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Abbreviations

EGFR epidermal growth factor receptor

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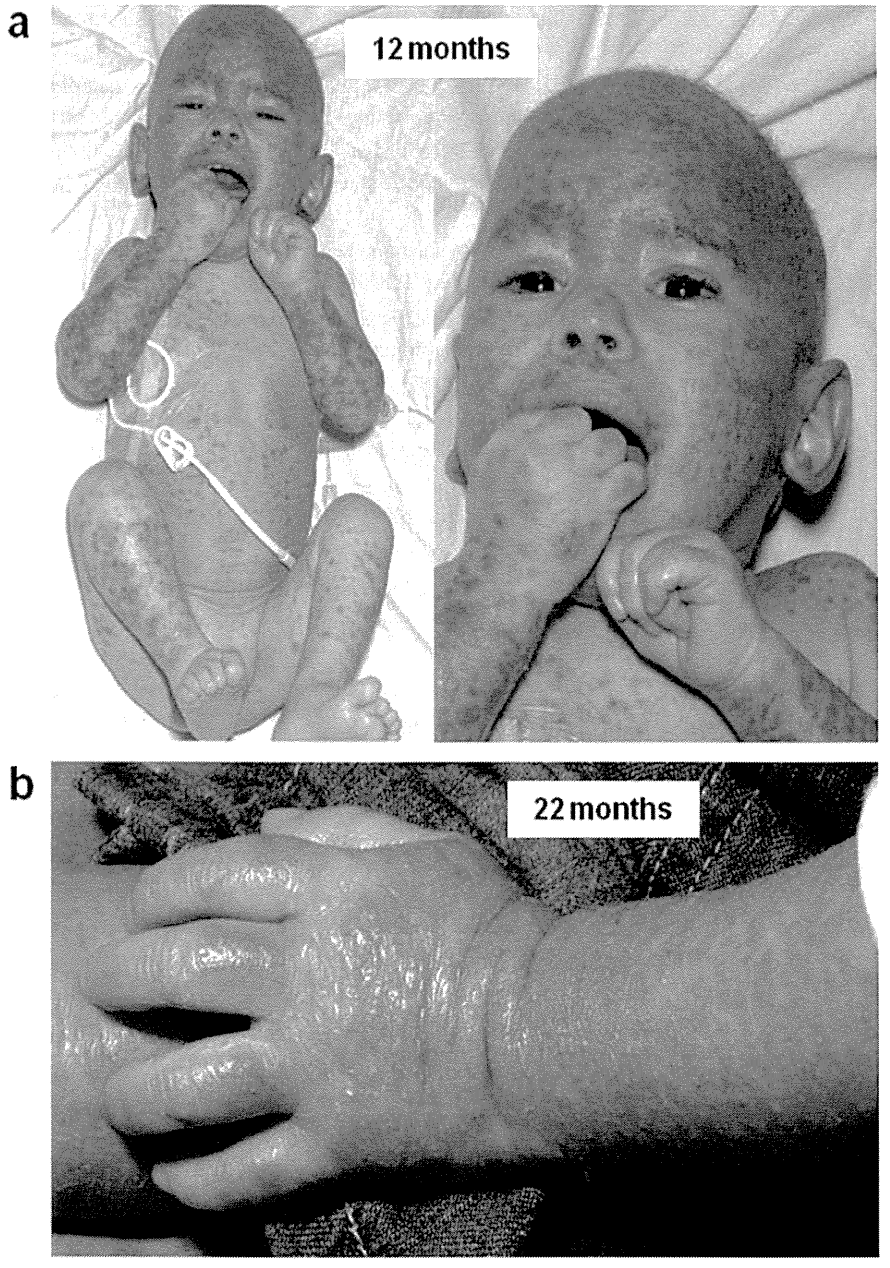

Figure 1. Clinical features of the patient demonstrating inflammation, erosions, papules and pustules

(**a**) At 12 months of age the infant has extensive erosions and markedly reduced scalp hair and eyebrows. He is also receiving total parenteral nutrition. (**b**) At 22 months there is a confluent papular eruption, particularly on the limbs, with numerous pustules. Consent to publish these photographs was obtained from the infant's mother.

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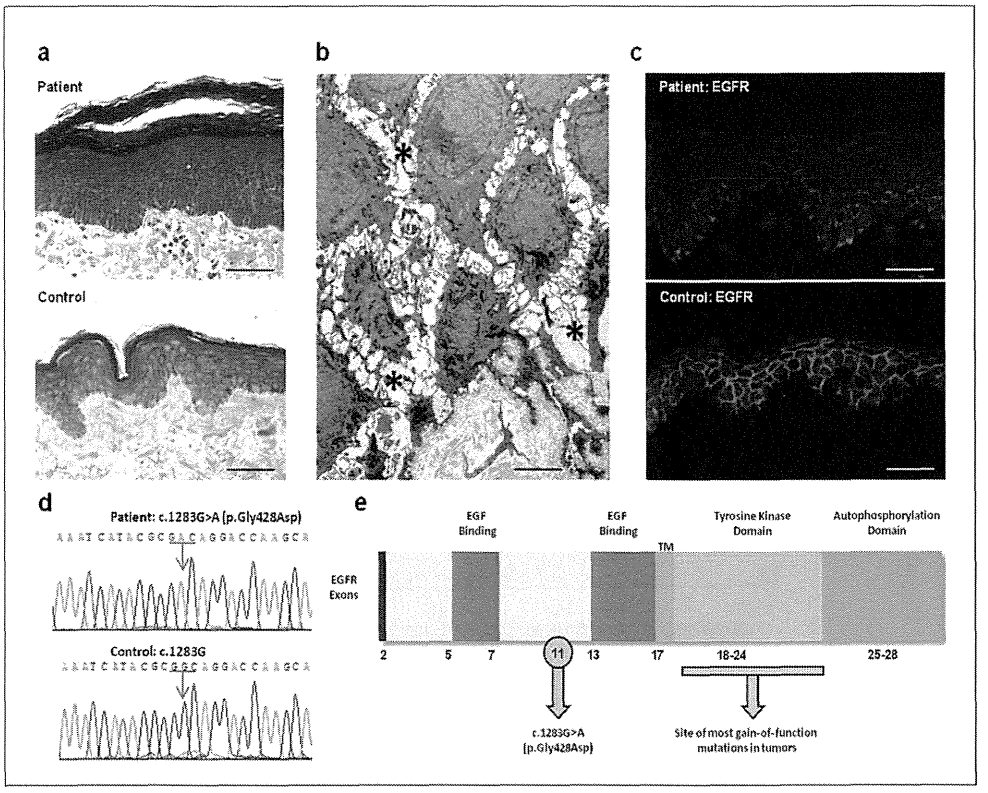

Figure 2. The homozygous mutation p.Gly428Asp results in acanthosis, intra-epidermal edema, and loss of keratinocyte cell membrane labeling for EGFR

(**a**) Semithin section of patient skin from the thigh reveals acanthosis and hyperkeratosis compared to age and site-matched control skin (scale bars = 50 μm). (**b**) Ultrastructurally, there is widening of spaces between adjacent keratinocytes in the lower epidermis (asterisks; scale bar = 2 μm). (**c**) Immunostaining for EGFR in patient epidermis shows loss of keratinocyte membrane staining compared to control skin (scale bars = 50 μm). (**d**) Sanger sequencing reveals a homozygous missense mutation in *EGFR*. (**e**) Schematic representation of the functional domains and encoding exons and the site of the pathogenic mutation in this patient. TM = transmembranous domain.

