connective tissue disease even if the new set of RA criteria [52] and the preliminary criteria for the very early diagnosis of SSc [53] were used, we applied the preliminary classification criteria of UCTD suggested by Mosca and colleagues [27]. Four UCTD patients with anti-PM/Scl were all so-called 'stable UCTD'. Their disease courses were stable over a period of more than three years without internal organ involvement, except for one, whose ILD was nonspecific interstitial pneumonia that did not exacerbate for more than ten years. Interestingly, Cordiali-Fei and colleagues [54] reported that anti-PM/Scl responses were mainly associated with Italian patients with UCTD, which was defined by the same criteria used in our study. They found 5 patients with anti-PM/Scl in 23 patients with UCTD (22%), a frequency that is almost the same as that of our study.

Of second importance are the three anti-PM/Scl-positive DM patients. Myalgia or muscle weakness varied, and the levels of creatine kinase ranged from normal levels to more than 2,000 IU/L. Patient C in this study is the second reported case of CADM, to the best of our knowledge, following the first case described by Lega and colleagues [55]. A previous study of 20 PM/DM patients with anti-PM/Scl demonstrated that anti-PM-Scl is not necessarily a marker for good prognosis in patients with PM/DM, because lung and esophageal involvement were found (in 75% and 20%, respectively), as was internal malignancy (in 15%) [56]. Also in our study, all three patients were complicated with ILD and required combined therapy of steroids and immunosuppressive agents. Although they were all alive during the observation periods, the prognosis of ILD in anti-PM/Scl-positive DM patients cannot be determined due to the very limited numbers examined and the limited observation periods (maximum 45 months). Two of these patients were also complicated with localized cancer without metastasis. In a previous study, two out of twelve antibody-positive patients with DM had mechanic's hands [56]. Very interestingly, also in our study, two patients exhibited mechanic's hands in addition to sole hyperkeratotic rhagadiform symptoms.

Conclusions

Our study of Japanese patients with various systemic autoimmune diseases confirms that anti-PM/Scl antibodies also exist in these patients. ELISA with PM/Scl-100 recombinant protein was useful in detecting anti-PM/Scl antibodies. Anti-PM/Scl was not always specific for DM or SSc; it was also present in various autoimmune conditions, including UCTD. All the anti-PM/Scl-positive DM cases were complicated with ILD and/or cancer, while no life-threatening internal organ involvement was found in other anti-PM/Scl-positive cases. Considering the higher prevalence of anti-PM/Scl in UCTD, this autoantibody may be more important in systemic autoimmune disease clinics than we expected. Further studies in larger cohorts are necessary to define the clinical significance of anti-PM/Scl antibodies in Japanese patients with each autoimmune condition. Future collaborative studies for evaluating our sera with LIA and PM-1α ELISA promise to be interesting.

Abbreviations

ACR: American College of Rheumatology; ANoA: anti-nucleolar antibody; ARS: aminoacyl tRNA synthetase; CADM: clinically amyopathic dermatomyositis; CIE: counter immunoelectrophoresis; DM: dermatomyositis; ELISA: enzyme-linked immunosorbent assay; EULAR: European League Against Rheumatism; ID: immunodiffusion; IIF: indirect immunofluorescence; ILD: interstitial lung disease; IPP: immunoprecipitation; LIA: line immunoassay; OL: overlap syndrome; PM: polymyositis; RA: rheumatoid arthritis; RLU: relative luminescence unit; Scl: scleroderma; SD: standard deviation; SLE: systemic lupus erythematosus; SS: Sjögren's syndrome; SSc: systemic sclerosis; TnT: *in vitro* translation and transcription product; UCTD: undifferentiated connective tissue disease.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

YM performed the serological analyses and the analysis of the data. YH and TM performed immunoprecipitation with the radiolabeled extract and interpreted the data. KS and YO provided sera for the analyses. MA made intellectual contributions and helped to prepare the manuscript. All authors were involved in the conception, design, and interpretation of data and in drafting the article and revising it critically for important intellectual content. All authors read and approved the final manuscript.

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SHORT COMMUNICATION

Symmetrical Giant Facial Plaque-type Juvenile Xanthogranuloma Persisting Beyond 10 years of Age

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Juvenile xanthogranuloma (JXG) is a non-Langerhans histiocytosis occurring predominantly in infancy and early childhood. Resolution usually occurs over a period of months to several years, and it is rare for the condition to persist beyond late childhood (1). The symmetrical giant facial plaque variant of JXG (SGFP-JXG) is very rare. It was originally reported by Gunson & Birchall. (2). Herein we report a case of SGFP-JXG that persisted beyond 10 years of age.

CASE REPORT

A 10-year-old Japanese boy was referred to our clinic with facial yellowish papules. He was a fraternal twin, the result of a normal pregnancy and delivery, and was otherwise completely healthy and taking no regular medications. The twin brother showed no skin lesions. There was no particular family history, such as neurofibromatosis. The plaques were first noticed as red macules at approximately 6 months of age, which slowly evolved during the first year of life. He had also had yellowish nodules on the bilateral arms. He had come to a nearby dermatology clinic at 2 years of age (Fig. 1A). At that time, a biopsy from a skin lesion on the left arm showed mixed inflammatory cell infiltration containing small lymphocytes and histiocytes from the superficial dermis, extending into the deep dermis. Touton giant cells and numerous foam cells were present (Fig. 2). The nodules on the arms had been diagnosed as JXG. Although the JXG on the arms gradually decreased in size and mostly resolved, the facial lesions did not improve. Physical examination at the age of 10 revealed symmetrical yellowish indurated

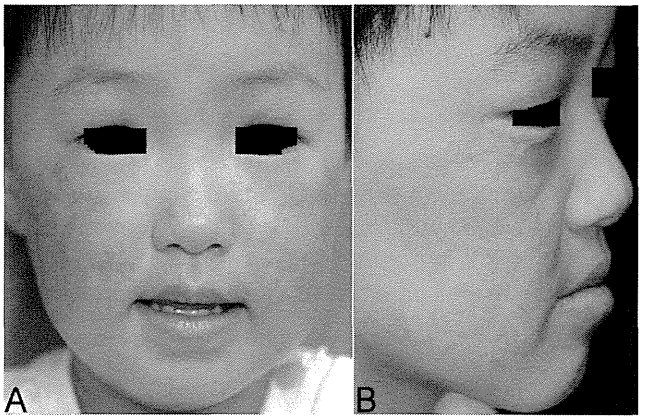

Fig. 1. Multiple yellowish plaques are distributed symmetrically on the upper and lower eyelids and on the cheeks. (A) At 2 years of age. (B) At 10 years of age.

plaques with smooth surface on the bilateral upper and lower eyelids and on the cheeks (Fig. 1B). Similar yellowish nodules were also seen on the left elbow. There was neither ophthalmic or oral mucosal involvement, or palpable hepatosplenomegaly nor lymphadenopathy. Pathological findings following a skin biopsy at the age of 10 from the lesion on the right cheek were consistent with JXG (Fig. 3A). In addition, CD68 was positive (Fig. 3B), but S-100, CD1a, and langerin were negative (data not shown) by immunohistochemistry, which is also consistent with JXG. Hence, the patient was diagnosed with SGFP-JXG.

