EGFR signaling is overactive in Pachyonychia congenita: effective treatment with oral erlotinib

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Title:

EGFR signaling is overactive in Pachyonychia congenita: effective treatment with oral erlotinib

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Short title:

EGFR pathway in pachyonychia congenita
Abbreviations used:

AREG: amphiregulin

DLQI: Dermatology Life Quality Index

EGFR: epidermal growth factor receptor

EREG: epiregulin

p-ERK: phosphorylated extracellular-signal regulated kinase

FFI: foot function index

HC: healthy control

HER: human epidermal growth factor receptor

HB-EGF: heparin binding epidermal growth factor

KRT: keratin

MAPK: mitogen-activated protein kinase

mTOR: mammalian target of rapamycin

OS: Olmsted syndrome

PC: pachyonychia congenita

PPK: palmoplantar keratoderma

p-RPS6: phosphorylated ribosomal protein S6

SC: stratum corneum

TG: transglutaminase

TGF-α: transforming growth factor alpha

TRPV3: Transient receptor potential vanilloid-3
Abstract:

Pachyonychia congenita (PC) is a rare keratinizing disorder characterized by painful palmoplantar keratoderma (PPK) for which there is no standard current treatment. PC is caused by dominant mutations in keratin 6A, 6B, 6C, 16, and 17 genes involved in stress, wound healing, and epidermal barrier formation. Mechanisms leading to pain and PPK in PC remain elusive. Here, we show overexpression of EGFR ligands epiregulin and TGF-α as well as HER1-EGFR and HER2 in the upper spinous layers of PC lesions. EGFR activation was confirmed by upregulated MAPK/ERK and mTOR signaling. Abnormal late terminal keratinization was associated with elevated transglutaminase-1 (TG1) activity. Additionally, the Ca²⁺ permeable channel TRPV3 was significantly increased in PC-lesional skin suggesting a predominant role of the TRPV3/EGFR signaling complex in PC. We hypothesized that this complex contributes to promoting TG1 activity and induces the expression and shedding of EGFR ligands. To counteract this biological cascade, we treated 3 PC patients with oral erlotinib for 6 to 8 months. The treatment was well tolerated and led to an early, drastic, and sustained reduction of neuropathic pain with a major improvement of quality-of-life. Our study provides evidence that targeted pharmacological inhibition of EGFR is an effective strategy in PC.
Introduction

Pachyonychia congenita (PC) is a rare autosomal dominant disorder of keratinization caused by mutations in keratin 6A (KRT6A), 6B (KRT6B), 6C (KRT6C), 16 (KRT16), or 17 (KRT17) genes. PC is characterized by severe and disabling plantar pain, palmoplantar keratoderma (PPK), nail abnormalities, oral leukokeratosis, cysts, follicular hyperkeratosis and hyperhidrosis. Plantar pain is the most severe and debilitating clinical feature, considerably affecting the quality-of-life of patients, especially by limiting activity in their daily life (Krupiczkojc and O’Toole 2018). PC is a disease with unmet medical needs since no satisfactory therapies are currently available. Frequently used management for PC-PPK includes regular grooming such as paring/trimming and shaving of calluses, although this only partially alleviates pain and over-shaving is paradoxically painful. Topical application of products containing salicylic acid, lactic acid or urea produces only minimal improvement (Kaspar et al. 2011). Botulinum toxin improved blistering and pain (Swartling et al. 2010), but is expensive and necessitates anaesthesia. Oral painkillers, steroids, and nonsteroidal anti-inflammatory drugs can partially alleviate pain, but their prolonged use can expose to toxic effects. Topical and systemic retinoids can reduce calluses size and thickness in some patients, but they are often poorly tolerated (Gruber et al. 2012).

The pathogenic mechanisms involved in PC are complex and not fully understood. Although previous studies provided evidence that pain is mediated by alterations in sensory innervations and neuropathic pathways (Brill et al. 2018; Pan et al. 2016; Wallis et al. 2016), the fact that only PC-affected skin is painful suggests that keratinocyte altered functions may drive nerve damage (Pang et al. 2015; Weinberg et al. 2020). Recent studies have shown that krt16/- mice footpad lesions and PC-PPK lesions exhibit significant dysregulation of genes implicated in skin inflammation, innate immunity and skin barrier maintenance (Cao et al. 2015; Lessard et al. 2013). These changes could be in part induced by increased oxidative stress associated with...
reduced activity of nuclear factor erythroid 2-related factor 2 (NRF2), a master cellular regulator of oxidative stress (Kerns et al. 2016; Zieman and Coulombe 2020). Pre-clinical studies have shown that the mammalian target of rapamycin (mTOR) pathway translationally regulates KRT6A expression and the mTOR inhibitor, sirolimus, selectively down-regulates KRT6A expression through inhibition of translation of mRNA containing a 5’untranslated oligopyrimidine tract (Hickerson et al. 2009). Of note, mTOR signaling has been suggested to contribute to persistent pain in PC patients as mTOR is involved in pain processing (Cao et al. 2015; Kersten et al. 2015; Martin et al. 2017). A proof-of-concept study of systemic sirolimus treatment showed a marked reduction of pain and improved PC symptoms. However, severe side effects including gastrointestinal and mucocutaneous toxicities, led to treatment discontinuation (Hickerson et al. 2009). Recently, a preliminary case study reported that topical sirolimus treatment improved thickness of plantar keratoderma and reduced pain without toxicity in two patients with KRT6A mutations (Teng et al. 2018). A phase 2 clinical trial using topical rapamycin achieved significant improvement and good tolerance supporting the central role of mTOR in PC. However, the mechanisms leading to mTOR pathogenic activity are unclear.