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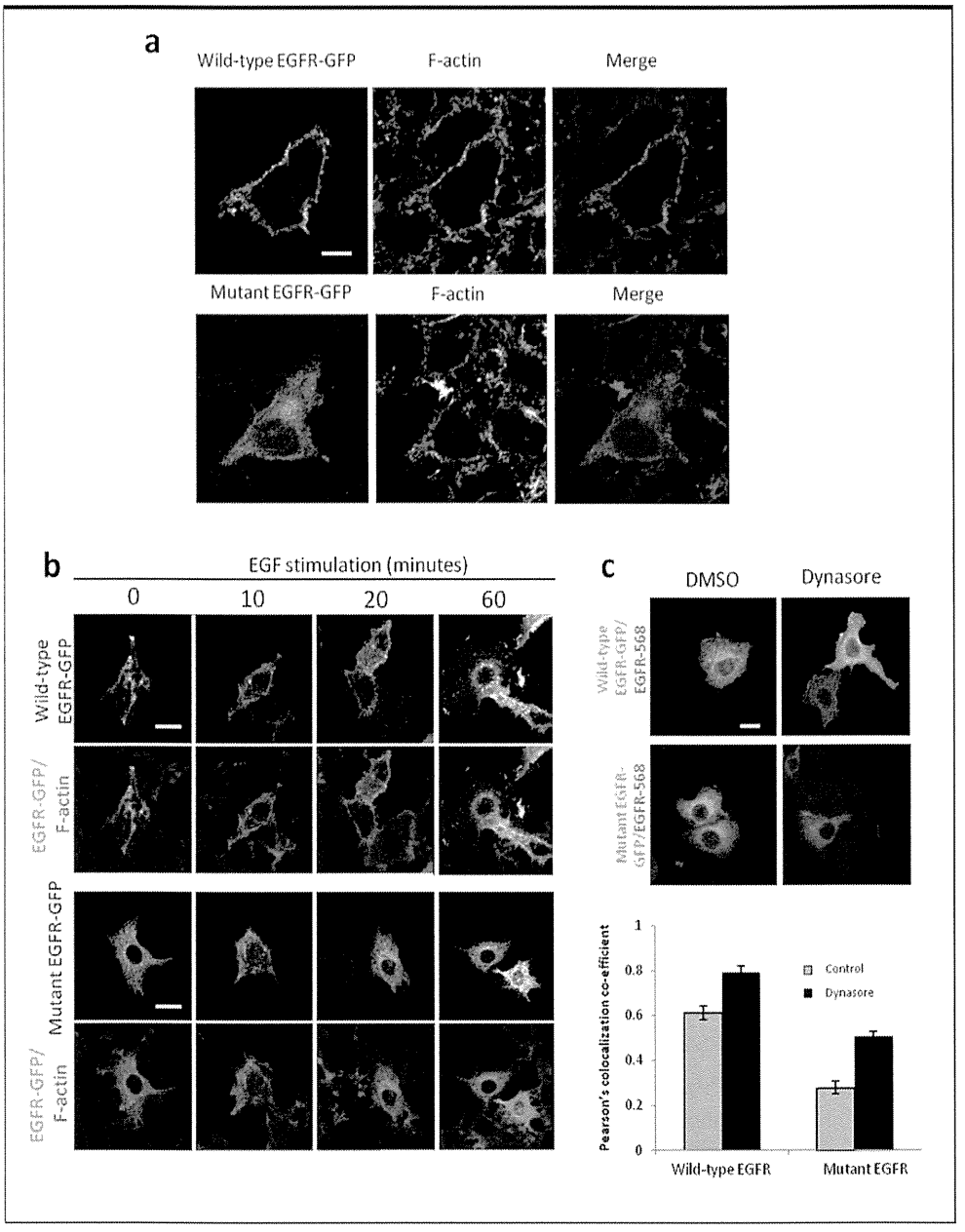

Figure 3. The mutation in *EGFR* renders the receptor unstable and susceptible to endocytosis

(**a**) Confocal microscopy was performed using GFP-tagged constructs of wild-type or mutant EGFR (green) and F-actin (red) in MCF-7 cells under normal growth conditions. (**b**) Images were then taken following stimulation of the cells with EGF at specified time points to assess the localization of EGFR within the cell cytoplasm or at the cell membrane. (**c**) Confocal microscope images were also taken for wild-type and mutant EGFR-GFP constructs (green) in cells stained for surface EGFR (red) in DMSO (dimethyl sulfoxide) control or Dynasore-treated cells (to inhibit endocytosis). The colocalization between EGFR antibody staining and either wild-type or mutant EGFR-GFP constructs was then quantified in DMSO control or Dynasore-treated cells using Pearson's correlation coefficient (Panel D lower). * $p<0.001$ vs WT-EGFR; scale bars = 10μm.

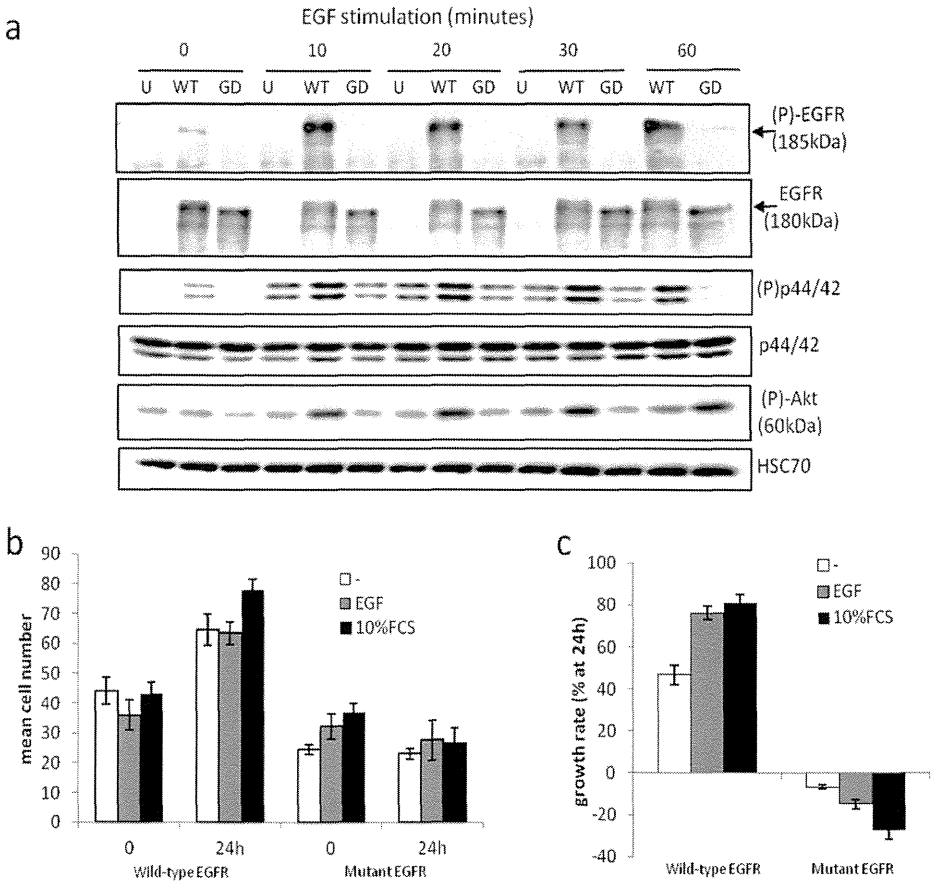

Figure 4. The mutation in *EGFR* reduces signal transduction and cell proliferation

(**a**) Western blotting was performed for p-EGFR, EGFR, p-ERK, ERK, p-Akt and Akt on lysates from untransfected (U), wild-type EGFR (WT) or mutant EGFR (MUT) transfected MCF-7 cells after EGF stimulation for the indicated times. MCF-7 cells express very low endogenous EGFR and therefore EGFR is undetectable in UT cells. Arrows indicate phospho and total EGFR species in top and second row blots respectively. Note the higher molecular weight species of EGFR in the WT samples in the second row blot are phosphorylated receptor and directly correlate with phosphorylated EGFR as detected in the top row blot. (**b**) Cell proliferation was quantified in CHO-K1 cells transfected with GFP, wild-type EGFR-GFP or mutant EGFR-GFP in starved (−), normal growth (10% fetal calf serum) or EGF-stimulated (EGF) conditions. (**c**) GFP-positive cells were counted and normalized against total cell number, and the cell growth rate was then calculated from these data.

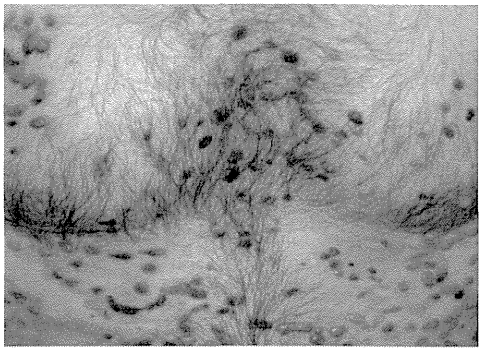

Fig 1. A 52-year-old man with bullous pemphigoid demonstrating numerous large, tense bullae and urticarial plaques on the chest.

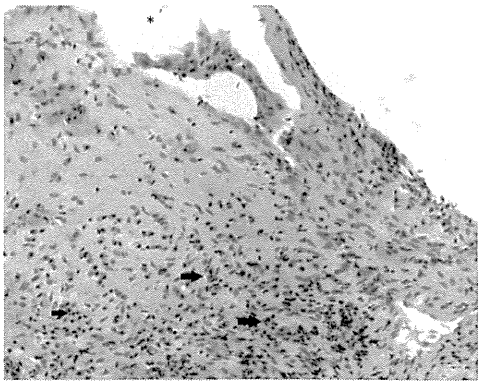

Fig 2. Bladder tissue from 52-year-old man with bullous pemphigoid and cystitis showing many eosinophils (*broad arrow*) and a subepithelial split (*asterisk*). (Hematoxylin-eosin stain; original magnification: ×100.)

histology alone. In a 2010 study,[3] 14 patients with newly diagnosed autoimmune bullous dermatoses, including 4 individuals with BP, underwent cystoscopy; of these patients, 13 had lower urinary tract lesions. On pathology 2 patterns predominated: nonkeratinizing squamous metaplasia and mucosal inflammation of the bladder base. Among patients with BP, the pathologic pattern of mucosal inflammation prevailed ($P = .003$).[3]

Limitations of this case include that the subepithelial split on bladder biopsy specimen could have been caused by trauma from the biopsy; the biopsy specimen did however demonstrate numerous eosinophils, which would be consistent with BP. Direct immunofluorescence studies were not performed on the bladder tissue. In addition, a follow-up cystoscopy was not available to evaluate whether the bladder lesions had cleared. An interesting consideration is the atypical nature of this patient's BP. Given his age of onset (42 years), concomitant psoriasis, and response to Enbrel (as was initially used to treat his psoriasis, but also helped resolve his BP), it is possible that this is a variant of pemphigoid that is not yet described in the literature.

