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Patients with Recessive Dystrophic Epidermolysis Bullosa Develop Squamous-Cell Carcinoma Regardless of Type VII Collagen Expression

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Recent data suggest that individuals with recessive dystrophic epidermolysis bullosa (RDEB) only develop squamous-cell carcinoma (SCC) in the presence of the NC1 domain of type VII collagen. This conclusion was based on experimental work in which cryosections of SCCs from 10 people with RDEB all showed positive type VII collagen immunostaining and observations in a murine model of SCC development in which tumors only occurred using keratinocytes from RDEB subjects that expressed detectable levels of the NC1 domain of the type VII collagen protein. To assess whether the clinical interpretation was valid in another cohort of RDEB patients, we examined expression of type VII collagen in 17 SCC tumors excised from 11 patients. Indirect immunofluorescent staining of SCC cryosections and Western blotting of cultured keratinocyte lysates identified two RDEB individuals who did not express detectable levels of type VII collagen. Mutation analysis revealed that these two patients harbor compound heterozygous nonsense mutations within the region of the *COL7A1* gene encoding the NC1 domain. These data suggest that individuals with RDEB can develop SCC regardless of type VII collagen expression and that additional factors have a role in explaining the high incidence of tumors complicating this genodermatosis.

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INTRODUCTION

Epidermolysis bullosa (EB) is a heterogeneous group of skin disorders associated with blisters, erosions, and chronic wounds. Among the subtypes of EB, dystrophic EB can be either dominantly (DDEB) or recessively (RDEB) inherited and is caused by mutations in the type VII collagen gene, *COL7A1* (reviewed by Varki *et al.*, 2007). Type VII collagen is the main component of anchoring fibrils, attachment structures within the basement membrane between the epidermis and the dermis (Uitto *et al.*, 1992; Burgeson, 1993). The *COL7A1* gene is approximately 32 kb in size and contains 118 exons (Christiano *et al.*, 1994). Over 250 mutations (point mutations, deletions, insertions) in *COL7A1* have been identified in dystrophic EB. To some extent, the severity of blistering and epidermal involvement can be correlated with the mutation profile in *COL7A1* (Jarvikallio *et al.*, 1997). In mild forms of dystrophic EB, mutated type VII collagen is secreted but the morphology of the anchoring fibrils is abnormal (Bruckner-Tuderman *et al.*, 1999). In the more severe subtypes, especially the Hallopeau–Siemens form of RDEB, type VII collagen is very poorly expressed or absent (McGrath *et al.*, 1993).

Individuals with dystrophic EB are at greater risk of developing squamous-cell carcinoma (SCC) than other EB patients. This risk appears to correlate with the severity of skin blistering, with 55% of Hallopeau–Siemens RDEB subjects dying from metastatic SCC by the age 40 years (Fine *et al.*, 1999). Research over the past 14 years supports the hypothesis that the severity of the disease is inversely proportional to the amount of functional type VII collagen expression present in the skin (McGrath *et al.*, 1993; Christiano *et al.*, 1994). However, the dogma that less type VII collagen leads to more severe disease coupled with a greater risk of SCC was challenged by a recent paper in

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Abbreviations: DEB, dystrophic epidermolysis bullosa; EB, epidermolysis bullosa; RDEB, recessive dystrophic epidermolysis bullosa SCC, squamous-cell carcinoma

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Science (Ortiz-Urda *et al.*, 2005). In this report, the authors demonstrated that type VII collagen, and more precisely the NC1 domain, is required for Ras-driven skin SCC development (Ortiz-Urda *et al.*, 2005). RDEB patients in this study who did not express detectable levels of type VII collagen failed to develop SCC and keratinocytes isolated from these patients did not form SCC in the experimental model. This observation has significant implications for patient monitoring and counseling as it suggests that individuals with the most severe forms of RDEB may not be predisposed to developing SCC.

In this study, we determined if there is a correlation between RDEB SCC development and type VII collagen expression. Therefore, we studied type VII collagen expression in 11 unrelated RDEB patients who had developed SCC. We found that, in our cohort of patients, RDEB SCC develops regardless of type VII collagen expression. Mutation analysis revealed that patients who did not express detectable type VII collagen were compound heterozygotes for nonsense mutations within the NC1 coding domain of the *COL7A1* gene. In addition, we were unable to detect *Ras* gene mutations by direct sequencing of DNA isolated from cultured SCC keratinocytes.

