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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 (tgagtc) 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 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 corneum^{2–4}. 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}. 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 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 intact⁷. In such cases, verification of decreased *ABCA12* mRNA expression in keratinocytes taken from the patient's skin or the hair follicles confirms the diagnosis⁹. 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 keratinocytes¹². 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 browser¹² 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

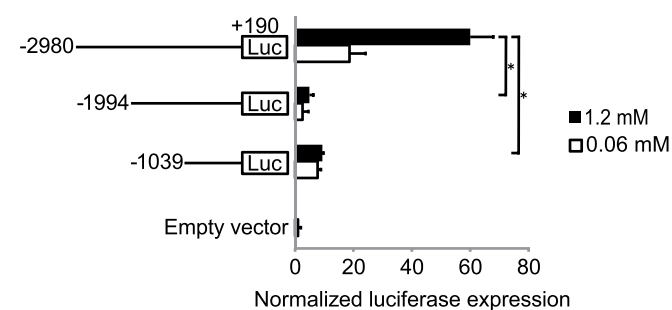


Figure 1 | Position -2980 to position -1994 of the *ABCA12* gene has promoter activity. Dual luciferase assay was performed using firefly luciferase reporter vectors that contain $-2980/+190$, $-1994/+190$ or $-1039/+190$ fragments of the *ABCA12* gene. The culture medium contained either 0.06 mM or 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.

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. 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 1¹⁴; these AP1-regulated genes are involved in keratinocyte differentiation and are mostly expressed in the late stage of terminal differentiation¹⁵. 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 $\Delta 1$ (tga-tca), mutant $\Delta 2$ (tga--ca) and mutant $\Delta 3$ (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 $\Delta 1$, $\Delta 2$ and $\Delta 3$, 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

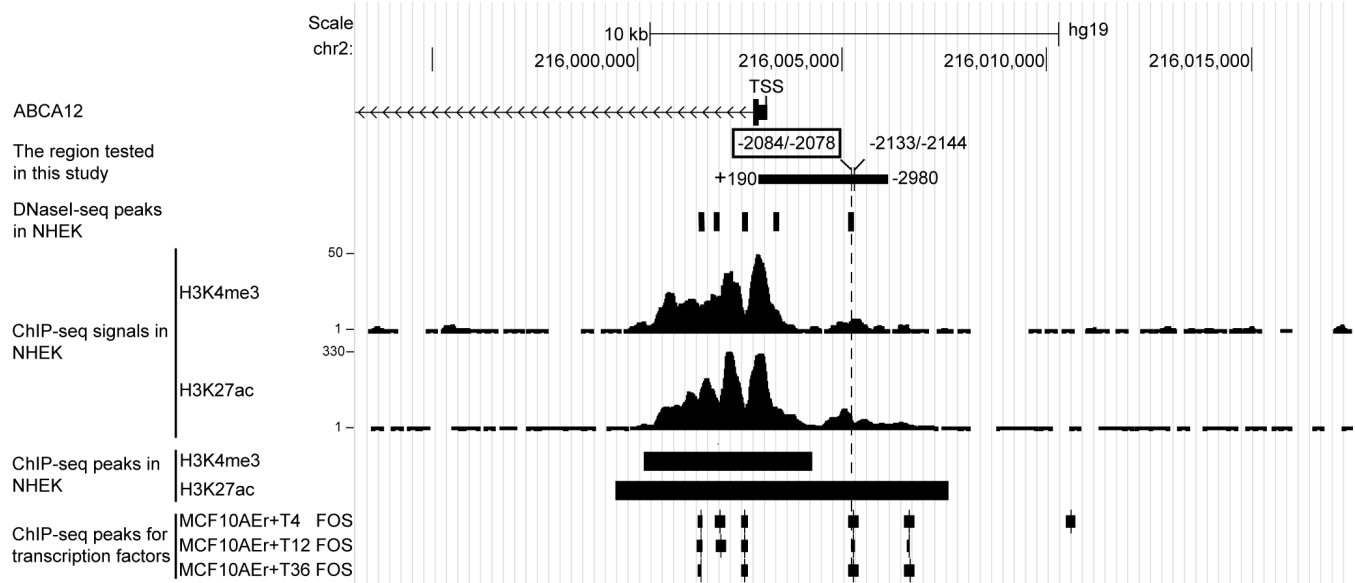


Figure 2 | Annotation of the *ABCA12* upstream gene using the ENCODE dataset. The *ABCA12* upstream genomic region (chr2:215,993,140–216,017,439:GRCh37/hg19) is illustrated using the UCSC genome browser. ChIP-seq signals and peaks of H3K4me3 and H3K27ac, and DNaseI-seq peaks in NHEK were extracted from 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 database¹⁶ 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 loricrin¹⁷. 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.

