Table 4. Immunohistochemical stainings of infiltrating cells in thin melanomas.

patient No	CD3	CD4	CD5	CD8	CD20	CD56	TIA-1	Perforin	FOXP3
36	2+	−	2+	2+	−	−	+	−	−
37	2+	−	2+	2+	+	−	2+	−	+
38	2+	+	2+	2+	−	−	+	−	−
39	2+	+	2+	2+	−	−	+	−	−
40	2+	−	2+	2+	−	−	+	−	−
41	2+	−	2+	2+	+	−	2+	−	−
42	2+	+	2+	2+	+	−	+	−	−
43	2+	+	2+	2+	−	−	+	−	−
44	2+	−	2+	+	−	−	−	−	−
45	2+	−	2+	2+	+	−	2+	−	−
46	2+	−	+	2+	−	−	+	−	−
47	2+	−	2+	2+	−	−	2+	−	−
48	2+	−	2+	2+	−	−	+	−	+
49	2+	−	2+	2+	+	−	2+	−	−
50	2+	−	2+	2+	−	−	+	−	−
51	2+	−	2+	2+	+	−	2+	−	−

2+, the number of stained cells was more than 60%; +, from 30% to 60%; −, less than 30%.

Table 5. Positive-staining rates of various markers in lymphocytes infiltrated in primary tumors.

	CD3	CD4	CD5	CD8	CD20	CD56	TIA-1	Perforin	FOXP3
RLM (+) case (n=1) (%)	100	0	100	100	0	0	100	0	0
RLM (-) cases (n=15) (%)	100	27	100	100	40	0	93	0	13

RLM, regional lymph node metastasis.

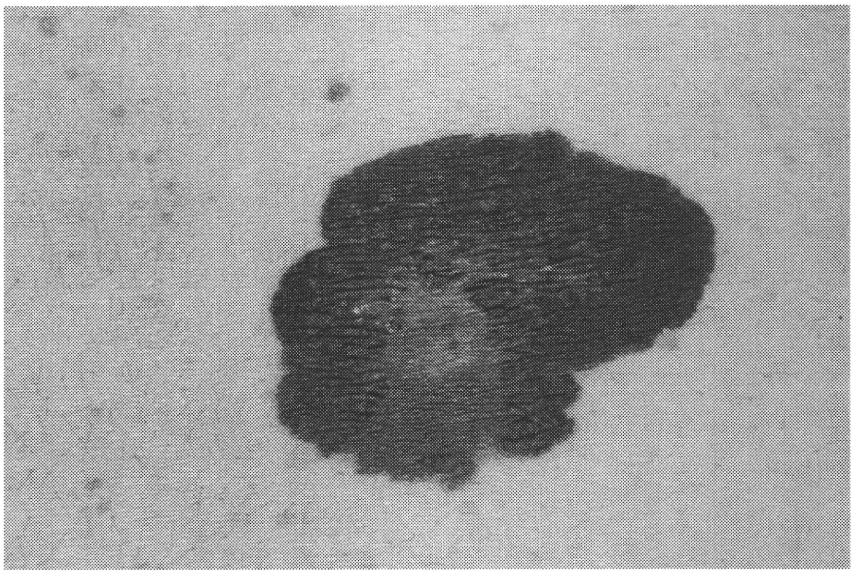

Fig. 1. Clinical feature of case No.34. This case showed positive sentinel lymph node metastasis (×4).