RESULTS

Immunofluorescence microscopy identifies RDEB patients who have developed SCC but who are negative for type VII collagen expression

We first examined type VII collagen expression in non-EB SCC frozen tumors using immunostaining of cryosections with a commercially available polyclonal antibody raised against the NC1 domain of type VII collagen. In sections from 20/20 non-EB SCC tumors, we saw type VII collagen expression restricted to the basement membrane (Figure 1a).

We then examined type VII Collagen expression in 16 separate SCC tumors excised from 10 individual RDEB patients. In 8/10 patients (13 tumors), we observed type VII collagen expression. In the remaining 2/10 patients (three tumors), we saw no detectable type VII collagen expression (Figure 1b). We then examined type VII collagen expression

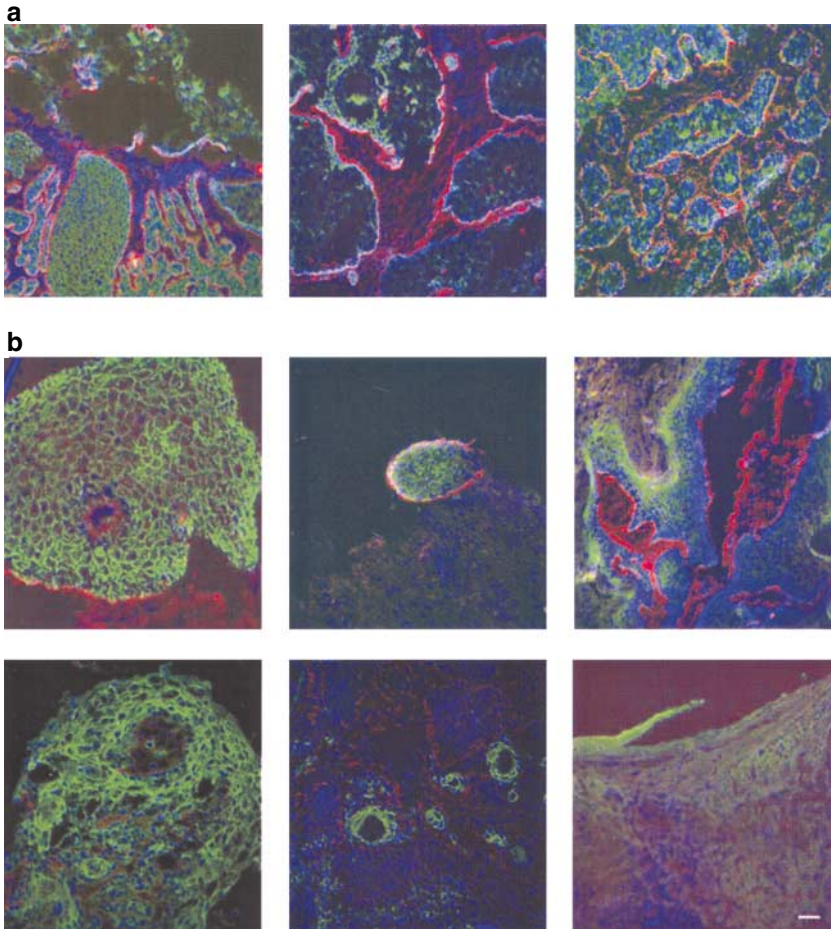

Figure 1. Immunofluorescence staining of RDEB SCC tumors reveals the presence or absence of type VII collagen immunoreactivity. Cryosections were stained with antibodies to the type VII collagen NC1 domain (red) and desmoplakin (green). DAPI (blue) was used for nuclear staining. (**a**) Representative data from 20 non-EB SCC tumors all of which showed positive staining for type VII collagen. (**b**) Top panel: representative data from 13 RDEB SCC tumors showing positive staining for type VII collagen (left = RDEB7-SCC3, middle = RDEB8-SCC1, right = RDEB9-SCC1). (**b**) Bottom panel: three RDEB SCC tumors from two patients negative for type VII collagen staining (left = RDEB1-SCC1, middle = RDEB1-SCC2, right = RDEB2-SCC1). Bar = 200 μm.

in normal skin or, where possible, keratinocytes isolated from normal skin from nine RDEB patients. Either immunostaining, immunoblotting from isolated keratinocytes or both experimental techniques using non-SCC skin from nine out of nine of our RDEB patients gave an identical pattern of type VII collagen expression as seen in SCC tumors (Figure 2).

