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Verrucous carcinoma in epidermolysis bullosa simplex is possibly associated with a novel mutation in the keratin 5 gene

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Summary

Epidermolysis bullosa simplex (EBS) is mainly caused by mutations in the KRT5 and KRT14 genes. Squamous cell carcinoma (SCC) represents the second most frequent skin neoplasia with complex aetiology. The molecular events disrupting the orchestrated interplay between the cytoskeleton, cell adhesion molecules and signalling proteins are ill understood in SCC. We describe the molecular background and the unusual course of the disease in a patient with EBS Dowling–Meara, severe keratoderma and a massive verrucous carcinoma. Skin and tumour samples from the patient were analysed using light microscopy, immunohistochemistry and immunofluorescence mapping. Mutation analysis of the KRT5 and KRT14 genes identified the novel KRT5 mutation p.E477D. Invasive tumour areas were characterized by downregulation of keratins 5 and 14, reduced and irregular desmocollin-2 expression and increased expression of keratins 6, 16 and 17. Levels of Ki-67 were increased and levels of E-cadherin strongly reduced in the tumour tissue. In this case a novel KRT5 mutation led to increased fragility of keratinocytes. Desmosome and adherens junctions were destabilized, which may trigger keratinocyte-mediated inflammation, possibly via p120-catenin-dependent signalling, suggesting a link between a keratin mutation and SCC, which adds weight to the hypothesis that disturbance of the cytoskeleton represents a major cause in the appearance of the malignant phenotype. Some individuals with EBS may be at risk of developing secondary SCC.

Epidermolysis bullosa simplex (EBS) represents a group of inherited skin disorders characterized by intraepidermal blistering.[1] Mutations in the KRT5 and KRT14 genes coding for keratin 5 (K5) and keratin 14 (K14) cause most EBS cases, but rare EBS mutations have been disclosed in at least seven other genes.[1–3] Of all the EBS subtypes caused by dominant keratin mutations, EBS Dowling–Meara (EBS-DM) represents the most severe subtype, usually present at birth or developing in early infancy. Clinical hallmarks include circinate clustering of blisters, mucosal blistering and palmoplantar keratoderma. The course of the disease may improve in later childhood or in adult life. Keratoderma at the palms and soles may be accompanied by painful fissures, and impede walking and normal hand function.

Keratin intermediate filament proteins consist of an α-helical rod domain, flanked by head and tail domains, and form intermediate filaments in epithelia from heterodimers of a type I and a type II keratin subunit. Most mutations in EBS-DM are located at the amino or carboxy end of the central rod. The KLLEGE domain is a highly conserved motif in different species and is located at the end of the coil 2B α-helical rod domain. Mutations in KRT5 or KRT14 affect the integrity of the cytoskeleton and cause formation of keratin aggregates in severe disease forms. As a result, basal keratinocytes cytolyse, accompanied by continuous cycles of tissue renewal and disruption.

Causative EBS mutations can be amino acid substitutions, premature stop codons or frame-shift mutations.[4] Despite an increasing number of mutations reported in this patient group, no clear genotype–phenotype correlations can be made, as the same mutation can be found in mild localized EBS, generalized EBS and in EBS-DM.[4–7] This suggests the contribution of modifier loci.

It is well established that mechanical trauma is the main cause for blistering in the EBS epidermis, which is weakened by the expression of mutant keratins. The development of keratoderma and skin inflammation in EBS-DM underlines the fact that inflammatory processes and altered cell proliferation add to the structural weakness of the cytoskeleton. Additional pathomechanisms have emerged, initially from studies in animal models, indicating that proinflammatory cytokines are involved.[8,9] In EBS, in addition to mutant keratins, altered cytokine signalling and recruitment of distinct immune effector cells contribute to the EBS phenotype.[10]

Patients with junctional and dystrophic epidermolysis bullosa subtypes have an increased risk of developing SCC, and a case of recessive EBS associated with SCC of the tongue has been reported.[11,12] However, the general risk of developing SCC in EBS subtypes has been estimated to be the same as in the general population.[12] Here we report the case of a patient with EBS-DM with severe blistering, keratoderma and the development of a massive verrucous carcinoma (VC) on the base of a plantar keratoderma. The underlying mutation is located in the highly conserved KLLEGE motif of K5. To the best of our knowledge, such a constellation has not previously been reported. In the tumour the distribution of keratins, desmosomal proteins, matrix metalloproteinase-7 (MMP-7) and E-cadherin were found to be modified.