mTOR is one of the primary targets of the human epidermal growth factor receptor (HER) family including the epidermal growth factor receptor (EGFR) which regulates cellular growth, survival, proliferation and differentiation. Several ligands can bind and activate HER members such as transforming growth factor-alpha (TGF-α), amphiregulin (AREG), epiregulin (EREG), heparin-binding EGF (HB-EGF), betacellulin (BTC), and epigen (EPGN). HER receptors and particularly EGFR signaling play a critical role in skin homeostasis by regulating basal keratinocyte proliferation and late terminal differentiation in suprabasal cells that have lost their interactions with the extracellular matrix (Cheng et al. 2010; Wakita and Takigawa 1999). We and others previously reported that blocking EGFR with oral erlotinib significantly reduced
pain and improved PPK and quality-of-life for patients with Olmsted syndrome (OS), another rare skin disorder characterized by painful PPK most often caused by gain-of-function mutations in the transient receptor potential vanilloid-3 (TRPV3) gene (Greco et al. 2020; Zhang et al. 2020). TRPV3 is a heat-sensitive Ca$^{2+}$ channel notably involved in skin barrier function, skin homeostasis, wound healing, and pain under inflammatory conditions (Sulk and Steinhoff 2015). TRPV3 forms a signaling complex with TGF-α and EGFR which controls keratinocyte proliferation and differentiation (Cheng et al. 2010; Schneider et al. 2008). In particular, this complex regulates the activity of transglutaminase-1 (TG1) to induce epidermal terminal differentiation (Lorand and Graham 2003).

In this study, we show evidence for EGFR signaling pathway overactivity in PC-lesional skin associated with increased TRPV3 expression and report successful treatment of 3 PC patients with oral erlotinib.

RESULTS

EGFR signaling is overactive in PC-calluses of the sole

To evaluate whether TRPV3 is involved in painful PC lesions, we first examined its expression in PC-calluses of the sole. *In situ* mRNA hybridization revealed a strong and significant increase in TRPV3 mRNA levels in all spinous layers of PC-calluses compared to non-lesional skin (by 2 fold) (Figure 1) and anatomically-matched healthy control (HC) skin (Supplementary Figure S1). We next investigated the expression of EGFR-ligands *EREG, HB-EGF, TGF-α*, and *AREG* whose transcript levels are induced by a rise in intracellular Ca$^{2+}$ (Cheng et al. 2010; Denning et al. 2000; Dlugosz et al. 1994). We found a considerable increase in EREG mRNA expression in the upper spinous layers of PC-lesional skin while its expression was lower and restricted to the granular layer in non-lesional skin and HC (Figure 1, Supplementary Figure
S1). TGF-α mRNA were also significantly increased in the granular and upper spinous layers of PC-lesional skin (Figure 1). HB-EGF and AREG expression also appeared to be upregulated in PC-lesional skin but at a lower level (Supplementary Figure S2). We next investigated the pattern of HER1-4 expression by immunostaining. We observed that both HER1/EGFR and HER2 expression, but not HER3 and HER4 (data not shown), was increased in all spinous layers of PC-lesional skin compared to non-lesional skin and HC (Figure 2a, 2b). To determine whether EGFR was activated, we assessed phosphorylation of the ribosomal protein S6 (RPS6), a downstream target of mTOR kinase, and the extracellular signal-regulated kinase (ERK1/2). Immunostaining showed a strong increase in both p-RPS6 and p-ERK1/2 in the spinous layers of PC-lesional skin, while only a few cells were detected in non-lesional skin and HC (Figure 2c). We also analyzed VEGF-A mRNA expression in PC-lesional skin and showed a significant increase in basal and all spinous layers as compared to non-lesional skin and HC suggesting that angiogenesis is promoted in PC-lesional skin (Figure 2d, 2e, Supplementary Figure S1). Overall, these results provided evidence that EGFR signaling is overactive in PC-lesional skin, could promote angiogenesis, and is associated with increased TRPV3 expression.

