Table 1. Clinical and laboratory features of the IIM patients and disease control patients who were found to be positive for antibodies to MMRE*

	Patient																	
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R
Age, years	48	56	70	22	19	28	40	30	47	25	15	4	40	44	63	38	15	28
Sex	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F
Diagnosis	PM	PM	PM	DM	DM	DM	DM	DM	CADM	CADM	JDM	JDM	PM–SSc	PM–SSc	PM–SLE	SLE	SLE	SLE
Anti-MLH1	+	–	–	+	+	–	–	+	–	+	+	–	+	+	+	+	+	+
Anti-PMS1	–	+	+	+	–	+	+	–	+	+	+	+	+	–	–	+	–	–
Anti-PMS2	+	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Anti-MSH2	+	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–	+	–
Other autoantibodies	–	Jo-1, SSA	–	–	–	TIF-1γ	MDA-5	MDA-5	PL-7	MDA-5	TIF-1γ	TIF-1γ	–	CENP-B	PL-12, SSA, dsDNA	U1 RNP, SSA, dsDNA	U1 RNP, Sm, SSA, dsDNA	U1 RNP, SSA
Muscle symptoms†	+	+	+	+	+	+	+	+	–	–	+	–	+	+	+	+	–	–
Highest CK level, IU/liter	339	393	3,405	1,263	150	6,554	209	220	202	101	130	65	1,016	195	437	252	87	95
Gottron's sign	–	–	–	+	–	+	+	+	+	+	+	+	–	–	–	–	–	–
Heliotrope rash	–	–	–	–	+	+	–	+	–	+	+	+	–	–	–	–	–	–
Mechanic's hands	–	–	–	–	–	–	–	+	+	–	–	–	–	–	+	–	–	–
Arthralgia	–	+	+	+	+	–	+	+	–	+	–	–	+	+	+	+	+	+
ILD	–	+	+	–	–	–	+	+	+	–	–	–	–	–	+	–	–	–
Malignancy	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

* IIM = idiopathic inflammatory myopathy; MMRE = DNA mismatch repair enzyme; PM = polymyositis; DM = dermatomyositis; CADM = clinically amyopathic DM; JDM = juvenile DM; SSc = systemic sclerosis; SLE = systemic lupus erythematosus; TIF-1γ = transcription intermediary factor 1γ; MDA-5 = melanoma differentiation–associated protein 5; dsDNA = double-stranded DNA; CK = creatine kinase; ILD = interstitial lung disease.
† Muscle weakness and/or myalgia.

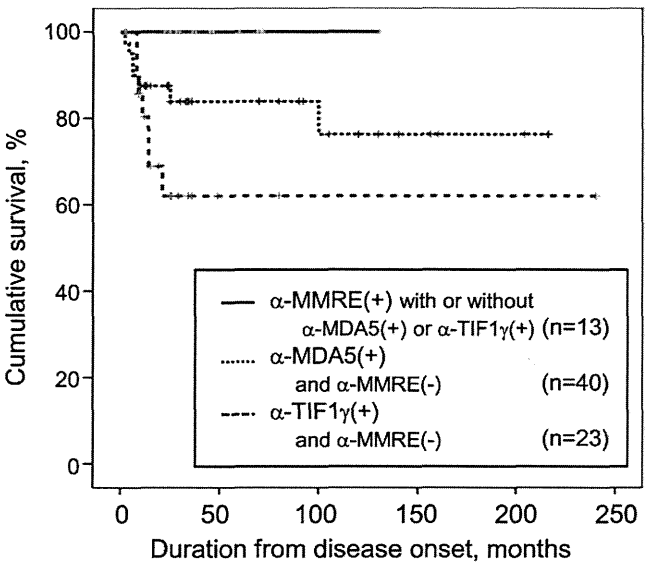

Figure 2. Cumulative survival rates from the time of disease onset in 76 Japanese patients with polymyositis/dermatomyositis (including clinically amyopathic dermatomyositis and myositis overlap syndrome) who were positive for serum anti–mismatch repair enzyme (anti-MMRE), anti–melanoma differentiation–associated protein 5 (anti-MDA-5), or anti–transcription intermediary factor 1γ (anti–TIF-1γ). The analysis did not include anti-MMRE–positive patients with juvenile dermatomyositis or systemic lupus erythematosus, although these patients were also still alive at the time of the analysis. The anti-MMRE–positive group included some patients who were also positive for anti–MDA-5 (n = 3) or anti–TIF-1γ (n = 1); no patients in the other 2 groups were positive for anti-MMRE. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.38866/abstract.

MMRE–positive patients were younger than antibody-negative patients ($P < 0.001$).

MSAs or systemic autoimmune disease–associated autoantibodies were found in 13 of the 18 IIM or SLE patients who were anti-MMRE positive. The prevalences of anti-MMREs in anti–MDA-5–positive patients (3 of 43 [7%]) and in anti–TIF-1γ–positive patients (3 of 30 [10%]) were not significantly higher than the prevalence of anti-MMREs in anti–Jo-1–positive patients (1 of 22 [4.5%]). Anti-aaRS and anti–MDA-5 antibodies were found in 3 patients and 2 patients, respectively, all of whom had interstitial lung disease. Three patients with anti–TIF1-γ were either juvenile or young adult patients with DM. Patients who were positive for anti–U1 RNP and/or anti-dsDNA were all diagnosed as having SLE. Of the 5 patients who were positive for at least 1 anti-MMRE antibody but for no other autoantibodies, all had IIMs.

Since all of the patients with anti-MMREs were still living at the time of the present analysis, we compared the survival rates among 3 groups: 1) patients who were positive for anti-MMRE with or without anti–MDA-5 or anti–TIF-1γ (excluding those with SLE or juvenile DM), 2) patients who were positive for anti–MDA-5 and negative for anti-MMRE, and 3) patients who were positive for anti–TIF-1γ and negative for anti-MMRE (Figure 2). Cumulative survival rates were lower among patients with anti–MDA-5 and in patients with anti–TIF-1γ compared to patients with anti-MMRE ($P = 0.136$ [not significant] and $P = 0.016$, respectively).

DISCUSSION

The present study is the first in which autoantibodies to 7 different types of MMREs were investigated. There are 2 previous reports describing the detection of anti-MMRE antibodies in patients with IIMs (3,4). Casciola-Rosen reported 6 patients with IIM and anti-MMRE positivity (3); anti-PMS1, anti-MLH1, and anti-PMS2 antibodies were found in 4, 3, and 2 patients, respectively. Japanese investigators initially detected anti-PMS1 and anti-MSH2 antibodies in a patient with pancreatic cancer (4). Subsequently, they detected anti-PMS1 in 13.5% of pancreatic cancer patients and in 6.7% of PM/DM patients by immunoprecipitation with TnT protein, and anti-MSH2 in 8.1% of pancreatic cancer patients and in 4.9% of PM/DM patients by immunoblotting with bacterial recombinant protein. Although detailed clinical information on the PM/DM patients was not reported, it was noted that 1 of the anti-PMS1–positive PM/DM patients had breast cancer.

In our study, anti-MLH1, anti-PMS1, anti-MSH2, and anti-PMS2 antibodies were detected, whereas no subject was positive for anti-MSH2, anti-HSH6, or anti-MLH3. Interestingly, nearly half of the antibody-positive patients (8 of 18) had multiple anti-MMRE antibodies, with the titers of the different antibodies changing in parallel within individual patients. We performed homology searches, using Pearson's online *lalign* program (http://www.ch.embnet.org/software/LALIGN_form.html), among amino acid structures of MSH1, PMS1, PMS2, and MSH2. These revealed no significantly homologous regions in long stretches among the 4 proteins. However, we found a highly homologous sequence with a 12–amino acid stretch ($T^{Y}/_{F}GFRGEAL^{A}/G/_{S}S^{I}/_{L}$) at the N-terminus of MLH1, PMS1, and PMS2, which MSH2 does not have. Thus, we cannot completely exclude the possibility of cross-reactivities among MLH1, PMS1, and/or PMS2.

Since only patients with IIM (n = 5) were positive for anti-MMRE but not for other autoantibodies, anti-MMREs can be considered as MAAs. The clinical features of the other 13 patients were closely associated with coexistent autoantibodies. In a previous study, 1 DM patient also had anti–Mi-2 and 1 PM patient had serologic evidence of SLE (autoantibodies to poly[ADP-ribose] polymerase and to catalytic subunit of DNA-dependent protein kinase) (3). Several MSAs have been discovered in recent years, and some of these autoantibodies may have been concomitantly present, but not tested for, in IIM patients who were found to be positive for anti-MMRE before the other MSAs were identified.

All of the IIM patients in whom anti-MMREs had been identified were still living at the time of the present analysis. The cumulative survival rate among these patients was significantly better than among patients with anti–TIF-1γ, which is a serologic marker for cancer-associated DM (1,13); the increased survival among patients with anti-MMREs compared to those with anti–MDA-5, which was originally defined as a serologic marker for CADM complicated by rapidly progressive interstitial lung disease (1,12,13), was not significant. There are conflicting reports regarding outcomes among anti–MDA-5 antibody–positive patients with IIM (15), but recent therapeutic advances are resulting in improved survival for this group (Muro Y, et al: unpublished observations).

In summary, anti-MMREs are considered to be myositis-associated antibodies, but clinical subsets are strongly influenced by coexistent autoantibodies. Inclusion of a larger number of disease control patients in the present study would likely have improved our ability to assess this in greater detail. Further study is needed to investigate whether the antibodies described herein might have prognostic, in addition to diagnostic, value.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Muro had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Muro, Nakashima, Mimori, Akiyama.