Case report

Soon after birth the patient started to develop blistering after mechanical stress, primarily on the hands, feet and lower legs. Episodes of circinate blistering on the trunk and the extremities were noted from early childhood. At the age of 5 years the patient developed keratoderma on the palms and soles. As an adult, keratoderma, appearing as verrucous epidermal tumours, developed on the lower legs and on both arms (Fig. 1). The patient's parents and a half-brother were free of skin features.

A slowly growing verrucous tumour on the left ankle increased gradually during the patient's mid-thirties. Carbon dioxide laser treatment was performed, but new keratodermas developed gradually. Acitretin was prescribed over a 5-year period, but the drug was taken irregularly. Numerous biopsies were obtained but only when the patient was 41 years old did they reveal a massive malignant VC infiltrating the left foot and the lower leg. A computer tomography scan indicated possible bone infiltration. Widespread wounds were present on the proximal lower leg. An above-knee amputation of the left leg was performed. Inguinal ultrasound examination showed enlarged lymph nodes suspicious of tumour infiltration, but subsequent lymph node dissection revealed no lymphatic metastasis.

To prevent the right leg from malignant tumour development, removal of benign verrucous tumours was performed several times, but recurrence was found within a few months.

Materials and methods

The ethics committee of the University Freiburg approved the study. After informed consent, skin samples and ethylenediaminetetraacetic acid (EDTA)-blood were obtained from the patient.

Tissues

The tissue material consisted of formalin-fixed, paraffin-embedded samples and frozen tissue from: (i) blistering skin (sample 1); (ii) verrucous epidermal hyperplasia (Fig. 1b,c, sample 2); and (iii) VC samples from the left lower leg (Fig. 1a, samples 3 and 4). Skin biopsies of blistered skin were additionally analysed by transmission electron microscopy (TEM) using standard methods.[5]

Immunohistochemistry and immunofluorescence microscopy

The paraffin-embedded samples were pretreated with antigen retrieval buffer, pH 6 (Dako, Hamburg, Germany) for 10 min. The primary antibody to E-cadherin (clone 36B5, Novocastra Laboratories, Newcastle, U.K.), targeting the N-ter-

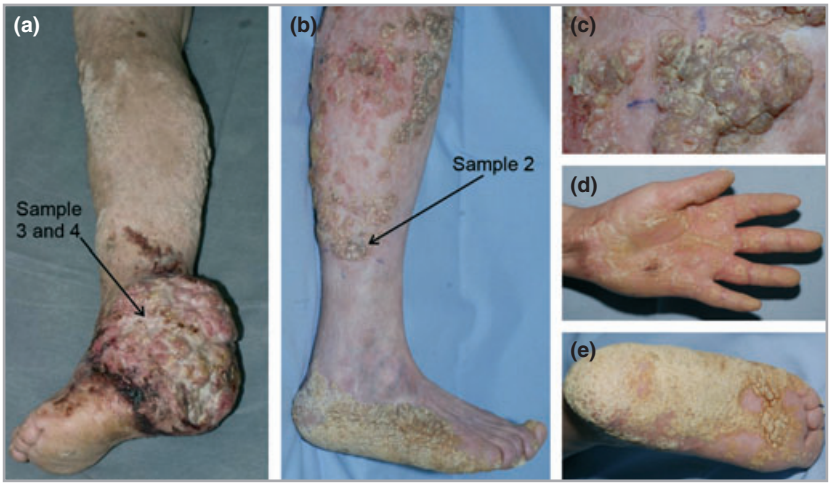

Fig 1. Clinical presentation at the age of 42 years. Verrucous carcinoma in succession to keratoderma on the left foot (a, samples 3 and 4), verrucous tumours and blisters on the right leg (b, sample 2), verrucous tumour/epidermal hyperplasia on the right lower leg (c), keratoderma on the left hand (d), and on the right foot (e).