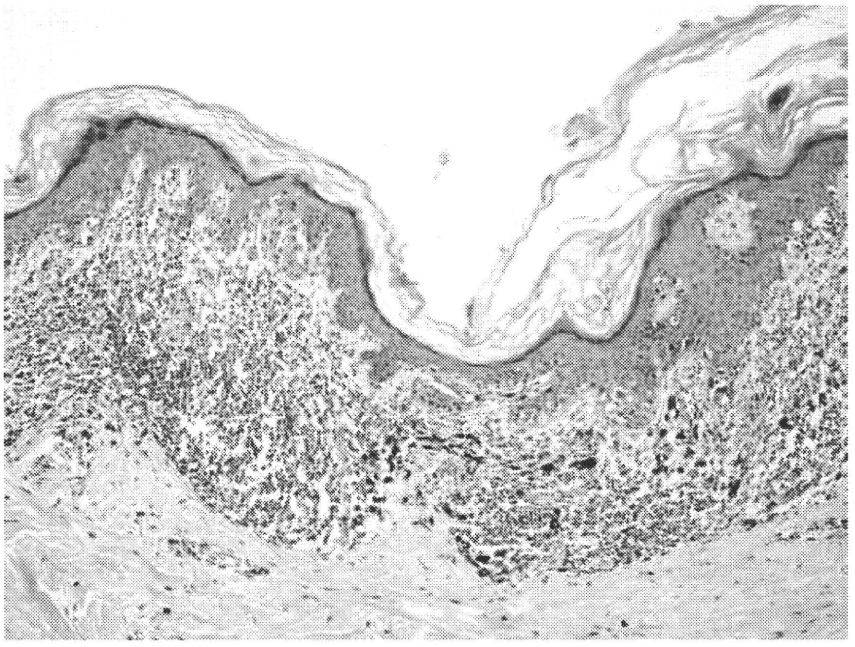

Fig. 2. Histopathological feature of case No.34. It shows intensely infiltrated type in mid and upper dermis (HE staining, ×100).

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Letter to the editor

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AKT plays an anti-apoptotic role in ABCA12-deficient keratinocytes

Teruki Yanagi[1], Masashi Akiyama[1, 2], Hiroshi Nishihara[3], Yuki Miyamura[1], Kaori Sakai[1], Shinya Tanaka[4] and Hiroshi Shimizu[1]

[1]Department of Dermatology, [3]Laboratory of Translational Pathology, [4]Laboratory of Cancer Research, Department of Pathology, Hokkaido University Graduate School of Medicine, Sapporo, Japan

[2]Department of Dermatology, Nagoya University Graduate School of Medicine, Nagoya, Japan

Correspondence and reprint requests to: Teruki Yanagi, MD, PhD

Hokkaido University Graduate School of Medicine, N15 W7, Kita-ku, Sapporo, Japan

TEL: +81-11-706-7387, FAX: +81-11-706-7820

E-mail: yanagi@med.hokudai.ac.jp

Short title: AKT in ABCA12-deficient keratinocytes

Abbreviations: ABCA12, ATP-binding cassette transporter A12; **HI**, harlequin ichthyosis; **IF**, immunofluorescence; **LXR**, liver X receptor; **PPAR**, peroxisome proliferator-activated receptor; **RXR**, retinoid X receptor

Key words: harlequin ichthyosis, ABCA12, apoptosis

Tables: 0; **Figures**: 2; **References**: 13

Harlequin ichthyosis (HI) is a hereditary skin disorder characterized by severe hyperkeratosis and impaired skin barrier function (Akiyama *et al.*, 2005; Moskowitz *et al.*, 2004). We have identified the ATP-binding cassette transporter A12 (*ABCA12*) as the causative gene of HI and, furthermore, demonstrated that ABCA12 is essential for keratinocyte lipid transport (Akiyama *et al.*, 2005; Yanagi *et al.*, 2008). Loss of ABCA12 function causes lipid transport to be defective in keratinocytes of the upper spinous and granular layers, resulting in the deposition of numerous intracellular lipid droplets and malformation of intercellular lipid layers (Akiyama *et al.*, 2005; Yanagi *et al.*, 2010). Recently, we have shown that gangliosides accumulate in the differentiated keratinocytes of HI patients (Mitsutake *et al.*, 2010). Based on evidence that lipid accumulation is involved in keratinocyte apoptosis (Uchida *et al.*, 2010; Wang *et al.*, 2001), we investigated apoptotic and anti-apoptotic parameters in skin samples from HI patients and *Abca12*$^{-/-}$ harlequin ichthyosis model mice.