DISCUSSION

JXG usually manifests as an asymptomatic, reddish-brown nodule which slowly grows to a diameter of 1–2

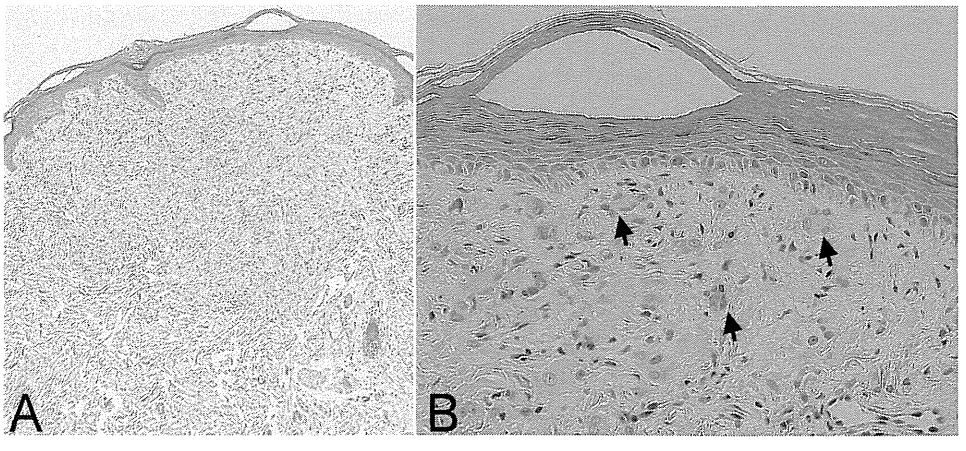

Fig. 2. Haematoxylin-eosin staining of the yellowish nodule on the left arm at 2 years of age. Numerous foam cells are seen throughout the dermis (original magnification ×4) (A). Giant cells and numerous foam cells are seen. Arrows indicate Touton giant cells (original magnification ×20) (B).

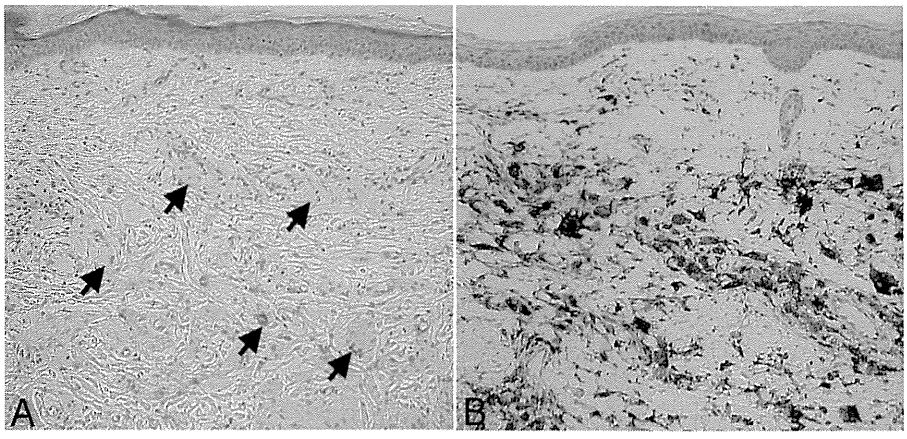

Fig. 3. Haematoxylin-eosin staining of the yellowish plaque on the right cheek at 10 years of age. Many foam cells are seen in the dermis (A). Immunohistochemistry of CD68 on the lesion of the right cheek. The histiocytes are CD68 positive (B) (original magnification × 10). Arrows: Indicates gaint cells and foam cells.

cm. Evolution into a yellowish-brown papule, plaque or nodule often occurs, followed by spontaneous resolution that may leave an atrophic scar.

Plaque and clustered types of JXG at extrafacial sites have been reported (3, 4). However, despite the atypically large lesions, these types of JXG usually reduce with scarring and pigmentation within a year.

SGFP-JXG was first reported by Gunson & Birchall. (2) in 2008. To the best of our knowledge, our report is the second reported case of SGFP-JXG. Gunson & Birchall described SGFP-JXG lesions as multiple large, flat, symmetrically distributed plaques of over 2 cm in diameter (2). The case in the literature had facial lesions, without any JXG lesions on other body sites. The lesions had been present for >6 years and had shown no sign of spontaneous resolution. The present case has also had the facial lesions for 10 years without any noticeable tendency of spontaneous resolution. Interestingly, he had also had JXG on the arms, and it tended to spontaneously resolve. Disseminated JXG, which has multiple cutaneous lesions, is sometimes associated with visceral JXG in infancy (5, 6). Both the present SGFP-JXG case and the patient in the literature showed no involvement of internal organs. Unfortunately, the therapeutic options are limited given the large and cosmetically sensitive area involved.

In conclusion, we report herein the second case of SGFP-JXG, and both cases suggest that this type of JXG may persist beyond the age of 10 years but that it is not associated with visceral lesions.

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Generalized Pustular Psoriasis Triggered by Amoxicillin in Monozygotic Twins with Compound Heterozygous *IL36RN* Mutations: Comment on the Article by Navarini *et al.*

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TO THE EDITOR

We read with great interest the recent report regarding *IL36RN* mutations in acute generalized exanthematous pustulosis (AGEP) by Navarini *et al.* (2013). They analyzed *IL36RN* mutations in 96 cases with AGEP and found only one homozygous mutation (p.Leu27Pro) and three heterozygous mutations. p.Leu27Pro is a founder mutation causing generalized pustular psoriasis (GPP) homozygously in African populations, and the AGEP patient described by Navarini *et al.* (2013) with the homozygous mutation p.Leu27Pro is an African woman (Marrakchi *et al.*, 2011; Navarini *et al.*, 2013). She had a previous history of drug-induced type-unknown skin reaction triggered by uncertain antibiotics. She showed pustules covering 85% of her body surface, including the mouth, and a 39.4 °C fever triggered by amoxicillin. A positive patch test to amoxicillin was noted. Treatment of the patient was not described. No recurrence was observed during the 18-month follow-up.

In a recent report, we analyzed 11 patients with GPP not accompanied by psoriasis vulgaris (PV) (GPP-alone; Sugiura *et al.*, 2013). Among the 11 GPP-alone patients, there was a monozygotic twin with the compound heterozygous mutations p.Arg10X and p.Arg10ArgfsX1 in *IL36RN* whose pustules and high fever had primarily been triggered by amoxicillin.

The patients were 6-year-old Japanese male identical twins. At the age of 2 years, they had erythema with pustules on the whole body and fever over 38 °C after amoxicillin intake. In one of the twins, blood examination revealed a white blood cell count of 24,300 μl^{-1} and a C-reactive protein concentration of 1.17 mg dl^{-1}. Bacterial culture for the pustules was negative. A skin biopsy from a pustular eruption on the trunk revealed a spongiform pustule of Kogoj in the epidermis (Figure 1c). The results of these examinations were similar to those of the other twin. Patch tests were conducted on one of the twins, and it was found to be positive for amoxicillin. Skin eruptions including pustules of the twins were improved with a dosage of 6 mg kg^{-1} oral cyclosporine. After the first episode, each twin has episodic systemic pustules unrelated to amoxicillin intake (Figure 1a and b). Neither twin has ever showed typical scaly erythema corresponding to PV. The two patients, monozygotic twins, had the compound heterozygous mutations p.Arg10X and p.Arg10ArgfsX1 in *IL36RN* (Sugiura *et al.*, 2013). Thus, they were diagnosed as having GPP-alone with *IL36RN* mutations that had once been triggered by amoxicillin.