minal external domain, was diluted 1 : 25 in antibody diluent (Dako). Immunolabelling was performed on tissue sections using ready-to-use streptavidin peroxidase (BioGenex, Fremont, CA, U.S.A.). 3-Amino-9-ethylcarbazol (Dako) was used as a chromogen, and Mayer's haematoxylin was applied as a light counterstain. To determine the level of skin cleavage, immunofluorescence mapping of blistered skin was performed as described previously.[13]

Extended immunofluorescence analyses of the tumour-affected skin were carried out as described, using antibodies as listed in Table S1 (see Supporting Information).[10] Images were acquired with an Axioplan 2 fluorescence microscope (Carl Zeiss, Göttingen, Germany) equipped with Zeiss Plan-Apochromat 63×/1·4 oil and Plan-Neofluar 40×/1·3 oil immersion objectives, and were recorded with an AxiocamHR camera (Carl Zeiss). Image analysis and processing were performed using the AxioVision 4.6 software (Carl Zeiss). Confocal images were recorded on an LSM710 microscope equipped with a Zeiss 63× LCI Plan Neofluar objective (Carl Zeiss, Jena, Germany). Image analysis and processing were performed with the Zen software (Carl Zeiss). Images were cropped and analysed in Adobe Photoshop CS4 (Adobe, San Jose, CA, U.S.A.); Adobe Illustrator CS4 was used for figure design.[10]

Mutation detection

Genomic DNA was extracted from EDTA-blood, and all *KRT5* and *KRT14* exons and exon–intron boundaries were amplified and sequenced as described.[4] The mutation was confirmed by resequencing. The missense mutation p.E477D in the *KRT5* gene was excluded in 100 control chromosomes by direct sequencing. Tumour material dissected from the paraffin-embedded tissue was used to extract DNA for mutation analysis of exon 1 of *KRAS* as described.[14]

Results

KRT5 and *KRAS* mutation analysis

A novel mutation in the *KRT5* gene c.1431G>C, p.E477D, was identified in a heterozygous state (Fig. 2). No further mutations were found in *KRT5* and *KRT14*. Mutation analysis of tumour material (samples 3 and 4) disclosed no mutation in exon 1 of the *KRAS* gene.

Morphological analyses of the skin and tumour

EBS was confirmed by immunofluorescence mapping, light microscopy and TEM. TEM revealed cytolysis in the basal layer with clumped keratin filaments in basal keratinocytes, showing a reduced density and appearing as lacuna-like structures typical of EBS-DM. The dermal–epidermal junction zone, with regular hemidesmosomes and anchoring fibrils, appeared normal (not shown). Intraepidermal split formation (sample 1) was detected by immunofluorescence microscopy in blistered but otherwise unaffected skin (not shown).