We studied the skin of two HI patients and that of *Abca12*$^{-/-}$ mice. The *ABCA12* mutations of the two HI patients have been previously reported: One patient has the homozygous splice acceptor site mutation c.3295-2A>G, and the other has the homozygous nonsense mutation p.Arg434X (Akiyama *et al.*, 2005). The procedure for generating *Abca12*$^{-/-}$ mice, the establishment of primary-cultured keratinocytes, immunofluorescence (IF) staining, immunoblotting, and real-time reverse transcriptase PCR analysis has been previously described (Yanagi *et al.*, 2008; Yanagi *et al.*, 2010). First, we investigated the apoptosis of HI patient epidermis by hematoxylin-eosin stain and TUNEL assay (*In situ* Apoptosis Detection Kit, Takara Bio Inc.). In the HI patients, the nuclei of the granular-layer keratinocytes were condensed (Figure 1b) and they show positive for TUNEL labeling (Figure 1d), although apoptotic nuclei are rare in the

normal human epidermis (Figure 1a, c). The histopathological findings and results of TUNEL staining of the *Abca12*$^{-/-}$ mice were similar to those in the skin of the HI patients (Figure 1f, h). TUNEL staining in the epidermis of 18.5-day embryos indicated that the apoptosis of keratinocytes started during fetal skin development (Figure 1j).

We assessed the degree of AKT activation of *Abca12*$^{-/-}$ skin and keratinocytes using anti-AKT antibody #4691 and anti-phosphorylated AKT (Ser473) #4060 antibody (Cell Signaling). By immunoblot analysis, differentiated primary-cultured keratinocytes and the epidermis of *Abca12*$^{-/-}$ mice showed higher expression levels of Ser-473 phosphorylated AKT (pAKT) than those of the control wild-type mice (Figure 1o). IF staining detected pAKT in the upper-granular-layer keratinocytes of the *Abca12*$^{-/-}$ mouse skin (Figure 1l), but not in control wild-type mouse skin (Figure 1k). Cell proliferation was assessed by Ki-67 IF (Figure 1m, n). Ki-67 stain was similar in the wild-type and the *Abca12*$^{-/-}$ samples, indicating that the granular-layer keratinocytes of the *Abca12*$^{-/-}$ neonatal mice showed no excessive cell proliferation. To clarify whether AKT activation has anti-apoptotic effects on *Abca12*$^{-/-}$ keratinocytes, we performed TUNEL staining of keratinocytes treated with AKT inhibitor, which blocks AKT phosphorylation (#124017; InSolution Akt Inhibitor VIII, Calbiochem). *Abca12*$^{-/-}$ keratinocytes incubated with 10μM #124017 AKT inhibitor showed a notably greater number of TUNEL-positive cells than both wild-type keratinocytes with AKT inhibitor and *Abca12*$^{-/-}$ keratinocytes without AKT inhibitor (Figure 2). These results suggest that AKT activation helps *Abca12*$^{-/-}$ keratinocytes to avoid apoptosis. Furthermore, mRNA and protein levels of peroxisome proliferator-activated receptor (PPAR)-delta from *Abca12*$^{-/-}$ epidermis were shown to be significantly higher than those from wild-type epidermis (Taqman Gene Expression Assay, probe ID; Mm00803184_m1,

Mm99999915_g1, Applied Biosystems, anti-PPAR-delta antibody H-74, Santa Cruz) (Supplementary Figure S1), which suggests up-regulation of PPAR-delta as a candidate pathway for AKT activation.

Herein, we have suggested that apoptosis is involved in the pathomechanism of HI. Defective lipid transport due to loss of ABCA12 function leads to the accumulation of intracellular lipids, including glucosylceramides and gangliosides (Akiyama *et al.*, 2005; Mitsutake *et al.*, 2010). Studies by Wang *et al.* (2001) and Sun *et al.* (2002) showed that the elevation of ganglioside levels leads to keratinocyte apoptosis. Thus, we are able to speculate that the accumulation of gangliosides leads to the apoptosis of *Abca12*$^{-/-}$ keratinocytes, although the exact mechanism of apoptosis in *Abca12*$^{-/-}$ keratinocytes remains unclear.