Acquisition of data. Muro, Nakashima, Hosono, Sugiura.

Analysis and interpretation of data. Muro, Nakashima, Hosono, Sugiura, Mimori, Akiyama.

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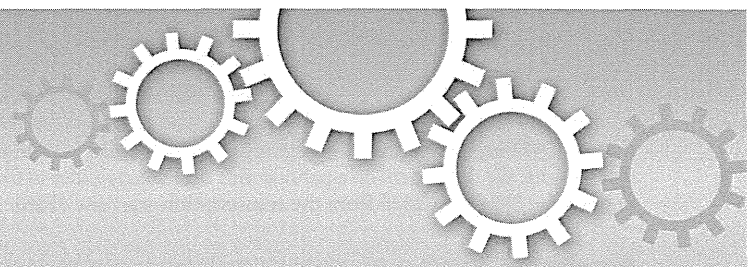

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A Palindromic Motif in the −2084 to −2078 Upstream Region is Essential for ABCA12 Promoter Function in Cultured Human Keratinocytes

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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

that *ABCA12* expression in differentiating cultured human keratinocytes is critically dependent on a palindromic motif that resides in the region of −2084 to −2078 from the transcription start site (TSS).

Results

Identification of the critical region of −2200 to −1934 in the upstream promoter of the *ABCA12* gene. To locate the essential elements in the upstream region of ABCA12, we first cloned a −2980/+190 fragment (the base position +1 is the TSS of *ABCA12*, chr2: 216,003,151) and performed a dual luciferase reporter assay to assess its promoter activity with respect to keratinocyte differentiation status. We utilized cultured normal human epidermal keratinocytes (NHEK). It is well established that increased cellular calcium level is a key signal to promote the differentiation of keratinocytes in culture as well as in normal skin[10,11]. Accordingly, NHEKs differentiate under the high-calcium condition, and ABCA12 expression increases in differentiated keratinocytes[1,2]. As expected, the −2980/+190 fragment showed 3.2 times as much activity when NHEKs were cultured under the high-calcium condition (1.2 mM) compared to the low-calcium condition (0.06 mM) (Fig. 1, Supplementary Fig. S1). We performed an *in silico* search of the ENCODE dataset using the UCSC genomic browser[12] throughout this region and found increased ChIP-seq signals and peaks of H3K4me3 and H3K27ac, and increased DNAseI-seq peaks in NHEK (Fig. 2). These results were considered to support the bona fide promoter function of this genetic region. Additionally, an *in silico* analysis using JASPAR CORE database (http://jaspar.genereg.net/)[13] found 398 putative transcription factor binding sites within this region.

We then aimed to narrow down the region that contains the essential promoter elements experimentally. For this purpose, increasing lengths of the upstream region of the *ABCA12* gene were cloned and their promoter activities were assessed by a dual luciferase reporter assay (Fig. 1, Supplementary Fig. S1). Under the high-calcium condition, the highest promoter activity was detected with the −2980/+190 fragment, whereas −1994/+190 and −1039/+190 fragments showed drastically decreased promoter activity (−12.2 fold, $P < 0.01$ and −6.41 fold, $P < 0.01$ compared to the −2980/+190 fragment, respectively). These results suggest that one or more critical promoter elements may reside within the region from −2980 to −1994 of the *ABCA12* promoter.

To further narrow the region containing the putative element(s), promoter activity was analyzed for five serially truncated fragments consisting of the −2980/+190 to −1934/+190 *ABCA12* upstream region (Fig. 3a, Supplementary Fig. S2). Under the high-calcium condition, the 2700/+190 fragment showed roughly comparable promoter activity to the −2980/+190 fragment (−1.43 fold compared to the −2980/+190 fragment, not significant), whereas the −1994/+190 fragment showed markedly reduced promoter activity (−16.1 fold compared to the −2980/+190 fragment, $P < 0.01$). Promoter activities of the −2400/+190 to −2200/+190 fragments were decreased compared with that of the −2980/+190 fragment (−2.28 fold, $P < 0.01$ and 35.3%, $P < 0.01$, respectively). Therefore, we considered that the region from −2700 to −1994 is critical for the promoter activity of the ABCA12 upstream region in NHEK.

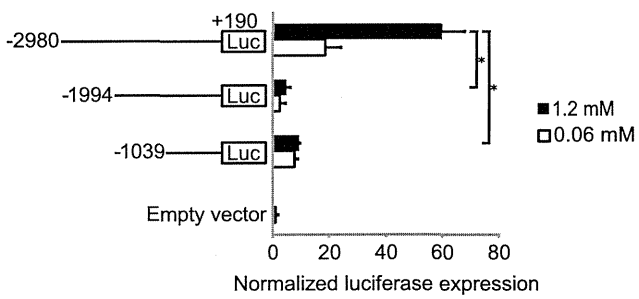

Figure 1 | Position −2980 to position −1994 of the *ABCA12* gene has promoter activity. Dual luciferase assay was performed using *firefly* luciferase reporter vectors that contain −2980/+190, −1994/+190 or −1039/+190 fragments of the *ABCA12* gene. The culture medium contained either 0.06 mM or 1.2 mM calcium. The promoter activities are normalized using the empty vector as a standard. The bars represent the standard deviation (n = 3). *$P < 0.01$ compared with the −2980/+190 fragment.

To identify the most critical region between −2700 and −1994, four short overlapping fragments spanning the region were cloned and their promoter activities were assessed (Fig. 3b, Supplementary Fig. S2b). The −2200/−1934 fragment showed the highest promoter activity, suggesting that this region may contain the critical element(s) (Fig. 3b, Supplementary Fig. S2).

Interestingly, the promoter activity of this fragment was not dependent on the increased calcium concentration, suggesting that an unknown element outside the −2200/−1934 region may exist to suppress the activity under low-calcium conditions. The neighboring −2400/−2106 fragment also showed some promoter activity, although not as much as the −2200/−1934 fragment.

A palindromic motif in the −2084 to −2078 region is essential for the promoter activity. To pinpoint the critical bases that could be used for the genetic diagnostic screening of congenital ichthyosis patients, we searched for consensus sequences for transcription factor binding elements within the region from −2200 to −1934. An *in silico* analysis using the JASPAR CORE database found a predicted specificity protein 1 (Sp1) binding motif (−2133 to −2144, reverse strand, chr2:216,005,284–216,005,295) and a palindromic motif that matches an AP1 binding sequence (tgagtca, −2084 to −2078, chr2:216,005,235–216,005,228) within the −2200/−1934 fragment (Fig. 2, Supplementary Fig. S3).

The AP1 elements may be able to tether AP1 transcription factors to the promoter region of the genome and regulate downstream gene expression. In keratinocytes, AP1 regulates the expression of various genes, such as involucrin and transglutaminase 1[14]; these AP1-regulated genes are involved in keratinocyte differentiation and are mostly expressed in the late stage of terminal differentiation[15]. When these motifs were mapped in the ENCODE dataset annotations using the UCSC genome browser, the palindromic −2084/−2078 motif was found to reside within the increased ChIP-seq signals of H3K4me3 and H3K27ac, and within the DNAseI-seq peak in NHEK (Fig. 2).

These considerations lead us to hypothesize that the −2084/−2078 motif may regulate the expression of ABCA12. Therefore, we introduced one to three nucleotide deletion mutations into the wild-type sequence (tgagtca): mutant Δ1 (tga-tca), mutant Δ2 (tga--ca) and mutant Δ3 (tg---ca). We investigated the effect of the mutations on the promoter activity of the −2980/+190 fragment (Fig. 4, Supplementary Fig. S4). All three mutants showed strikingly reduced promoter activity compared to the wild type (−6.10 fold, $P < 0.01$, −9.71 fold, $P < 0.01$ and −11.7 fold, $P < 0.01$ for Δ1, Δ2 and Δ3, respectively) (Fig. 4, Supplementary Fig. S4). No significant differences in promoter activity were found between the three mutants.