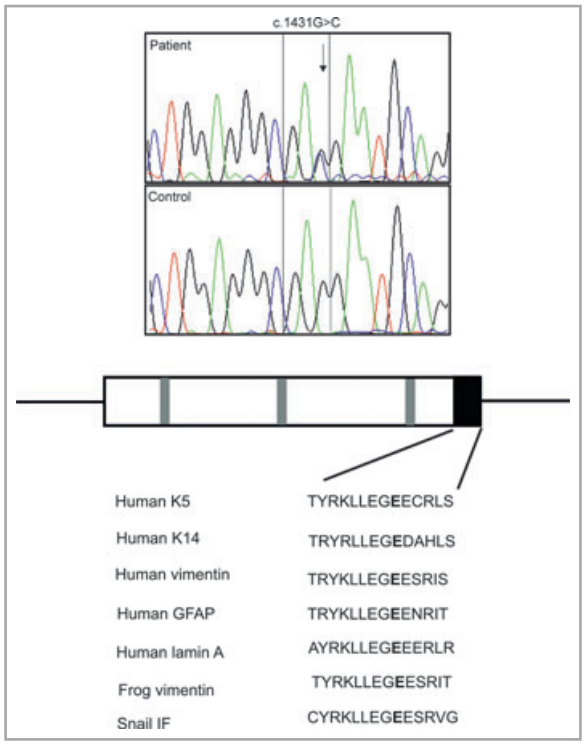

Fig 2. The *KRT5* mutation and the conserved KLLEGE motif. The mutation c.1431G>C in the *KRT5* gene causing the amino acid substitution p.E477D (upper panel), graphic representation of K5, showing the highly conserved KLLEGE motif at the end of the 2B domain in the central α-helical rod in black (middle). Evolutionary comparison of KLLEGE sequences in additional intermediate filament proteins reveals conservation of -E- at position 477 (lower panel).

Several skin samples were assessed for human papillomavirus (HPV) DNA in three different specialized laboratories with primer sets covering all currently known pathogenic HPV subtypes. The analyses did not detect HPV DNA in any sample.

Haematoxylin and eosin-stained samples 3 and 4 showed irregular hyperplasia (Fig. S1a, b, see Supporting Information) in the upper epidermal part of the tumour samples. The lower part of the sample at the invasive tumour front revealed a highly differentiated VC (Fig. S1c, d). The staining of the acanthotic epidermis in the upper part of the tumour (sample 4) showed increased K5 and K14 staining in the upper epidermal layers (Fig. S2b, e, h, see Supporting Information), in agreement with a hyperthickened and hyperproliferative epidermis. Compared with normal human skin, where K5 and K14 expression was strong in the basal and lower spinous layer (Fig. S2a, d, g), the patient's skin revealed a reduced overall intensity. Specifically, the invasive tumour front revealed very low keratin staining (Fig. S2c, f, i). Outside the blister areas, the overall organization of the keratin cytoskeleton appeared normal, without detectable aggregates.

A spectrum of antibodies was employed to characterize the epidermis in affected and nonaffected skin areas. The staining results are summarized in Table S1. E-cadherin showed

pericellular staining in the blistered skin of the patient (Fig. 3b, sample 1), similar to the epidermis of normal human skin (Fig. 3a). In sample 2 with keratoderma (Fig. 3c), the signal was reduced, in particular in the central tumour areas (Fig. 3d, sample 3). In the normal epidermis, E-cadherin antibodies recognizing the extracellular and intracellular domains revealed strong staining (Fig. S3a, d, see Supporting Information), whereas in the tumour areas with irregular hyperplasia, markedly reduced or irregular staining (Fig. S3b, e, respectively) was detected. Tumour segments displaying invasive growth were characterized by weak staining with both antibodies (Fig. S3c, f).

Desmocollin-2 and desmoplakin were localized to the plasma membrane of keratinocytes in all layers of the verrucous tumour (Fig. 4b, e, h), similar to normal human skin (Fig. 4a, d, g). However, in invasive tumour areas, desmocollin-2 staining was reduced and irregular, with increased cytoplasmic staining, indicative of partial redistribution (Fig. 4c, f). Desmoplakin showed irregular membrane staining and diffuse cytoplasmic distribution (Fig. 4i). Pericellular p120-catenin staining was found in the normal epidermis (Fig. 4j), but a reduced signal was detected in the upper part of the tumour with irregular epidermal hyperplasia (Fig. 4k, sample 3), and in central tumour areas (Fig. 4l, sample 4).

The distribution of integrins was unaltered. Typically, β1- and β4-integrin subunits were restricted to the basolateral membrane regions in both normal skin and the tumour of the patient.

In the normal epidermis the expression of MMP-7 was very low (Fig. S4a, d, see Supporting Information), but was focally increased in areas with irregular epidermal hyperplasia (sample 3, Fig. S4b, e) and strongly increased in invasive tumour areas (sample 4, Fig. S4c, f).