Although *Abca12*$^{-/-}$ granular-layer keratinocytes show characteristics of apoptosis, including condensed nuclei and positive TUNEL labeling, they are able to form epidermal stratification. In several disorders involving keratinocyte apoptosis, e.g. toxic epidermal necrolysis, the apoptotic epidermal keratinocytes show not only TUNEL-positive nuclei but also defective epidermal stratification (Abe *et al.*, 2003). Thrash *et al.* (2006) reported that AKT1 activation is an essential signal for keratinocyte cell survival and stratification, by experiments with gene silencing and three-dimensional cell cultures. Thus, we hypothesized that the AKT pathway might work as a compensatory mechanism against apoptosis in *Abca12*$^{-/-}$ keratinocytes. We have clearly shown that AKT activation occurs in *Abca12*$^{-/-}$ granular-layer keratinocytes, which suggests that AKT activation serves to prevent the cell death of *Abca12*$^{-/-}$ keratinocytes. By immunoblot analysis using anti-AKT1/2/3 antibodies (#2938/3063/3788, Cell Signaling), *Abca12*$^{-/-}$ epidermis showed expression of AKT1

97 and AKT2, but not AKT3 (Supplementary Figure S2). Compared to wild-type epidermis,
98 *Abca12*$^{-/-}$ epidermis seemed to have more AKT1 than AKT2. From our data and the
99 literature (Thrash *et al.*, 2006), we are able to speculate that AKT1 is the major isoform
100 of phosphorylated AKT in *Abca12*$^{-/-}$ epidermis.

101 We have shown that PPAR-delta is a candidate molecule in the upstream of the
102 AKT activation pathway in *Abca12*$^{-/-}$ keratinocytes. Di-Poi *et al.* (2002) reported that
103 PPAR-delta has an anti-apoptotic role in keratinocytes via transcriptional control of the
104 AKT1 signaling pathway. PPAR-delta also regulates the expression of ABCA12 (Jiang
105 *et al.*, 2008). From these studies, we can speculate that up-regulation of PPAR-delta is
106 in response to apoptosis or decreased ABCA12 expression. To ascertain PPAR-delta's
107 function, we performed the experiments using a PPAR-delta-specific antagonist
108 (GSK0660, Santa Cruz). Differentiated *Abca12*$^{-/-}$ keratinocytes treated with 1μM
109 GSK0660 for 48 hours showed TUNEL-positive nuclei, from which we are able to
110 speculate an anti-apoptotic role for PPAR-delta in *Abca12*$^{-/-}$ keratinocytes
111 (Supplementary Figure S1). From our studies and the literature (Di-Poi *et al.*, 2002),
112 PPAR-delta has been shown to have at least an anti-apoptotic role in *Abca12*$^{-/-}$
113 keratinocytes; however, it remains unclear whether the up-regulation of PPAR-delta is
114 in response to apoptosis or decreased ABCA12 expression.

115 Furthermore, we have measured mRNA expression levels of other nuclear
116 hormone receptors including PPAR-alpha, PPAR-gamma, retinoic acid receptor-alpha,
117 liver X receptor (LXR)-alpha, LXR-beta, retinoid X receptor (RXR)-alpha, and
118 RXR-gamma (Applied Biosystems). The mRNA level of RXR-alpha from *Abca12*$^{-/-}$
119 epidermis was shown to be significantly higher than that from wild-type epidermis
120 (Supplementary Figure S1). The interaction between up-regulation of RXR-alpha and

AKT activation in keratinocytes has not been reported. However, Wang *et al.* (2011) reported that RXR-alpha ablation in the epidermis enhances UV-induced apoptosis, which suggests that RXR-alpha has an anti-apoptotic function in keratinocytes. Thus up-regulation of RXR-alpha may also have an anti-apoptotic function in *Abca12*$^{-/-}$ keratinocytes.

In conclusion, the present data suggest that keratinocyte apoptosis is involved in the pathomechanisms of HI and that the AKT signaling pathway helps *Abca12*$^{-/-}$ keratinocytes to survive during the keratinization process. In light of this, activation of the AKT signal pathway may be a novel strategy for treating keratinization disorders, including ichthyosis.

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Conflict of Interest Statement

The authors declare no conflicts of interest.

Figure Legends

Figure 1 ABCA12-deficient keratinocytes show TUNEL-positive nuclei and AKT activation.

(a-d) In the HI patients, the nuclei of the granular-layer keratinocytes are condensed (b, white arrows) and they show positive TUNEL labeling (d, white arrows), although apoptotic nuclei are rare in the normal human epidermis (a, c). Data shown are representative of those from the two harlequin ichthyosis patients.