Discussion

The present results clearly demonstrate that disruption of the −2084/−2078 motif alone can critically decrease the promoter activity of the entire 3 kb upstream sequence of *ABCA12*. In addition, the 267 bp fragment containing this motif (−2200/−1934) is sufficient to establish potent promoter activity. This experiment using short genomic fragments may have the shortcoming that the assays are done without the context of their original core promoters or other minimal promoters, and the interpretation of the results may be

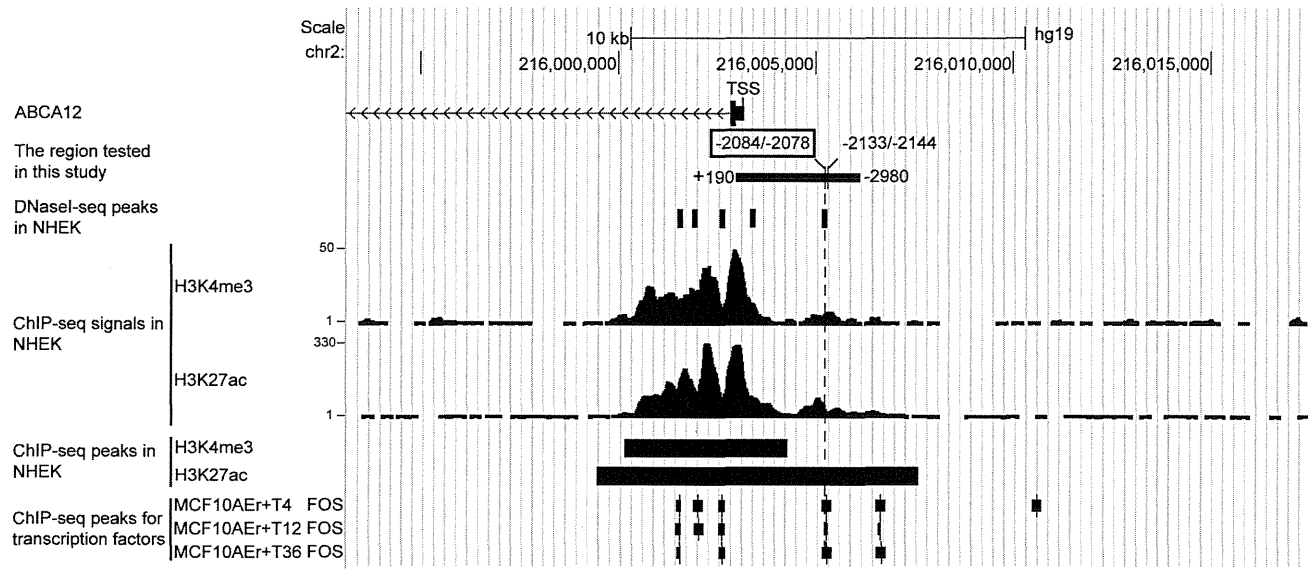

Figure 2 | Annotation of the ABCA12 upstream gene using the ENCODE dataset. The ABCA12 upstream genomic region (chr2:215,993,140–216,017,439:GRCh37/hg19) is illustrated using the UCSC genome browser. ChIP-seq signals and peaks of H3K4me3 and H3K27ac, and DNaseI-seq peaks in NHEK were extracted from the ENCODE dataset and annotated. The read density of ChIP-seq signals is indicated in the Y-axis. ChIP-seq peaks of c-Fos/c-Jun in 91 human cell types from the ENCODE dataset are also shown. c-Fos peaks for MCF10A-Er-Src cells stimulated with tamoxifen for 4 hr (MCF10AEr + T4 FOS), 12 hr (MCF10AEr + T12 FOS), or 36 hr (MCF10AEr + T36 FOS) were found in the database search. The −2084/−2078 palindromic motif is boxed, and its genetic location is highlighted with a dotted line. The predicted −2133/−2144 Sp1 binding element is also indicated.

limited to the demonstration of a possible capability of each fragment to recruit transcription factors. The following mutagenesis experiment thus confirmed the essential role of the −2084/−2078 motif in its native genomic context. In support of these results, the ENCODE dataset search showed increased ChIP-seq signals for H3K4me3 and H3K27ac, which mark active promoters, and a DNaseI-seq peak that demonstrates the open chromatin state in NHEK. Interestingly, in MCF10A-Er-Src cells stimulated with tamoxifen, the ChIP-seq tag containing this motif is associated with a peak for c-Fos, a component of AP1 (Fig. 2), showing that the motif may indeed recruit these transcription factors in certain cells, although it is not yet proven whether c-Fos binds the −2084/−2078 motif in NHEK. In agreement with the experimentally proven significance of this motif, a database search shows a high conservation of the −2084/−2078 sequence among multiple eutherian species (Supplementary Fig. S5). We searched for previously reported SNPs in the HAPMAP database[16] in this genetic region, but we found no SNP in the −2084/−2078 sequence in populations with European or Asian ancestry.

Apart from the −2084/−2078 motif, the −2200/−1934 fragment harbors a predicted −2133/−2144 Sp1 binding element. It is reported that AP1 and Sp1 cooperatively regulate the expression of target genes in keratinocytes, such as *LOR*, which encodes loricrin[17]. Therefore, it may be assumed that the expression of ABCA12 is also regulated by the putative −2133/−2144 element. Nevertheless, in the actual context of the entire promoter sequences, the predicted element may play rather an adjunctive role in *ABCA12* expression, because the disruption of the −2084/−2078 motif alone is sufficient to reduce the promoter activity of the −2980/+190 fragment to one-tenth of its original activity.

In the present study, we are aware of the limitation of depending solely on luciferase assays performed on NHEK, as they do not provide direct evidence for the function of the −2084/−2078 motif in its true genomic context. However, the existing ChIP-seq and DNaseI-seq results obtained from NHEK are in accordance with the proposed function of this motif as the essential promoter element and can reinforce the conclusions obtained from our experiments.

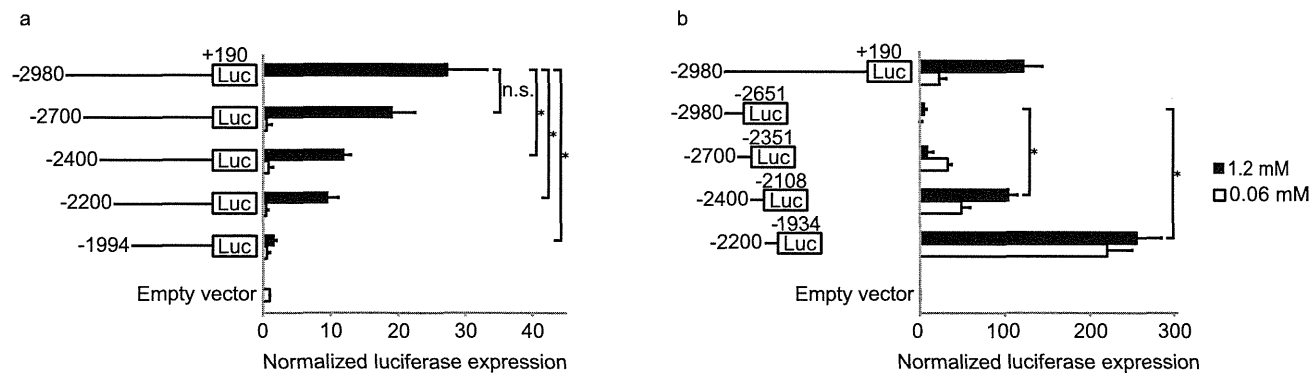

Figure 3 | Position −2200 to position −1934 of the *ABCA12* gene exhibits potent promoter activity. Promoter activities of −2980/+190, −2700/+190, −2400/+190, −2200/+190, or −1994/+190 fragments (a), and −2980/−2651, −2700/−2351, −2400/−2108, or −2200/−1934 fragments (b) were assayed. *P < 0.01 compared with the −2980/+190 fragment (a) or the −2980/−2651 fragment (b).

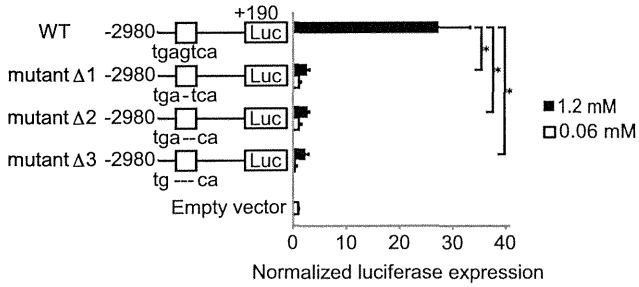

Figure 4 | Mutants of the −2084/−2078 motif in *ABCA12* show striking reduction of promoter activity. Three different mutants of the −2084/−2078 motif were generated using the −2980/+190 fragment as a template, and the promoter activity of each fragment was assessed. The mutants lacked 1–3 bases from the wild-type sequence (tgagtca): mutant Δ1 (tga-tca), mutant Δ2 (tga--ca) and mutant Δ3 (tg---ca). The promoter activities of the mutants were compared to that of the wild type. *$P < 0.01$ compared with the wild type.

From these considerations, we propose that future routine genetic screening for the diagnosis of congenital ichthyosis patients include the sequencing of the −2084/−2078 upstream region of *ABCA12*, especially if *ABCA12* mutation is detected in one of the patient's allele. We sequenced the −2200/−1934 *ABCA12* upstream region of five autosomal recessive congenital ichthyosis patients. These patients showed decreased *ABCA12* mRNA expression; however, known pathogenic mutations within *ABCA12* exons or exon-intron boundaries were found in only one allele of each patient. Although no mutations were detected in this limited number of patients (data not shown), the screening of future patients could benefit from sequencing of the genetic region.

This report describes the −2084/−2078 palindromic motif upstream of the *ABCA12* gene as a promoter element. However, it may be more precisely termed an enhancer element, because it is farther upstream of the core promoter that neighbors the TSS of *ABCA12*. Nevertheless, there is no widely accepted standard for differentiating between a promoter element and an enhancer element. Considering the comparative proximity of the motif (2.1 kb from TSS), we propose that it may be reasonably termed a promoter element.

It is of interest that the −2200/−1934 fragment exhibited potent promoter activity in the low-calcium condition as well as in the high-calcium condition. Since the −2980/+190 fragment shows a marked calcium-dependent upregulation of its promoter activity, it may be suggested that an unknown suppressor element(s) exists outside the −2200/−1934 region and functions specifically under low-calcium conditions. However, suppressor elements would not warrant the genetic sequencing of autosomal recessive congenital ichthyosis patients and is beyond the scope of the present study.