The expression of keratin 6 (K6), keratin 16 (K16) and keratin 17, indicative of hyperproliferation, was low and restricted to basal keratinocytes in normal skin (Fig. S5a, c, see Supporting Information). In contrast, it was clearly increased in invasive tumour regions (Fig. S5b, d). In line with these findings, the proliferation marker Ki-67 showed increased expression in invasive tumour areas (Fig. S5f), compared with the normal skin. Staining of B-cell lymphoma 2 (BCL2), an antiapoptotic regulator, was nearly absent in the tumour (Fig. S5h), but positive in normal skin (Fig. S5g).

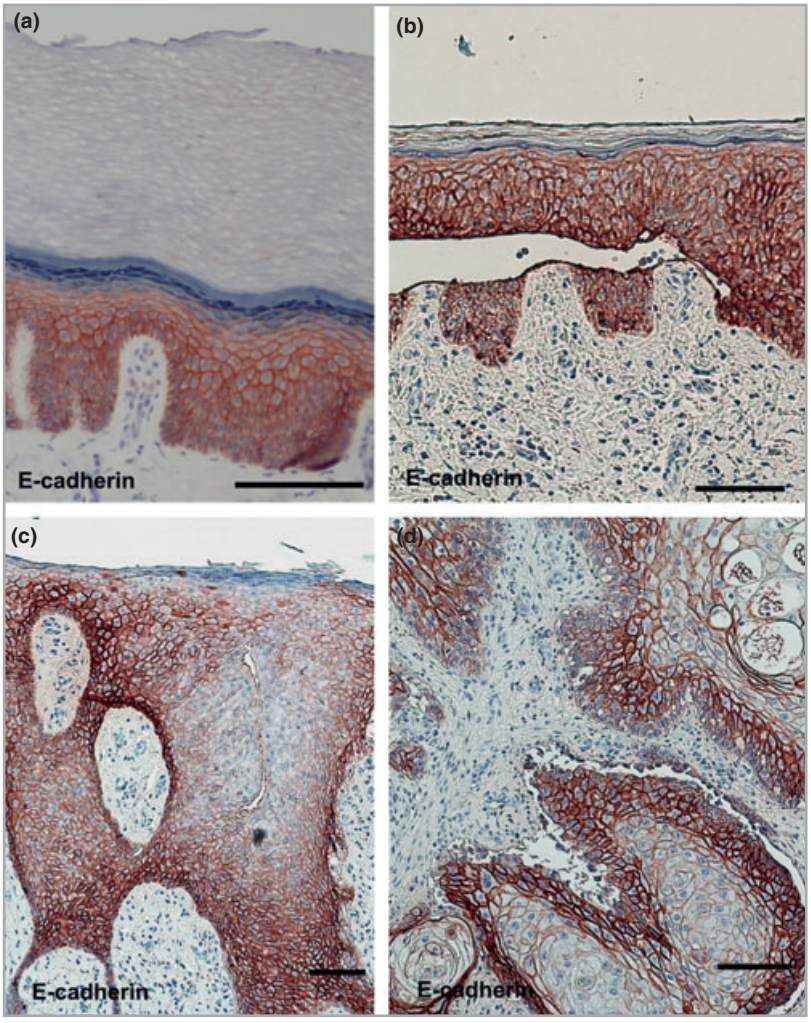

Fig 3. Reduced E-cadherin in invasive tumour areas. Pericellular staining in the epidermis of normal human skin (a) and a similar picture in blistering skin of the patient (b, sample 1); a reduced signal was detected in the upper part of the tumour with irregular epidermal hyperplasia (c, sample 3); note the near absence of staining in central tumour areas (d, sample 4). Scale bar: 50 μm.

