TO THE EDITOR

Ectodermal dysplasia (ED) comprises a large heterogeneous group of inherited disorders defined by developmental defects in two or more tissues derived from embryonic ectoderm, including the skin, its appendages (i.e., hair follicles, eccrine glands, sebaceous glands, and nails) and teeth (Itin, 2014). The marked heterogeneity of ED hinders accurate genotype-phenotype correlation, although the application of next generation sequencing has added further insights (Lin et al., 2012; Petrof et al., 2014; Raykova et al., 2014). Clinical assessment and accurate diagnosis are further compounded by observations that congenital nail or hair disorders may occur as isolated phenomena (Khan et al., 2015; Shimomura, 2012). Here, we investigate a case presenting with congenital anonychia and uncombable sparse hair, and use whole exome sequencing to demonstrate that the phenotype results from two separate autosomal recessive disorders rather than a single variant of ED. Permission from the subject's guardian (and also the guardians of the other children mentioned in this report) was given for the publication of clinical images and data.

The proband, a 4-year-old Kuwaiti boy born to consanguineous parents, had a complete absence of all 20 nails from birth (Figure 1a). In addition, he had somewhat sparse scalp hair that was slow to grow, of wavy texture, and difficult to comb (Figure 1b). There was no other abnormality apart from a congenital squint. The proband is shown as individual IV-3 in the pedigree (Figure 1c). Clinical abnormalities were also present in his cousins (individuals IV-5, IV-6, and IV-7 in Figure 1c), again born to consanguineous parents. Those siblings had a similar absence of all 20 nails but lacked any hair abnormalities (Figure 1d). The clinical conundrum was whether the proband with anonychia and hair abnormalities had a different ED disorder or whether he might have two separate autosomal recessive ectodermal conditions. With regard to the anonychia, biallelic mutations in R-spondin 4 (Online Mendelian Inheritance in Man, OMIM. Johns Hopkins University, Baltimore, MD. MIM Number: 610573. http://www.ncbi.nlm.nih.gov/omim/) have been identified in autosomal recessive congenital anonychia/hyponychia (Bergmann et al., 2006; Blaydon et al., 2006). The encoded protein, R-spondin 4, is expressed in nail mesenchyme and acts as an activator of Wnt/β-catenin signaling, notably for nails (Blaydon et al., 2006). After written informed consent and institutional ethics approval, we Sanger-sequenced R-spondin 4, using published methods (Blaydon et al., 2006), and identified a homozygous donor splice-site mutation, IVS1+1G>A, in all anonychic individuals, including the proband and his three cousins (Figure 2a). To date, 18 mutations in R-spondin 4 have been identified in isolated congenital anonychia/hyponychia (Khan et al., 2015). The particular splice-site mutation in our pedigree previously has been reported as pathogenic (Blaydon et al., 2006).

To delineate the genetic basis of the hair abnormality, whole exome sequencing was performed on the Illumina NextSeq 500 platform using genomic DNA samples from the proband and both his unaffected parents (trio). Using various in silico pathogenicity prediction tools, including SIFT, PolyPhen-2, MutationTaster, CADD and DANN, and models of dominant or de novo dominant inheritance, we identified 11 possible autosomal recessive mutations and 3 de novo dominant changes (Supplementary Tables S1 and S2 online). A nonsynonymous homozygous mutation in peptidyl arginine deiminase, type III (PADI3) (c.1372C>A; p.Pro458Thr) was thought to be the most likely causative finding (SIFT: 0; Polyphen-2: 0.989; MutationTaster: 0.997; CADD: 26.3; DANN: 0.998). This mutation is very rare in the general population (25 heterozygous allele counts among 121,406 alleles in the ExAC Browser, http://exac.broadinstitute.org/) with no homozygotes. Sanger sequencing confirmed this homozygous missense mutation in PADI3 in the proband’s DNA. Only the proband was homozygous; the other unaffected family members were wild-type (WT) or heterozygous carriers (Figure 2b and c).