Late terminal differentiation is enhanced in PC-lesional skin

The biological effect of EGFR activation differs between basal keratinocytes and epidermal spinous layers. It is thought that EGFR signaling in basal keratinocytes mainly supports proliferation and survival but prevents differentiation while it contributes to terminal epidermal differentiation and skin barrier formation in suprabasal keratinocytes (Cheng et al. 2010; Wakita and Takigawa 1999; Wolf et al. 2016). To investigate the effects of EGFR activity in spinous layers of PC-lesional skin, we examined early and late differentiation markers. We found that the early differentiation marker KRT10 was reduced in PC-lesional skin with many
cells of the spinous layers devoid of KRT10 expression compared to HC. Conversely, the expression of the late terminal differentiation marker filaggrin was increased in PC-lesional skin as compared to non-lesional skin and HC (Figure 3a). Filaggrin was also seen in the nucleus of some keratinocytes from the spinous PC-lesional layers, an event described in nuclear dissolution during terminal differentiation (Pearton et al. 2002). TG1 activity, a direct target of EGFR signaling (Wolf et al. 2016) and regulated by TRPV3-mediated Ca\(^{2+}\) rise, was also investigated. We observed a strong TG1 activity in the granular and spinous layers and the stratum corneum (SC) of PC-lesional skin compared to non-lesional skin and HC in which TG1 activity was restricted to the granular layers and the SC (Figure 3b). These results corroborated with increased EGFR/TRPV3 signaling in the spinous layers of PC-lesional skin, promoting late terminal differentiation, and thus contributing to the thickening of the SC of PC-calluses.

**Patient treatment**

Given the strong EGFR activity detected in PC-lesional skin, we treated 3 adult PC patients with oral erlotinib for 6 to 8 months. P1 was treated with a daily dose of 75mg and P2 and P3 received 50mg/day according to their weight. A summary of patient characteristics, baseline weights and genotypes is included in Supplementary Table S1.

The Visual scale analogue for pain, Dermatology Life Quality Index (DLQI), Skindex-16 quality-of-life survey, and foot function index (FFI) were used to assess subjective clinical and quality-of-life improvements. Patients also kept a daily diary to document pain intensity when standing or walking (0-10 scale), level of physical activity, use of analgesics, and the occurrence of side effects. All three patients reported a remarkable and rapid reduction in foot pain, from an initial score of 8-9/10 to 4/10 within the first week of treatment. The pain steadily declined to a plateau of 2/10 within 6 weeks (Figure 4a, Supplementary Figure S3). All patients also
showed a rapid decrease in their DLQI scores (from 21/30 to 6/30 on average) and Skindex-16 scores (from 64/96 to 20/96 on average) within 1 month of treatment (Figure 4b, 4c, Supplementary Figure S3). Such an improvement allowed patients to ambulate better and to increase their walking range without support or pain. We evaluated and quantified PC-related activity before and during treatment using the FFI which objectively measures the severity of pain and disability in foot pathology (Budiman-Mak et al. 2013). The 3 patients demonstrated a remarkable decrease in their FFI scores at 1 month (initial scores of 74% for P1, 50% for P2, and 74% for P3 vs 42%, 27.6% and 52.7% respectively at one month), and their scores were even lower by month 6 (11,75% for P1, 7,64% for P2, 21,76% for P3) illustrating the drastic pain reduction and increased ability to walk and exercise (Figure 4d). Activities that were impossible before treatment could be performed regularly without inducing mechanical hyperalgesia (long-distance walks, motorcycle racing, bowling, walking barefoot/in socks at home, shopping, walking briskly, walking up and down 3 flights of stairs) (Figure 4e, 4f, and 4g, Supplementary Figure S3a, S3b, S3c). Nocturnal neuropathic-like pain also quickly disappeared, considerably improving the patient’s sleep quality and asthenia. They discontinued their pain medication and reported disappearance of antalgic posture.

The size and thickness of plantar calluses had markedly decreased and some of them had disappeared (Figure 5a, 5b, Supplementary Figure S4a, S4b). Patients reported they became softer and painless to pressure with disappearance of their inflammatory borders (Figure 5c) and marked reduction or disappearance of painful subcorneal neurovascular structures (Supplementary Figure S4c). The thickness of P1 fingernails became apparently normal and his toenails were markedly thinner (Supplementary Figure S5). Shaving of their calluses became less frequent (every 2-3 weeks instead of every week before treatment).

Mean erlotinib trough plasma concentrations were lower than the reported toxic concentration (781ng/mL for P1; 225ng/mL for P2 and 388ng/mL for P3). Erlotinib was well tolerated in all
patients despite moderate acneiform lesions on the face and thoracic area in P1 and P3 which improved upon classical topical (benzoyl peroxide 5%) and oral (doxycycline 100 mg/day for P3) treatment for acne.

DISCUSSION

In this study, we show increased EGFR activity in the upper spinous layers of PC-lesional skin which most likely originates from overexpression of EGFR ligands EREG and TGF-α. TRPV3 expression was elevated in the same epidermal layers, implicating the EGFR/TRPV3 signaling complex in impaired epidermal differentiation. Oral treatment with the EGFR inhibitor erlotinib resulted in quick and remarkable pain reduction, enhanced patient physical activities, and improved quality-of-life with a decrease in size and thickness of several plantar calluses in the three patients treated. Overall, our study provides strong evidence for the importance of the EGFR pathway in the pathophysiology of painful plantar keratoderma in PC.