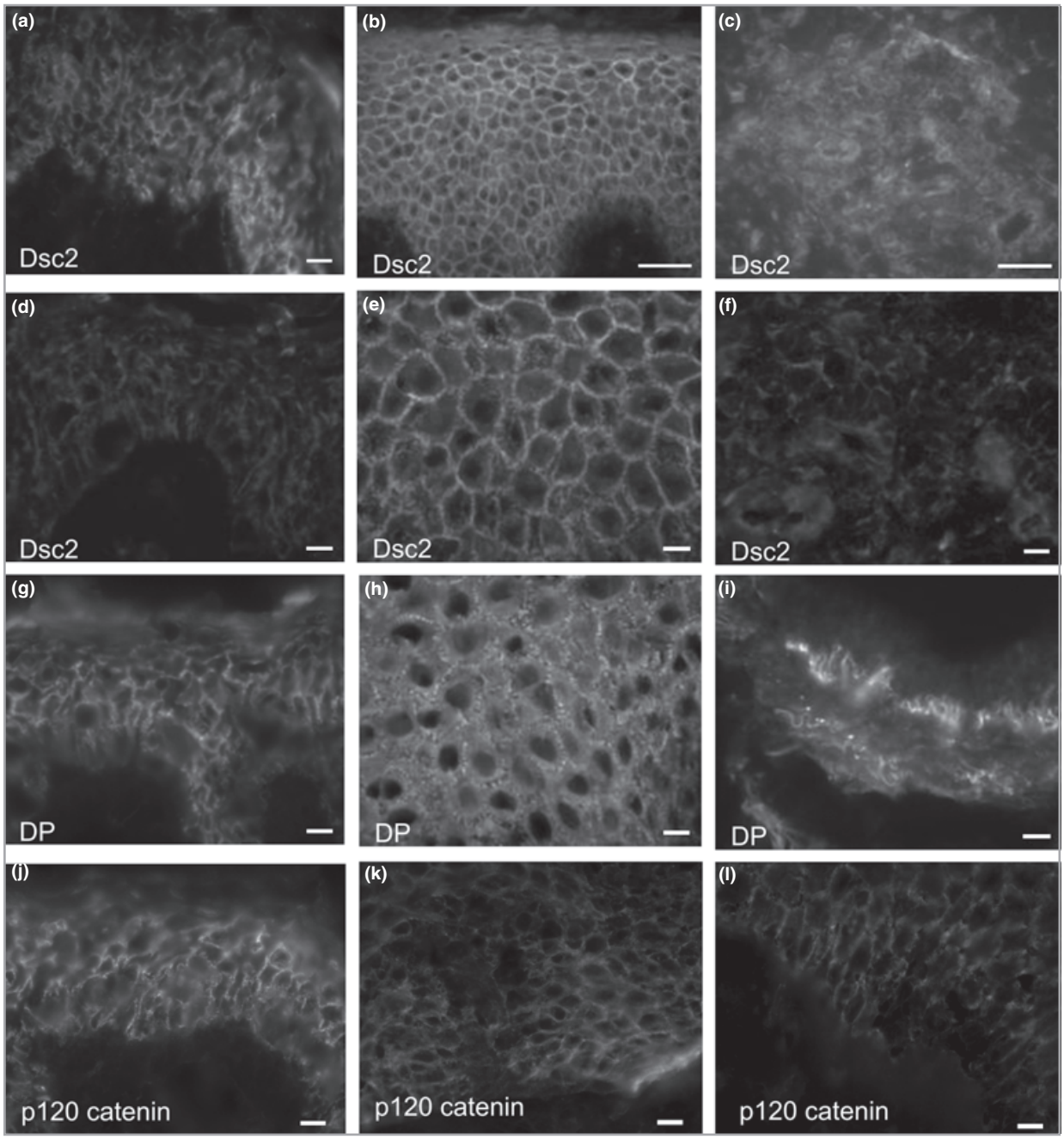

Fig 4. Expression of desmosomal proteins. Desmocollin-2 (Dsc2) and desmoplakin (DP) localize at the plasma membrane of keratinocytes in all layers in normal human skin (a, d, g), and showed a similar pattern in the verrucous upper part of the tumour (b, e, h, sample 3). However, in invasive tumour areas desmocollin-2 staining was reduced and irregular, with an increased cytoplasmic staining, indicative of a partial redistribution (c, f), and desmoplakin showed irregular membrane and diffuse cytoplasmic distribution (i, sample 4). Pericellular p120-catenin staining in the epidermis of normal human skin (j) and a reduced signal in the upper part of the tumour with irregular epidermal hyperplasia (k, sample 3), and in central tumour areas (l, sample 4). Scale bar: 50 μm (a–c, j–l); 10 μm (d–f).