Amoxicillin- or penicillin-related drug-induced GPP has been reported for a long time, although the pathomechanisms of how penicillin induces GPP have never been addressed (Ryan and Baker,

Abbreviations: AGEP, acute generalized exanthematous pustulosis; DITRA, deficiency of IL-36 receptor antagonist; GPP, generalized pustular psoriasis; IL36RN, IL-36 receptor antagonist; PV, psoriasis vulgaris

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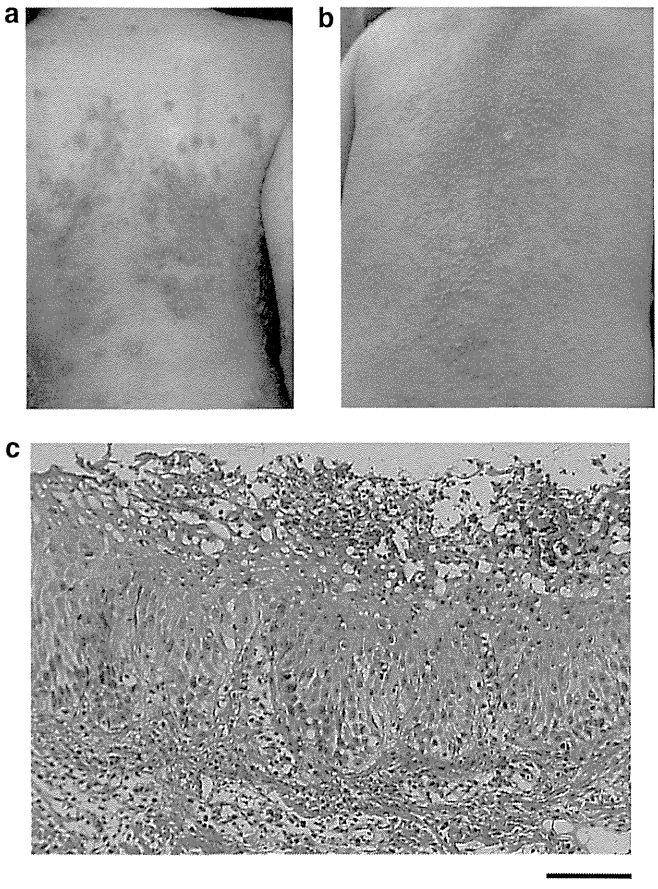

Figure 1. Clinical features and pathological findings of pustules of the generalized pustular psoriasis-alone patients. The clinical features of the identical twin patients (**a**, **b**) are shown. Pustules on background erythema are seen on the trunk. The pathology of the pustules is indicated for one of the patients (**c**). Spongiosis of Kogoj and acanthosis are observed in the epidermis of the pustular erythematous lesions on the trunk. Bar = 100 μm.

1971; Lindgren and Groth, 1976; Katz *et al.*, 1987). Our monozygotic twins and the Navarini case suggest that the combination of amoxicillin and IL36RN deficiency due to *IL36RN* homozygous or compound heterozygous mutations induces pustule formation on the whole body with high fever.

Penicillin treatment of *Streptococcus pneumoniae* increases the production of tumor necrosis factor-α and IL-1β by human monocyte–derived macrophages (Moore *et al.*, 2005). IL-36α, IL-36β, and IL-36γ are also proinflammatory cytokines in the IL-1 family (Dinarello *et al.*, 2010). Thus, oral amoxicillin or penicillin in combination with bacterial infection may induce the production of IL-36 by inflammatory cells in the skin. Homozygotes or compound heterozygotes for *IL36RN* loss-of-function mutations are thought to be sensitive to IL-36 signals and to be intolerant to excessive IL-36 production. Thus, increased production of IL-36 induced by amoxicillin may trigger the onset of generalized pustular eruptions. Korber *et al.* (2013) recently reported that 7 out of 19 GPP cases (GPP-alone and GPP preceding or accompanied by PV) had homozygous or compound heterozygous *IL36RN* mutations. Very recently, we reported that the majority of GPP-alone cases are caused by homozygous or compound heterozygous mutations of *IL36RN*, although a few cases with GPP preceding or accompanied by PV were also found to have *IL36RN* mutations (Sugiura *et al.*, 2013). The majority of GPP-alone cases are individuals with deficiency of IL36RN (DITRA) and, among such patients, not a few patients have amoxicillin or penicillin intolerance as a trigger of pustular eruptions (Korber *et al.*, 2013; Sugiura *et al.*, 2013). In this context, we speculate that in cases with *IL36RN* mutations, even if the initial diagnosis may be amoxicillin- or penicillin-related drug-induced AGEP, the final diagnosis might really be GPP-alone condition, in which amoxicillin- or penicillin-related drug was only triggering the primary episode.

In conclusion, we suggest that we should not administer amoxicillin- or penicillin-related drugs to patients with DITRA nor to carriers of *IL36RN* mutations, in order to avoid generalized pustular eruptions with high fever.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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CARD14 c.526G>C (p.Asp176His) Is a Significant Risk Factor for Generalized Pustular Psoriasis with Psoriasis Vulgaris in the Japanese Cohort

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TO THE EDITOR

Generalized pustular psoriasis (GPP) often presents in patients with existing or prior psoriasis vulgaris (PV), although cases of GPP have been known to arise without a history of PV. We recently reported that the majority of the cases of GPP without a history of PV ("GPP alone") are caused by homozygous or compound heterozygous mutations of *IL36RN* (Sugiura *et al.*, 2013). In contrast, only a few exceptional cases of GPP that were preceded or accompanied by PV ("GPP with PV") were found to have *IL36RN* mutations. Thus, GPP with PV is genetically different from GPP alone.

CARD14 encodes caspase recruitment domain family member 14 (CARD14), which is an activator of nuclear factor of κ-light-chain-enhancer of activated B cells within the epidermis. Jordan *et al.* (2012a, b) identified rare gain-of-function variants and mutations in *CARD14*, including p.Gly117Ser, in two large multiplex families affected by Mendelian forms of psoriasis and psoriatic arthritis (Figure 1). Autosomal dominant pityriasis rubra pilaris, which is phenotypically related to psoriasis, was also reported to be caused by mutations in *CARD14* (Fuchs-Telem *et al.*, 2012), including p.Glu138del and p.Leu156Pro. Moreover, *CARD14* p.Arg820Trp (rs11652075) was found to be a PV-susceptible variant in a large psoriasis cohort (Tsoi *et al.*, 2012; Jordan *et al.*, 2012a). Therefore, *CARD14* variants and mutations are closely related to various types of psoriasis and psoriasis-related diseases. Jordan *et al.* (2012b) identified the rare *de novo CARD14* gain-of-function variant p.Glu138Ala in a child with severe early-onset GPP. They found three other rare *CARD14* gain-of-function variants in large PV cohorts including p.Asp176His. There was no association of the p.Asp176His variant with PV because the rare variant was found not only in the large PV cohorts but also in the large control cohorts.

The present study aimed to investigate the presence of *CARD14* variants in GPP with PV. We analyzed the entire coding regions of *CARD14* in 19 cases of GPP with PV and in 11 cases of GPP alone. Then, we analyzed exon 4 of *CARD14* in the 100 cases of PV and the 100 healthy controls that we had previously studied (Sugiura *et al.*, 2013). All cases and controls were Japanese. It was previously reported that 9 out of 11 cases of GPP alone had homozygous or compound heterozygous *IL36RN* mutations (Sugiura *et al.*, 2013). The clinical characteristics of the 30 patients with GPP and the 100 patients with PV are indicated in Supplementary Table S1 and S2 online, respectively. Following approval from the Ethics Committee of Nagoya University, written informed consent was obtained from all participants in compliance with the Declaration of Helsinki guidelines.