Our results suggest a pathogenic role of EGFR pathway leading to aberrant TG1 activity and late terminal differentiation of keratinocytes in PC. TGF-α is known to promote late terminal differentiation by markedly enhancing TG1 activity and CE formation while inhibiting KRT10 expression both in vitro and in vivo (Dlugosz et al. 1994; Wakita and Takigawa 1999). Recently, ADAM17-EGFR signaling has been suggested to mediate proteolytic processes involved in the maintenance of a functional epidermal barrier (Tholen et al. 2016) by directly inducing TG1 expression and activity in keratinocytes committed to differentiation most likely through PKCα and PLCγ1, but not PKCδ (Kerns et al. 2016; Wolf et al. 2016). Interestingly, constitutive activation of ADAM17-mediated EGFR signaling has previously been reported in tylosis, an autosomal dominant syndrome with focal PPK and oesophageal cancer caused by mutations in the iRHOM2 gene (Blaydon et al. 2012; Brooke et al. 2014). Tylosis skin also shows elevated
TG1 activity in hyperkeratotic lesions (Brooke et al. 2014), suggesting that ADAM17/EGFR may also contribute to increased TG1 activity in PC.

PKC activity was not examined in PC skin, however, we observed a strong increase in PI3K/Akt/mTOR and MAPK/ERK signaling, reflected by high levels of both p-RSP6 and p-ERK. EGFR-mediated PI3K activation is known to phosphorylate Akt which in turn inactivates tuberous sclerosis complex 1 and 2 (TSC1/2) and prevents TSC1/2-dependent inhibition of mTOR (Tee et al. 2002). Interestingly, TSC2 is a transcription factor that negatively regulates EREG expression (Pradhan et al. 2014). Elevated levels of EREG have been found in patients with tuberous sclerosis in whom mTOR signaling is hyperactive due to TSC1/2 loss-of-function mutations (Li et al. 2008). Thus, it is possible that upregulation of EREG observed in PC patients is due to the abrogation of transcriptional repression following TSC2 inactivation. Therefore, EGFR activation may lead to uncontrolled EREG synthesis, resulting in a self-amplifying signaling loop.

VEGF-A is an angiogenic factor that has also been identified as an upregulator of KRT6, KRT16 and KRT17 expression (Jiang et al. 2017). The significant increase in VEGF-A observed in PC-lesional skin could therefore contribute to PC pathogenesis through the aberrant overexpression of PC-associated genes. In addition, VEGF-A was shown to enhance primary keratinocyte proliferation in vitro and to induce epidermal thickening in vivo (Jiang et al. 2017; Yang et al. 2006). Ki67 and KRT6 immunostaining confirmed that proliferating keratinocytes were increased in PC-lesional skin compared to non-lesional and HC (Supplementary Figure S6) suggesting that VEGF-A may promote hyperproliferation in PC.

TRPV3 was mainly overexpressed in the upper spinous layers of PC-lesional skin. A pathological role of TRPV3 in the skin was recently discovered by identifying gain-of-function mutations leading to constitutive activation of the Ca²⁺ channel in OS (Duchatelet and Hovnanian 2015). TRPV3 is expressed in healthy keratinocytes, is upregulated during wound
healing, e.g., in hypertrophic post-burn scars (Kim et al. 2016), and activated by physical stress (heat, pressure…) (Sulk and Steinhoff 2015). The significant increase in TRPV3 found in PC-lesional skin correlates with the important upregulation of wound repair-associated genes \(KRT6A, KRT6B, KRT6C, KRT16,\) and \(KRT17\) (Cao et al. 2015; Lessard et al. 2013) (Supplementary Figure S6). Additionally, the predominant location of painful calluses at pressure points and their exacerbation in summer suggest TRPV3 overactivity in PC-lesional skin. The increase in intracellular \(\text{Ca}^{2+}\) level controls both EGFR ligands production and release, the latter being mediated by the metalloproteinase ADAM17 (Cheng et al. 2010; Denning et al. 2000; Wakita and Takigawa 1999). As ADAM17 activity also depends on the TRPV3-induced rise of cytosolic \(\text{Ca}^{2+}\) in keratinocytes (Cheng et al. 2010), a positive-feedback loop involving TRPV3, ADAM17, EGFR ligands EREG/TGF-\(\alpha\), and EGFR could drive PPK and pain in PC (Figure 6).