Immunoblotting of SCC keratinocyte lysates confirms immunohistochemical results: RDEB patients who do not express type VII collagen can develop SCC

Next, we isolated keratinocytes from non-EB SCC tumors and six RDEB SCC tumors isolated from six patients; five of these tumors were analyzed by immunohistochemistry (as above). Lysates were prepared and immunoblotting was performed using an affinity-purified polyclonal antibody raised against recombinant NC1 domain of type VII collagen (Chen *et al.*, 1997). The pattern of type VII collagen expression seen with frozen sections of normal skin and SCC tissue from both RDEB and non-EB individuals were confirmed by these experiments (Figure 3a and b), thus two of six RDEB patient SCC keratinocytes did not express type VII collagen.

***COL7A1* mutation analysis correlates with nonsense mutations within the NC1 domain in patients lacking type VII collagen expression**

Direct sequencing of all 118 exons of the *COL7A1* gene revealed R525X and Q905X mutations in DNA from patient RDEB2 (data not shown). Mutation analysis of the remaining RDEB patients has been described previously (Whittock *et al.*, 1999; Kern *et al.*, 2006). Of the two RDEB patients who did not express detectable type VII collagen only nonsense mutations within the NC1 domain were found (Table 1). Of the RDEB patients who did express detectable type VII collagen and whose *COL7A1* genotype had been determined, all were found to harbor at least one missense, one nonsense, or one mutation that predicts a premature termination codon distal to the NC1 domain of the *COL7A1* gene.

Real-time PCR analysis of *COL7A1* relates to the level of type VII collagen expression

Taqman® Gene Copy Number Assays were used to assess the level of *COL7A1* transcripts relative to *GAPDH* in cDNA constructed from total RNA isolated from normal cultured keratinocytes, as well as cultured keratinocytes from RDEB1-SCC1, RDEB3-SCC1, RDEB6-SCC1, and non-EB-SCC1. RDEB patient SCC keratinocytes showed less *COL7A1* transcripts relative to *GAPDH* than did normal keratinocytes and non EB-SCC1, whereas patient RDEB1 keratinocytes that did not express type VII collagen showed the lowest levels of *COL7A1* expression (Figure 3c).

No *Ras* mutations were found in DNA isolated from SCC keratinocytes

We analyzed DNA isolated from RDEB SCC keratinocytes of six subjects and DNA isolated from SCC keratinocytes of four non-EB subjects by PCR amplification and direct sequencing and did not find any mutations in codons 12, 13, or 61 of the *N*, *K*, and *Ha Ras* genes (data not shown).