(e, f) Granular-layer keratinocytes of *Abca12*$^{-/-}$ mice show more condensed nuclei (f, white arrows) than those of wild-type mice (e).

(g-j) Granular-layer keratinocytes of *Abca12*$^{-/-}$ mice, a neonate (h) and a 18.5-day embryo (j) show TUNEL-positive nuclei. No TUNEL-positive cells are seen in the epidermis of the control wild-type mice (g, i). Dotted lines indicate the basement membrane. Non-specific staining is seen on the skin surface (white arrowheads).

(k, l) By immunofluorescence (IF) staining, AKT activation (Ser-473 phosphorylated AKT; green) is observed in granular-layer keratinocytes of *Abca12*$^{-/-}$ mice.

(m, n) IF staining for the Ki-67 proliferation marker shows similar staining patterns of basal keratinocytes in wild-type (m) and *Abca12*$^{-/-}$ (n) samples.

(a, b, e, f; hematoxylin-eosin stain. Scale bars of c, d, g, h, i, j, k, l, m, n = 20μm. Scale bars of a, b, e, f = 5μm.)

(o) Immunoblot analysis shows that levels of serine-473-phosphorylated AKT (pAKT) in neonatal epidermis and differentiated keratinocytes of *Abca12*$^{-/-}$ mice are higher than those of wild-type mice.

Figure 2 Inhibition of AKT activation leads to apoptosis of *Abca12*$^{-/-}$ keratinocytes.

(a) Immunoblot analysis indicates that the AKT inhibitor can inhibit AKT activation (pAKT synthesis) in differentiated keratinocytes.

(b, c, d, e) TUNEL staining of keratinocytes cultured under high Ca^{2+} condition treated with/without the AKT inhibitor. Neither wild-type cells (b) nor *Abca12*$^{-/-}$ cells (c) are TUNEL positive. *Abca12*$^{-/-}$ keratinocytes with the AKT inhibitor (#124017) (10μM) show many TUNEL-positive nuclei (e), although only a small number of wild-type cells with the AKT inhibitor are TUNEL-positive (d). (Scale bars = 20μm)

(f) Percentage of TUNEL-positive keratinocytes. *Abca12*$^{-/-}$ keratinocytes with AKT inhibitor shows a significantly greater number of TUNEL-positive nuclei than wild-type keratinocytes with/without the AKT inhibitor and *Abca12*$^{-/-}$ keratinocytes without the AKT inhibitor. (n=3, mean ± SD, *$p<0.05$)

Supplementary Figure S1

(a) The mRNA level of PPAR-delta in *Abca12*$^{-/-}$ epidermis is significantly higher than that in wild-type epidermis. (n=4, mean ± SD, *$p<0.05$)

(b) Immunoblotting of epidermal extracts shows that protein expression of PPAR-delta is higher in *Abca12*$^{-/-}$ epidermis (right lane) than in the wild-type epidermis (left lane).

(c) The mRNA level of retinoid X receptor (RXR)-alpha in *Abca12*$^{-/-}$ epidermis is significantly higher than that in wild-type epidermis. (n=4, mean ± SD, *$p<0.05$)

(d, e, f, g) TUNEL staining of keratinocytes cultured under high Ca^{2+} condition treated with/without the PPAR-delta-specific antagonist (GSK0660). The wild-type cells (d), the *Abca12*$^{-/-}$ cells (e) or wild-type cells with the PPAR-delta-specific antagonist (1μM) (f) are not TUNEL positive. *Abca12*$^{-/-}$ keratinocytes with the PPAR-delta antagonist show TUNEL-positive nuclei (g, white arrows). (Scale bars = 20μm)

(h) Percentage of TUNEL-positive keratinocytes. *Abca12*$^{-/-}$ keratinocytes with PPAR-delta-specific antagonist show a significantly greater number of TUNEL-positive nuclei than wild-type keratinocytes with/without PPAR-delta specific antagonist and *Abca12*$^{-/-}$ keratinocytes without the PPAR-delta specific antagonist. (n=3, mean ± SD, *$p<0.05$)

Supplementary Figure S2

Immunoblot analysis with anti-AKT1/2/3 antibodies. *Abca12*$^{-/-}$ epidermis shows AKT1 and AKT2, but not AKT3 expression. Compared to wild type epidermis, *Abca12*$^{-/-}$ epidermis seems to have more AKT1 than AKT2.

