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Title page

Reduction of keratin aggregates in epidermolysis bullosa simplex keratinocytes after pretreatment with trimethylamine N-oxide

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Key words: Epidermolysis bullosa simplex, keratin aggregates, TMAO, 4-PBA.

BACKGROUND

Epidermolysis bullosa simplex (EBS) is a dominantly inherited skin disease caused by mutations in the keratin 5 (*KRT5*) or *KRT14* genes (1). Some reports suggested that fever and/or hot weather may exacerbate EBS phenotype (2). Effective EBS therapies are still lacking. Molecular chaperones are proteins whose main function is to promote the correct folding of polypeptides (s1). Molecules such as trimethylamine N-oxide (TMAO) and sodium 4-phenylbutyrate (4-PBA) act as chemical chaperones (s2) with protein folding and stabilization activities (s3, s4, s5). Treatment of affected epidermal cells by chemical chaperones to correct the misfolded and aggregated keratins that characterize EBS seems a viable therapeutic option (3). Furthermore, the type I keratins K16 and K17 polymerize with K5, and upregulation of these proteins could replace the mutant K14 in the heterodimer and improve disease pathology (s6). Hence, chemical chaperones which can reduce keratin aggregates formation and upregulate K16 and K17 in EBS affected cells would be ideal therapeutic candidates for EBS.

QUESTIONS ADDRESSED

Investigated the potential therapeutic effects of two chemical chaperones, TMAO and 4-PBA, in the treatment of EBS by comparing keratin cytoskeleton abnormalities in cells of EBS patients and non-EBS participants in a heat shock model; an ideal model as EBS patients usually suffer from disease exacerbation in a hot and humid climate (2).

EXPERIMENTAL DESIGN

See supplementary information.

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RESULTS

TMAO reduced heat-induced keratin aggregates

To determine if TMAO or 4-PBA had a beneficial effect on EBS keratinocytes, cytoplasmic keratin aggregates were evaluated by immunofluorescence. Unstressed or heat stressed CTRL cells showed very few or no keratin aggregates (Fig. 1a, b). Of the four cell lines, EBS21 cells had the highest increase in the percentage of cells with aggregates (42.5%) after heat shock (Fig. S1). EBS21 keratinocytes showed keratin aggregates in resting conditions (Fig. 1c) and transient heat-shock led to a collapse of the keratin cytoskeleton and an increase of keratin aggregates (Fig. 1d). Pre-treatment of EBS21 cells with TMAO at 150mM prior to heat-shock reduced keratin aggregation (Fig. 1e). Percentage of cells with aggregates decreased from 42.5% (after heat shock) to 19.5% ($p=0.03$). Replication assays showed a significant reduction of cells with aggregates in EBS10 ($p=0.002$) but not in EBS1 (Fig. S1). Non-significant aggregates reduction was observed in the three EBS cells with pre-treatment of 4-PBA at 10mM (data not shown).

TMAO decreased Hsp70 and Hsp40 protein expression in heat shocked EBS cells

Protein levels of Hsp70 and Hsp40 were assessed by Western blot to determine if pre-treatment with TMAO affected their levels in EBS21 and CTRL cells. Our data revealed that Hsp70 was 5.5 fold more expressed in unstressed condition in EBS21 compared to CTRL and that Hsp40 was not induced (Fig. 2). Heat shock or pre-treatment with TMAO alone had no effect on Hsp70 or Hsp40 levels. Pre-treatment with TMAO in heat shocked EBS21 cells reduced Hsp70 and Hsp40 expression significantly compared to unstressed EBS21 cells ($p=0.03$ and $p=0.05$ respectively) (Fig. 2b, d).

KRT16 and *KRT17* expression after TMAO pre-treatment

Pre-treatment of TMAO in unstressed EBS21 cells did not significantly change *KRT16* and *KRT17* gene expression from those without pretreatment. Similar results were observed for control cells (data not shown).

CONCLUSION

Our data demonstrated a promising potential for TMAO as a therapeutic agent for EBS patients. In this study, TMAO (150mM) partially reduced the percentage of heat-shocked EBS cells with aggregates, although it was not able to eliminate all aggregates. TMAO may prevent aggregate formation by restoring misfolded proteins (4) or may promote protein degradation of misfolded proteins through the ubiquitin-proteasome pathway (5).

The increase in Hsp70 protein expression observed in unstressed EBS21 as compared to control cells may be due to other genetic variations than the *KRT14* mutation. However, the reduction of Hsp70 and Hsp40 protein level by TMAO and heat shock was not observed in control cells suggesting that the mutation is associated with an environment in which TMAO functions beneficially. Our data of TMAO reducing Hsp70 and Hsp40 protein levels is in line with results observed in red blood cells (6). A reduced expression of molecular chaperones by TMAO in cells under stress may be explained by a preventing effect of TMAO from heat shock damages, reducing the need to elicit the cellular heat shock response (6).