Discussion

Here we report the occurrence of a massive VC in a patient with EBS-DM and severe keratoderma and verrucous hyperplasia. A new heterozygous *KRT5* mutation (p.E477D) was disclosed, located in the KLLEGE motif at the end of the 2B domain in the central α-helical rod. Missense mutations in the highly conserved helix boundary motifs of K5 and K14 are most disruptive for keratin filament organization and often cause severe EBS phenotypes.[4,15] However, the type of amino acid exchange at the same position in K5 and K14 can result in variable disease severity, as mutations at amino acid posi-

tion 477, as in this case, were reported both in localized EBS (p.E477G) or severe general EBS-DM (p.E477K, http://www.interfil.org).[4,6,15–19] The presence of apparently normal keratin filaments in nonlesional keratinocytes is compatible with the fact that the ratio between mutant and intact keratins determines the extent of filament vs. aggregate formation. Factors affecting this ratio are not well understood.

Based on our findings, we suggest that the E477D mutation affects the integrity of the epidermal cytoskeleton with subsequent weakening of intercellular adhesion, possibly accompanied by local inflammation. The latter is compatible with elevated cytokine levels in an EBS mouse model and patient skin.[10] Furthermore, Liovic *et al.*[20] showed that keratinocytes with *KRT5* or *KRT14* mutations activated stress-signalling pathways (mitogen-activated protein kinases), in correlation with the EBS severity. The irregular or reduced presence of E-cadherin in the upper part of the tumour and its near absence in central tumour areas are consistent with reduction and mislocalization of p120-catenin (Figs 3, 4 and S3).[21,22] The loss of E-cadherin during tumour progression is a well-known phenomenon, and reduced E-cadherin expression was shown to increase epithelial cell invasiveness, dedifferentiation and metastasis in human carcinomas.[23,24]

In invasive tumour areas, a strong increase in MMP-7 (Fig. S4), compared with normal human epidermis became evident, and expression of K6 and K16 was elevated (Fig. S5d). Together with the promigratory function of these keratins, elevated MMP-7 levels might initiate a cascade of enhanced migration and invasion.

Downregulation of desmosomal proteins may occur first in adherens junctions to drive tumour development and early invasion, suggesting a two-step model of adhesion dysfunction in cancer progression.[25] In a cell culture model of EBS, transcriptional downregulation of desmosomal proteins was previously reported.[20] Here, we found reduced or irregular desmocollin-2 staining in invasive tumour areas, indicative of a partial redistribution. At the same time, desmoplakin staining appeared increased. Reduced expression or altered distribution of desmosomal proteins conceivably weakens cell junctions, which may alter cell behaviour and predispose EBS skin to neoplasia.

This study revealed a strongly reduced BCL2 signal in invasive tumour areas. Apart from poor prognosis and a tendency of some cancers lacking BCL2 expression to metastasize, a recent study revealed that loss of BCL2 resulted in enhanced motility in mouse embryonic fibroblasts, possibly via regulation of cell adhesion involving complex formation and actin polymerization.[26] Furthermore, increased Ki-67 in invasive tumour areas compared with normal skin reflects high cell proliferation. High expression of Ki-67 can be associated with a poor prognosis. A connection to HPV infection, a well-known predisposing factor for epithelial cancer, or to *KRAS* mutations, was ruled out in this case.[27]