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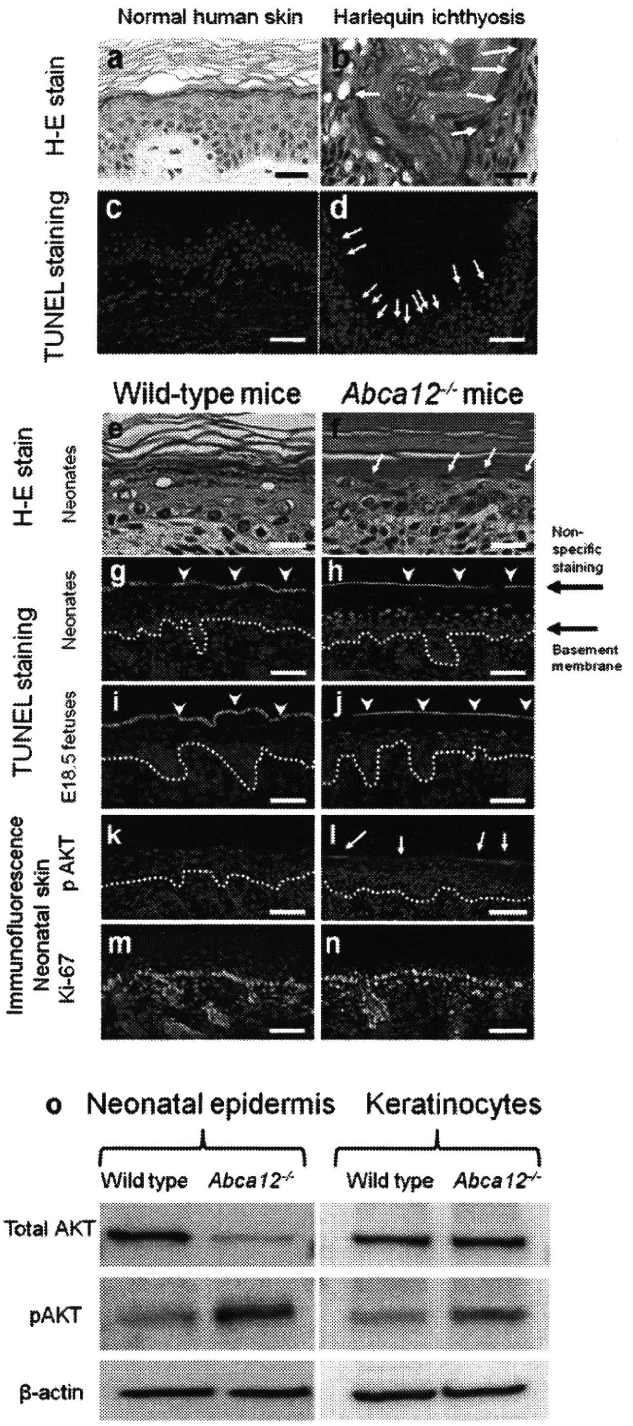