The sequence primers for analysis of *CARD14* are indicated in Supplementary Table S3 online. Direct-sequencing analysis of the entire coding regions of *CARD14* with exon–intron boundaries revealed 4 of the 19 cases of GPP with PV to be heterozygous for c.526G>C (p.Asp176His) (Figure 2). We found no other mutations or variants. However, none of the 11 cases of GPP alone had any variants. Among the cases

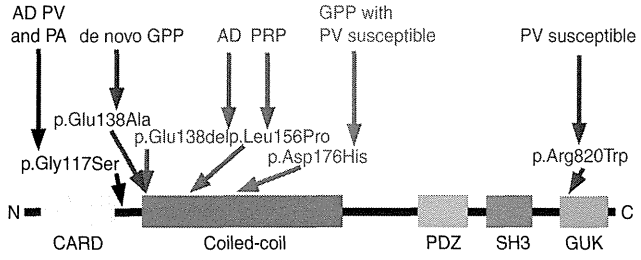

Figure 1. Caspase recruitment domain family, member 14 (CARD14) protein domain structure, locations of amino acid substitutions and deletions, and related psoriatic diseases. CARD and coiled-coil domains are essential for nuclear factor of kappa-light-chain-enhancer of activated B cells (NF-κB) activation. The PDZ, SH3, and GUK domains are proposed as being essential for membrane localization and lipid raft recruitment of CARD14 protein (Scudiero *et al.*, 2011). Rare and common variants and mutations found are indicated. p.Asp176His is the generalized pustular psoriasis (GPP) with psoriasis vulgaris (PV)-susceptible variant shown in the present study. p.Arg820Trp is the PV-susceptible variant (Tsoi *et al.*, 2012; Jordan *et al.*, 2012a). p.Gly117Ser is a mutation found in the family of Mendelian autosomal dominant (AD) PV and psoriatic arthritis (PA; Jordan *et al.*, 2012a). p.Glu138Ala was found in a patient with early-onset GPP. p.Glu138del and p.Leu156Pro indicate that the mutations found in AD pityriasis rubra pilaris (RPR; Fuchs-Telem *et al.*, 2012). CARD, caspase recruitment domain; GUK, guanylate kinase; PDZ, a domain from the initial letters of the PSD-95, Dlg, and ZO-1 proteins; SH3, src homology 3.

Abbreviations: CARD14, caspase recruitment domain family, member 14; GPP, generalized pustular psoriasis; PV, psoriasis vulgaris

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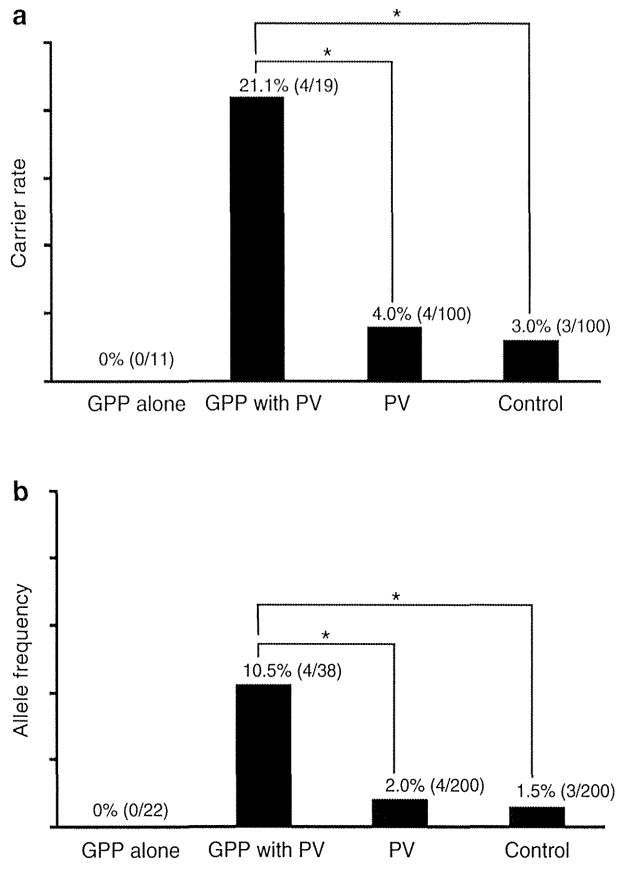

Figure 2. Carrier rate and allele frequency of p.Asp176His in generalized pustular psoriasis (GPP) with psoriasis vulgaris (PV), and in PV. The carrier rate (**a**) and allele frequency (**b**) of p.Asp176His in GPP with PV are significantly higher compared with those in PV and in healthy controls. *$P<0.05$.

with homozygous or compound heterozygous *IL36RN* mutations, no cases had the variant c.526G>C (p.Asp176His). Direct-sequencing analysis of exon 4, which encodes p.Asp176, revealed 4 out of the 100 cases of simple PV and 3 out of the 100 healthy controls to have the heterozygous variant c.526G>C (p.Asp176His). The carrier rate of the p.Asp176His variant in GPP with PV (4/19: 21.1%) was significantly higher compared with that in the controls (3/100: 3%; Fisher exact test: $P<0.0123$; odds ratio of 8.62; confidence intervals between 1.75 and 42.4; power calculation of 0.609). However, there was no significant difference in the carrier rate of p.Asp176His between GPP alone (0/11: 0%) and the healthy controls. Jordan *et al.* (2012a) reported that p.Asp176His was found in 2 out of 1609 controls in European populations. The carrier rate of p.Asp176His in the Japanese individuals in the present study is significantly higher compared with that in the European cohort ($P<0.01$; Jordan *et al.*, 2012a). The Ensembl genome browser indicates that 3 out of 178 alleles in the Japanese population have c.526G>C. This frequency is very close to the 3 out of 200 alleles of the present study. We conclude that the *CARD14* variant p.Asp176His is an important predisposing factor for GPP with PV in the Japanese population.

Because of the low number of patients, the current study is underpowered to detect variants at low frequencies, such as pGlu138Ala, in this population (Jordan *et al.*, 2012a). pGlu138Ala was reported in an early-onset GPP patient, although the mutant was not found in any of the 30 GPP patients in the present study. HLA-Cw*602 is the main genetic predisposing factor for psoriasis. We analyzed HLA-C in the 30 patients with GPP and the 100 patients with PV using Micro SSPTM HLA typing trays (One Lambda, Canoga Park, CA; Supplementary Tables S1 and S2 online). No interaction was found between the *CARD14* variant c.526G>C and HLA-Cw*602.

The haplotype block structure flanking the *CARD14* gene was constructed using genotype data from the HapMap database. We analyzed the haplotypes of 200 alleles of the controls. The haplotype block was represented by eight haplotypes (Supplementary Figure S1 online). In all, 11 individuals carrying the *CARD14* variant c.526G>C (p.Asp176His), the chromosome containing c.526G>C, had an identical haplotype, haplotype III (GGACCTC CA), which is seen in 11.5% of the Japanese population. Thus, the variant c.526G>C (p.Asp176His) in the present Japanese individuals appears to represent founder effects of the prevalent variant *CARD14* alleles among individuals in this island nation.

In this study, we found that *CARD14* p.Asp176His is a prevalent founder variant in the Japanese population and is a predisposing factor for GPP with PV. In contrast, *CARD14* p.Asp176His is not associated with PV in the Japanese population. These findings suggest that GPP with PV has a genetic background different from that of simple PV. In addition, no patient with GPP alone has had *CARD14* p.Asp176His, although the majority have had *IL36RN* mutations, as previously reported (Sugiura *et al.*, 2013). In this context, the present results further support the idea that GPP with PV is genetically different from GPP alone.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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Epidermal Growth Factor–Functionalized Polymeric Multilayer Films: Interplay between Spatial Location and Bioavailability of EGF

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TO THE EDITOR

Wound healing is a complex process involving cell–cell and cell–matrix interactions modulated by the surrounding microenvironment. One of the key events in this process is re-epithelialization: the directed migration and proliferation of epithelial cells to resurface the wound. Numerous studies have documented that the migration of cells into the wound site is regulated by autocrine and paracrine signaling of growth factors (Gurtner *et al.*, 2008). Epidermal growth factor (EGF) is an extensively investigated growth factor, which is implicated in keratinocyte adhesion and migration, as well as fibroblast function, and granulation tissue formation (Hardwicke *et al.*, 2008). Conflicting reports exist regarding the ability of EGF alone to modulate wound healing (Brown *et al.*, 1989; Cohen *et al.*, 1995). A systematic review concluded that EGF is effective as an adjuvant but not as monotherapy for diabetic foot ulcers (Buchberger *et al.*, 2011). The reason for this lack of efficacy of EGF in healing wounds may be derived from the mechanism of delivery, in that it needs to be intimately associated with the extracellular matrix to limit proteolysis (Macri and Clark, 2009).