Despite these limitations, given the cystoscopy findings of bullae by the urologist, pathology of the bladder epithelium on biopsy, and that resolution of the patient's urinary symptoms coincided with resolution of his cutaneous bullae, and typical immunofluorescence pattern on salt-split skin for BP, this case seems to be most consistent with symptomatic bladder involvement of BP.

Alison Launhardt, BS, Jayantha Thiyanaratnam, MD, and Kristen Fernandez, MD

Department of Dermatology, University of Missouri, Columbia

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Correspondence to: Kristen Fernandez, MD, Department of Dermatology, University of Missouri, 1 Hospital Dr, MA111, Columbia, MO 65212

E-mail: fernandezk@health.missouri.edu

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Successful topical adapalene treatment for the facial lesions of an adolescent case of epidermolytic ichthyosis

To the Editor: Epidermolytic ichthyosis (EI) is a keratinopathic ichthyosis that is characterized by generalized multiple blisters and erosions with erythroderma at birth. EI is caused by autosomal dominant rarely recessive mutations in the keratin 1 gene (*KRT1*) or the keratin 10 gene (*KRT10*). Their mutations lead to cytoskeletal fragility in the upper epidermis. Here we report the successful use of topical adapalene for treating a patient with EI.

A 16-year-old Japanese male adolescent was given the diagnosis of EI at birth. He was born with erythrodermic skin, blisters, and erosions on the whole body. As previously reported,[1] mutation analysis using the patient's genomic DNA samples revealed the heterozygous missense mutation c.457C>G (p.Leu153Val) in *KRT10*. During the first

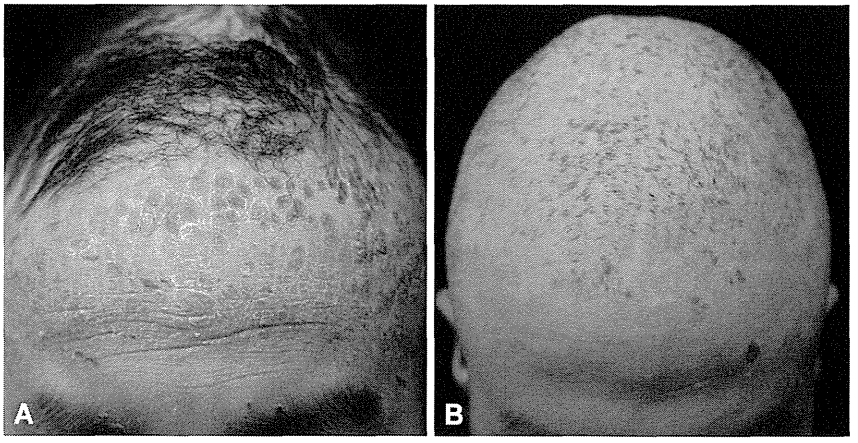

Fig 1. Epidermolytic ichthyosis. Clinical features of our patient. **A**, Before topical adapalene treatment, thick dark-gray and brown scales are seen on the forehead. **B**, After topical adapalene treatment, the hyperkeratosis is improved and thick scales are not seen on the forehead.

year of life, blister formation became less frequent, and the erosions and blisters were replaced with diffuse hyperkeratosis on almost the entire body surface. The palms and soles were spared, as is often the case with patients whose EI is caused by *KRT10* mutations. He experienced diffuse pruritus on the whole body. During his course, various treatments implemented with modest efficacy included topical vitamin D3, urea, steroids, and sulfadiazine.

When he was 14 years old, he began applying topical adapalene gel 0.1% to his face. After 6 months of treatment, his facial skin, mainly on the forehead, became smoother and his facial appearance greatly improved. He was satisfied with the results, and he has continued to use topical adapalene. The skin changes have persisted (Fig 1).

For hyperkeratotic lesions in EI, topical emollients containing glycerin, lactic acid, urea, and white petrolatum, along with topical vitamin D3 analogues are often used. Systemic retinoids are a beneficial treatment, despite their several adverse effects, for example, cheilitis, conjunctivitis, and liver dysfunction. For pediatric cases, we sometimes hesitate to use systemic retinoids because long-term administration carries the risk of bone hypoplasia. In light of this, topical application of retinoids is a beneficial treatment for EI, especially for pediatric patients, because a topical retinoid leads to less systemic exposure to the agent than a systemic treatment.

A tretinoin derivative, adapalene is a third-generation retinoid. To our knowledge, there are no case reports in which ichthyosis was treated with topical adapalene, although tazarotene, another third-generation retinoid, was reported to be effective for several types of ichthyosis, including X-linked ichthyosis, lamellar ichthyosis, and ichthyosis vulgaris.[2,3] In the current case, we used topical adapalene because topical adapalene causes less irritation than topical tazarotene, and adapalene is the only topical retinoid available in Japan.

Adapalene works to suppress the proliferation of keratinocytes. Unlike tretinoin, adapalene does not bind to the cytosolic retinoic acid binding proteins, but selectively binds to the nuclear retinoic acid receptors β and γ. The retinoic acid receptor γ subtype is predominantly expressed in the epidermis.[4] Several studies have suggested that, because of this selectivity, adapalene may demonstrate greater suppressive effect against hyperkeratosis with milder side effects than other retinoids demonstrate.[5]

For pediatric patients with EI, instead of systemic retinoids, topical adapalene might be a useful and powerful tool for improving facial lesions and quality of life.

Mariko Ogawa, MD, and Masashi Akiyama, MD, PhD

Department of Dermatology, Nagoya University Graduate School of Medicine, Japan

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Correspondence to: Masashi Akiyama, MD, PhD, Department of Dermatology, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya, Aichi 466-8550, Japan

E-mail: makiyama@med.nagoya-u.ac.jp

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T_{RM} then initiated cytotoxic responses against keratinocytes that resulted in epidermal necrolysis. Further analyses are needed to reveal the precise mechanisms.

This case shows that TEN can be evoked even in the absence of circulating T cells, and emphasizes the importance of T_{RM} during skin inflammation including drug hypersensitivity.

Hisato Iriki, MD,[a] Takeya Adachi, MD,[a] Mariko Mori, MD,[a] Keiji Tanese, MD, PhD,[a] Takeru Funakoshi, MD, PhD,[a] Daiki Karigane, MD,[b] Takayuki Shimizu, MD, PhD,[b] Shinichiro Okamoto, MD, PhD,[b] and Keisuke Nagao, MD, PhD[a]

Departments of Dermatology[a] and Hematology,[b] Keio University School of Medicine, Tokyo, Japan

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Correspondence to: Keisuke Nagao, MD, PhD, Dermatology Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bldg 10, Rm 12N238, 9000 Rockville Pike, Bethesda, MD 20892

E-mail: keisuke.nagao@nih.gov

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Varicella zoster virus–associated generalized pustular psoriasis in a baby with heterozygous IL36RN mutation

To the Editor: A 2-month-old otherwise healthy boy presented with erythema and pustules without vesicles on the face, trunk, and all limbs. There was no improvement following treatment with oral antibiotics 3 weeks earlier. On admission to our hospital he had a slight fever and a generalized crusted pustular eruption (Fig 1). Laboratory data showed the following abnormal values: white blood cell count 19.14 × 10^9/L (normal range: 7-15 × 10^9/L); C-reactive protein level 108.5 mg/L (normal range: <3 mg/L). Bacterial culture of the pustules and microscopy for fungal infection were negative. Histopathologic examination of a skin biopsy specimen revealed spongiosis with neutrophil infiltration in the upper epidermis (Fig 2).