Peptidylarginine deiminases (protein name abbreviated to PAD; gene name abbreviated to PADI) are Ca2+-dependent enzymes responsible for the formation of protein-bound citrulline, first detected in the hair follicles of the guinea pig (Rogers and Taylor, 1977). In humans, five different isoforms (PADI1–4 and PADI6) have been identified in various tissues and all are clustered on 1p35–36 (Vossebaar et al., 2003). PADI3 is involved in deaminating trichohyalin in the hair follicles medulla and Henle layer (Nachat et al., 2005). The relevance of PADI3 to hair biology has been further emphasized by the recent discovery of biallelic PADI3 mutations in nine cases/families with uncombable hair syndrome (Basmanav et al., 2016). The mutations reported

Abbreviations: ED, ectodermal dysplasia; PADI3, peptidyl arginine deiminase, type III; WT, wild type

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comprise three missense variants: c.335T>A (p.Leu112His), c.881C>T (p.Ala294Val), and c.1813C>A (p.Pro605Thr) (Figure 2d), either as homozygous or compound heterozygous findings (Basmanav et al., 2016). Our homozygous amino acid substitution, p.Pro458Thr, occurs in the PAD domain, which is important for protein-arginine deiminase activity and calcium ion binding.

To examine the functional significance of p.Pro458Thr, we engineered a mutant construct by performing site-specific mutagenesis on the vector that was previously used to study the other PADI3 mutations (see Basmanav et al., 2016, for full details). The construct contains the WT PADI3 sequence (1995 bp) inserted into the pcDNA3.1 V5/His TOPO TA vector (Invitrogen, Carlsbad, CA). Mutagenesis was performed using the QuikChange II Site-Directed Mutagenesis kit according to the manufacturer’s instructions (Agilent Technologies, Foster City, CA) with verification by Sanger sequencing. Western blotting of extracts from HaCaT cells transfected with the WT and mutant PADI3 constructs showed a 4.7-fold reduction in PADI3 mutant protein (Figure 2e). Immunofluorescence microscopy of HaCaT cells revealed stark differences in the distribution of WT and mutant proteins. Although WT PADI3 was homogeneously distributed in the cytosol, the mutant protein formed aggregates, which were diffuse in the cytoplasm and particularly evident around the nucleus (Figure 2f). These results were similar to the cell pathology shown for the reported p.Ala294Val and p.Pro605Thr constructs, although our mutant construct (p.Pro458Thr) was associated with more aggregation than was seen for the previously assessed mutant p.Leu112His (Basmanav et al., 2016). In contrast, no aggregates at all were observed in cells transfected with WT constructs.

The hair phenotype in our proband was also uncombable, due to its wavy textural change, but was predominantly sparse and slow growing, thus differing slightly from all but one of the other reported PADI3 cases (Basmanav et al., 2016) and underscoring the range of hair pathologies that can result from PADI3 mutations. Our case also highlights the value of whole exome sequencing in identifying coinheritance of two distinct autosomal recessive conditions in consanguineous pedigrees that, for one individual herein, jointly gave rise to an ED phenotype.

CONFLICT OF INTEREST
The authors state no conflict of interest.

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Figure 2. Coinheritance of two separate autosomal recessive disorders accounts for the ectodermal dysplasia phenotype. (a) Sanger sequencing identifies a recurrent homozygous donor splice-site mutation in RSPO4 (IVS1+1G>A) in all subjects with anonychia, including the proband and three cousins; (b) Sanger sequencing confirms the proband’s homozygous missense mutation in PADI3 (c.1372C>A; p.Pro458Thr), originally identified by WES trio analysis. The proband is homozygous for the PADI3 mutation, whereas other unaffected family members are wild-type (WT) or heterozygous carriers; (c) verification of the mutation by HpyCH4III restriction enzyme digestion (New England Biolabs). The mutation c.1372C>A creates a new cut site such that the 452-bp band is cleaved into 300-bp and 152-bp products. For the patient (IV-3) two cleaved bands are present, whereas in carriers (III-3, III-4, and III-8) three discrete bands are seen. For the WT only the single undigested upper band is visible. (d) Schematic illustration of the PAD-3 protein and the mutations identified thus far (new mutation boxed in red); (e) immunoblot analysis of protein extracts from transiently transfected HaCaT cells shows a reduced expression of mutant PADI3 protein level compared with WT; immunoblotting was performed with anti-V5 antibody. (f) Immunofluorescence analysis in HaCaT cells transiently expressing WT and mutant PADI3 shows the homogeneous cytosolic expression of WT PADI3, whereas the mutant protein is observed in the form of large aggregates (scale bar = 10 μm). MW, molecular weight ladder; NC, negative control; PADI3, peptidyl arginine deiminase, type III; PC, positive control; RSPO4, R-Spondin 4; WES, whole exome sequencing.
Are Idiopathic Stevens-Johnson Syndrome/Toxic Epidermal Necrolysis Related to Drugs in Food? The Example of Phenylbutazone