We provided evidence that inhibition of EGFR by the treatment of 3 patients with oral erlotinib resulted in early, drastic, and sustained reduction in pain and improved patient’s quality-of-life. Recently, treatment of a limited number of PC patients with topical or systemic EGFR inhibitors, erlotinib or lapatinib (Greco et al. 2022; Lee and Lowe 2022), also led to clinical improvement in hereditary \(KRT16\) or \(KRT6A\) PPK, confirming a key role for EGFR signaling in PC pathogenesis. The analgesic effect we observed is consistent with previous reports for erlotinib efficacy in neuropathic pain (Borges et al. 2021; Kersten et al. 2015; Kersten et al. 2013) and OS-related pain. PC patients experienced a reduction in their calluses without the formation of new neurovascular structures, but the improvement in PC-PPK appears to be slower than in OS patients treated with oral erlotinib (Greco et al. 2020; Zhang et al. 2020).
Interestingly, mTOR inhibition by topical sirolimus ointment (Teng et al. 2018) alleviated pain in PC patients, suggesting that EGFR may enhance nociception through the mTOR pathway. Although the EGFR pathway may regulate KRT6 and KRT16 expression (Jiang et al. 1993; Wang and Chang 2003) through an EGF-responsive element within their promoter, the mechanisms by which EGFR mediates pain in PC-PPK are still unclear. We hypothesize that the EGFR-TRPV3 signaling complex is involved. In addition, TRPV3 mediates neuronal sensation via the release of nociceptors, in particular ATP and PGE2 (Huang et al. 2008; Mandadi et al. 2009). Notably, TRPV3 has been shown to be involved in pain in inflammatory conditions, especially in mechanical hyperalgesia (Huang et al. 2008), which is an important and common feature in PC patients (Brill et al. 2018).

Despite relatively low trough concentrations in 2 patients, all 3 patients showed a remarkable improvement of their quality-of-life, suggesting that low doses of erlotinib could be sufficient for clinical benefit. The patients did not complain about the persistence of painless calluses, despite the aesthetic discomfort. A combination of low systemic dosing and topical application of erlotinib could improve local efficacy while minimizing systemic exposure and side effects. Other EGFR inhibitors with other specific targets (EGFR vs HER2) and pharmacological properties should also be considered.

Together, our results show that EGFR signaling contributes to PC pathogenesis and provide evidence that pharmacological inhibition of EGFR is an efficient strategy to treat PC keratoderma and its neuropathic/nociceptive components.

Material and methods

Patients:
The 4 patients developed life-long and painful plantar calluses in pressure areas (P1, P2 and P3) or diffuse plantar keratoderma (P4) with fissures, painful blisters and neuropathic pain since infancy. All patients except P1 had oral leukokeratosis. None of them had neonatal teeth. Their condition was aggravated in summer by heat and sweating and was improved during winter and cold. They all described mechanical hyperalgesia.

P1 is a 43-year-old male heterozygous for the KRT16 pathogenic variation c.362T>A, (p.Met121Lys). Fingernails and toenails were thickened with splinter haemorrhages. He described very painful subcorneal neurovascular structures on his heels, lower back pain and headaches. Pain was present as soon as he put his feet on the ground in the morning and led to antalgic postures. His treatments consisted of paracetamol, non-steroidal anti-inflammatory drugs and anaesthetic lidocaine plaster for PC symptoms, and β-blockers and platelet aggregation inhibitor for coronary insufficiency.

P2 and P3 are mother and son. P2 is a 52-year-old female and P3 is a 24-year-old male carrying the heterozygous c.1403T>C (p.Leu468Pro) KRT6A pathogenic variant. All their nails were markedly thickened with multiple fissures. Both patients had insomnia and described subcorneal painful neurovascular structures of their calluses. P2 treatment consisted of pedicure care, paracetamol, and oral non-steroidal anti-inflammatory drugs. She has been on disability since the age of 31, used a wheelchair and moved around at home on her knees since the age of 38. P3 had repetitive whitlow of multiple fingers during infancy and puberty. His past treatment included shaving of calluses and paracetamol.

P4 is a 44-year-old female heterozygous for the c.395T>C (p.Leu132Pro) pathogenic variant in the KRT16 gene. She showed marked thickening of all fingernails and toenails since the age of 15 months. Hyperkeratosis of her soles was transgredient with inflammatory borders. She also developed localised linear hyperkeratosis of her palms and fingers. She usually travelled by
bicycle to avoid the pain triggered by walking. Past therapies included paracetamol, acitretin, oral steroids and nonsteroidal anti-inflammatory drugs, calcipotriol/betamethasone dipropionate cream, which were not effective.

The study protocol was approved by the Ethical Review Boards of the Imagine Institute and the French National Healthcare Insurance agency. All patients provided written informed consent for participation in this study. Patients consented to the publication of their images.

**Erlotinib treatment and trough levels:**

P1 received a daily oral dose of 75mg (0.91mg/kg), P2 and P3 were treated with 50mg/day (0.74mg/kg and 0.67mg/kg respectively). Blood samples were collected at the trough point just before the next administration to quantify plasma concentrations of erlotinib.