The pustulosis did not improve with application of a potent topical steroid and vitamin D_3-containing ointment. Oral cyclosporine was started and gradually increased to 4 mg/kg at 64 days from the disease onset. One week later, the pustules had almost cleared. Administration of 4 mg/kg cyclosporine was continued for more than 9 months. Extensive pustules reappeared with development of an upper respiratory tract infection

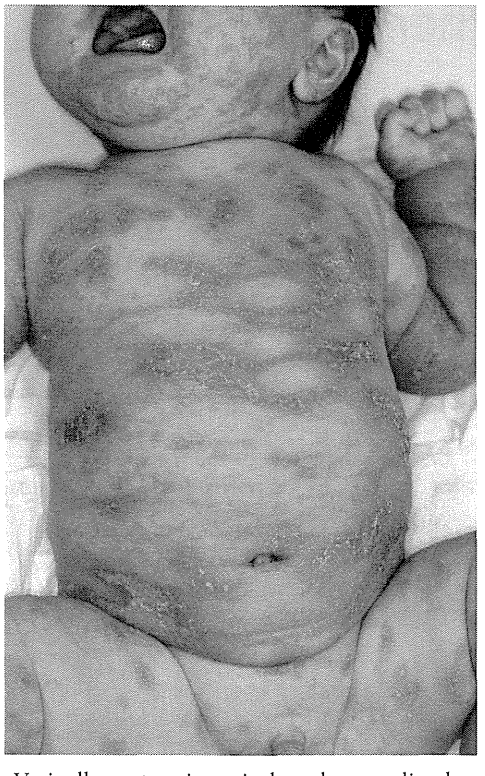

Fig 1. Varicella zoster virus–induced generalized pustular psoriasis in an infant. Pustulosis and crusts on the face, trunk, and limbs are seen.

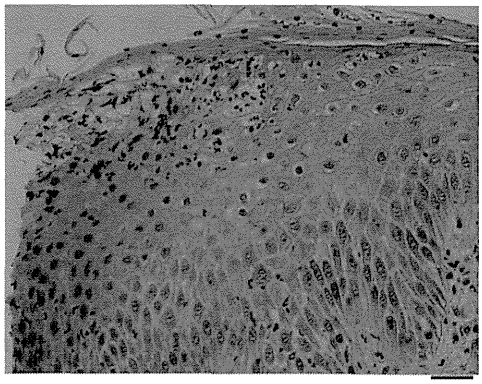

Fig 2. Varicella zoster virus–induced generalized pustular psoriasis: hematoxylin and eosin staining of the pustules; Bar: 40 μm.

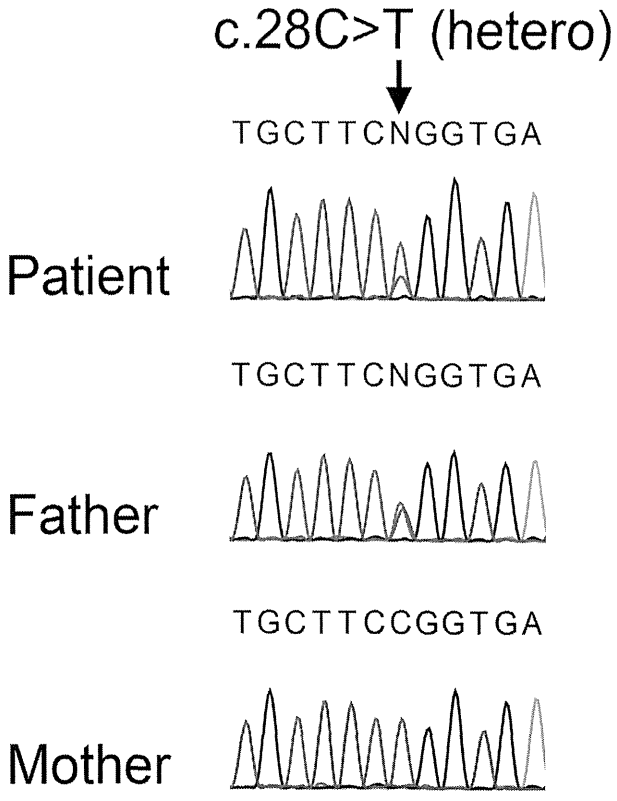

Fig 3. Varicella zoster virus–induced generalized pustular psoriasis: IL36RN sequence data of the patient and the parents. Arrow indicates the heterozygous c.28C>T mutation.

but subsequently regressed without a change in cyclosporine dosage.

There was no family history of a similar eruption. Before the skin manifestations developed, chickenpox was diagnosed in the patient's brother. Serum anti-varicella zoster virus (anti-VZV) immunoglobulin M tested negative in the baby initially but was positive 46 days after the disease onset. Anti-VZV immunoglobulin G antibodies were positive and thought to be derived from the baby's mother. There was no serologic evidence of herpes simplex virus infection.

After ethical approval was granted, written informed consent was obtained from the baby's parents in compliance with the Declaration of Helsinki. The entire coding regions of *IL36RN* including the exon/intron boundaries were sequenced using genome DNA samples from the patient and his parents. The patient and his father had a heterozygous c.28C>T (p.Arg10X) mutation in *IL36RN*, one of the generalized pustular psoriasis (GPP)-causing founder mutations in the Japanese cohort, whereas his mother did not have an *IL36RN* mutation (Fig 3).[1]

Diagnosis was VZV-induced GPP, a rare type of psoriasis that periodically recurs. Infection is one of its triggers. Mutation of *IL36RN*, which encodes interleukin-36 receptor antagonist (IL-36RN), has been associated with GPP in both its heterozygous and homozygous forms.[1-3] IL-36 is not present in normal skin but is induced by inflammatory cytokines including tumor necrosis factor-α (TNF-α).[4] When functional IL-36RN is underproduced, IL-36 can induce neutrophil-rich infiltration. TNF-α is elevated in the blood of VZV-infected individuals.[5] It is possible that this patient could not produce enough IL-36RN to antagonize the excessive IL-36 induced by VZV infection, an imbalance that resulted in GPP.

To our knowledge, this is the first report of VZV-induced GPP and of GPP triggered by infection in a patient with a heterozygous *IL36RN* mutation. Clinicians should consider IL-36RN deficiency in the setting of prolonged viral-induced generalized pustulosis.

Kazumitsu Sugiura, MD, PhD,[a] Ryuhei Uchiyama, MD,[b] Ryuhei Okuyama, MD, PhD,[b] and Masashi Akiyama, MD, PhD[a]

Department of Dermatology,[a] Nagoya University Graduate School of Medicine; Department of Dermatology,[b] Shinshu University Graduate School of Medicine, Japan

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Correspondence to: Kazumitsu Sugiura, MD, PhD, 65 Tsurumai-cho, Showa-ku, Nagoya, 466-8550, Japan

E-mail: kazusugi@med.nagoya-u.ac.jp

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Skin manifestations associated with chronic recurrent multifocal osteomyelitis in a 9-year-old girl

To the Editor: A 9-year-old girl presented with a 15-month history of severe joint pain limited to the right ankle. She had been treated for fatigue fracture and epiphysitis, but continued to require the use of crutches. The patient had pronounced muscular atrophy of the right leg, swelling and hyperthermia at the heel, and plantar pustulosis. She had mild paronychia on most fingers of the right hand and progressive changes in the fingernails, which were characteristic of nail psoriasis (Fig 1). Thus, psoriatic arthritis or osteitis was suspected, and oral naproxen (200 mg twice daily) therapy was prescribed. Whole-body magnetic resonance imaging (MRI) was performed and demonstrated inflammatory bone lesions, osteolysis, and sclerotic lesions (Fig 2). Laboratory parameters were within normal ranges. Her family history was unremarkable for similar cutaneous or musculoskeletal pathology. Chronic recurrent multifocal osteomyelitis (CRMO) with multifocal bone lesions, plantar pustulosis, and nail involvement was diagnosed. Oral methotrexate therapy (15 mg/week) was initiated and naproxen was continued. After 6 months, the joint pain resolved, and muscular atrophy, palmar pustulosis, and nail lesions improved.

CRMO is an acquired aseptic autoinflammatory bone disease that presents predominantly in girls and is characterized by pain that is worse at night, with or without fever. Typically there is a discrepancy between the mild symptoms and extensive bone inflammation. Sedimentation rate and C-reactive protein (CRP) values may be elevated,

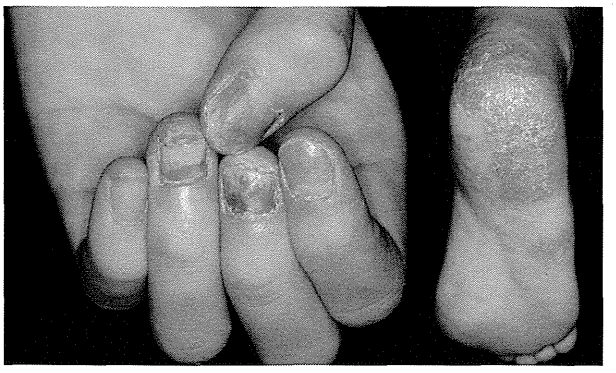

Fig 1. Chronic recurrent multifocal osteomyelitis. Onycholysis, nail pits, oil spots, and discoloration of the nails as well as erythema, hyperkeratosis, pustules on the sole of our 9-year-old female patient.

while the white blood cell count and other laboratory parameters are usually normal. The diagnosis of CRMO is mainly reliant on imaging studies. Conventional radiography initially shows osteolytic bone lesions with development of peripheral sclerosis in the course of the disease. MRI may show early lesions such as edema of bone marrow and inflammation of soft tissue. In order to diagnose CRMO, two major or one major and three minor criteria must be fulfilled.[1-3] Major criteria are osteolytic or sclerotic bone lesions, multifocal bone lesions, palmoplantar pustulosis or psoriasis, and sterile bone biopsy with signs of inflammation, fibrosis, or both. Minor criteria are normal blood cell count, good general health, slightly to moderately elevated CRP and erythrocyte sedimentation rate, clinical course of at least 6 months, hyperostosis, association with autoinflammatory diseases other than palmoplantar pustulosis or psoriasis, and a first- or second-degree relative with nonbacterial osteitis, or autoimmune or autoinflammatory disorders.