Various approaches for delivering EGF to the wound have been attempted with the goal of maximizing therapeutic effects with minimal dose (to minimize potential adverse effects). EGF conjugated to copolymers composed of poly(ε-caprolactone) (PCL) and poly (ethyleneglycol) (PEG) improved healing in diabetic mouse wounds, compared with treatment with the copolymers alone or no treatment (Choi *et al.*, 2008). Recent advances in materials science have led to the development of engineered systems such as polymeric microparticles for controlled release of cytoactive factors (CFs; Lee *et al.*, 2011). Another method that has received limited attention is the covalent immobilization of CFs within polyelectrolyte multilayer (PEM) films. PEM films, which are formed by the layer-by-layer deposition of polyelectrolytes, provide numerous advantages for biomedical applications, such as ease of preparation, biocompatibility, tunable mechanical properties, spatio-temporal control over film organization, and most importantly, the sustained and controlled contact of embedded factors with tissue (Guthrie *et al.*, 2013). In particular, we speculate that the nanoscopic thickness of PEMs enables their intimate contact with the tissue surface upon application. When CFs are conjugated to the PEMs, it also limits, if not eliminates, the systemic release of embedded CFs and the associated potential for systemic toxicity (Agarwal *et al.*, 2010). Another key advantage of the PEM films is that they can also be easily immobilized to complex geometries such as wound bed (Jain *et al.*, 2013). However, the efficacy, bioavailability, and bioactivity of CFs such as growth factors, when conjugated to PEMs, have not been reported.

To address the question of the bioavailability of growth factors within PEMs, we fabricated PEM films with EGF that was covalently immobilized at varying depths within the PEMs. The accessibility and bioactivity of EGF immobilized within the PEM films was determined by measuring the migratory and proliferative responses of

Abbreviations: CF, cytoactive factor; EGF, epidermal growth factor; HKGS, human keratinocyte growth supplement; NHK, neonatal human keratinocyte; PAA, poly(acrylic acid); PAH, poly(allylamine hydrochloride); PEM, polyelectrolyte multilayer

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Epithelial inflammation resulting from an inherited loss-of-function mutation in *EGFR*

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Abstract

Epidermal growth factor receptor (EGFR) signaling is fundamentally important for tissue homeostasis through EGFR/ligand interactions that stimulate numerous signal transduction pathways. Aberrant EGFR signaling has been reported in inflammatory and malignant diseases but thus far no primary inherited defects in EGFR have been recorded. Using whole-exome sequencing, we identified a homozygous loss-of-function missense mutation in *EGFR* (c. 1283G>A; p.Gly428Asp) in a male infant with life-long inflammation affecting the skin, bowel and lungs. During the first year of life, his skin showed erosions, dry scale, and alopecia. Subsequently, there were numerous papules and pustules – similar to the rash seen in patients receiving EGFR inhibitor drugs. Skin biopsy demonstrated an altered cellular distribution of EGFR in the epidermis with reduced cell membrane labeling, and *in vitro* analysis of the mutant

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CONFLICT OF INTEREST
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receptor revealed abrogated EGFR phosphorylation and EGF-stimulated downstream signaling. Microarray analysis on the patient's skin highlighted disturbed differentiation/premature terminal differentiation of keratinocytes and upregulation of several inflammatory/innate immune response networks. The boy died aged 2.5 years from extensive skin and chest infections as well as electrolyte imbalance. This case highlights the major mechanism of epithelial dysfunction following EGFR signaling ablation and illustrates the broader impact of EGFR inhibition on other tissues.

INTRODUCTION

EGFR signaling represents a key activity in several biologic processes underpinning tissue development and homeostasis (Jutten and Rouschop, 2014). Thus far, 7 EGFR ligands have been identified: EGF, transforming growth factor-α, amphiregulin, heparin-binding EGF-like growth factor, betacellulin, epiregulin, and epigen (Schneider and Wolf, 2009; Nanba *et al.*, 2013). Interaction between EGFR and these ligands leads to stimulation of numerous transduction pathways that includes the RAS/RAF/MEK/ERK, PLC-gamma/PKC, PI3K/AKT, JAK/STAT, and NF-κB cascades (Jost *et al.*, 2000; Silbilia *et al.*, 2007). Acquired abnormalities of EGFR signaling have been observed in various inflammatory and malignant diseases (Jost *et al.*, 2000; Gschwind *et al.*, 2004; Dreux *et al.*, 2006; Zhang *et al.*, 2014). Of note, aberrant EGFR activation is found in many tumor cells, and humanized neutralizing antibodies and synthetic small compounds against EGFR are in clinical use today. These drugs may cause skin and hair toxicities, indicating a key role for EGFR in cutaneous homeostasis (Lacouture, 2006; Liu *et al.*, 2013). These side-effects have been recapitulated in a mouse model, with evident abnormalities in cutaneous chemokines associated with early infiltration of macrophages and mast cells and later infiltration of eosinophils, T cells and neutrophils (Mascia *et al.*, 2013). *EGFR* mutations in tumors are typically gain-of-function and located within the tyrosine kinase domain of the protein, but thus far there are no reports of naturally occurring loss-of-function mutations in humans. Knockout and transgenic murine models and *in vitro* studies have demonstrated the essential role of EGFR in multiple organs (Miettinen *et al.*, 1995; Silbia and Wagner, 1995; Threadgill *et al.*, 1995; Jost *et al.*, 2000; Schneider *et al.*, 2008), but clinical correlates in patients are currently lacking. In this study, we used whole-exome sequencing to identify an inherited loss-of-function mutation in *EGFR* in an individual with inflammatory skin, lung and bowel disease.

RESULTS AND DISCUSSION

Clinical presentation with cutaneous erosions and inflammation

We investigated a male infant born to parents of Polish Roma origin who presented to us at 12 months of age with extensive skin inflammation. Initially, there were widespread erosions affecting his trunk and limbs that had been present since birth (Figure 1a), but subsequently we observed papules and pustules (Figure 1b). He also had life-long watery diarrhea and respiratory difficulties. He was born at 34 weeks' gestation, weighing 1560 grams; the pregnancy had been complicated by polyhydramnios and maternal hypertension. Clinically, at 12 months his weight was only 5.52kg with evident failure to thrive. His

inflamed skin was frequently infected with *Staphylococcus aureus*. He had loss of scalp hair but long eyelashes (trichomegaly). No nail abnormalities were observed. He had previously undergone surgery for probable coarctation of the aorta and was noted to be hypertensive. Other abnormalities included recurrent bronchiolitis and pulmonary infection with *Pseudomonas aeruginosa* (requiring tracheostomy and oxygen). Renal ultrasound showed bilateral renal enlargement but no obstruction. The child had no specific food allergies but was unable to tolerate solids due to diarrhea and vomiting and severe dehydration. We observed low albumin, zinc, vitamin A, iron, magnesium, profound hypokalaemia (persistent), and hypernatraemia - though barium meal showed no evidence of malabsorption. He preferred to drink water rather than milk and subsequently required total parenteral nutrition. Blood eosinophil counts were slightly elevated during the first year (observed levels 0.4-1.0 × 10^9/l). Total IgE was found to be raised as a neonate (100 IU/l; range for <1 year 0-15 IU/l) and at 15 months (107 IU/l; range for this age 0-60 IU/l). IgM was borderline normal/low; IgG and IgA were normal. Venous access lines frequently became clotted and he developed deep vein thromboses. He died aged 2.5 years as a result of cutaneous and pulmonary infections and electrolyte imbalance. Further details of the clinical history can be found in the Supplementary Material.