Methods

Ethics statement. This study was approved by the Medical Ethics Committee of Nagoya University (#1088), and performed according to the Declaration of Helsinki Principles. The participants gave written informed consent. Written informed consent was obtained from the guardians on behalf of the children enrolled. We recorded participant consent in paper. The ethic committee approved the consent procedure.

Construction of plasmids. To determine the promoter region of the *ABCA12* gene, a series of luciferase reporter plasmids were generated. PCR fragments containing upstream regions of the human *ABCA12* gene (NCBI Reference Sequence: NG_007074.1, http://www.ncbi.nlm.nih.gov/) of increasing lengths were amplified using specific primers (Table 1). The fragments were subcloned using the In-Fusion HD Cloning Kit (Clontech) into a pGL4.10 basic vector (Promega) that contains the *firefly* luciferase gene but does not contain any eukaryotic regulatory elements. All of the produced vectors were verified.

Cell culture and transfection. Normal human epidermal keratinocytes (NHEKs) were cultured in EpiLife (Gibco) supplemented with human keratinocyte growth supplement (HKGS; Gibco) at 37°C under 5% CO_2. For transfection of the plasmid vectors, the NHEKs were seeded on 24-well plates (1.0×10^5 cells/ml). At 50–70% confluence, the cells were washed once with PBS. Transfection was then performed by adding 0.5 μg of *firefly* luciferase reporter vector and 0.05 μg of pGL4.74 [*hRluc*/TK] *renilla* luciferase reporter vector (Promega) mixed with TransIT-Keratinocyte Transfection Reagent (Mirus) in EpiLife, and incubating for 20 min at 25°C. The transfected NHEKs were incubated for 6 h at 37°C with the mixture, and then the medium was changed to EpiLife/HKGS supplemented with either 0.06 mM or 1.2 mM calcium.

Dual-luciferase reporter assay. The NHEKs were incubated in either low (0.06 mM) or high (1.2 mM) calcium condition for 48 h after transfection. Then, the promoter activity was measured by Dual-Luciferase Reporter Assay System (Promega) using the GloMax-Multi Detection System (Promega) according to manufacturer's protocol. All experiments were performed in triplicate. The promoter activity was calculated as the ratio of *firefly* luciferase reporter expression to Renilla luciferase reporter expression that is driven by the *Herpes simplex* virus thymidine kinase promoter contained in the pGL4.74[*hRluc*/TK] vector.

In silico analysis of transcription factors. We performed *in silico* analysis to reveal potential transcription binding elements in the upstream region of the *ABCA12* gene using the JASPAR CORE database (http://jaspar.genereg.net/)[13]. The sequence was scanned with JASPER CORE vertebrata matrix models with a relative profile score threshold of 90%.

ENCODE and HAPMAP data set search and comparative multiple alignments. A series of database searches was performed using the UCSC Genome Browser database; ENCODE dataset for ChIP-seq signals and peaks of H3K4me3 and H3K27ac in NHEK, DNAseI-seq peaks in NHEKs, transcription factor ChIP-seq peaks of c-Fos/c-Jun in 91 human cell types, and SNPs from HAPMAP datasets[12,16,18]. All genomic annotations are mapped on genome assembly GRCh37/hg19. The multiple alignments of the conserved AP1 element among 55 vertebrae species were also obtained and the alignment and consensus logo was generated with CLC Main Workbench software (CLC Bio).

Table 1 | PCR Primers for cloning of the promoter region of *ABCA12*

Fragment	Forward primer	Reverse primer
−2980/+190	gctcgctagcctcgaacttgttgcacatttccacc	cgccgaggccagatctcttctttttctccactccac
−1994/+190	gctcgctagcctcgagtattctagcagatggcagg	cgccgaggccagatctcttctttttctccactccac
−1039/+190	gctcgctagcctcgaactagccttggctccagtag	cgccgaggccagatctcttctttttctccactccac
−2980/−2651	gctcgctagcctcgaacttgttgcacatttccacc	cgccgaggccagatctctcagcactggttg
−2700/−2351	gctcgctagcctcgacaaacaaacaaagtt	cgccgaggccagatcctaagagctggtctg
−2400/−2108	gctcgctagcctcgaggtccctaactctcc	cgccgaggccagatcttttccagcacttcc
−2200/−1934	gctcgctagcctcgactatctgtgcgaaca	cgccgaggccagatctgccagcttcatcag
−2980/+190	gctcgctagcctcgaacttgttgcacatttccacc	cgccgaggccagatctcttctttttctccactccac
−2700/+190	gctcgctagcctcgacaaacaaacaaagtt	cgccgaggccagatctcttctttttctccactccac
−2400/+190	gctcgctagcctcgaggtccctaactctcc	cgccgaggccagatctcttctttttctccactccac
−2200/+190	gctcgctagcctcgactatctgtgcgaaca	cgccgaggccagatctcttctttttctccactccac
mutant Δ1	acacttgatcagtcatttgagaatgagcacaat	atgactgatcaagtgtaacttcccctctgaagc
mutant Δ2	tacacttgtcagtcatttgagaatgagcacaat	aatgactgacaagtgtaacttcccctctgaagc
mutant Δ3	ttacacttgcagtcatttgagaatgagcacaat	aaatgactgcaagtgtaacttcccctctgaagc

Mutation analysis. To confirm the promoter activity of the −2084/−2078 motif (TGAGTCA), three different mutants were constructed by inverse PCR using the pGL4.10 −2980/+190 reporter as a template (Table 1). The three mutants lacked 1–3 bases from the wild-type sequence (wild-type AP1, tgagtca; mutant Δ1, tga-tca; mutant Δ2, tga--ca; mutant Δ3, tg---ca). Promoter activities of reporter vectors containing the mutants were analyzed and compared with that of the wild type.

Statistics. Statistical analyses were performed with the GraphPad Prism software version 5.04 (GraphPad Software). One-way analysis of variance (ANOVA) with Tukey post hoc test was used to determine statistical significance. A P value of <0.05 was considered significant.

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Author contributions

Y.S., T.Y., A.K., D.S., S.H. and M.A. designed the experiments. Y.S., K.S.-S. and T.Y. performed the experiments. Y.S., J.T. and K.S. performed genetic analyses. K.S. and M.A. provided clinical samples. Y.S., Y.O., K. Sakai, T.Y. and M.A. analyzed the data. Y.S., Y.O. and M.A. wrote the paper.

Additional information

Supplementary information accompanies this paper at http://www.nature.com/scientificreports

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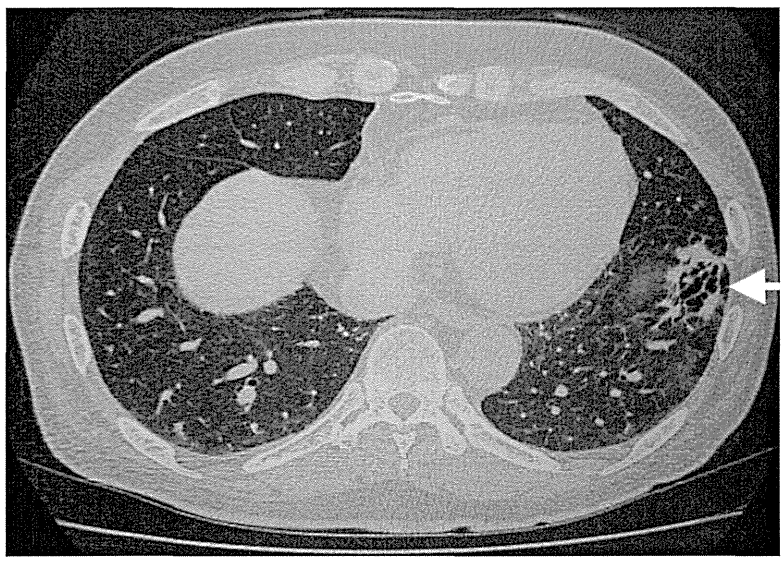
Clinical Image: Solitary organizing pneumonia mimicking lung adenocarcinoma in systemic sclerosis



The patient, a 57-year-old man, presented to our hospital with Raynaud's phenomenon. On examination, prominent bilateral skin sclerosis of the fingers, hands, and forearms was observed. Skin biopsy revealed swollen collagen bundles in the dermis, and autoantibody profiling revealed U1 RNP antibody positivity. He was diagnosed as having limited cutaneous systemic sclerosis (SSc). Radiography showed a nodule in the left lower lung. Computed tomography revealed a 39 × 27–mm nodule with multiple cystic structures (**arrow**) in the left lower lung and ground-glass opacities in both lower lungs. Percutaneous lung biopsy showed no evidence of carcinoma. However, open-chest partial excision was performed because lung adenocarcinoma with cystic structures was suspected clinically. The pathologic finding was fibrosis with inflammatory cell infiltration containing lymphocytes and plasma cells. Ziehl-Neelsen staining and Grocott staining were negative. Thus, solitary organizing pneumonia was diagnosed. Organizing pneumonia is a pathologic and clinical entity that is often cryptogenic or secondary to various diseases, including rheumatic conditions. Solitary organizing pneumonia frequently presents as an isolated focal lesion. Organizing pneumonia is considered a rare disease with an incidence of 1.96 cases/100,000, of which ~10–15% are solitary organizing pneumonia. Solitary organizing pneumonia is often misdiagnosed and removed surgically as it is rare and difficult to distinguish from lung carcinoma. The risk of cancer, particularly of the lung, liver, hematologic system, and bladder, is increased among patients with SSc, especially male patients. The present case suggests that solitary organizing pneumonia should be considered when an isolated nodular lung lesion is noted in a patient with SSc.