**Evaluation of efficacy and adverse effects of erlotinib:**

Quality-of-life and pain were assessed using the Dermatology Life Quality index, Skindex-16 and Visual Analog Score for pain. Foot function was assessed using the Foot function Index which measures the severity of pain and disability. Physical activity was monitored daily using a step counter. Adverse effects were evaluated throughout the study.

**Statistics:**

All summary data are presented as means ± SD. Statistical analyses were performed using Mann-Whitney test or unpaired t-test depending on Normality of the data. P < 0.05 is considered statistically significant. Graphs were generated by GraphPad Prism.

**Data availability statement:**

No datasets were generated or analyzed during the current study.
Conflict of Interest statement:

The authors state no conflict of interest.

Acknowledgements:

We are grateful to Janice Schwartz and Holly Evans from Pachyonychia Congenita (PC) project for genetic testing of the PC patients and for their constant support. We are indebted to Dr Emmanuelle Bourrat for insightful discussions. We thank Dr. Michael McGrath and Dr. Kavita Subramaniam for critical reading of the manuscript, the SFR Necker Cell Imaging Platform for technical support and Dr Benoit Blanchet laboratory from Cochin hospital, Paris, for measurement of erlotinib trough levels.

Author contributions statement:

References


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Figure Legends:

Figure 1: TRPV3 and EGFR ligands EREG and TGF-α are upregulated in PC-lesional skin. **a.** Representative in situ RNA hybridization showing the expression level of TRPV3 and EGFR ligands EREG, and TGF-α, in PC-calluses of the sole and PC non-lesional skin. Cell nuclei are stained in blue and target mRNA are stained in red. Scale bars represent 250µm (50µm in insets). **b.** Quantification of the average number of TRPV3, EREG, or TGF-α probe signals per area detected in PC-lesional skin and PC non-lesional skin. Data are presented as mean ± s.d. from at least 2 patients (5 to 10 images each). ***, P < 0.01, **** P < 0.0001.

Figure 2: EGFR-mTOR signaling complex is overactive in suprabasal layers of PC-lesional skin. **a-c.** Representative immunostaining for HER1-EGFR (a), HER2 (b) and phospho-RPS6 (a biomarker of mTOR activation), and phospho-ERK (c) in sections from PC-lesional skin (plantar calluses), PC non-lesional skin and healthy control. Scale bars represent 50µm in panel. **d.** Representative in situ RNA hybridization of VEGF-A in PC-lesional skin and PC non-lesional skin. Cell nuclei are stained in blue and target mRNA are stained in red. Scale bars represent 250µm (50µm in insets). **e.** Quantification of the average number of VEGF-A probe signals/area detected in PC-lesional skin and non-lesional skin. Data are presented as mean ± s.d. from at least 2 patients (5 to 10 images each). **** P < 0.0001.

Figure 3: Late terminal differentiation is promoted in PC-lesional skin. **a.** Representative immunostaining for early differentiation marker keratin 10 (KRT10) and late terminal differentiation marker filaggrin (FLG) in PC-lesional skin (calluses), PC non-lesional skin, and
healthy control. The nuclei were stained with DAPI (blue) and KRT10 and FLG were stained in green. Scale bars represent 50µm. b. Representative analysis of transglutaminase 1 (TG1) activity in PC-lesional skin, PC non-lesional skin and feet healthy control. Scale bars in panels A and B represent 50µm.

**Figure 4:** Erlotinib significantly improves quality-of-life indexes, pain scores, and physical activity of patients. a-d. Visual Analog Scale scores of pain (a), Dermatology Life Quality Index (DLQI) (b), Skindex-16 quality-of-life survey (c), and foot function index (FFI) (d), were collected during treatment. Data are presented as a percentage of the scores at Erlotinib initiation. Each patient is represented by a colour and shape-coded line (P1 = black circle, P2 = yellow square, P3 = blue triangle). e-g. Average steps per day and pain scores show an inverse correlation during treatment for patient 1 (e), patient 2 (f), and patient 3 (g). The average steps are presented in blue and the average pain scores are shown in red.

**Figure 5:** Representative clinical pictures of calluses over the course of Erlotinib treatment. a-b. Treatment with systemic erlotinib for 6 months reduced painful plantar keratoderma in all 3 patients. a. Evolution of plantar keratoderma of patient 1 during treatment. b. Evolution of plantar keratoderma of patients 2 and 3 during treatment. Arrows show reduction and thinning of keratoderma. c. Representative clinical pictures showing decreased inflammation of the side edge of the right foot in patient 3. Patients consented to the publication of images.