Some authors believe CRMO to be a juvenile variant of the SAPHO syndrome (synovitis, acne, pustulosis, hyperostosis, osteitis). However, to what extent CRMO and SAPHO present a spectrum of one disease or separate entities remains controversial.[2]

There is no standard therapy of CRMO; however, nonsteroidal antiinflammatory drugs (NSAIDs) are considered to be first-line treatment with a favorable response rate in up to 80% of patients. Patients may require therapy to control skin and bone lesions, and NSAIDs can be used during attacks or to prevent attacks.[5] NSAID therapy is usually continued until patients are symptom-free for at least 3 months. When NSAID therapy is inadequate, primary treatment options are bisphosphonates and tumor necrosis factor antagonists,[5] and strong data

Mutations in *GRHL2* Result in an Autosomal-Recessive Ectodermal Dysplasia Syndrome

Gabriela Petrof,[1] Arti Nanda,[2] Jake Howden,[3] Takuya Takeichi,[1,4] James R. McMillan,[5] Sophia Aristodemou,[5] Linda Ozoemena,[5] Lu Liu,[5] Andrew P. South,[6] Celine Pourreyron,[6] Dimitra Dafou,[7] Laura E. Proudfoot,[1] Hejab Al-Ajmi,[2] Masashi Akiyama,[4] W.H. Irwin McLean,[6] Michael A. Simpson,[8] Maddy Parsons,[3] and John A. McGrath[1,6,*]

Grainyhead-like 2, encoded by *GRHL2*, is a member of a highly conserved family of transcription factors that play essential roles during epithelial development. Haploinsufficiency for *GRHL2* has been implicated in autosomal-dominant deafness, but mutations have not yet been associated with any skin pathology. We investigated two unrelated Kuwaiti families in which a total of six individuals have had lifelong ectodermal defects. The clinical features comprised nail dystrophy or nail loss, marginal palmoplantar keratoderma, hypodontia, enamel hypoplasia, oral hyperpigmentation, and dysphagia. In addition, three individuals had sensorineural deafness, and three had bronchial asthma. Taken together, the features were consistent with an unusual autosomal-recessive ectodermal dysplasia syndrome. Because of consanguinity in both families, we used whole-exome sequencing to search for novel homozygous DNA variants and found *GRHL2* mutations common to both families: affected subjects in one family were homozygous for c.1192T>C (p.Tyr398His) in exon 9, and subjects in the other family were homozygous for c.1445T>A (p.Ile482Lys) in exon 11. Immortalized keratinocytes (p.Ile482Lys) showed altered cell morphology, impaired tight junctions, adhesion defects, and cytoplasmic translocation of GRHL2. Whole-skin transcriptomic analysis (p.Ile482Lys) disclosed changes in genes implicated in networks of cell-cell and cell-matrix adhesion. Our clinical findings of an autosomal-recessive ectodermal dysplasia syndrome provide insight into the role of GRHL2 in skin development, homeostasis, and human disease.

Grainyhead-like 2 (GRHL2) is a mammalian homolog of *Drosophila* protein grainy head (GRH), which, along with GRHL1 and GRHL3, has a role in epithelial morphogenesis.[1,2] This family of transcription factors controls the development and differentiation of multicellular epithelia by regulating genes germane to cell junction formation and proliferation.[3,4] Biologically, GRHL2 contributes to formation of the epithelial barrier and wound healing, as well as neural-tube closure, maintenance of the mucociliary airway epithelium, and tumor suppression.[5–11] *GRHL2* (MIM 608576) has been shown to regulate *TERT* (MIM 187270) expression and to enhance proliferation of epidermal keratinocytes; it also impairs keratinocyte differentiation through transcription inhibition of genes clustered at the epidermal differentiation complex[12] and regulates epithelial morphogenesis by establishing functional tight junctions.[13]

GRHL2 is also present in the cochlear duct,[14] and mutations in human *GRHL2* have been found in progressive autosomal-dominant hearing loss (DFNA28 [MIM 608641]),[15,16] and other polymorphic sequence variants in *GRHL2* have been implicated in age-related hearing impairment and noise-induced hearing loss.[17–19] To date, however, the role of GRHL2 in skin biology has not been well established. Causing severe facial and neural-tube defects, *Grhl2* knockout is embryonically lethal in mice,[17,20] and mutant zebrafish display inner-ear defects and abnormal swimming positions.[18] In contrast, *Grhl1*$^{-/-}$ mice show hair loss and palmoplantar keratoderma, as well as abnormal desmosome cell junctions and dysregulated terminal differentiation in keratinocytes.[21] Moreover, *Grhl3*$^{-/-}$ embryos fail to establish a normal epidermal barrier and display defective embryonic wound repair.[22] Thus, unlike for GRHL1 and GRHL3, there is currently a lack of data associating GRHL2 with skin pathology. In this report, however, we have identified two families in which affected subjects have developmental defects affecting skin, oral mucosa, and teeth (as well as hearing and lungs), thus implicating *GRHL2* in an autosomal-recessive ectodermal dysplasia syndrome.

We investigated two unrelated Kuwaiti families, both consanguineous, in which clinically similar features were present in a total of six affected individuals (Figures 1A and 1B). The clinical features were noted in early infancy and comprised short stature ($\leq 25^{th}$ percentile), nail dystrophy and/or loss, oral mucosa and/or tongue pigmentation, abnormal dentition (delay, hypodontia, enamel hypoplasia), keratoderma affecting the margins of the palms

[1]St. John's Institute of Dermatology, King's College London, Guy's Campus, London SE1 9RT, UK; [2]As'ad Al-Hamad Dermatology Center, Al-Sabah Hospital, Kuwait City 13001, Kuwait; [3]Randall Division of Cell and Molecular Biophysics, King's College London, Guy's Campus, London SE1 9RT, UK; [4]Department of Dermatology, Nagoya University Graduate School of Medicine, Nagoya 466-8560, Japan; [5]National Diagnostic Epidermolysis Bullosa Laboratory, Viapath, St. Thomas' Hospital, London SE1 7EH, UK; [6]Dermatology and Genetic Medicine, College of Life Sciences and College of Medicine, Dentistry, and Nursing, University of Dundee, Dundee DD1 5EH, UK; [7]Department of Genetics, Development, and Molecular Biology, School of Biology, Aristotle University, Thessaloniki 54124, Greece; [8]Department of Medical and Molecular Genetics, King's College London School of Medicine and Guy's Hospital, London SE1 9RT, UK

*Correspondence: john.mcgrath@kcl.ac.uk

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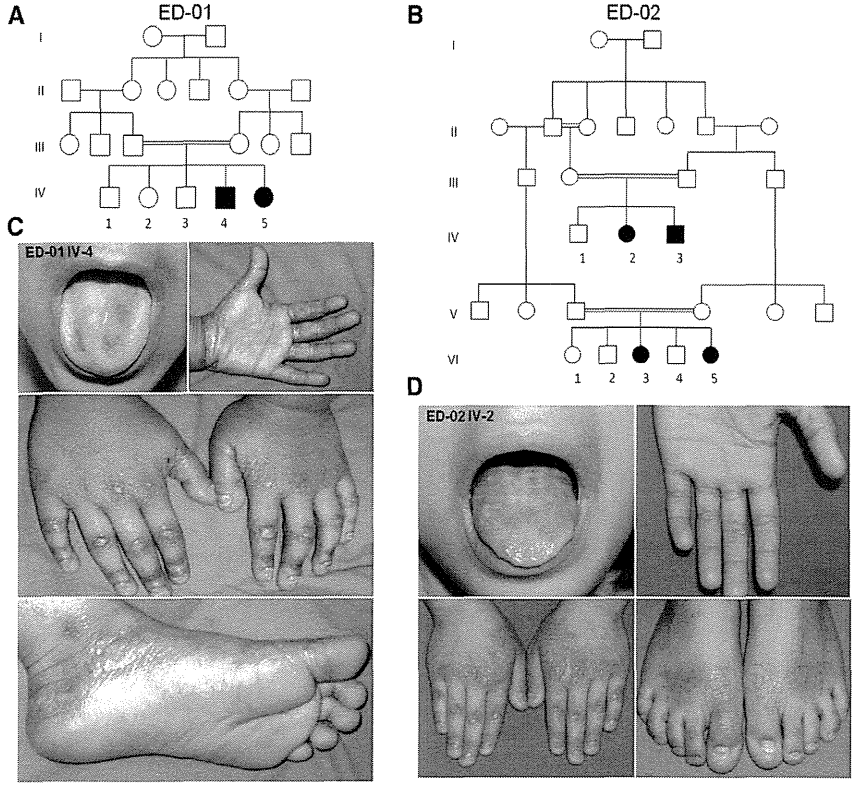

Figure 1. Pedigrees and Clinical Features of This Autosomal-Recessive Ectodermal Dysplasia Syndrome

(A and B) Two unrelated consanguineous pedigrees with a total of six affected individuals.