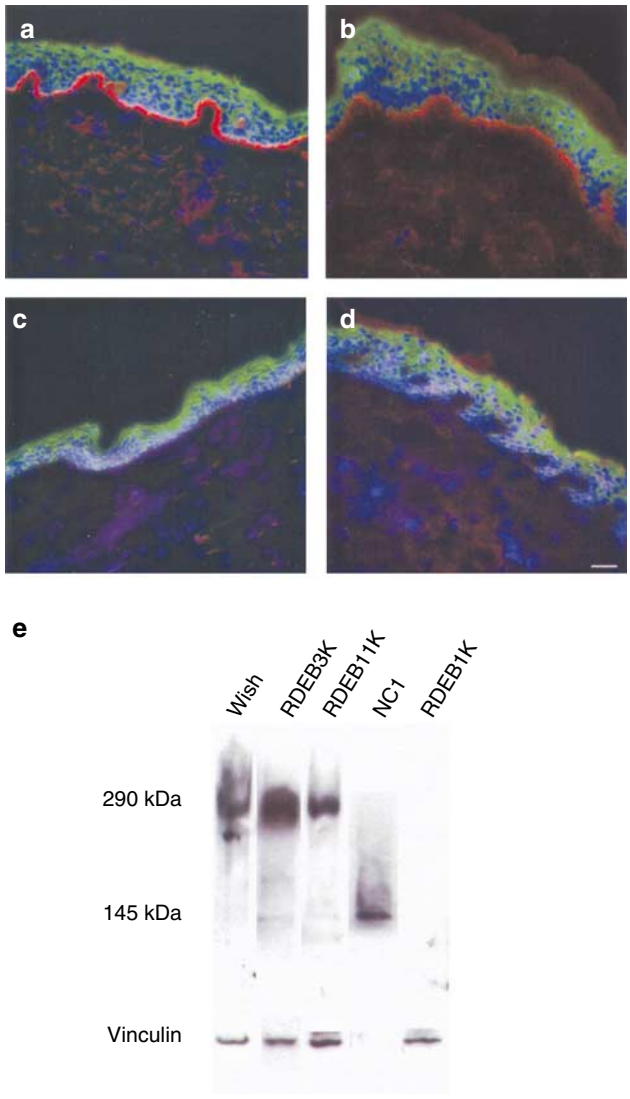

Figure 2. Expression pattern of type VII collagen. The expression pattern of type VII collagen in normal skin and/or keratinocytes isolated from RDEB patients who have developed SCC is identical to that seen in SCC tumors by immunofluorescence and immunoblotting methodology. (**a–c**) Cryosections were stained with antibodies to the type VII collagen NC1 domain (red) and desmoplakin (green). DAPI (blue) was used for nuclear staining. **a** = non-EB normal skin. **b** = normal skin from patient RDEB9. **c** = normal skin from patient RDEB1. **d** = type VII collagen negative control, normal skin. Bar = 200 μm. (**e**) Immunoblotting of lysates from keratinocytes isolated from normal skin using a purified polyclonal antibody to the NC1 domain of type VII collagen. WISH = human amniotic cell line. NC1 = recombinant NC1 domain. RDEB11K, RDEB3K, RDEB1K = normal keratinocytes from patients RDEB11, RDEB3, and RDEB1. Note negative immunofluorescence and immunoblotting for type VII collagen in the normal skin or normal keratinocytes isolated from patient RDEB1 who has developed two SCC tumors during the course of this study.

DISCUSSION

Patients with the most severe form of RDEB are predisposed to a high rate of aggressive SCC, which is the main cause of premature death in this disorder (Fine *et al.*, 1999). The severity of disease in individuals with RDEB is correlated inversely with type VII collagen expression, with the most

severely affected patients expressing little or no detectable protein (Christiano *et al.*, 1994). Recent data published in the journal *Science*, have suggested that SCC can only develop in the presence of the NC1 domain of type VII collagen (Ortiz-Urda *et al.*, 2005). If this is correct then RDEB patients with no type VII collagen expression would be free from the life-threatening complication of SCC and as such could be counseled appropriately and monitored less frequently for tumor development. In addition to clinical management, if type VII collagen is essential for SCC development this protein would become an attractive target for the development of anticancer therapies.

In this study, we sought to clarify whether in our cohort of RDEB patients only those that express type VII collagen develop SCC. Two out of eight hsRDEB patients analyzed here did not express type VII collagen and have gone on to develop one or more SCC tumors. Lack of detectable type VII collagen expression was confirmed using two independent experimental methods and is supported by mutation analysis; patients with no detectable type VII collagen are compound heterozygous for nonsense mutations within the NC1 domain coding portion of the *COL7A1* gene.

SCC is a heterogeneous cancer characterized by multiple genetic alterations (reviewed by Backvall *et al.*, 2005). In DNA isolated from 10 separate SCC keratinocyte populations (six RDEB and four non-EB), we were unable to detect any Ras mutations by direct sequencing. Although we cannot rule out Ras-activating events in these SCC cells, these data suggest that in these samples Ras mutations are not a major event. Likewise, we cannot exclude that contributing