**Figure 6:** Proposed model for the role of EGFR signaling in Pachyonychia congenita.
Upon physical stress (heat, pressure...), cellular stress and/or wound healing, *KRT6, KRT16* and *KRT17* alongside with *TRPV3* are upregulated. TRPV3 overexpression induces intracellular Ca\(^{2+}\) rise resulting in EGFR ligands EREG and TGFα synthesis and ADAM17-mediated shedding. EGFR ligands in turn activate EGFR and its downstream signaling pathways PI3K/Akt/mTOR, MAPK/ERK, and PKC. PI3K/Akt inhibits TSC1/2 inhibitor complex allowing mTOR activation and EREG and VEGF-A upregulation. VEGF-A and ERK promote cell proliferation and PC-associated genes expression. PKC together with increased Ca\(^{2+}\) level induces TG1 expression and activity resulting in abnormal terminal differentiation. Thus, a positive feedback loop involving TRPV3/ADAM17/EGFR ligands EREG-TGFα/EGFR signaling would drive PPK and pain in PC.
Supplementary Table:

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<th>Patient ID</th>
<th>Gender</th>
<th>Age (years)</th>
<th>Weight</th>
<th>Mutated gene</th>
<th>Mutation</th>
<th>Disease severity</th>
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<td>KRT6A</td>
<td>p.Leu468Pro</td>
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<td>M</td>
<td>24</td>
<td>76 kg</td>
<td>KRT6A</td>
<td>p.Leu468Pro</td>
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<td>P4</td>
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<td>44</td>
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<td>p.Leu132Pro</td>
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Supplementary Table S1: Patient characteristics
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<td>Abcam</td>
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Supplementary Table S2: List of antibodies and probes used
Supplemental

Supplementary figure S1:

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<table>
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<th>VEGF-A</th>
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Supplemental

Supplementary figure S2:

(a) Comparison of HB-EGF and AREG expression in Non-Lesional and Lesional tissues.

(b) Statistical analysis showing significant differences in HB-EGF and AREG expression between Non-Lesional and Lesional tissues.
Supplemental figure S3:
Supplemental

Supplementary figure S4:

a  Before Erlotinib  Month 6 of Erlotinib
P1 (KRT16)

b  Before Erlotinib  Month 6 of Erlotinib
P2 (KRT16)

P3 (KRT16)

c  Before Erlotinib  Month 4 of Erlotinib  Month 8 of Erlotinib
P1 (KRT16)
Supplemental

Supplementary figure S5:

**a**

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<td>![Month 8 Photo]</td>
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**b**

<table>
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Supplemental

Supplementary figure S6:

**a**

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<td><img src="image4" alt="Image" /></td>
</tr>
<tr>
<td>Leisional</td>
<td><img src="image5" alt="Image" /></td>
<td><img src="image6" alt="Image" /></td>
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</tbody>
</table>

**b**

%Ki67+ cells

- Healthy control
- Non-Leisional
- Leisional

Graph showing %Ki67+ cells with statistical significance indicated by asterisks.
### Figure 1

<table>
<thead>
<tr>
<th>Protein</th>
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<tr>
<td>Epiregulin</td>
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<tr>
<td>TGF-β</td>
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<td><img src="image6" alt="Image of TGF-β Lesional" /></td>
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</table>

**Graph:**

- **TRPV3**
- **EREG**
- **Average SGR**
- **Average SGR - Challenging**
- **Average SGR - Challenging**
- **TGF-β**

Bar charts showing the comparison between Non-Lesional and Lesional conditions for each protein.
Figure 3

(a) KRT10 and Filaggrin expression in Healthy, Non-Lesion, and Lesional skin.

(b) TG1 activity in Healthy, Non-Lesion, and Lesional skin.
Figure 4
Figure 5

a  Patient 1 (KRT16)  
   Before Erlotinib  Month 4 of Erlotinib  Month 6 of Erlotinib

b  Patient 2 (KRT18)  
   Before Erlotinib  Month 3 of Erlotinib  Month 4 of Erlotinib  Month 6 of Erlotinib

c  Patient 3 (KRTGA)  
   Before Erlotinib  Month 3 of Erlotinib
Figure 6
Supplementary Material

Skin biopsies:

Skin samples were obtained from patient 1, 2 and 4 with KRT6A or KRT16 pathogenic variants and healthy volunteers after informed consent. Biopsy samples were taken from calluses of the sole and an adjacent non-lesional area. The healthy controls correspond to biopsies taken from the sole of a healthy subject. Biopsies were then fixed in 4% formaldehyde in phosphate-buffered saline (PBS) pH 7.4 for 24h and embedded in paraffin or immediately fresh-frozen in optimal cutting temperature compound (Tissue Tek O.C.T).