While the causal role of *KRT5* and *KRT14* mutations in EBS is undisputed, there is compelling evidence for additional pathomechanisms contributing to the EBS phenotype. Epidemiological studies have not reported an increased risk for SCC in EBS; however, the cumulative risk of developing basal cell carcinoma (BCC) was increased in EBS-DM.[12] The *KRT5* p.G138E mutation affects susceptibility to BCC,[28] and one previous case of recessive EBS resulting from a *KRT14* mutation (p.E392X) was associated with a SCC of the tongue.[11] The notably severe keratoderma in this case might well be related to the specific amino acid substitution caused by the novel mutation, and inflammation and hyperproliferation might pave the way to tumour pathogenesis in this case. Other EBS-DM patients may therefore be at risk of developing SCC secondary to keratoderma or verrucous lesions. Close clinical monitoring is recommended in these cases.

What's already known about this topic?

- Epidermolysis bullosa simplex Dowling–Meara (EBS-DM) is caused by mutations in the *KRT5* or *KRT14* genes disrupting the keratin cytoskeleton.
- Verrucous carcinoma (VC) represents a rare variant of a squamous cell carcinoma, one of the most common cancers in humans.

What does this study add?

- EBS-DM caused by a novel *KRT5* mutation p.E477D, associated with severe keratoderma and VC, is presented.
- A possible link between a novel keratin mutation, mechanical stability and keratinocyte-mediated inflammation and VC is discussed.

Acknowledgments

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Supporting Information

Additional Supporting Information may be found in the online version of the article:

Fig. S1. Light microscopy of the tumour. A whole view of a sample from the excised tumour shows irregular epidermis hyperplasia in the upper part and an invasive tumour area detached from the rest due to processing in the lower part (a, sample 3). Magnification of the upper part with the irregular epidermis hyperplasia of the tumour (b) and invasive tumour in the lower part (c, d, sample 4).

Fig. S2. Immunostaining of keratin 5 (K5) and keratin 14 (K14). K5 and K14 staining revealed a reduced intensity in the patient's skin samples from the upper part of the tumour with irregular epidermal hyperplasia and an increased intensity in the upper strata (b, e, h, sample 4). As a control, normal human skin with strong K5 and K14 expression in the basal epidermis persistent to the spinous and lower granular layer is shown (a, d, g). Downregulation of both keratins in sections from the invasive tumour front (c, f, i, sample 4); note the normal organization of the keratin cytoskeleton without aggregates. Scale bar: 50 μm.

Fig. S3. Extracellular and intracellular E-cadherin in invasive tumour areas. Extracellular E-cadherin (detected with the antibody DECMA-1) in the upper panel vs. intracellular E-cadherin in the lower panel (detected with the antibody EP700Y); left: normal human epidermis shows strong staining (a, d), middle: upper part of the tumour with irregular epidermal hyperplasia staining is reduced (b) or irregular (e, sample 3), right: the lower part of the tumour with invasive growth shows strongly reduced staining (c, f, sample 4). Nuclei are stained in blue with 4′,6-diamidino-2-phenylindole.

Fig. S4. Increased matrix metalloproteinase-7 (MMP-7) expression in invasive tumour. Weak MMP-7 staining in control skin (a, d). In the upper part of the tumour with irregular epidermal hyperplasia the MMP-7 staining signal is increased (b, e, sample 3) and strongly increased in invasive tumour areas (c, f, sample 4). Scale bar: 50 μm, upper panel; 10 μm, lower panel.

Fig. S5. Hyperproliferation and reduced antiapoptotic regulator. In normal human skin, expression of keratin 6, keratin 7, keratin 17 and Ki-67, indicative of hyperproliferation, was low and restricted to basal keratinocytes (a, c, e). In contrast, expression was highly increased in invasive tumour regions (b, d, f sample 4). The signal of B-cell lymphoma 2, an antiapoptotic regulator, was found strongly reduced to negative in the tumour (h, sample 4) compared with normal human skin (g). Scale bar: 10 μm, upper four pictures; 50 μm, lower four pictures.

Table S1. Antibodies used for immunofluorescence analyses and the results of the staining.

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