(C and D) An affected 8-year-old male (from pedigree ED-01) and an affected 12-year-old female (from pedigree ED-02) both show features of tongue hyperpigmentation, skin thickening around the margins of the palms and soles, hypoplastic finger and toe nails, knuckle pads on the fingers, and atrophic wrinkling on the dorsal aspects of the hands and feet. Additional clinical images from other subjects are shown in Figure S1.

and soles, and focal hyperkeratosis of the dorsal aspects of the hands and feet (Figures 1C and 1D; Figure S1, available online). No individual showed any wound-healing defect, blistering tendency, hair or sweating abnormalities, or other developmental anomalies. Two affected sisters (ED-02 VI-3 and VI-5) had dysphagia with evident esophageal strictures. Three individuals (ED-01 IV-4 and IV-5 and ED-02 VI-3) developed sensorineural deafness in early infancy, and three others (ED-01 IV-4 and IV-5 and ED-02 IV-2) had bronchial asthma. One individual (ED-01 IV-4) had severe iron-deficiency anemia requiring blood transfusion. Laboratory tests (full blood count, serum biochemistry, immunoglobulin levels, and thyroid-function tests) were otherwise within the normal range for all affected individuals. None of the parents had any skin, hair, teeth, nail, or hearing abnormalities.

To investigate the etiology of the condition, we first assessed lesional skin biopsies taken from three affected individuals (ED-01 IV-4 and ED-02 VI-3 and VI-5) by using immunohistochemistry and transmission electron microscopy. The subjects' legal guardians provided written informed consent according to a protocol approved by the St. Thomas' Hospital Ethics Committee (Molecular basis of inherited skin disease: 07/H0802/104). Blood and skin samples (ellipse of skin taken under local anesthesia by 1% lignocaine) were obtained in adherence to the Declaration of Helsinki guidelines. Light microscopy showed mild acanthosis and hyperkeratosis (Figure S2), but transmission electron microscopy of the skin was unremarkable—it showed no clear abnormalities in keratinocytes, hemidesmosomes, or desmosome cell-cell junctions. Likewise, skin immunolabeling using a panel of antibodies that target basement membrane extracellular matrix proteins (collagen IV and collagen VII), epidermal-adhesion-associated proteins (desmoglein 1, keratin 1, keratin 14, and desmoplakin), and markers of terminal differentiation (filaggrin) showed normal intensity and labeling patterns when affected skin from two affected individuals was compared to skin from a normal control subject (Figure S3; see Supplemental Data for methods and antibody details). However, we noted increased staining for the proliferation marker Ki-67 in the epidermis (Figure S4). Collectively, the skin-biopsy findings were not diagnostic for any known inherited skin disease.

We then used whole-exome sequencing to identify a candidate gene or genes. We extracted genomic DNA from peripheral blood from two affected individuals (ED-01 IV-4 and ED-02 VI-3). We performed whole-exome capture by using in-solution hybridization (Agilent All Exon Kit V4) and generated sequencing on the Illumina HiSeq 2000. Resulting reads were aligned to the reference human genome (UCSC Genome Browser hg19, GRCh37) with the Novoalign software package (Novocraft Technologies). Duplicate reads, resulting from PCR clonality or optical duplicates, and reads mapping to multiple locations were excluded from downstream analysis. A summary of exome-coverage data is presented in Table S1. Sixteen previously unreported homozygous mutations were identified (ten in family ED-01 and six in family ED-02). The only gene containing homozygous variants common to both subjects was *GRHL2* (Table S2). The respective mutations were c.1192T>C (p.Tyr398His) in exon 9 and c.1445T>A (p.Ile482Lys) (RefSeq NM_024915.3) in exon 11. These mutations were confirmed by Sanger sequencing (Figures 2A and 2B) and were also shown to segregate with the disease phenotype in other affected pedigree members. Both mutations are located in the DNA binding site of *GRHL2* (Figure 2C) and are predicted to be "probably damaging" by PolyPhen-2 analysis (scores 0.984 and 0.994 for

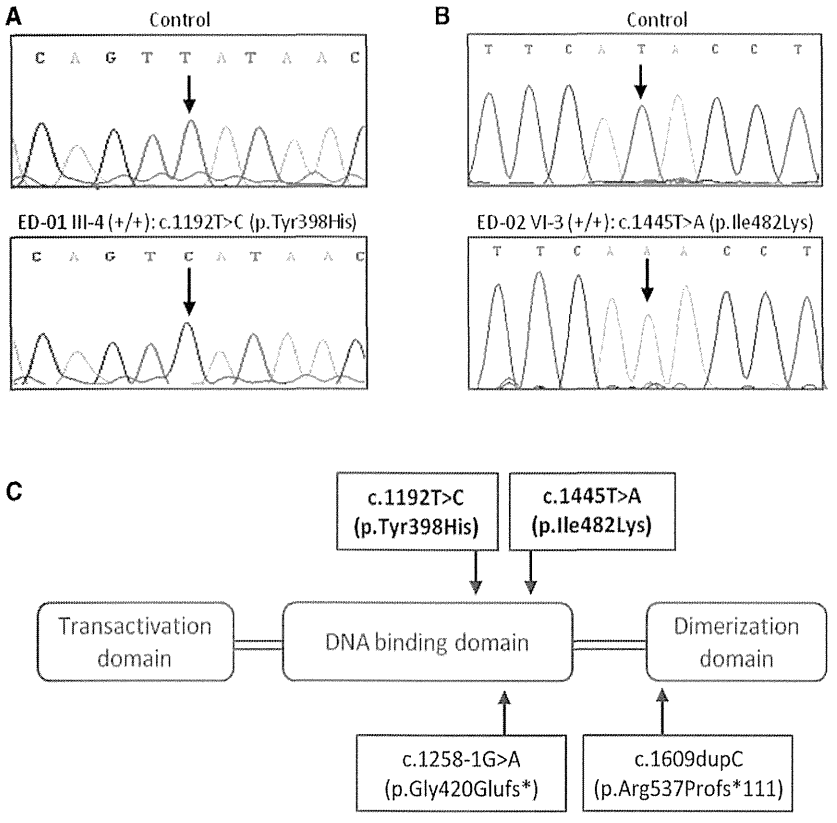

Figure 2. Autosomal-Recessive Mutations in *GRHL2* Lead to Reduced Gene Expression and Protein Levels

(A and B) Sanger sequencing confirmed the presence of different homozygous missense mutations in *GRHL2* in affected subjects from both pedigrees.

(C) Schematic representation of the functional domains of GRHL2. The recessive missense mutations we identified (top) are located within the DNA binding domain; the previously reported heterozygous splice-site or deletion mutations that cause autosomal-dominant deafness are also illustrated (bottom).

(D) qPCR for *GRHL2* expression in cultured keratinocytes showed reduced expression in two affected subjects from pedigree ED-02 (*$p < 0.05$ in comparison to control cells). Error bars represent the SD from three independent experiments.

(E) Immunoblotting using cultured keratinocyte whole-cell lysates revealed markedly reduced or undetectable amounts of GRHL2 in these same individuals.

c.1192T>C and c.1445T>A, respectively). Neither variant has been observed by the 1000 Genomes Project or detected in ~1,200 control in-house exomes or in 260 ethnically matched control chromosomes.

GRHL2 can be detected in the nuclear fraction of normal human keratinocytes in culture (but is subsequently lost in cell senescence).[23] We cultured keratinocytes from two of the skin biopsies (ED-02 VI-3 and ED-02 VI-5) by standard methods and used these cells to examine *GRHL2* expression and GRHL2 localization (see Supplemental Data for methods); both were found to be reduced (Figures 2D and 2E). We then isolated primary keratinocytes from one of the affected individuals (ED-02 VI-5) and immortalized these cells at passage 1 (see Supplemental Data for methods). The phenotype of these cells was assessed by confocal microscopy. *GRHL2* mutant cells showed a less cuboidal, elongated phenotype and failed to form intact cell junctions, as seen in control immortalized keratinocytes. Notably, there was a reduction in cell membrane labeling for E-cadherin (adherens junctions) and zona-occludens-2 (tight junctions) (Figure S5). GRHL2 staining in control keratinocytes was seen both at cell-cell contact areas and within the nucleus (Figure 3A), whereas in mutant cells, the signal was not at the periphery and instead showed a fragmented punctate nuclear localization (Figure 3B). To assess the effects of the mutations on keratinocyte cell function, we also performed assays of cell adhesion and de-adhesion (see Supplemental Data for methods). No differences were noted for cell adhesion between mutant and control cells (Figure 3C), but mutant cells detached from fibronectin much faster than normal human keratinocyte controls after exposure to trypsin (Figure 3D).