TO THE EDITOR

Stevens-Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN) are rare life-threatening mucocutaneous reactions associated with a high mortality risk (8–23%, according to hospital centers) (Duong et al., in press; Sekula et al., 2013). SJS and TEN are drug-related reactions for approximately two thirds of SJS-TEN patients (Sassolas et al., 2010). For 36% of patients, no culprit drug is clearly identified, and pathology is named “idiopathic” SJS-TEN. Major drugs responsible of these reactions are some nonsteroidal anti-inflammatory drugs (NSAIDs) (Mockenhaupt et al., 2008; Roujeau et al., 1995). In the 1990s, NSAIDs represented 38% of culprit drugs. Phenylbutazone (PBZ) was found in 55% of cases (Guillaume et al., 1987). Today, PBZ is no longer marketed for human medicine but is still widely used in the veterinary field (Dubreil-Cheneau et al., 2011). Recently, this drug was banned from food in Europe after fraud related to horse meat: 6% of horse carcasses tested in 2012 were positive for PBZ (ec.europa.eu, 2013). These meats are consumed by humans. PBZ and oxyphenbutazone (OPB), its major active metabolite (Lees et al., 1986; Lees and Toutain, 2013; Tobin et al., 1986), or suxibuzone (SBZ), a PBZ prodrug usually used in veterinary medicine, could be found in individuals who consumed meat and milk containing PBZ. Because susceptible individuals could then develop SJS/TEN, our objective was to quantify drug-induced SJS-TEN, who were controlling subjects according to the same protocol and guidelines, for whom one culprit drug was clearly identified thanks to algorithm of drug causality for epidermal necrolysis (ALDEN) score considered as probable or very probable (score ≥ 4).

Seven patients (five women), treated for idiosyncratic SJS-TEN were included: one patient without drug intake at all during the month before the start of SJS-TEN and six patients with a drug intake during the month before the start of symptoms that was possibly, unlikely, or very unlikely related to the reaction, based on an ALDEN score of 3 or less. They were compared with 33 patients (20 women) treated for drug-induced SJS-TEN. In this group, drugs responsible were allopurinol (n = 7), lamotrigine (n = 10), nevirapine (n = 9) and sulfamethoxazole (n = 7). Demographic and clinical data are described (Table 1). All plasma samples were analyzed by liquid chromatography coupled with mass spectrometry (QuantumUltra, ThermoFisher, San Jose, CA) using an electrospray ionization in negative mode. Specific multiple reaction mode parameters were 307.13/278.9 (130.9) for PBZ, 323.13/295.0 (134.0) for OPB, and 437.14/306.8 (130.7) for SBZ. The standard concentrations ranged from 0.01 to 5 mg/L. The method validation was based on the availability of standards and quality control samples. The limits of quantification (LLOQ) were 10 ng/L for PBZ, 3 ng/L for OPB and 2 ng/L for SBZ. The method was validated by a linear regression analysis of different concentration levels (n = 3) and a recovery study of 80% (n = 3).

We tested for the presence of PBZ (OPB, SBZ) in the plasma samples of 18 patients with drug-induced SJS-TEN (16 women) treated for drug-induced SJS-TEN, who were controlling subjects according to the same protocol and guidelines, for whom one culprit drug was clearly identified thanks to algorithm of drug causality for epidermal necrolysis (ALDEN) score considered as probable or very probable (score ≥ 4).

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Abbreviations: LLOQ, lower limit of quantification; NSAID, nonsteroidal anti-inflammatory drug; OPB, oxyphenbutazone; PBZ, phenylbutazone; SBZ, suxibuzone; SJS, Stevens-Johnson syndrome; TEN, toxic epidermal necrolysis

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