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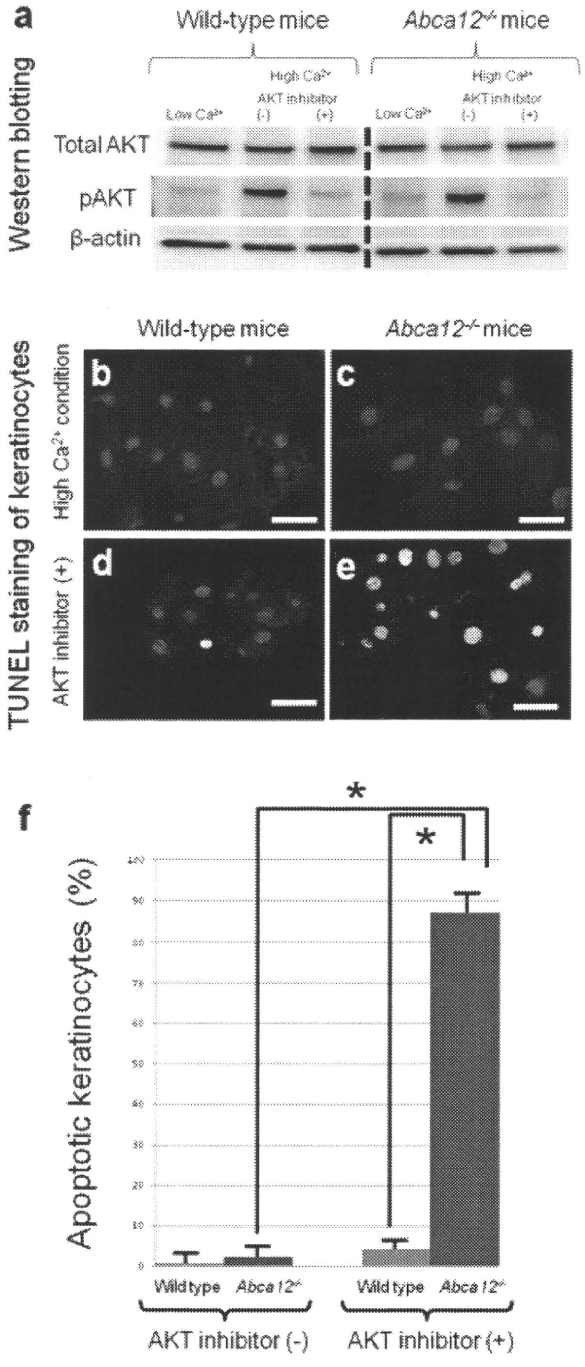

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259 Figure 2.

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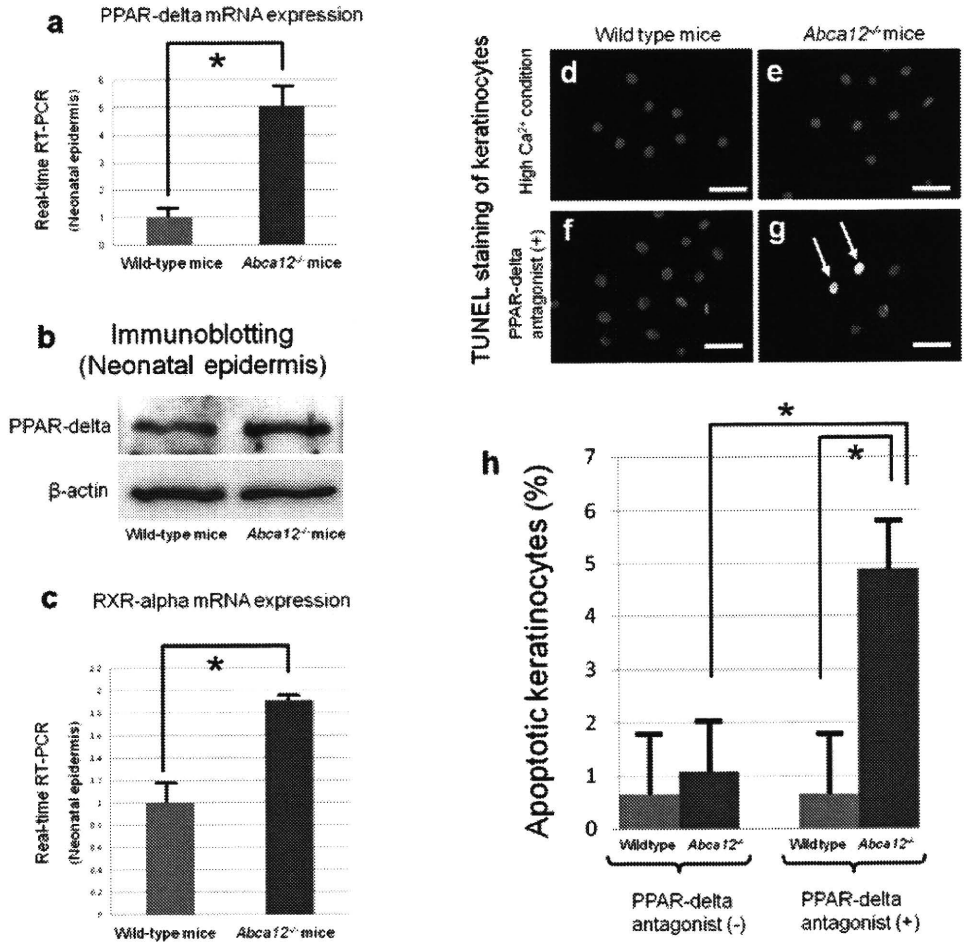