Next, we assessed the transcriptome profile by using RNA extracted from whole skin from two individuals in pedigree ED-02. RNA from healthy control skin was obtained from discarded abdominoplasty tissue from plastic surgeons and used as four pooled samples. RNA extraction was performed with the Ambion mirVana miRNA Isolation kit (Invitrogen) according to the manufacturer's instructions. RNA was amplified with the Illumina TotalPrep RNA Amplification Kit, and subsequent gene-expression profiling was performed with the Illumina array HumanHT-12 v4 Expression BeadChip Kit according to the manufacturer's instructions. Gene-expression data were then analyzed with GenomeStudio software (Illumina). A prefiltering set was determined for significantly modulated expression (detection p value < 0.01; signal intensity fold change ≥ 2.0) between affected and control skin. A comprehensive functional-enrichment analysis was then performed with (1) the Database for Annotation, Visualization, and Integrated Discovery (v.6.7), based on the Gene

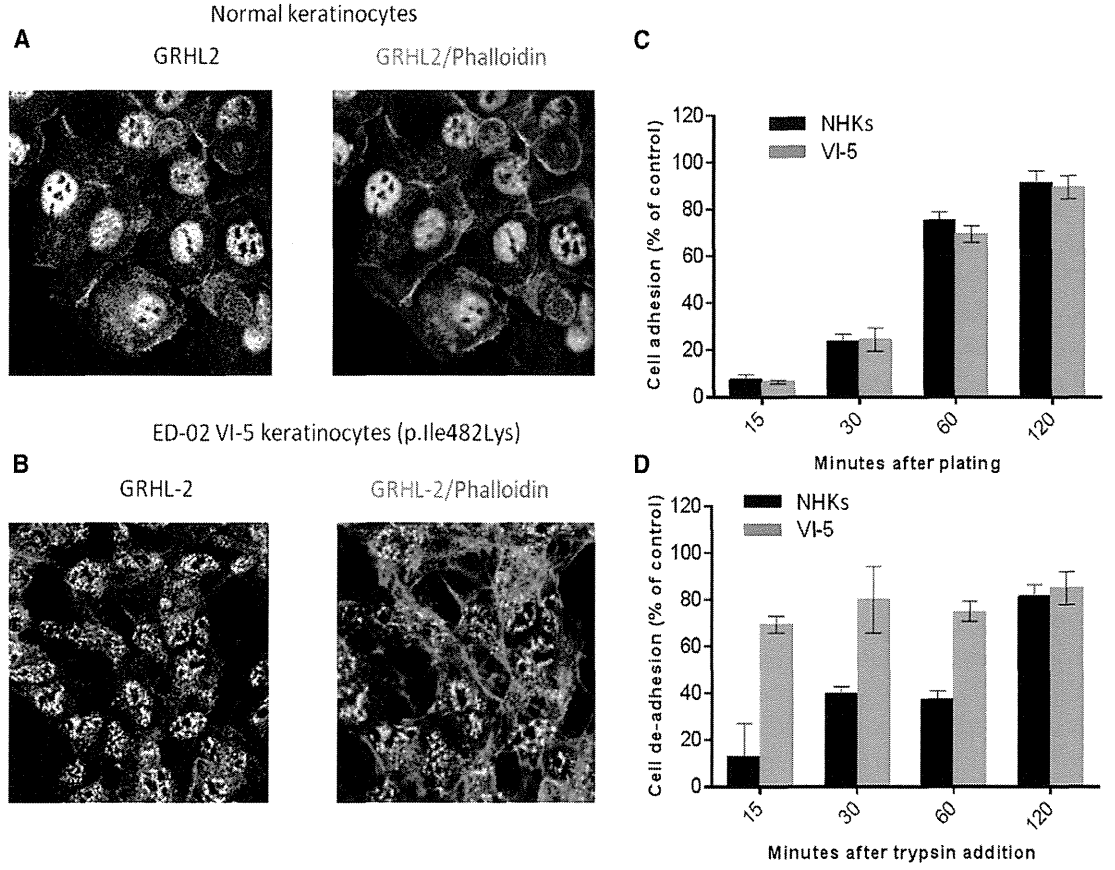

Figure 3. Impact of *GRHL2* Mutations on Keratinocyte Cell Biology
(A) Confocal microscopy in normal keratinocytes revealed nuclear, cytoplasmic, and membranous labeling for an antibody raised against GRHL2.
(B) In contrast, keratinocytes from an affected subject showed an altered pattern of antibody localization within the nucleus and a lack of any cell membrane labeling.
(C) Cell-adhesion assays showed no difference between wild-type and mutant keratinocytes. Error bars represent the SD from three independent experiments.
(D) In contrast, mutant cells showed more rapid detachment in trypsin de-adhesion assays. Error bars represent the SD from three independent experiments. NHK stands for normal human keratinocyte.

Ontology (GO) database (see Web Resources), and (2) the GeneGo Metacore software (Thomson Reuters), a systems-biology analysis tool based on a curated database of human protein-protein and protein-DNA interactions, transcription factors, signaling, and metabolic pathways. Comparison of the affected individuals' skin with the skin of healthy age- and site-matched control individuals identified 1,457 gene transcripts that were significantly altered: 668 upregulated ($\geq$2-fold change) and 789 downregulated ($\leq$0.5-fold change) transcripts for ED-02 VI-5. For ED-02 VI-3, 1,141 gene transcripts were altered: 466 upregulated and 675 downregulated. Of these changes in gene expression, 359 upregulated and 344 downregulated gene transcripts were common to both affected subjects. Evaluation of the changes in gene expression by functional-enrichment analysis identified several enriched GO pathways, processes, networks, and disease-associated transcripts, some of which are germane to the known functions of GRHL2. The top three upregulated GO pathways were linked to protein-folding maturation, cytoskeleton remodeling, and transcriptional control of lipid biosynthesis (involving genes encoding proopiomelanocortins and mitochondrial enzymes involved in metabolic pathways) (Tables S3–S10). Among the most significantly upregulated GO networks were the signal-transduction pathways and intermediate-filament remodeling (Table S7). Conversely, immune-response signaling; migration-inhibitory-factor-induced cell adhesion, migration, and angiogenesis; and networks of cell-cell and cell-matrix adhesion were downregulated (Table S8).

With regard to skin differentiation and barrier formation, selected alterations in gene expression are presented in Table S11. We also verified potential changes by performing quantitative PCR (qPCR) with RNA from whole skin of three individuals from the two pedigrees, as well as immortalized keratinocytes and primary fibroblasts from one affected person (see Supplemental Data for methods and controls). We observed reduced expression of *GRHL2* for all templates and a contrasting increase in *GRHL1* (MIM 609786) and *GRHL3* (MIM 608317) expression: unique and cooperative roles for this transcription factor family have been previously documented.[24] The most marked skin-barrier-associated gene changes were upregulation of aquaporin-encoding genes *AQP5* (MIM

600442) and *AQP7* (MIM 602974), the latter of which was expressed 50×–100× more in affected skin than in control skin. Gain-of-function mutations in *AQP5* have previously been associated with a form of autosomal-dominant nonepidermolytic palmoplantar keratoderma (MIM 600962).[25] Two-fold or greater reduction in gene expression was noted for *S100A8* (MIM 123885) and *S100A9* (MIM 123886), known targets for *GRHL1*. Previously, it has also been shown that GRHL2 enhances skin-barrier function by upregulating the tight-junction components claudins 3 and 4 and also Rab25, which localizes claudin 4 to tight junctions.[26] In affected people, we noted increases in *CLDN3* (MIM 602910), *CLDN4* (MIM 602909), and *RAB25* (MIM 612942) expression in whole skin (transcriptome and qPCR) and cultured keratinocytes (qPCR). Increased claudin 4 immunolabeling was also noted in the skin of two affected individuals (Figure S5).

