.) undergoes a medical consultation (S.A.L.) in order to establish the diagnosis and determine the appropriateness of genetic testing. If clinical suspicion for PC is confirmed, the participant receives genetic counseling and is referred for mutation testing. Mutation status is determined by sequence analysis in a research laboratory (W.H.I.M. and F.J.D.S.) and is verified in the clinical laboratory of GeneDx (Gaithersburg, Maryland). As with the case reports, the percentage of cases affected was calculated by utilizing the number of affected cases divided by the total number reported; the undocumented cases were not included in the calculation.

NRIRD The NRIRD has for more than 10 y been an IRB-approved research registry (FWA#: 00006878) that has been collecting data according to the Declaration of Helsinki principles on participants with ichthyotic skin diseases.

Tissue collection Five IPCRR participants, two with mutation N171K in K6a, one with mutation L469P in K6a, one with mutation N125D in K16, and a normal control participant, provided tissue specimens for the study. Biopsy sites included the buccal mucosa, knee, foot, normal buttock, axillary and inguinal cysts, and follicular keratosis of the buttock. Each tissue was split into two sections and either formalin fixed and paraffin embedded or frozen in optimal cutting temperature Tissue-Tek embedding medium (Sakura Finetek, Torrance, CA) embedding medium for frozen sectioning. Each sample was stained or immunostained as described below.

Histology Routine hematoxylin and eosin (H&E) staining was performed to evaluate morphologic features of each specimen. Immunoperoxidase staining of frozen and paraffin-embedded sections utilized the Dako Envision system (catalog number K4007, DakoCytomation, Denmark). This is a sensitive system based on horseradish peroxidase-conjugated secondary antibodies to reduce non-specific staining. Paraffin and frozen 7 μ m sections were cut and placed on electrostatically charged slides for improved binding (Superfrost slides, catalog number 406/0179/00, VWR International, Bristol, CT). Paraffin sections were deparaffinized and endogenous peroxidase activity was blocked by incubation in 0.3% hydrogen peroxide in methanol. Sections were rehydrated and antigen retrieval was accomplished by autoclaving at 120°C for 20 min in antigen unmasking solution (H-3300, Vector Labs, North Holland) using the 2100 Retriever autoclave (Pickcell Laboratories, Peterborough, UK, the Netherlands) and allowing sections to cool for at least 2 h.

Frozen and antigen-retrieved paraffin-embedded sections were rinsed in phosphate-buffered saline (PBS) and incubated in primary antibody solution in a humidified chamber. One hour incubations of the following primary antibodies were performed: (1) 1:10 dilution anti-cytokeratin 10 (LHP1, Novocastra Laboratories Ltd, Newcastle-

upon-Tyne, UK; (2) 1:10 dilution anti-cytokeratin 14 (LL002, from E.B.L. Laboratory, Dundee, UK); (3) 1:20 dilution anti-cytokeratin 6 (Ks6.KA12, Progen Biotechnik GmbH, Heidelberg, Germany); (4) 1:10 dilution anti-cytokeratin 16 (LL025, from E.B.L. Laboratory); (5) 1:20 dilution anti-cytokeratin 17 (E3, from Vector Laboratories, Burlingame, California). After primary antibody incubation, the sections were washed in PBS. Anti-mouse peroxidase-labeled polymer (Dakocytomation Envision System, Cambridgeshire, UK) was applied undiluted for 30 min, washed in PBS, and developed with the DAB/substrate-chromogen system from Dakocytomation. Slides were counterstained with hematoxylin.

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