Skin biopsy reveals acanthosis and intra-epidermal edema

We obtained written informed consent from the subject's legal guardian with approval from the St. Thomas' Hospital Ethics Committee. Elliptical skin biopsies were obtained under local anesthetic from a non-lesional area on the right thigh of the child (aged 12 months) as well as the abdomens of healthy control patients undergoing cosmetic ("tummy tuck") surgery. The initial clinical diagnosis was a subtype of inherited skin fragility, a heterogeneous group of disorders known collectively as epidermolysis bullosa (EB) (Fine *et al.*, 2008). Light microscopy of non-lesional skin revealed mild acanthosis compared to age- and site-matched control skin, as well as a slight widening between adjacent keratinocytes (Figure 2a). A few neutrophils were noted within the superficial dermis with some infiltration of follicular epithelium and folliculitis, although Gram stain for bacteria was negative. The changes within the epidermis resembled those of a primary inherited abnormality of desmosomes (Petrof *et al.*, 2012), although transmission electron microscopy showed intercellular edema from the basal layer to the mid-spinous layer and a slight decrease in the number of gap junctions but no structural or numerical abnormalities in hemidesmosome or desmosome cell junctions (Figure 2b). Skin immunolabeling using a panel of antibodies that target basement membrane and epidermal proteins implicated in inherited skin fragility disorders (see Supplementary Material for antibody details) demonstrated a marked reduction in staining intensity for the desmosomal proteins desmoglein 1 and plakophilin 1, as well as altered labeling patterns for profilaggrin, involucrin and keratin 10, markers of terminal differential in the epidermis (illustrated in Supplementary Material). Some of the clinicopathologic features also resembled Netherton syndrome, an autosomal recessive disorder associated with loss-of-function mutations in a serine protease inhibitor (*SPINK5*) that encodes lympho-epithelial Kazal-type related inhibitor (LEKT1) (Chavavas *et al.*, 2000; Stoll *et al.*, 2001). Immunostaining for LEKTI in our patient's skin, however, revealed a slight increase compared to control (illustrated in Supplementary Material)– in contrast to findings of reduced LEKTI expression that usually

accompany Netherton syndrome. Taken together, these findings were not in keeping with any known form of EB, Netherton syndrome or any other recognized inherited or acquired skin disease.

Whole-exome sequencing reveals a homozygous missense mutation in *EGFR*

Next, after obtaining approval from the ethics committee and informed consent, we extracted genomic DNA from peripheral blood samples from the child, other family members and controls in compliance with the Helsinki Guidelines. Using the patient's DNA, whole-exome sequencing was performed on the Illumina HiSeq 2000 (San Diego, CA, USA). After excluding pathogenic mutations in genes implicated in different forms of EB (Fine *et al.*, 2008), the exome variant profile was filtered to look for novel homozygous mutations (based on probable consanguinity). This disclosed 6 possible gene mutations (see Supplementary Material). Within these genes, *EGFR* was deemed to be of potential relevance given that some clinical features (e.g. papulo-pustular skin eruptions, alopecia and trichomegaly) resembled known side-effects of EGFR inhibitor drugs (Lacouture, 2006). Immunofluorescence microscopy to assess EGFR expression in the affected infant's skin showed a markedly altered staining pattern with loss of the cell peripheral membrane labeling and a more cytoplasmic or peri-nuclear region distribution, compared to strong cell membrane localization of EGFR throughout the epidermis, with minimal intracellular labeling, in control skin (Figure 2c). The non-synonymous substitution identified in exon 11 of *EGFR*, c.1283G>A, replaces a neutral glycine with a negatively charged aspartic acid molecule at a highly conserved residue, p.Gly428Asp. The pathogenicity of this missense mutation was supported by both the 'Sorts Intolerant From Tolerant' (SIFT; score 0, probably damaging) and Polymorphism Phenotyping v2 (polyphen-2; score 1, probably damaging) programs (Ng and Henikoff, 2003; Adzhubei *et al.*, 2010). The *EGFR* mutation was also validated by Sanger sequencing (Figure 2d; primers for *EGFR* amplification are given in Supplementary Material) and found to be homozygous in the affected patient, heterozygous in the mother, and homozygous wild-type sequence in the unaffected older sibling; DNA from the father was not available. The mutation is located in extracellular domain III of EGFR (Figure 2e), in contrast to the tyrosine kinase domain mutations commonly found in tumors (Kumar *et al.*, 2008). The region surrounding the mutation has been postulated to be involved in intra-molecular interactions that promote dimerization of EGFR and receptor activation (Ogiso *et al.*, 2002; Dawson *et al.*, 2005). We were unable to find the observed mutation in the dbSNP database, the 1000 Genomes database, the 13,005 exomes in the Exome Variant Server or in 900 unrelated European in-house control exomes.

Aberrant cutaneous expression of genes germane to keratinocyte differentiation, inflammatory response and innate immunity

Microarray data have been deposited in the Gene Expression Omnibus repository; accession number GSE54162. Comparison of the affected infant's skin with healthy control skin identified 2-fold or greater differential expression for 2,157 gene transcripts: 1004 up-regulated and 1153 down-regulated. These genes did not include *EGFR*, which was insignificantly up-regulated in the patient's skin (fold change=1.36; p=0.012). Evaluation of the changes in gene expression by functional enrichment analysis identified a multitude of enriched gene ontology (GO) pathways, processes, networks and disease-associated

transcripts germane to EGFR signaling (see Supplementary Materials for tabulated results). The top 3 up-regulated GO processes were linked to known EGFR functionality: keratinocyte differentiation, keratinization and epidermis development (involving genes such as transglutaminases, small proline rich genes, S100 genes, involucrin and late cornified envelope genes). Reports have also indicated a role for EGFR in regulating skin inflammation and cutaneous defence (Lichtenberger *et al.*, 2013; Mascia *et al.*, 2013). Thus, innate inflammatory response gene components (specifically PLA2, NF-κB, and JNK1) were among the most significantly up-regulated GO networks. Notably, *CCL2* expression was increased 2.2 fold, in keeping with reported findings in mouse models lacking epidermal *Egfr* expression (Lichtenberger *et al.*, 2013). Additionally, regulation of epithelial-to-mesenchymal transition and angiogenesis, processes known to be controled by EGFR activation (Perrotte *et al.*, 1999; Avraham and Yarden, 2011), were identified as significantly down-regulated GO pathways and processes, respectively. Of note, there was also down-regulation of transcripts that are normally increased in tumors which show gain-of-function mutations in *EGFR* (e.g. non-small cell lung cancer, colorectal cancer, and glioblastoma).

Mutant EGFR at the plasma membrane is highly unstable and more susceptible to constitutive endocytosis

To determine whether the mutation p.Gly428Asp in *EGFR* was responsible for mislocalization of EGFR in the affected infant, we generated DNA constructs containing mutant or wild-type *EGFR* sequence linked to green fluorescent protein (GFP) and expressed these constructs in MCF7 cells that express low endogenous EGFR and analyzed EGF-dependent localization of EGFR using confocal microscopy. Under normal growth conditions, the mutant *EGFR* construct localized diffusely throughout the cytoplasm of the cell in contrast to the wild-type construct, which was present mostly on the plasma membrane (Figure 3a). EGF stimulation resulted in robust translocation of wild-type EGFR to the plasma membrane within 10 minutes of EGF treatment (Figure 3b). Conversely, the mutant EGFR remained in the cytoplasm following EGF stimulation with no clear recruitment to the plasma membrane (Figure 3b), suggesting this mutation prevents or diminishes EGFR membrane localization, supporting the immunostaining findings in patient skin (Figure 2c).