Immunostaining and mRNA in situ hybridization:

Immunofluorescence staining and mRNA in situ hybridization were performed on 5µm paraffin sections mounted on Superfrost® Plus Slides (Epredia) or Superfrost® Plus Slides Gold (Epredia). For immunofluorescence, rehydrated sections were washed in PBS, blocked in 5% FBS or 3% BSA-0,1% triton (p-ERK staining) for 1h at room temperature (RT), incubated with primary antibody solution overnight at 4°C, washed in PBS, incubated with secondary antibody for 1h, counterstained with DAPI (ThermoFischer), mounted (Fluoromount, SouthernBiotech) and imaged using a Leica TCS SP8 confocal microscope. Antibodies used for all staining are presented in Supplementary Table S2. For mRNA in situ hybridization, RNA scope procedure from ACD’s RNA scope kit (Biotechne) was followed. All probes used are available in Supplementary Table S2. Deparaffinized and rehydrated sections were incubated with RNA scope® Hydrogen Peroxide reagent (BioTechne) at RT for 10min. Slides were washed in deionized water and antigen retrieval steps were performed using RNAscope® Target Retrieval Reagents (BioTechne) at 98°C for 15min followed by RNA scope® Protease Plus (BioTechne) at 40° for 30min. Slides were washed and incubated with target probes for 2h at 40°C. Sections were then incubated with amplifier 1 for 30min at 40°C and washed in 1X Wash Buffer (BioTechne). The same process was repeated for amplifier 2 (15min), amplifier 3 (30min),
amplifier 4 (15min), amplifier 5 (30min, RT) and amplifier 6 (15min, RT). Chromogenic detection was performed using Fast RED reagents (BioTechne) for 10min at RT. Slides were counterstained using 50% Gibbs hematoxylin, dipped in 0.02% ammonia water, dried 15min at 60°C and mounted with EcoMount (BioCare Medical). All sections were scanned by the SFR Necker Histology platform using the Nanozoomer 2.0 HT, Hamamatsu at objective 40x.

**Transglutaminase 1 activity assay:**

*In situ* detection of TG1 activity in skin sections was assessed using the biotinylated amine donor substrate monodansylcadaverine (MDC) as described earlier (Raghunath et al. 1998). 5µm thick cryosections were air dried and pre-incubated in 0.1M Tris-HCl, 1% BSA, pH 7.4 for 30min at RT. The sections were then incubated with 0.4µg/µL MDC (ThermoFischer #A1594), 5mM CaCl2, 0.1M Tris-HCl pH 7.4 for 2h. After stopping the reaction with 10mM EDTA and PBS washing, the sections were stained with Streptavidin-conjugated Alexa-Fluor 488 (Invitrogen, #S11223) and DAPI (ThermoFischer) before mounting (Fluoromount, SouthernBiotech #0100-20).

**Qupath Analysis**

Qupath analysis guidelines obtained from ACD (Biotechne) were followed to analyze each section scanned. Briefly, on each scan, the nucleus color was selected and associated with hematoxylin stain, the color of the spots was selected and associated with the mRNA probe. The magnification for analysis was then set at 40x on the Qupath software, the epidermis surface was selected, and parameters were set for cell and spot detections. When the size and quality of the section allowed it, 10 different areas were analyzed. For each area, the average area for one single spot was determined and the area of each cluster was divided by the average area of one single spot. The number of spots in each cluster was therefore estimated and allowed
for the average number of mRNA per area to be calculated. Numbers were compared between all areas analyzed.

Supplementary results Legends:

Supplementary Figure S1: Expression of TRPV3, EREG, TGF-α, HB-EGF, AREG and VEGF-A in healthy control skin. Representative in situ RNA hybridization showing levels of TRPV3, EREG, TGF-α, AREG, HB-EGF or VEGF-A in healthy control skin of a sole. Cell nuclei are stained in blue and target mRNA in red. Scale bars represent 100µm.

Supplementary Figure S2: Expression of AREG and HB-EGF mRNA in PC skin. a. Representative in situ RNA hybridization showing levels of AREG or HB-EGF in PC-lesional skin and PC-unaffected skin. Cell nuclei are stained in blue and target mRNA in red. Scale bars represent 250µm (50µm in insets). b. Quantification of the average number of AREG or HB-EGF probe signals per area detected in PC-lesional skin and PC-unaffected skin. Data are presented as mean s.d. from at least 2 patients (5 to 10 images each). **, P < 0.01, ****, P<0.0001

Supplementary Figure S3: Individual quality of life indexes and pain scores of the three patients. a. Visual Analog Scale scores of pain are presented as blue lines. b. Dermatology Life Quality Index (DLQI) are shown in red. c. Skindex-16 quality of life surveys are presented in green.

Supplementary Figure S4: Clinical pictures of plantar calluses over the course of erlotinib treatment. a. Clinical aspect of the right sole of the three patients. b. Clinical aspect of the left sole of the three patients. c. Evolution of subcorneal neurovascular structures under treatment in P1. Patients consented to the publication of images.
Supplementary Figure S5: Clinical course of fingernails and toenails thickening over erlotinib treatment in P1. a. Clinical aspect of fingernails. b. Clinical aspect of toenails. The patient consented to the publication of images.

Supplementary Figure S6: Expression of Ki67 and KRT6 in PC skin. a. Representative immunostaining for Ki67 (left panels) and KRT6 (right panels) in sections from PC-lesional skin (plantar calluses), PC non-lesional skin and healthy control. Scale bars represent 50µm in panel. b. Quantification of the % of cell positive for Ki67 signals/area detected in PC-lesional skin and non-lesional skin. Data are presented as mean s.d. ** P < 0.01.