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CASE REPORT

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Progressive hyperpigmentation in a Taiwanese child due to an inborn error of vitamin B12 metabolism (cblJ)

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Summary

The physiology of human skin pigmentation is varied and complex, with an extensive melanogenic paracrine network involving mesenchymal and epithelial cells, contributing to the regulation of melanocyte survival and proliferation and melanogenesis. Mutations in several genes, involving predominantly the KIT ligand/c-Kit and Ras/mitogen-activated protein kinase signalling pathways, have been implicated in a spectrum of diseases in which there is hyperpigmentation, hypopigmentation or both. Here, we report on a 12-year-old girl from Taiwan with a 6-year history of diffuse progressive skin hyperpigmentation resulting from a different aetiology: an inborn metabolic disorder of vitamin B12 (cobalamin), designated cblJ. Using whole-exome sequencing we identified a homozygous mutation in *ABCD4* (c.423C>G; p.Asn141Lys), which encodes an ATP-binding cassette transporter with a role in the intracellular processing of cobalamin. The patient had biochemical and haematological evidence of cobalamin deficiency but no other clinical abnormalities apart from a slight lightening of her previously black hair. Of note, she had no neurological symptoms or signs. Treatment with oral cobalamin (3 mg daily) led to metabolic correction and some reduction in the skin hyperpigmentation at the 3-month follow-up. This case demonstrates that defects or deficiencies of cobalamin should be remembered in the differential diagnosis of diffuse hyperpigmentary skin disorders.

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Conflicts of interest
None declared.

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What's already known about this topic?

- Inherited defects affecting vitamin B12 (cobalamin, cbl) metabolism can result in various haematological and neurological abnormalities, as well as occasional changes in the skin.
- Nine different metabolic defects in the intracellular processing of cbl have been reported, which led to isolated methylmalonic aciduria and/or isolated homocystinuria.
- The most recently described disease subtype is cblJ, resulting from mutations in *ABCD4*; three individuals with *ABCD4* mutations have been described.

What does this study add?

- We identified a homozygous missense mutation in *ABCD4* in a 12-year-old girl with progressive hyperpigmentation.
- *ABCD4* mutations can lead to skin hyperpigmentation in the absence of any neurological abnormalities, thus *ABCD4* is a further candidate gene for familial progressive hyperpigmentation.
- Treatment with oral cbl can reduce the hyperpigmentation that results from *ABCD4* mutations over several months.

Progressive skin hyperpigmentation is a genetically heterogeneous disorder with two key signalling cascades, the KIT ligand/c-Kit and Ras/mitogen-activated protein kinase pathways, implicated in the pathophysiology of several clinical syndromes.[1] Hyperpigmentation can also result from vitamin B12 (cobalamin, cbl) deficiency, although the associated hyperpigmentation is not usually diffuse. Typically, it is more evident on the palms, soles and mucosae and sites of pressure, and there may be additional clinical abnormalities such as glossitis.[2–6] In this report, we describe a patient with diffuse progressive skin hyperpigmentation resulting from an autosomal recessive disorder of cbl metabolism, further expanding the differential diagnosis in such cases.

Case report

A 12-year-old girl, the only child of nonconsanguineous Han Chinese parents, presented with a 6-year history of asymptomatic progressive generalized skin hyperpigmentation. Examination revealed diffuse mottled hyperpigmentation affecting all of her skin, including sun-covered sites; her hair was dark brown rather than black (Fig. 1a,b). No mucosal or nail pigmentation was noted and her tongue was normal. Biopsy of hyperpigmented skin showed increased melanin within basal keratinocytes and in numerous melanophages in the papillary dermis (Fig. 1c,d). Transmission electron microscopy revealed melanophages around capillaries (Fig. 1e), and heavily melanized melanosomes packed in phagocytic vacuoles (Fig. 1f). She was otherwise in good health with no neurological or cardiovascular symptoms. Her parents and other family members reported no similar skin changes.

To identify the genetic basis of the hyperpigmentation, and following informed consent, whole-exome capture was performed (peripheral blood genomic DNA) by in-solution hybridization using the SureSelect All Exon 50 Mb Version 4.0 (Agilent, Santa Clara, CA, U.S.A.) followed by massively parallel sequencing (HiSeq2000; Illumina, San Diego, CA, U.S.A.) with 100-bp paired-end reads. Over 8·4 gigabases of mappable sequence data were generated, such that > 93% of the coding bases of the exome defined by the GENCODE Project (http://www.gencodegenes.org/) were represented by at least 20 reads.

In total 25 454 single-nucleotide substitutions were identified in the patient: 10 925 homozygous and 14 529 heterozygous. Within these variants, a nonsynonymous homozygous mutation was identified in *ABCD4* (c.423C>G; p.Asn141Lys). This mutation has been reported previously, in a 14-year-old Taiwanese boy with skin hyperpigmentation and neurological abnormalities.[7] *In silico* analysis with PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) predicts the mutation to be 'probably damaging', and the SIFT program (http://sift.jcvi.org/) predicts it to be 'damaging', thus functionally relevant. We further confirmed the mutation by Sanger sequencing and restriction endonuclease digestion (MwoI) (Fig. 2); both parents were shown to be heterozygous carriers. Next, we screened for this missense change in unrelated Taiwanese DNA samples (MwoI digest), but the mutant allele was not detected in any of 498 unrelated control chromosomes.

Biochemically, the patient was found to have elevated plasma homocysteine (52·0 μmol L^{-1}, reference 3·7–17·2 μmol L^{-1}) and low serum cbl (187·7 pg mL^{-1}, reference 250–900 pg mL^{-1}); a macrocytic anaemia (mean corpuscular volume of 102·6 fL) and haemoglobin of 11·5 g dL^{-1} were also noted. Tandem mass spectrometry analysis revealed a slightly decreased methionine level (17·5 μmol L^{-1}, reference 18–42 μmol L^{-1}), and urine organic acid analysis revealed the presence of extremely high levels of methylmalonic acid (Fig S1; see Supporting Information). The plasma homocysteine and serum cbl in parental blood samples were normal. The patient was treated with 3 mg of vitamin B12 orally per day, and biochemical correction of homocysteine and methylmalonic acid levels was noted: after 12 weeks of therapy her serum cbl was 344·6 mg mL^{-1}, with a haemoglobin of 14·0 g dL^{-1} and mean corpuscular volume of 93·3 fL. Over 3 months of follow-up there was a slight reduction in skin pigment levels. Of note, in the other reported Taiwanese case with this mutation in *ABCD4*, the skin hyperpigmentation resolved after 12 months of vitamin B12 therapy.

Discussion

ABCD4 [ATP-binding cassette, subfamily D (ALD), member 4] encodes a member of the superfamily of ATP-binding cassette (ABC) transporters that transport various molecules across extra- and intracellular membranes. It also has a putative role in peroxisomal import of fatty acids and/or fatty acyl-CoAs in the organelle, and in peroxisome biogenesis. Its relevance to

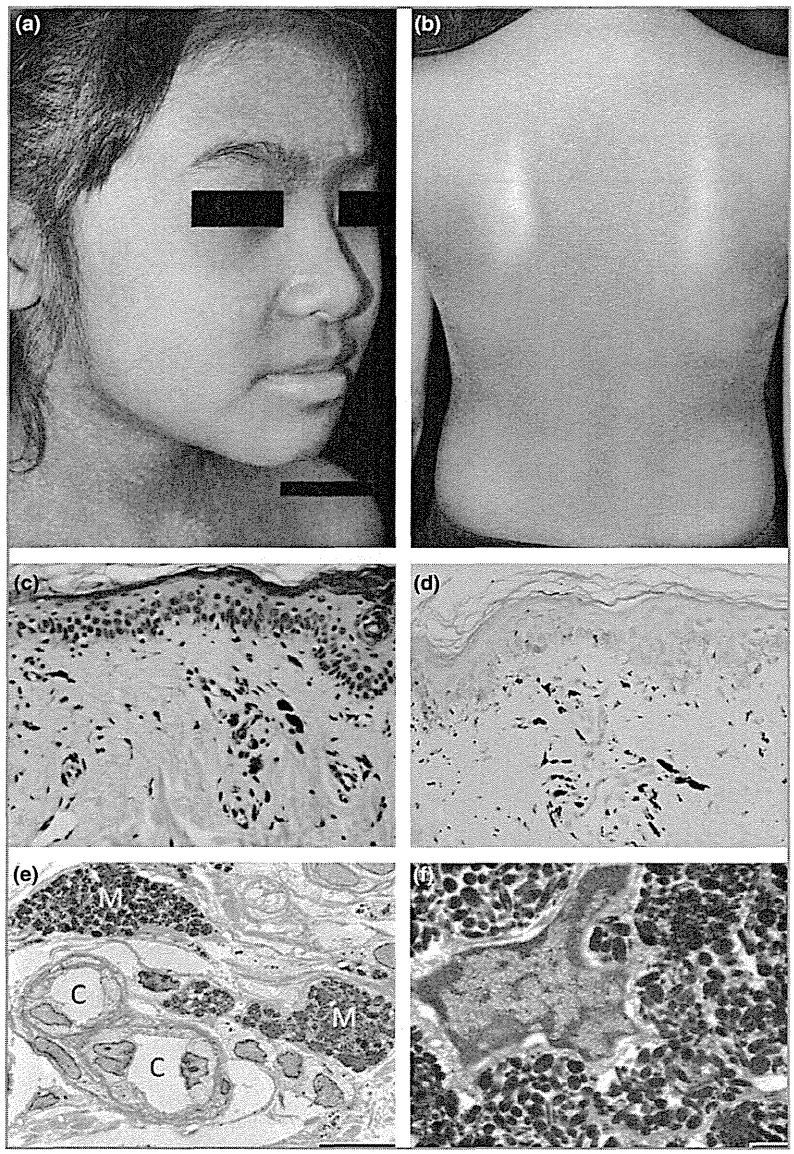

Fig 1. Clinicopathological studies of this 12-year-old Taiwanese girl. (a, b) Diffuse mottled hyperpigmentation on the face, neck and trunk. (c) Light microscopy reveals increased melanization in the lower epidermis and numerous melanophages in the papillary dermis. (d) Fontana–Masson staining highlights the presence of increased melanin. (e) Ultrastructural images showing melanophages near capillary vessels. C, capillary; M, melanophages. Magnification ×2500. (f) High magnification revealing heavily melanized melanosomes packed in phagocyte vacuoles (×30 000).