Labeling for the proliferation marker Ki-67 was increased in the affected subjects' skin (Figure S4). This indicates that suprabasal keratinocytes are subject to an abnormal terminal-differentiation program, which provides a possible explanation for thickening of the epidermis and impairment of the epidermal barrier in our affected individuals with mutant *GRHL2*, although it is unclear why the most prominent skin scaling was found around the margins of the soles. We also noted reduced expression of *TERT* in the affected individuals' skin and keratinocytes. Overexpression of *GRHL2* in normal keratinocytes increases telomerase activity and increases replicative life span (*TERT* and *PCNA* [MIM 176740]). In contrast, knockdown of *GRHL2* represses the expression of these genes.[12]

The impact of *GRHL2* mutations on cell morphology has been previously described.[4] Lung epithelial cells transduced with *Grhl2* small hairpin RNA flatten in culture and lose their cuboidal morphology into an expanded cell phenotype. Knocking down *Grhl2* in lung epithelial cell lines leads to downregulation of *Cldn4* and *Cdh1*.[9] In our subjects' keratinocytes, immunostaining with E-cadherin showed reduced expression and qPCR showed downregulation of this transcript (Figure S6).

In addition to being expressed in skin, *Grhl2* and *GRHL2* are highly expressed in the inner ear, the lung epithelium, the ureteric bud of the kidney, the olfactory epithelium, the urogenital tract, the gastric mucosa, and human breast cancer cells.[4,8–11,18,27,28] With regard to the clinical phenotype in our affected individuals, aside from the changes affecting the skin and oral mucosa, the other main features comprised deafness and asthma, although this was variably present. Three subjects (ED-01 IV-4 and IV-5 and ED-02 VI-3) had deafness that developed in early infancy (c.f. the later-onset deafness in other families with GRHL2 haploinsufficiency).[15,16] Of note, none of the heterozygous carriers of either missense mutation in *GRHL2* had any deafness. The significance of GRHL2 in vertebrate inner-ear development is well established,[16] but the lack of deafness in the heterozygotes (and some homozygotes) in our pedigrees indicates a different functional effect of the missense mutations. Deafness is not a common feature of ectodermal dysplasia syndromes, although hearing loss can result from abnormalities in p63 and Notch signaling (morphological defects in organ of Corti)[29] and mutations in connexins 26 and 30 (altered endolymph ion homeostasis).[30] In contrast, mutagenesis studies in *Grhl2* have indicated a probable different pathophysiology for deafness with enlarged otocysts, absent otoliths, and malformed semicircular canals.[16]

The observation that three of the affected individuals (ED-01 IV-4 and IV-5 and ED-02 IV-2) had clinical symptoms of asthma is also noteworthy because the top enriched GO disease among the downregulated transcripts in our microarray data was asthma (Table S10). Previous in situ hybridization analyses have indicated that *Grhl2* is the only family member that is highly expressed in distal lung epithelium throughout development, although the particular cells expressing *Grhl2* have not been identified, nor has its functional role in the lung epithelium been fully established.[4] *Grhl1* and *Grhl3*, in contrast, are expressed in the embryonic lung epithelium, but later their expression is reduced in bronchi and bronchioles and is undetectable in the alveolar lung epithelium.[4,27] The potential relevance of other sequence variants in *GRHL2* to sporadic or familial cases of human asthma and other obstructive-airway diseases remains to be determined.

In summary, GRHL2, a member of a family of highly conserved transcription factors, is implicated in epithelial morphogenesis across a number of species. We have used whole-exome sequencing to identify *GRHL2* mutations underlying an ectodermal dysplasia syndrome in two families, and our data expand the current knowledge about the role of *GRHL2* in human disease and epithelial cell biology.

Supplemental Data

Supplemental Data include 6 figures and 13 tables and can be found with this article online at http://dx.doi.org/10.1016/j.ajhg.2014.08.001.

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Web Resources

The URLs for data presented herein are as follows:

Ensembl Genome Browser, http://www.ensembl.org/index.html
Gene Expression Omnibus, http://www.ncbi.nlm.nih.gov/geo/
Gene Ontology Consortium, http://www.geneontology.org/
Online Mendelian Inheritance in Man (OMIM), http://www.omim.org/
Primer3, http://frodo.wi.mit.edu/primer3/
PubMed, http://www.ncbi.nlm.nih.gov/PubMed/
RefSeq, http://www.ncbi.nlm.nih.gov/RefSeq
UCSC Genome Browser, http://genome.ucsc.edu/

Accession Numbers

The Gene Expression Omnibus accession number for the microarray data reported in this paper is number GSE56486.

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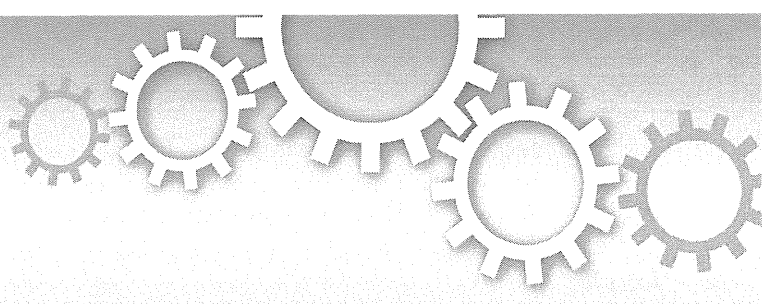

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* These authors contributed equally to this work.

A Palindromic Motif in the −2084 to −2078 Upstream Region is Essential for ABCA12 Promoter Function in Cultured Human Keratinocytes

Yoshitaka Shimizu[1]*, Yasushi Ogawa[1]*, Kazumitsu Sugiura[1], Jun-ichi Takeda[2], Kaori Sakai-Sawada[3], Teruki Yanagi[3], Atsushi Kon[4], Daisuke Sawamura[5], Hiroshi Shimizu[3] & Masashi Akiyama[1]

[1]Department of Dermatology, Nagoya University Graduate School of Medicine, Nagoya, Japan, [2]Division of Neurogenetics, Nagoya University Graduate School of Medicine, Nagoya, Japan, [3]Department of Dermatology, Hokkaido University Graduate School of Medicine, Sapporo, Japan, [4]Department of Nutrition, Aomori University of Health and Welfare, Aomori, Japan, [5]Department of Dermatology, Hirosaki University Graduate School of Medicine, Hirosaki, Japan.

ATP-binding cassette transporter family A member 12 (ABCA12) is a keratinocyte transmembrane lipid transporter that plays a critical role in preserving the skin permeability barrier. Biallelic loss of function of the *ABCA12* gene is causative of some forms of recessive congenital ichthyosis, an intractable disease marked by dry, thickened and scaly skin on the whole body. Genetic diagnosis is essential, although the results may occasionally be inconclusive, because some patients with low *ABCA12* expression have one mutant allele and one apparently intact allele. Aside from aberrant splicing or deletion mutations, one possible explanation for such discrepancy is loss of promoter function. This study aims to elucidate the promoter region of *ABCA12* and to locate the essential elements therein, thus providing the necessary information for genetic diagnostic screening of congenital ichthyosis. Close examination of the 2980-bp upstream regions of the *ABCA12* gene revealed that a palindromic motif (tgagtca) at −2084 to −2078 is essential for the promoter function, and a short fragment of −2200/−1934 alone has potent promoter activity. Identification of the key promoter element of *ABCA12* in this study may provide relevant information for genetic diagnosis of recessive congenital ichthyosis.

ATP-binding cassette transporter family A member 12 (ABCA12, OMIM 607800) is a keratinocyte transmembrane lipid transporter. ABCA12 is expressed in the stratum spinosum and stratum granulosum of the skin, where it is localized in lamellar granules (LGs), the cellular organelles that contain the lipids, proteins and enzymes needed for formation of the stratum corneum[1]. The function of ABCA12 is critical for keratinocyte differentiation as well as for maintenance of the skin permeability barrier via the formation of intercellular lipid layers in the stratum corneum[2–4]. In normal skin and in cultured human keratinocytes, the expression of ABCA12 parallels the differentiation of the keratinocytes[3]. Accordingly, mutations in the *ABCA12* gene underlie three distinct phenotypes of autosomal recessive congenital ichthyosis: harlequin ichthyosis (HI, OMIM 242500) and lamellar ichthyosis/congenital ichthyosiform erythroderma (LI/CIE, OMIM 601277)[5–7]. Since there are several causative genes whose mutation may cause congenital ichthyosis, identification of the pathogenic mutation in the patient's genome is essential for correct diagnosis[8].

There are cases, however, in which the results of genetic diagnosis are inconclusive, such as for patients of HI, LI or CIE who possess a recessive pathogenic *ABCA12* mutation in one allele but whose other allele is apparently intact[7]. In such cases, verification of decreased *ABCA12* mRNA expression in keratinocytes taken from the patient's skin or the hair follicles confirms the diagnosis[9]. Apart from anomalous splicing events due to intronic mutations or large deletion mutations, one possible reason for the decreased expression is loss of the promoter function due to mutations in key promoter elements.

To date, however, detailed functional promoter analysis of *ABCA12* has not been carried out, and key genetic elements that regulate *ABCA12* expression have not been described.

This study aims to identify the promoter region of *ABCA12* and to locate the essential elements therein, thus providing the necessary information for genetic diagnostic screening of congenital ichthyosis. The results show