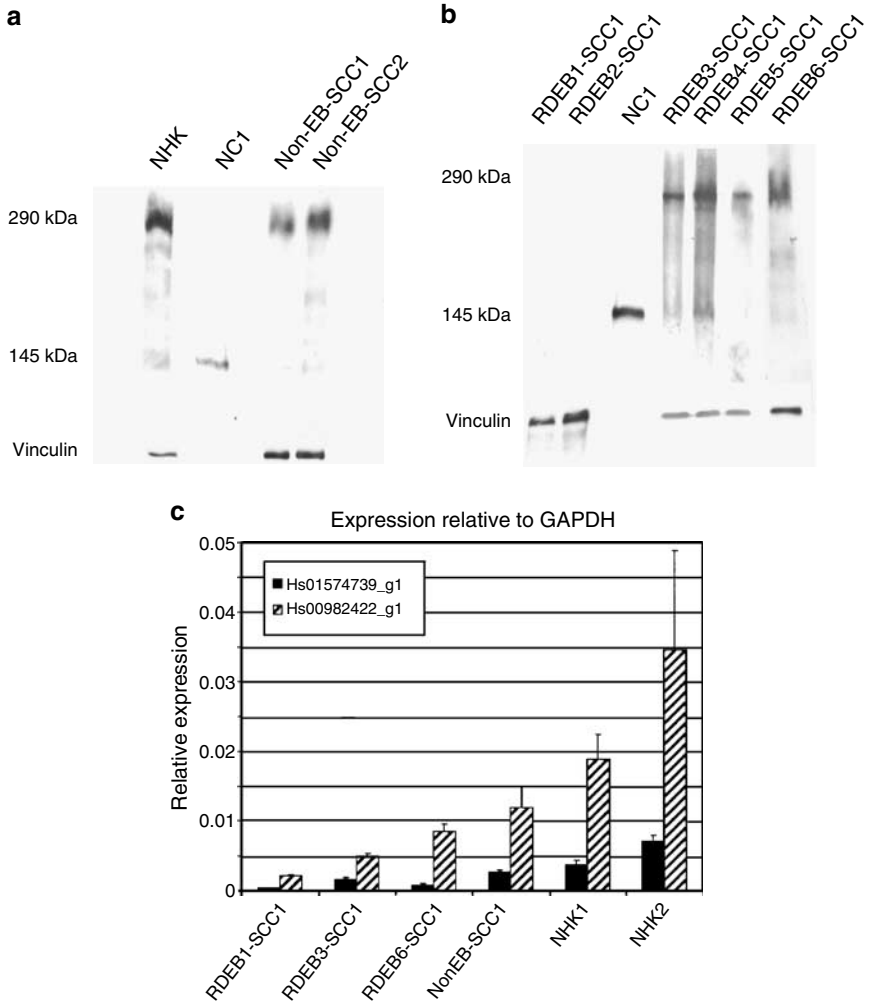

Figure 3. Immunoblotting reveals the presence or absence of type VII collagen expression in primary keratinocytes isolated from RDEB SCC. Primary keratinocytes were isolated from either normal skin, non-EB SCC, or RDEB SCC tissue and lysates were prepared as described. NC1 = recombinant NC1 domain. (**a**) Immunoblotting using affinity-purified polyclonal antibodies to the NC1 domain of type VII collagen shows expression from normal human keratinocytes and two representative keratinocyte lysates from four separate non-EB SCC samples. (**b**) Immunoblotting using affinity-purified polyclonal antibodies to the NC1 domain of type VII collagen shows presence or absence of expression in RDEB SCC keratinocyte lysates. (**c**) TaqMan® Gene Copy Number Assays for 5′ *COL7A1* mRNA sequence (Hs01574739_g1) and 3′ *COL7A1* mRNA sequence (Hs00982422_g1) relative to *GAPDH* relate to the level of type VII collagen expression seen in **a** and **b** ($n = 3$; $\pm$SD). cDNA was synthesized from mRNA of RDEB SCC cells (RDEB1-SCC1, RDEB3-SCC1, and RDEB6-SCC1), non-EB SCC cells (non-EB-SCC1), and normal keratinocytes (NHK1, NHK2).

Table 1. Clinical features, COL7A1 genotype, and type VII collagen expression pattern of RDEB patients in this study

					Type VII collagen expression			
					SCC		Normal skin	
Patient	Age	Gender	Mutation	Diagnosis	IMF	WB	IMF	WB
RDEB1	35	F	R525X/R578X	hsRDEB	− −	− N/A	−	−
RDEB2	29	F	R525X/Q905X	hsRDEB	−	−	−	N/A
RDEB3	54	M	3832−1G>A/unknown	hsRDEB	+	+	+	+
RDEB4	42	F	6075delC/unknown	RDEB	+	+	+	N/A
RDEB5	33	F	3839delC/6501G>A	RDEB	+	+	N/A	N/A
RDEB6	32	M	8244insC/8244insC	hsRDEB	N/A	+	N/A	N/A
RDEB7	43	M	R578X/7786delG	hsRDEB	+ + + +	N/A N/A N/A N/A	+	N/A
RDEB8	26	M	6501+1G>C/unknown	hsRDEB	+ +	N/A N/A	+	N/A
RDEB9	26	M	5572delG/G1703E	RDEB	+	N/A	+	N/A
RDEB10	57	F	Unknown	hsRDEB	+	N/A	+	N/A
RDEB11	38	F	G2073D/R578X	hsRDEB	+ +	N/A N/A	+	+