human health/disease was realized only in 2012 with the discovery of human mutations in *ABCD4* through whole-exome sequencing.[8] ABCD4 has been shown to colocalize with lysosomal proteins, contributing ATP-ase activity for the intracellular processing of cbl, and thus permitting its release from lysosomes into the cytoplasm.[1] Cbl is then converted into two active cofactors: methylcobalamin (needed for methylation of homocysteine to methionine) and adenosylcobalamin (which helps process methylmalonyl-CoA to succinyl-CoA). Biochemically, inborn errors of cbl therefore lead to elevated blood and urine levels of homocysteine and/or methylmalonic acid.[8,9] Mutations in *ABCD4* underlie the disease subtype cblJ (MIM 614857).[7]

The first two reported cases with mutations in *ABCD4* presented with feeding difficulties, hypotonia, developmental delay, bone marrow suppression and cardiovascular defects in early life.[8] Skin hyperpigmentation was not evident in these children. The main phenotypic abnormalities in the one further case reported (from Taiwan) were neurological (dizziness, headache and transient ischaemic attacks), although some skin hyperpigmentation and greying of the hair were noted as minor additional clinical features.[7] In contrast, skin hyperpigmentation was the predominant sign in our case.

With regard to genotype–phenotype correlation, the recurrent missense mutation in the Taiwanese cases may be less disruptive to the ABCD4 protein than the splice site and

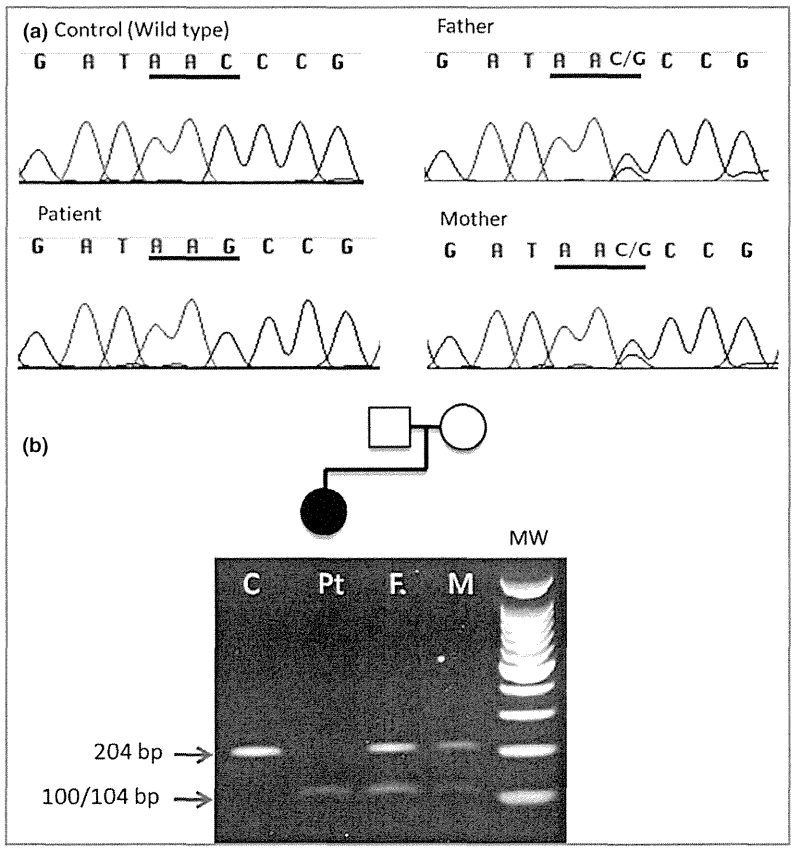

Fig 2. Molecular pathology of this case. (a) The proband has a homozygous single-nucleotide substitution, c.423C>G, in *ABCD4*, which leads to the amino acid change p.Asn141Lys; both parents are heterozygous carriers of this missense mutation. (b) Verification of the mutation by MwoI restriction enzyme digestion. The mutation c.423C>G creates a new (solitary) cut size such that the 204-bp band is cleaved into 104-bp and 100-bp fragments (which cannot be separated on this gel). For the patient (Pt) only the lower cleaved band is present, whereas in the father (F) and mother (M) two discrete bands are seen, consistent with heterozygosity for this mutation. For the control DNA (C) only the single undigested upper band is visible. MW, molecular weight ladder.

frameshift mutant alleles in more severely affected individuals, although clearly there is phenotypic disparity (so far unexplained) between the two Taiwanese subjects both homozygous for p.Asn141Lys. However, it is possible that early diagnosis and treatment in our case has pre-empted and prevented the other potential disease manifestations and complications. This amino acid substitution appears to be exclusive to Taiwanese subjects, as it has not been observed or reported elsewhere (or detected in > 1500 in-house exome datasets), although we failed to detect its presence in 498 control Taiwanese chromosomes. None of the parents of the Taiwanese cases was known to be related to one another, and all came from different parts of Taiwan; therefore c.423C>G in *ABCD4* may represent a rare mutant allele in this population.

Skin pigmentation is dependent on the type and amount of melanin present; this is regulated by tyrosinase and tyrosinase-related enzyme activity and by other proteins that influence the size, number and distribution of melanosomes within keratinocytes.[1] For our case, the main differential diagnosis was familial progressive hyperpigmentation (MIM 614233; 145250), characterized by progressive hyperpigmentation, but without hypopigmentation. The latter is a feature of other related disorders such as familial progressive hyperpigmentation and hypopigmentation, and dyschromatosis universalis hereditaria type 2 (MIM 612715). Familial progressive hyperpigmentation appears to be genetically heterogeneous, although a mutation has previously been reported in *KITLG*.[10] In our case, the mechanism underlying how mutations in *ABCD4* led to skin hyperpigmentation is unknown. Possible aetiologies include cbl deficiency leading to lower levels of reduced-type glutathione (which has a tyrosinase-inhibiting effect), or disruption of lysosomes in melanophages, although both hypotheses are highly speculative.

In summary, our case highlights the protean nature of progressive hyperpigmentation, and emphasizes that disorders of vitamin B12 metabolism should also be remembered in the differential diagnosis. Early recognition of this clinicopathological disorder is important, as oral treatment with vitamin B12 may not only improve the appearance of the skin, but also prevent the major neurological and cardiovas-

cular complications associated with inborn errors of cbl metabolism.

Acknowledgments

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Fig S1. Raw laboratory data showing extremely high levels of methylmalonic acid. This is not given any precise quantification, but the peak extends to the top of the screen and is reported as 'extremely high'.

CASE REPORT

BJD
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Whole-exome sequencing diagnosis of two autosomal recessive disorders in one family

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Summary

Autosomal recessive congenital ichthyosis (ARCI) is a genetically heterogeneous disorder for which subtyping through molecular analysis can help determine the eventual phenotype and prognosis. We used whole-exome sequencing to identify a new homozygous splice-site mutation in *ST14* (IVS5+1G>A), encoding matriptase, in a 4-year-old girl with ARCI from a consanguineous Kuwaiti family. Clinically, she also had hypotrichosis, which supported a diagnosis of ARCI type 11. Only four previous examples of pathogenic mutations in *ST14* have been reported, and our findings expand the genotype–phenotype correlation for this subtype of ARCI. Our patient was the second child born to these parents; the first (deceased) and third children had congenital brain and eye abnormalities, of uncertain aetiology and with no precise diagnosis. Further analysis of our patient's exome dataset revealed heterozygosity for a splice-site mutation in *POMT1* (IVS4+1G>T), encoding the protein O-mannosyltransferase, a gene implicated in Walker–Warburg syndrome. DNA sequencing in the third child showed homozygosity for this mutation in *POMT1*. The first-cousin parents were both heterozygous for the splice-site mutations in *ST14* and *POMT1*. In this family, whole-exome sequencing provided accurate subtyping of a form of ARCI in one child and provide an explanation for an undiagnosed developmental disorder in two other children, findings that improve the prospects for diagnostic accuracy and genetic counselling, and demonstrate the impact of next-generation sequencing technologies on clinical genetics.

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Conflicts of interest
None declared.

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What's already known about this topic?

- Mutations in the suppression of tumorigenicity 14 gene (*ST14*), encoding matriptase, underlie autosomal recessive congenital ichthyosis type 11 (ARCI11). To date, only four pathogenic mutations in *ST14* have been reported.
- Walker–Warburg syndrome (WWS) is a rare autosomal recessive disorder with developmental malformations, resulting from mutations in *POMT1*, which encodes O-mannosyltransferase.
- Next-generation sequencing has the potential to reveal both anticipated and incidental mutations.