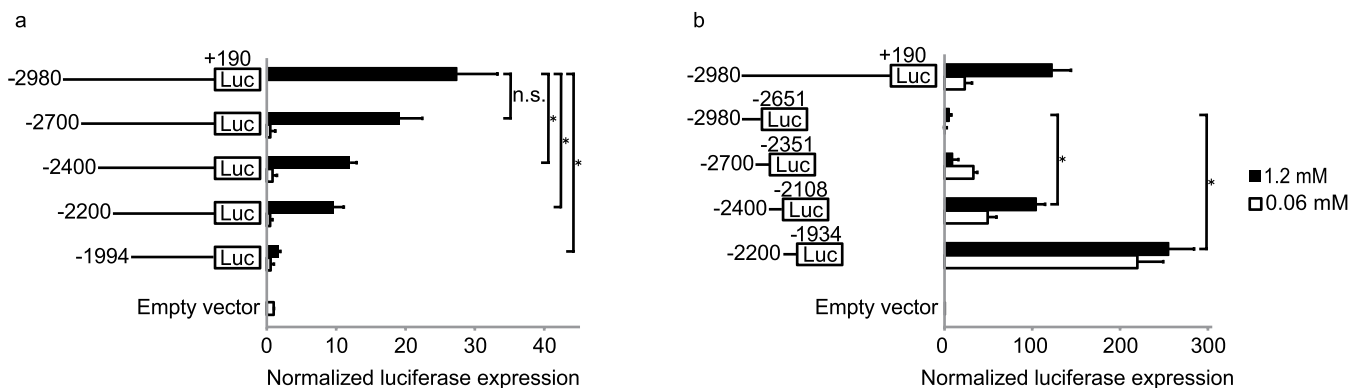


Figure 3 | Position -2200 to position -1934 of the *ABCA12* gene exhibits potent promoter activity. Promoter activities of $-2980/+190$, $-2700/+190$, $-2400/+190$, $-2200/+190$, or $-1994/+190$ fragments (a), and $-2980/-2651$, $-2700/-2351$, $-2400/-2108$, or $-2200/-1934$ fragments (b) were assayed.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

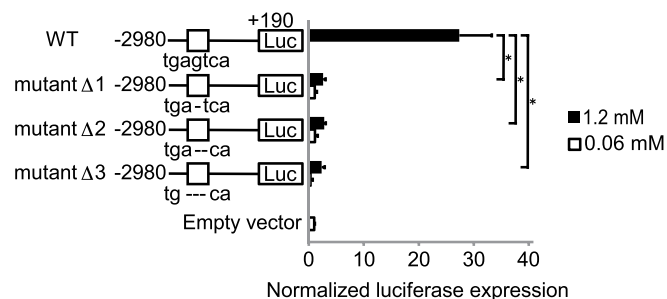


Figure 4 | Mutants of the $-2084/-2078$ motif in *ABCA12* show striking reduction of promoter activity. Three different mutants of the $-2084/-2078$ motif were generated using the $-2980/+190$ fragment as a template, and the promoter activity of each fragment was assessed. The mutants lacked 1–3 bases from the wild-type sequence (tgagtca): mutant $\Delta 1$ (tga-tca), mutant $\Delta 2$ (tga--ca) and mutant $\Delta 3$ (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 JASPAR CORE vertebrate 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 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
$-2980/+190$	gctcgtagcctcgactgtgacatctccacc	cgccgaggccagatctctctctccactccac
$-1994/+190$	gctcgtagcctcgactgtgacatgagcagg	cgccgaggccagatctctctctccactccac
$-1039/+190$	gctcgtagcctcgactgactgctcctcagtag	cgccgaggccagatctctctctccactccac
$-2980/-2651$	gctcgtagcctcgactgtgacatctccacc	cgccgaggccagatctctcagactggttg
$-2700/-2351$	gctcgtagcctcgacaacaacaagaagt	cgccgaggccagatctctcagactggttg
$-2400/-2108$	gctcgtagcctcgaggctcctaactctcc	cgccgaggccagatctctcagactggttg
$-2200/-1934$	gctcgtagcctcgactatctgtgcaaca	cgccgaggccagatctctcagactggttg
$-2980/+190$	gctcgtagcctcgactgtgacatctccacc	cgccgaggccagatctctctctccactccac
$-2700/+190$	gctcgtagcctcgacaacaacaagaagt	cgccgaggccagatctctctctccactccac
$-2400/+190$	gctcgtagcctcgaggctcctaactctcc	cgccgaggccagatctctctctccactccac
$-2200/+190$	gctcgtagcctcgactatctgtgcaaca	cgccgaggccagatctctctctccactccac
mutant $\Delta 1$	acactgatcagctcattgagaatgagcacaat	aatgactgacaagtgttaacttcccctgaagc
mutant $\Delta 2$	tacactgtcagctcattgagaatgagcacaat	aatgactgacaagtgttaacttcccctgaagc
mutant $\Delta 3$	ttacactgtcagctcattgagaatgagcacaat	aatgactgacaagtgttaacttcccctgaagc



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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