F, female; hsRDEB, Hallopeau–Siemens RDEB; IMF, immunostaining; M, male; N/A, sample not available; RDEB, recessive dystrophic epidermolysis bullosa; SCC, squamous-cell carcinoma; WB, immunoblotting; −, no expression of type VII collagen; +, expression of type VII collagen.
Multiple entries for SCC type VII collagen; expression for any given patient indicate multiple SCCs analyzed for that patient, that is, two SCC tumors from patient RDEB1 were analyzed, whereas four SCC tumors from patient RDEB7 were analyzed. Age refers to development of first SCC tumor analyzed in this study.

molecular events downstream of the Ras-driven oncogenesis (described by Dajee *et al.* (2003) and Ortiz-Urda *et al.* (2005)) have not been altered in the two patients described here who do not express type VII collagen and therefore we cannot speculate whether separate modes of tumor initiation and progression exist. However, from this study we can conclude that patients with RDEB who do not express type VII collagen are still at risk of developing SCC and as such these individuals should continue to be monitored closely and investigated under the same clinical criteria as RDEB patients who do express type VII collagen (Mallipeddi, 2002).

MATERIALS AND METHODS

Patients

This study was conducted according to the Declaration of Helsinki Principles and was approved by the East London and City Health Authority Research Ethics Committee and cooperating centres. The RDEB diagnosis was confirmed by characteristic immunofluorescence findings and clinical criteria. In our cohort of 11 RDEB patients, eight were diagnosed with Hallopeau–Siemens-type RDEB (Hs-RDEB). After informed consent, biopsies from normal skin or SCC tissue from non-EB and RDEB patients were obtained.

Tissue section preparation and immunohistochemistry

Skin or SCC tissue was washed immediately in phosphate-buffered saline (PBS) before being embedded in OCT compound (VWR, Lutterworth, UK) and snap-frozen in iso-pentane cooled by liquid nitrogen. Cryosections (6 μm thick) were re-hydrated in PBS for 2 minutes at room temperature before blocking of nonspecific immunoreactive sites with 3% bovine serum albumin in PBS for 20 minutes at 37°C. Sections were incubated in the primary antibodies for 1 hour at 37°C followed by three 5-minutes washes in PBS. They were then incubated with secondary antibodies; goat antimouse Alexa Fluore 488 conjugate and goat antirabbit Alex Fluore 568 conjugate (Molecular probes via Invitrogen, Paisley, UK) along with the nuclear counterstain DAPI (Molecular Probes) for 45 minutes at 37°C. After three 5-minutes washes in PBS, sections were rinsed in water, briefly air-dried and mounted with coverslips. Sections were imaged using Nikon eclipse TE2000-S microscope within 18 hours. Primary antibodies used were anti-collagen, type VII, human (rabbit polyclonal supplied by Calbiochem, Via Merck Chemicals Ltd, Nottingham, UK) and 115F (mouse monoclonal anti desomplakin I and II, a generous gift from David Garrod, University of Manchester, UK) (Parrish *et al.*, 1987). As control, tissue sections were processed in parallel without adding primary antibody. No reactivity for secondary antibodies was observed on control histological sections.

Keratinocyte isolation and culture

Primary keratinocyte cultures were isolated following a standard procedure (Rheinwald, 1989). Briefly, keratinocytes were obtained from biopsies of normal skin and SCC tissue from non-EB and RDEB individuals. After mechanical dissociation, the biopsy fragments were immersed for 1 hour at 37°C in a trypsin-EDTA solution. Then,

the solution was filtered through a 100 μm pore cell strainer (VWR) and medium supplemented with 10% fetal bovine serum was added to neutralized trypsin. Cells were isolated using a centrifuge (5 minutes, 1000 r.p.m.) and the pellet was resuspended in normal keratinocyte medium. Finally, the cells were seeded in T25 flasks containing feeders. The keratinocytes were maintained in DMEM/Ham's F12 medium supplemented with 10% fetal bovine serum, 5 μg/ml transferrin, 0.4 μg/ml hydrocortisone, 10^{-10} M cholera toxin, 10 ng/ml EGF1, 5 μg/ml insulin, and 2×10^{-11} M liothyronine. Fresh feeder cells were added to the keratinocytes twice a week. Feeder cells were NIH 3T3 cells treated with mitomycin (7 μg/ml during 3 hours).