EGFR membrane localization is regulated through a balance of endocytosis under the control of a number of intracellular signaling pathways that converge on the large GTPase dynamin (Lee *et al.*, 2006). To examine whether the lack of mutant EGFR localization to the cell membrane was due to defective endocytic traffic, MCF-7 cells expressing either wild-type or mutant EGFR were treated with an inhibitor of dynamin-mediated endocytosis, Dynasore (Macia *et al.*, 2006). Receptor localization was then examined by confocal microscopy. In the absence of ligand, membrane localization of both wild-type and mutant EGFR increased after treatment with Dynasore (Figure 3c). Similar results were observed in a normal human keratinocyte cell line expressing wild-type or mutant EGFR (see Supplementary Material). Immunostaining of MCF-7 cells expressing wild-type or mutant EGFR was performed using an antibody against the extracellular domain of EGFR to quantify membrane localization. Pearson's correlation coefficient analysis to quantify the

co-localization between wild-type or mutant EGFR with the antibody staining demonstrated that for both constructs the EGFR membrane localization increased significantly following Dynasore treatment (Figure 3c) but not following treatment with the chemical chaperone 4-PBA (data not shown). These data demonstrate that the mutant EGFR is able to undergo recycling to the plasma membrane but that it can only be retained at the membrane upon blockade of endocytic signaling. This suggests that the mutation p.Gly428Asp renders EGFR at the plasma membrane highly unstable and thus more susceptible to constitutive endocytosis.

Loss of downstream Akt and ERK signaling associated with mutant EGFR

Following EGF binding, EGFR undergoes rapid autophosphorylation and activation at a number of residues within the cytoplasmic domain of the receptor. These changes result in recruitment of a host of initiators and scaffolds of signaling leading to activation of MAP kinase family members including ERK (p44/42) and Akt (Avraham and Yarden, 2011). We therefore assessed the effect of the mutation p.Gly428Asp on EGF dependent phosphorylation of EGFR and downstream signaling. EGF-dependent ERK and Akt activation were assessed in untransfected MCF-7 cells or those expressing wild-type or mutant EGFR. EGF-stimulated activation of EGFR (monitored by a phospho-specific antibody) was robust in the cells expressing wild-type EGFR, but was undetectable in the mutant EGFR-expressing cells above levels seen in untransfected controls (Figure 4a). Similarly, neither Akt nor ERK activation was detected following ligand binding in mutant EGFR-expressing cells, in contrast to cells expressing wild-type EGFR which showed robust EGF-stimulated EGFR, ERK and Akt phosphorylation (Figure 4a). We observed similar results in parallel experiments in another cell line (CHO-K1 cells) (see Supplementary Material for data).

The suppression of EGFR-dependent activation and downstream signaling observed suggests that the EGFR mutation p.Gly428Asp results in loss-of-function as well as loss of plasma membrane localization. One of the primary functions of EGFR signaling following EGF binding is to promote cell proliferation (Jost *et al.*, 2000). To determine whether the loss of EGF-dependent signaling in p.Gly428Asp mutant EGFR-expressing cells results in functional defects, proliferation was analyzed in CHO-K1 cells expressing the wild-type or mutant EGFR constructs. EGF and serum-stimulated proliferation was induced in CHO-K1 cells expressing wild-type EGFR, but no induction of cell growth was detected in cells expressing the mutant EGFR (Figure 4b). Indeed, there was a negative growth rate of the mutant EGFR-expressing cells, consistent with other work showing inhibition of EGFR signaling induces apoptosis in keratinocytes (Figure 4c) (Rodeck *et al.*, 1997).

Clinicopathologic overlap with patients harboring mutations in *ADAM17*

The exact mechanism by which the missense mutation p.Gly428Asp leads to loss of EGFR function is unclear. Mutagenesis of the adjacent amino acid (p.Arg429Glu) was previously found to prevent EGFR homo-dimerization through disruption of an interface between domains II and III of the protein (Dawson *et al.*, 2005). However, altered homo-dimerization would not provide a sufficient explanation for the pathology as both monomeric EGFR and the p.Arg429Glu mutant were present at the plasma membrane (Ogiso *et al.*, 2002). In our

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experiments, we found that inhibition of endocytosis promoted levels of mutant EGFR at the cell surface. We hypothesize that the mutant receptor is unstable and rapidly undergoes internalization from the plasma membrane, perhaps implicating altered intracellular protein binding and subsequent aberrant tethering within membrane microdomains.

Functionally, failure of EGFR to correctly localize to cell membrane is expected to reduce ligand binding. In healthy people, several ligands require proteolytic cleavage to become capable of binding to EGFR – this ectodomain shedding is induced by at least 5 different metalloprotease enzymes, including ADAM17 (Blobel, 2005) – mutations in which may underlie an inflammatory skin and bowel disease that has overlap with the clinical features present in our patient (Blaydon *et al.*, 2011). We hypothesize that the lack of EGFR localization at the cell membrane in our patient has some similarity at a signaling level to that which occurs in the ADAM17-deficient patients, i.e. a common failure of EGFR-ligand interaction and altered downstream signal transduction.

Clinicopathologic similarities with mouse models of EGFR impairment

The clinical manifestations of the mutation p.Gly428Asp in terms of loss of EGFR function show some similarities with the phenotype of *Egfr* knockout or transgenic mice (Miettinen *et al.*, 1995; Silbia and Wagner, 1995; Threadgill *et al.*, 1995; Schneider *et al.*, 2008). Although the phenotype of *Egfr*-deficient mice depends on the strain of the mice (several are embryonic lethal), the abnormalities in the skin, lung and bowel in surviving mice are similar to many of the clinical manifestations in our patient. In the epidermis, some knockout mice show an initially thin skin that then becomes thicker but with defective barrier function (Miettinen *et al.*, 1995). Lack of *Egfr* in mice also affects hair growth, with wavy or reduced hair growth; EGFR is essential for normal hair follicle progression through the anagen, catagen, and telogen phases of the hair growth cycle (Hansen *et al.*, 1997; Schneider *et al.*, 2008). EGFR initiates hair growth and hair follicle organization, and EGFR inhibition leads to inflammation, follicular necrosis and alopecia, as well as slow hair growth with brittle hairs, as observed in our patient (Hansen et al., 1997; Schneider *et al.*, 2008). *Egfr*-deficient mouse lungs show condensed collapsed alveoli with a lack of surfactant leading to respiratory difficulty that has been likened to human neonatal respiratory distress syndrome (Miettinen *et al.*, 1995). In murine bowel lacking Egfr, there are fewer, shorter intestinal villi and reduced proliferation of jejunal enterocytes, leading to fluid loss (Miettinen *et al.*, 1995). Other abnormalities noted in the mice that show phenotypic similarities to our patient have included cystic dilatation of the collecting ducts in the kidneys (indicating that EGFR is vital for the differentiation of structures derived from the ureteric bud), aortic narrowing and a thrombotic tendency (Miettinen *et al.*, 1995). In addition, there are similarities in the phenotype of transgenic *Egfr*-and *Adam17* mice, thus supporting the clinical overlap in humans with germline autosomal recessive mutations in *EGFR* or *ADAM17* (Peschon *et al.*, 1998).