What does this study add?

- Using whole-exome sequencing, we identified a new homozygous splice-site mutation in *ST14* in a 4-year-old Bedouin girl with ARCI11.
- Bioinformatics analysis of the exome dataset revealed that this girl was also heterozygous for a mutation in *POMT1*, providing insight into WWS affecting two other siblings in this family.
- Whole-exome sequencing can provide both molecular diagnostics for known disorders, and data that can improve clinical diagnosis and genetic counselling.

Autosomal recessive congenital ichthyosis (ARCI) is an umbrella term used to describe a generic phenotype of red, scaly skin that presents at birth.[1] ARCI is genetically heterogeneous and its different subtypes have variable disease associations, complications and prognoses. One particular subtype of ARCI is ARCI11 (MIM #602400), also known as autosomal recessive ichthyosis with hypotrichosis, and follicular atrophoderma with hypotrichosis and hypohidrosis. ARCI11 is characterized by congenital ichthyosis and generalized hypotrichosis with curly, sparse hair with or without follicular atrophoderma.[2]

The molecular pathology of ARCI11 involves loss-of-function mutations in *ST14* (suppression of tumorigenicity 14), which encodes matriptase, a type II transmembrane serine protease of the S1 trypsin-like family.[3,4] Matriptase has an important role in keratinocyte proliferation and early differentiation of keratinocytes, and activates epidermal kallikreins. Thus far, four pathogenic homozygous mutations in *ST14* (two missense, one single-nucleotide deletion, one splice site) have been identified in ARCI11.[4–6] In this study, we used whole-exome sequencing (WES) to identify a new homozygous donor splice-site mutation in *ST14*, in a child with ARCI11 from a parent-related Kuwaiti family. We thus expand the molecular basis of this genodermatosis. In addition, we used the exome dataset to identify a second, previously undiagnosed autosomal recessive disorder in this family, a finding that demonstrates the value of next-generation sequencing in improving molecular diagnosis for rare monogenic diseases and providing better genetic counselling for families.

Case report

The patient is a 4-year-old Bedouin girl, the second of three siblings born to first-degree cousin parents. She was delivered at 36 weeks by normal spontaneous vaginal delivery with a birth weight of 2365 g. At birth, she was noticed to have generalized erythema, skin scaling and mild palmoplantar keratoderma. Scaling and redness gradually lessened over subsequent months, and currently there is only mild erythema and localized scaling on the back (Fig. 1a). From birth, she was also noted to have a mild diffuse hypotrichosis with hair that grows slowly, as well as a receding frontal hair line (Fig. 1b), partial loss of eyebrows and curled eyelashes.

On examination, she had frontal bossing, mild hypertelorism, a flat nasal bridge and widely spaced slanting eyes. Her hair was diffusely thin and light brown in colour (black in other family members), and light microscopy of cut and plucked hair did not reveal any gross aberrations. In addition, there were no nail or dental abnormalities, no mucosal changes and no visual or hearing difficulties. Her height was at the 5th percentile, her weight was below the 3rd percentile, and her head circumference was at the 25th percentile. Her mental development was grossly normal. There was no family history of any skin disorder.

Following informed consent, and in accordance with the Declaration of Helsinki principles, genomic DNA from the patient was used for WES analysis, using methodology described elsewhere.[7] The reason for using WES rather than Sanger sequencing of candidate genes was a lack of full clinical information at the time of the initial investigation, as well as our own recent positive experience in using WES to diagnose genetically heterogeneous inherited skin diseases.[8]

The resulting variant calls were filtered with the BCFtools utility (http://samtools.github.io/bcftools/), filtered for a minimum coverage (calls with fewer than four reads filtered) and hard filtered for quality (variants with quality < 20 filtered from further analysis). This high-quality call set was then annotated with respect to the genes, and for consequences on protein sequence and/or splicing with the ANNOVAR tool (http://www.openbioinformatics.org/annovar/). Further annotation regarding previously reported observation of specific variants and estimated population frequencies was achieved through further rounds of ANNOVAR annotation against dbSNP135 (http://www.ncbi.nlm.nih.gov/snp), population frequency estimates from the 1000 Genomes project (http://www.1000genomes.org/) and the National Institutes of Health Heart, Lung and Blood Institute Grand Opportunity Exome Sequencing Project (https://esp.gs.washington.edu/drupal/), and ~1500 control exomes that have been processed through the same bioinformatics analysis pipeline.[7]

In total, 375 novel mutations were identified by WES, 31 homozygous and 344 heterozygous. Within these variants, there was a previously unreported homozygous splice-site mutation in *ST14*, IVS5+1G>A, which was then confirmed by Sanger sequencing (Fig. 1c); both parents and one unaf-

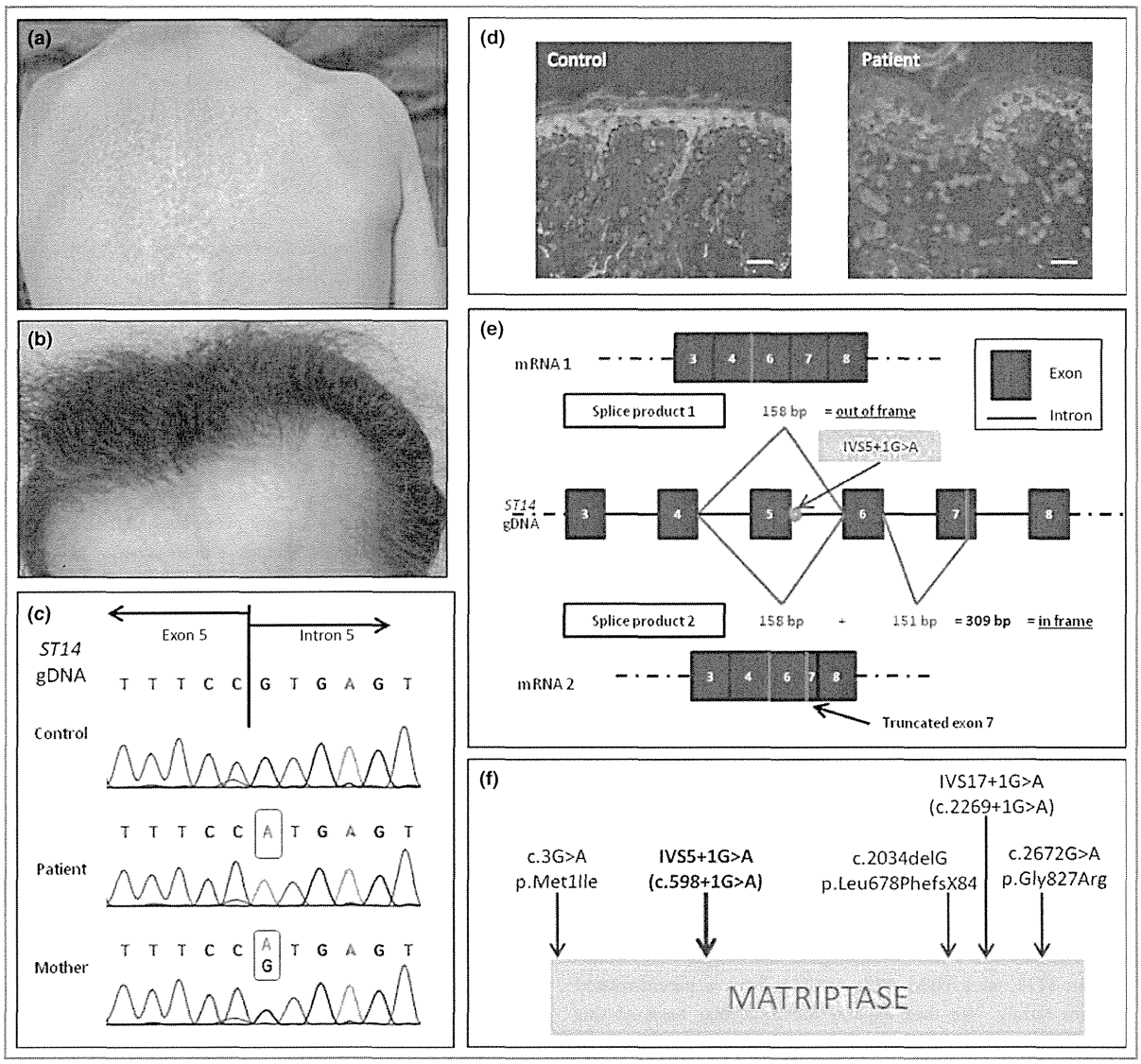

Fig 1. Clinicopathological and molecular characterization of our patient with autosomal recessive congenital ichthyosis type 11. (a) The patient has tan-coloured diffuse scaling on the upper back. (b) The hair shows evident frontal thinning and is curly and light brown. (c) Sanger sequencing confirms a homozygous mutation, IVS5+1G>A, in *ST14* in the patient. Both parents were shown to be heterozygous carriers (here illustrated for the mother). (d) Immunolabelling shows slightly reduced expression of matriptase in the affected individual compared with normal control skin (bar = 50 μm). (e) Schematic representation of transcripts identified as a result of the aberrant splicing: skipping of exon 5 is out of frame, but skipping of exon 5 with an additional partial deletion of exon 7 is in frame. (f) Schematic representation of the mutations reported thus far in *ST14*, with the new mutation from our case shown in bold.

fected younger sister were shown to be heterozygous carriers. At the protein level, there was reduced (but not absent) expression of immunoreactivity for ST14 (anti-ST14 antibody, ab28266; Abcam, Cambridge, U.K.) in the affected individual's skin compared with control skin (Fig. 1d). We then examined *ST14* mRNA expression by quantitative reverse-transcriptase polymerase chain reaction (RT-PCR) and found this to be significantly decreased (~25% expression vs. normal controls), consistent with RNA decay (data not shown).