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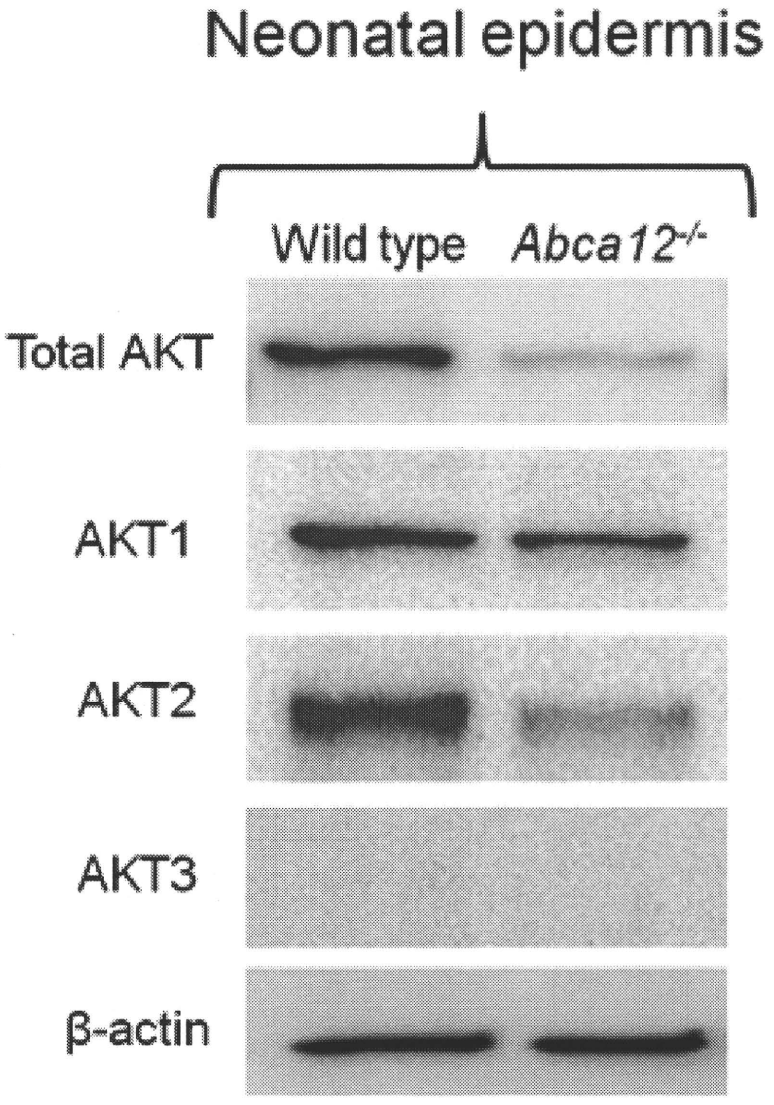

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267 Supplemental Figure S2.

Journal of Investigative Dermatology

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Original Article

Consequences of two different amino acid substitutions at the same codon in *KRT14* indicate definitive roles of structural distortion in epidermolysis bullosa simplex pathogenesis

Ken Natsuga[1*], Wataru Nishie[1], Brian J. Smith[2,3], Satoru Shinkuma[1], Thomasin A. Smith[4], David A.D. Parry[4], Naoki Oiso[5], Akira Kawada[5], Kozo Yoneda[6], Masashi Akiyama[1,7], Hiroshi Shimizu[1]

[1]Department of Dermatology, Hokkaido University Graduate School of Medicine, Sapporo, Japan; [2]The Walter and Eliza Hall Institute of Medical Research, Parkville, Australia; [3]Department of Medical Biology, The University of Melbourne, Parkville, Australia; [4]Institute of Fundamental Sciences, Massey University, Palmerston North, New Zealand; [5]Department of Dermatology, Kinki University Faculty of Medicine, Osaka, Japan; [6]Department of Dermatology, Kagawa University Faculty of Medicine,