COL7A1 mutation analysis

COL7A1 mutation analysis was performed previously or as described (Whittock *et al.*, 1999; Kern *et al.*, 2006) except in the case of patient RDEB2 where all 118 exons were analyzed by direct sequencing.

Immunoblotting

Keratinocytes were cultured for 2 days in keratinocyte serum free medium (Invitrogen) containing 1.2 mM $CaCl_2$ and 150 μM ascorbic acid. The cells were lysed with a chilled RIPA buffer containing antiproteases. An aliquot of each lysate (22.5 μg for non-EB-SCC1, non-EB-SCC2, RDEB1-SCC1, RDEB2-SCC1, and RDEB6-SCC1, 15 μg for normal human keratinocyte, RDEB3-SCC1, RDEB4-SCC1, and RDEB5-SCC1) was resolved on a 5% SDS-polyacrylamide gel. The fractionated proteins were transferred to Hybond-ECL™ nitrocellulose transfer membrane (Amersham Biosciences, Little Chalfont, UK). The membrane was blocked with 10% non-fat milk-TTBS during 2 hours at room temperature and incubated overnight at 4°C with a purified rabbit polyclonal antibody against Col VII NC1 domain (1/2,000) (Chen *et al.*, 1997). Antibody–antigen complexes were visualized by enhanced chemiluminescence (Amersham Biosciences), according to the manufacturer's instructions. As a loading control, the same membrane was reprobed with a mouse monoclonal antibody against human vinculin (1/10,000) (Sigma, Poole, UK) during 1 hour at room temperature. The exposure times were 5 minutes for collagen VII expression (using ECL+; Amersham Biosciences) and 2 minutes for vinculin expression (using ECL, Amersham Biosciences) for all samples.

mRNA isolation and Q-PCR

Total RNA was isolated from cultured cells using the RNAeasy Minikit according to the manufacturer's instructions (Qiagen, Crawley, UK). cDNA was constructed using standard protocols. Predesigned TaqMan® Gene Expression assays, which analyze the 5′ and 3′ end of the *COL7A1* transcript (Hs01574739_g1 and Hs00982422_g1, respectively), were cycled on the 7900HT as per manufacturer's recommendations (Applied Biosystems, Foster City, CA). Assays were set up in a total volume of 10.5 μl in 384-well clear optical plates (Applied Biosystems) using the Biomek FX liquid handling system (Beckmann Coulter, High Wycombe, UK) and data were analyzed using SDSv2.3 (Applied Biosystems).

Ras mutation detection

DNA was isolated from cultured keratinocytes using Blood & Cell Culture DNA Midi Kit (Qiagen) according to the manufacturer's instructions. Ras gene exons 1 and 2 were amplified either as one PCR product or two as separate fragments for *H-ras*, *K-ras*, and *N-ras*, using the following primers: Hrase × 1F cagatggccctgccagcagc; Hrase × 2R catccaggacatgcgcagag; KRase × 1F ggtactggtggagtatttg; KRase × 1R cagataacttaactttcagc; Krase × 2F gttgttgagttgtatataac; Krase × 2R gcatggcattagcaaagac; N-rase × 1F gtgaggccgatattaatccg; N-rase × 2R ctcatttccccataaagattc. Conditions for *K-ras* exons were as follows: 95°C 2 minutes followed by 34 cycles of 95°C 30 seconds, 55°C 30 seconds, 72°C 45 seconds. Conditions for *H-* and *N-ras* genes were as follows: 95°C 2 minutes followed by 34 cycles of 95°C 30 seconds, 50°C 30 seconds for *N-ras* or 65°C for *H-ras*, 72°C 180 seconds. PCR products were directly sequenced using BigDye™ Terminator Cycle Sequencing Ready Reaction; precipitated products were loaded onto an ABI/PE Biosystems 377 automated sequencer (ABI/PE, Warrington, UK).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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