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. 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 corneum1. 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 corneum2–4. In normal skin and in cultured human keratinocytes, the expression of ABCA12 parallels the differentiation of the keratinocytes3. 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.

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 diagnosis8. 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 intact9. In such cases, verification of decreased ABCA12 mRNA expression in keratinocytes taken from the patient’s skin or the hair follicles confirms the diagnosis. 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 recessive 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 the skin 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 in vivo.

Accordingly, NHEKs differentiate under the high-calcium condition, and ABCA12 expression increases in differentiated keratinocytes. 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 throughout this region and found increased ChIPseq signals and peaks of H3K4me3 and H3K27ac, and increased DNase1-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/) 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.

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. S2). 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 (tgagtczagatc, −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; these AP1-regulated genes are involved in keratinocyte differentiation and are mostly expressed in the late stage of terminal differentiation. 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 DNase1-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 ABCA12 promoter. 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 (tgagtczagatc, −2084 to −2078, chr2:216,005,235–216,005,228) within the −2200/−1934 fragment (Fig. 2, Supplementary Fig. S3).

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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 (tgagtczagatc): mutant Δ1 (tga-tca), mutant Δ2 (tgaca) and mutant Δ3 (tgaca). 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
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 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.

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. 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 DNAse-I-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.
known pathogenic mutations within the sequencing of the screening for the diagnosis of congenital ichthyosis patients include SCIENTIFIC

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 (tggatca): 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 includes the sequencing of the genetic region. In our study, we localized mutations in ABCA12 in 11 of 29 patients (38%). For currently affected patients, no mutations were detected in this limited number of patients (data not shown), the screening of future patients could benefit from sequencing of the 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% CO2. For transfection of the plasmid vectors, the NHEKs were seeded on 24-well plates (1.0 × 104 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 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 JASPAR 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 H3K2me3 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 datasets21,24. All genomic annotations are mapped on genome assembly GRCh37/hg19. The multiple alignments of the conserved API1 element among 55 vertebrate 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 | gctgcagctcctgaacatggcacoattccaccc | cggccggagcatccttttttttccacccccc |
| −1994/+190 | gctgcagctcctgaacatggcacoattccaccc | cggccggagcatccttttttttccacccccc |
| −1039/+190 | gctgcagctcctgaacatggcacoattccaccc | cggccggagcatccttttttttccacccccc |
| −2980/−2651 | gctgcagctcctgaacatggcacoattccaccc | cggccggagcatccttttttttccacccccc |
| −2700/−2351 | gctgcagctcctgaacatggcacoattccaccc | cggccggagcatccttttttttccacccccc |
| −2400/−2108 | gctgcagctcctgaacatggcacoattccaccc | cggccggagcatccttttttttccacccccc |
| −2200/−1934 | gctgcagctcctgaacatggcacoattccaccc | cggccggagcatccttttttttccacccccc |
| −2980/−190 | gctgcagctcctgaacatggcacoattccaccc | cggccggagcatccttttttttccacccccc |
| −2700/−190 | gctgcagctcctgaacatggcacoattccaccc | cggccggagcatccttttttttccacccccc |
| −2400/−190 | gctgcagctcctgaacatggcacoattccaccc | cggccggagcatccttttttttccacccccc |
| −2200/−190 | gctgcagctcctgaacatggcacoattccaccc | cggccggagcatccttttttttccacccccc |
| mutant Δ1 | tacactctgacatcattggaagtacgacacat | aatacgacagaacaattgtaacctcccccttcgaagcc |
| mutant A2 | tacactctgacatcattggaagtacgacacat | aatacgacagaacaattgtaacctcccccttcgaagcc |
| mutant Δ3 | tacactctgacatcattggaagtacgacacat | aatacgacagaacaattgtaacctcccccttcgaagcc |
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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. wrote the paper.

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