Next, we examined whether this splice-site mutation leads to aberrant or normal splicing by RT-PCR using RNA extracted from the patient's skin. Sequencing of cDNA from exon 2 to exon 7 of *ST14* demonstrated skipping of exon 5 (Fig. S1; see Supporting Information). Skipping of exon 5 (158 base pairs) causes a frame shift, generating a premature stop codon 36 codons downstream (p.Ser147Serfs*36). By contrast, sequencing of cDNA from exon 3 to exon 8 of *ST14* demonstrated skipping of exon 5, as well as a partial skipping of exon 7 (Fig. S2; see Supporting Information). The partial loss of exon

7 (151 base pairs), in combination with the skipping of exon 5, results in a 309-base pair deletion, which restores the reading frame. These splice transcripts are illustrated in Figure 1e. Collectively, these data identify a new pathogenic mutation in *ST14* underlying ARCI11, with functional loss at the RNA and protein levels. The mutation in our case and the previously reported mutations in *ST14* are depicted in Figure 1f.

With respect to genotype–phenotype correlation, the degrees of ichthyosis and hypotrichosis in our case are somewhat milder than those described in most other published reports, although considerable intra- and interfamilial variation in phenotypic severity may be found in ARCI11; for example, affected boys tend to have greater hair loss than affected girls. The milder phenotype in our case may reflect the consequences of the aberrant in-frame splicing transcript we detected, which resulted in some residual mRNA and protein expression. In contrast, the reported mutation c.2043delG in a clinically more severe Turkish pedigree led to a complete absence of ST14 protein expression on Western blotting.[6] Full comparative details relating to genotype–phenotype correlation for cases of ARCI11 are presented in Table S1 (see Supporting Information).

However, in addition to this child with ichthyosis, the first and third children born to these parents had multiple congenital abnormalities for which no precise diagnosis had been made. The first child was delivered at term by pump-assisted delivery with a birth weight of 2565 g. She had a large occipital encephalocele with protrusion of brain and evidence of holoprosencephaly and mild cleft palate. She died soon after birth. The third child (currently 4 weeks old) was born with a posterior occipital encephalocele and abnormal eye globes. To try to establish a diagnosis in these two children, we further examined the exome dataset from the child with ARCI11. The hypothesis was that the child with ARCI11, who had no brain or eye abnormalities, might be a heterozygous carrier of whatever mutant allele was underlying her siblings' brain and eye malformations.

Examining the filtered rare variant list generated by WES, we identified a heterozygous donor splice-site mutation (IVS4 +1G>T) in *POMT1*, which encodes O-mannosyltransferase, an enzyme present in endoplasmic reticulum that catalyses O-mannosylation of proteins, an important protein modification required for cell integrity and cell-wall rigidity. Mutations in *POMT1* have been reported in Walker–Warburg syndrome (WWS, MIM #236670), also called muscle–eye–brain disease, a rare autosomal recessive disorder associated with developmental malformations of the brain, skeletal muscle and eyes, as well as other abnormalities. Indeed, the mutation IVS4+1G>T has been reported previously as a pathogenic variant in individuals from Saudi Arabia and Spain.[9,10] Sanger sequencing revealed that the individual with ARCI11 and both parents were heterozygous carriers of this mutation in *POMT1*, and that the youngest child was homozygous for this mutation (Fig. 2a). DNA from the first child was not available for sequencing. Thus the likely diagnosis for the congenital developmental abnormalities in the first and third children in this family is WWS.

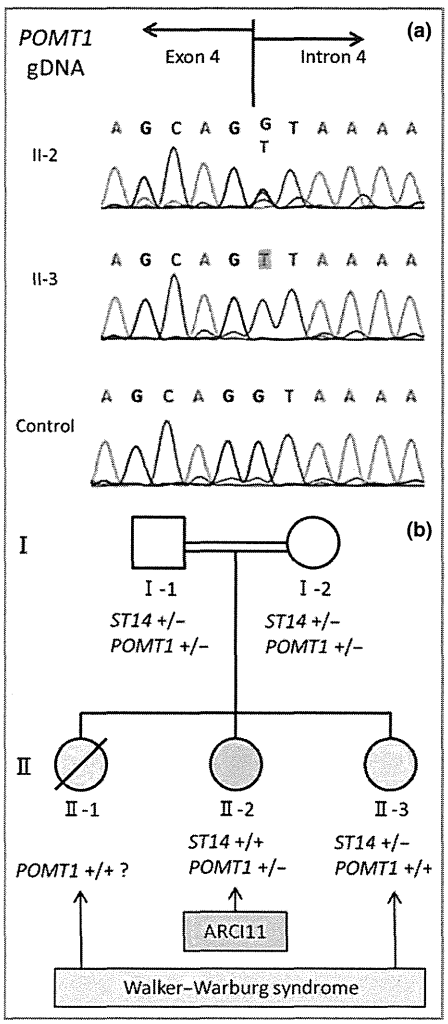

Fig 2. Sanger sequencing of *POMT1* and the family tree with two autosomal recessive disorders. (a) The individual with autosomal recessive congenital ichthyosis type 11 (ARCI11) (II-2) is also heterozygous for a donor splice-site mutation in *POMT1*, IVS4+1G>T. The younger sibling, II-3, is homozygous for this mutation, giving a presumptive diagnosis of Walker–Warburg syndrome (WWS). (b) Pedigree of the family showing genotypes for the mutations in *ST14* and *POMT1* and phenotypes (ARCI11 in green; WWS in yellow). For individual II-1, no DNA was available (deceased child) and therefore the genotype and phenotype are speculative.

Discussion

Rare, atypical and undiagnosed autosomal recessive disorders frequently occur in the offspring of consanguineous couples, and current standard diagnostic genetic tests often fail to establish a diagnosis in many cases. Recent studies in consanguineous families have shown that WES is both a powerful diagnostic technique and a valuable tool in establishing more detailed causative links between mutant genotypes and clinical phenotypes.[11] The findings in our family, with pathogenic mutations in *ST14* and *POMT1* (Fig. 2b), support the clinical

value of WES for both of these outcomes in accurately diagnosing two autosomal recessive diseases and thereby improving genetic counselling.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Fig S1. cDNA sequencing. Reverse-transcriptase polymerase chain reaction across the site of the splice-site mutation using RNA extracted from patient skin reveals skipping of exon 5, which is out of frame (see Fig. 1e for interpretation).

Fig S2. cDNA sequencing. Reverse-transcriptase polymerase chain reaction across the site of the splice-site mutation using RNA extracted from patient skin also reveals skipping of exon 5 along with a partial deletion of exon 7, which is in frame (see Fig. 1e for interpretation).

Table S1. Genotype–phenotype correlation. This table compares the clinical findings and ST14 mutations in all reported cases of autosomal recessive congenital ichthyosis type 11 published to date, including our patient.

CASE REPORT

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Founder mutation in dystonin-e underlying autosomal recessive epidermolysis bullosa simplex in Kuwait

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Summary

Only two homozygous nonsense mutations in the epidermal isoform of the dystonin gene, *DST-e*, have been reported previously in autosomal recessive epidermolysis bullosa simplex (EBS); the affected pedigrees were Kuwaiti and Iranian. This subtype of EBS is therefore considered to be a rare clinicopathological entity. In this study, we identified four seemingly unrelated Kuwaiti families in which a total of seven individuals had predominantly acral trauma-induced blistering since infancy. All affected individuals were homozygous for the mutation p.Gln1124* in *DST-e*, the same mutation that was identified in the originally reported family from Kuwait. Haplotype analysis in the five pedigrees (including the previous case) revealed a shared block of ~60 kb of genomic DNA across the site of the mutation, consistent with a founder effect. Most heterozygotes had no clinical abnormalities although one subject had mild transient skin fragility during childhood, an observation noted in the previously reported Iranian pedigree, suggesting that the condition may also be semidominant in some pedigrees rather than purely autosomal recessive. Our study reveals propagation of a mutant ancestral allele in *DST-e* throughout Kuwait, indicating that this subtype of EBS may be more common in Kuwait, and perhaps other Middle Eastern countries, than is currently appreciated.

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Conflicts of interest
None declared.

DOI 10.1111/bjd.13294

What's already known about this topic?

- The epidermal isoform of dystonin (*DST-e*), which encodes the 230-kDa bullous pemphigoid antigen (BP230), is a candidate gene for autosomal recessive epidermolysis bullosa simplex (EBS).
- Only two pedigrees with pathogenic mutations in *DST-e* have been reported, from Kuwait and Iran.
- The identified homozygous nonsense mutations led to a lack of BP230 protein at the dermoepidermal junction, poorly formed hemidesmosomal inner plaques and basal keratinocyte fragility.

What does this study add?

- We identified four new pedigrees from Kuwait, where seven individuals had recessive EBS due to the same homozygous nonsense mutation, c.3370C>T (p.Gln1124*), in *DST-e*.