Clinical resemblance to the side-effects of EGFR inhibitor medications

The clinical manifestations in the child homozygous for p.Gly428Asp in *EGFR* also show some resemblance to the side-effect profile in individuals taking EGFR inhibitors. The side-effects of these drugs include a distinctive acne-like rash with pustules, dry skin, alopecia,

trichomegaly, as well as mucositis, diarrhea, and, in rare instances, interstitial lung disease (Inoue *et al.*, 2013; Lacouture, 2006). All these clinical features were evident to some degree in our patient. Nevertheless, although skin papules and pustules developed during disease progression in our case, the early skin changes mostly consisted of erosions (which led to the erroneous initial clinical diagnosis of EB). EGFR signaling is known to be involved in the re-epithelialization phase of wound healing (Repertinger *et al.*, 2004; Pastore *et al.*, 2008), as well as the proliferation of keratinocyte stem cells (Jensen *et al.*, 2009), and we hypothesize that the missense mutation in our patient impeded these processes and was the primary cause of the erosive skin changes, with bacterial super-infection being a secondary contributing factor. The transcriptomic data from our patient illustrate the consequences of impaired EGFR signaling. The results (albeit based on RNA extracted from non-inflamed skin) reveal enhanced pro-inflammatory activation and disturbed differentiation/premature terminal differentiation of keratinocytes as key pathogenic mechanisms, thereby demonstrating a similar profile to that associated with the papulopustular rash associated with EGFR inhibitory drugs (Lacouture, 2006; Lichtenberger *et al.*, 2013; Mascia *et al.*, 2013). Notably, several inflammatory/innate response networks were significantly up-regulated, including NF-κB.

In conclusion, the EGFR network is one of the most influential and intricate signaling systems in biology. We believe the protean features in our patient offer clinical insight into the critical and diverse roles of EGFR, supporting many of the putative functions that have been ascribed to the receptor via murine models and *in vitro* studies. This case highlights the major mechanism of epithelial dysfunction following EGFR signaling ablation and illustrates the broader impact of EGFR inhibition on other tissues that might be under-appreciated in the context of concurrent malignancy in patients receiving EGFR inhibitor medications.

MATERIALS AND METHODS

Transmission electron microscopy

Small pieces of skin (<2mm^3) were prepared for transmission electron microscopy, as described in the Supplementary Material.

Immunofluorescence microscopy

A skin biopsy from the affected infant and control skin were collected in Michel's medium. The methods for immunofluorescence microscopy and primary antibodies used are listed in the Supplementary Material.

Whole-exome sequencing

Initially, 3 μg of genomic DNA was sheared with focused acoustic technology (Covaris, Woburn, MA, USA) to yield a mean fragment size of 150bp. Fragments ends were repaired and sequencing adaptors ligated. Biotinylated 120bp RNA probes (Agilent, Santa Clara, CA, USA), designed against the coding regions of the genome were hybridized with the sequence library for 24 hours. DNA bound to RNA probes was retained using streptavidin-coated magnetic beads; unbound DNA was washed off. The exome-enriched pool of DNA

was eluted and amplified with a low-cycle PCR. The DNA fragments were then sequenced with 100 bp paired-end reads. Novoalign (Novocraft technologies, Selangor, Malaysia) was used to align reads to the reference genome (hg19, National Center for Biotechnology Information build 37). With over 7.0 Gb of sequence generated, more than 90% of coding bases of the GENCODE-defined exome were represented by at least 20 reads (see Supplementary Material).

Sanger sequencing

We sequenced the exon and intron/exon boundaries of all 28 exons of *EGFR*, including the mutation present in exon 11, in the infant, mother, unaffected older brother and 3 controls. Each exon was amplified by PCR using AmpliTaq Gold® 360 Master Mix (Applied Biosystems, Foster City, CA, USA) and the primers listed in the Supplementary Material.

Whole-genome expression microarray analysis

Whole-genome expression microarray analysis was performed using RNA extracted from skin biopsies sampled from the affected child as well as 4 pooled, healthy controls. RNA extraction from cutaneous biopsies was performed using the Ambion mirVana miRNA Isolation kit (Invitrogen, Paisley, UK) according to the manufacturer's instructions. RNA was amplified using the Illumina TotalPrep RNA Amplification Kit (Illumina, San Diego, CA, USA) and subsequent gene expression profiling was performed using the Illumina array HumanHT-12 v4.0 Expression BeadChip kit according to the manufacturer's instructions (Illumina). Gene expression data were then analyzed using GenomeStudio software (Illumina). Control samples were pooled and compared to the affected individual.

Mutant construct transfection

Mutant complementary cDNA mimicking the mutation of the infant (c.1283G>A) was generated with green fluorescent protein (GFP) tagged to the C-terminus of EGFR. Wild type EGFR1-GFP was a gift from Dr Andrew Reynolds (Institute for Cancer Research, London, UK). The mutant construct, p.Gly428Asp EGFR-GFP was generated using a site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) as per the manufacturer's instructions using the following mutagenesis primer: 5′ GAGAACCTAGAAATCATACGCGACAGGACCAAGCAACATGGT-3′. The mutation in the plasmid was verified by sequencing. Transfection was carried out using Fugene (Roche Applied Science, Penzberg, Germany) or Lipofectamine (Invitrogen) reagents according to manufacturer's instructions.

Confocal microscopy

For confocal microscopy, cultured MCF-7 human breast adenocarcinoma cells were transfected with wild-type EGFR-GFP or p.Gly428Asp EGFR-GFP washed with PBS, fixed with 4% paraformaldehyde (PFA) in PBS for 10 mins and then permeabilized with 0.2% TritonX-100 for 10 mins. For EGF stimulation experiments, cells were incubated in serum-free media (Opti-MEM®; Gibco Life Technologies, Carlsbad, CA, USA) for 16h prior to stimulation with 100 ng/ml EGF for the indicated times. For endocytosis inhibition experiments, cells were treated for 1 hour with 10 μM Dynasore (Millipore, Billerica, MA,

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USA) or equivalent volume of dimethyl sulfoxide (DMSO) as a control prior to fixation. Where appropriate, cells were incubated with a primary antibody directed against the extracellular domain of EGFR for 2h followed by the relevant secondary antibodies conjugated to Alexafluor-568 and Phalloidin conjugated to Alexafluor 568 or 633 for 1h at room temperature.

Western blotting

Thirty-thousand MCF-7 or CHO cells per condition were cultured in either Dulbecco's modification of Eagle's medium (DMEM) alone or DMEM containing 10% fetal calf serum (10% FCS) and transfected with wild-type EGFR-GFP or p.Gly428Asp EGFR-GFP; 36h later, the cells were starved for 16h with Opti-MEM® (Gibco Life Technologies) before treatment with 100 ng/ml EGF for the appropriate times. Cells were then lysed in sample buffer containing 2-mercaptoethanol at room temperature. Lysates were immediately subjected to SDS-PAGE and blotted using nitrocellulose membrane. Blots were blocked and probed using p-ERK, ERK, p-Akt, Akt, p-EGFR and EGFR antibodies (Cell Signaling Technology, Beverly, MA, USA), as well as HSC-70 antibodies (Santa Cruz Biotechnology, Dallas, Texas, USA), using 3% milk/PBS-0.2%tween or 5%BSA/TBS-0.1%tween.

Growth assessment rates

Ten-thousand CHO cells per condition were cultured in DMEM containing 10% FCS for 24h before transfection with GFP alone, wild-type EGFR-GFP or p.Gly428Asp EGFR-GFP. Twenty-four hours later, the media was replaced with normal growth media (10% FCS), growth media minus FCS or growth media minus FCS supplemented with 100 ng/ml EGF. Cells were then either Hoechst treated and fixed immediately with 4% PFA or were fixed 24h later. Phase and Hoechst images of 5 fields of view per condition were taken using an Olympus IX71 widefield microscope with a 4× 0.13 NA air objective (Olympus, Tokyo, Japan). Images were then analyzed for total number of cells as well as GFP expressing cells. The number of GFP expressing cells was then normalized using the total number of cells per field of view and presented either as the average number of GFP expressing cells per condition or the growth rate per condition.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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