In contrast to our findings, TMAO treatment at 50 and 100mM in two cell lines with *KRT5* mutations had been shown to induce moderate upregulation of Hsp70 protein expression (5). These differences suggest that the mechanism of action of TMAO may be dependent on the mutations of the affected cells (7,8) or on the concentration of TMAO (5).

KRT16 and *KRT17* gene expression results shown no differences in contrast to the increase of K16 and K17 observed in a sulforaphane-treated K14-null mice (9). May be gene knock-out will elicit more pronounced biological consequences than a mutated gene.

Although TMAO demonstrated itself as a promising therapeutic candidate for EBS with its thermo stress protective role, particularly in those with mutations in *KRT5* and *KRT14*, it is worthy to mention that reduction of heat induced aggregates in EBS cells by TMAO could simply be with no therapeutic value and no potential benefit to EBS patients.

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Further mechanistic studies are needed to relate keratin aggregates to stronger and more resilient skin.

Acknowledgements:

We thank the participants in EBS biobank. This work was supported by Le Groupe Riverin Inc and by La Fondation du Grand Défi Pierre Lavoie.

Author contributions:

MB contributed to experiment design, performed the experiments with JL, TF and ML. MB and JL wrote the manuscript. JP, CM, AD, and CM performed skin biopsies. VLG contributed to study design and manuscript. CL leads the research program, built the biobank, designed and supervised the study and manuscript redaction.

Ethical approval:

All participants provided written informed consents. The study was approved by the Ethics committee from the Hôpital Sainte-Justine and from the Centre intégré universitaire de santé et de services sociaux du Saguenay–Lac-Saint-Jean. The Declaration of Helsinki protocols were followed.

Conflict of Interest: No conflict of interest to declare.

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References

1 Loffek S, Woll S, Hohfeld J, *et al*. The Ubiquitin Ligase CHIP/STUB1 Targets Mutant Keratins for Degradation. Hum Mutat 2010: 31: 466-476.

2 Morley S M, Dundas S R, James J L, *et al*. Temperature sensitivity of the keratin cytoskeleton and delayed spreading of keratinocyte lines derived from EBS patients. Journal of cell science 1995: 108 (Pt 11): 3463-3471.

3 Werner N S, Windorffer R, Strnad P, *et al*. Epidermolysis bullosa simplex-type mutations alter the dynamics of the keratin cytoskeleton and reveal a contribution of actin to the transport of keratin Subunits. Mol Biol Cell 2004: 15: 990-1002.

4 Bennion B J, Daggett V. Counteraction of urea-induced protein denaturation by trimethylamine N-oxide: a chemical chaperone at atomic resolution. Proceedings of the National Academy of Sciences of the United States of America 2004: 101: 6433-6438.

5 Chamcheu J C, Navsaria H, Pihl-Lundin I, *et al*. Chemical Chaperones Protect Epidermolysis Bullosa Simplex Keratinocytes from Heat Stress-Induced Keratin Aggregation: Involvement of Heat Shock Proteins and MAP Kinases. J Invest Dermatol 2011: 131: 1684-1691.

6 Kolhatkar A, Robertson C E, Thistle M E, *et al*. Coordination of chemical (trimethylamine oxide) and molecular (heat shock protein 70) chaperone responses to heat stress in elasmobranch red blood cells. Physiological and biochemical zoology : PBZ 2014: 87: 652-662.

7 Wagner M, Trost A, Hintner H, *et al*. Imbalance of intermediate filament component keratin 14 contributes to increased stress signalling in epidermolysis bullosa simplex. Exp Dermatol 2013: 22: 292-4.

8 Hamada T, Tsuruta D, Fukuda S, *et al*. How do keratinizing disorders and blistering disorders overlap? Exp Dermatol 2013: 22: 83-7.

9 Kerns M L, DePianto D, Dinkova-Kostova A T, et al. Reprogramming of keratin biosynthesis by sulforaphane restores skin integrity in epidermolysis bullosa simplex. P Natl Acad Sci USA 2007: 104: 14460-14465.

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Figures Legends

Fig. 1 Reduction of keratin aggregates in EBS21 derived keratinocytes by TMAO. Intermediate filaments were stained with an anti-K5 antibody in unstressed (a) and heat-stressed CTRL keratinocytes (b), unstressed (c) and heat-stressed EBS21 keratinocytes (d), and TMAO pretreated heat-stressed EBS21 keratinocytes (e). Keratin aggregates are marked with arrows. Scale bars=10µm.

Fig. 2 TMAO reduced Hsp70 and Hsp40 protein levels in heat-stressed EBS21 derived keratinocytes. A representative western blot (WB) revealed with anti-Hsp70 (a) and anti-Hsp40 (c) antibodies comparing cell extracts from CTRL and EBS21 derived keratinocytes (-/+) stress and/or (-/+) TMAO as indicated. Graphs show Hsp70 (b) and Hsp40 (d) fold difference (FD) compared to unstressed CTRL, for CTRL and EBS21 keratinocytes in unstressed (US), unstressed and pretreated with TMAO (US+TMAO), heat-stressed (HS) and heat-stressed and pretreated (HS+TMAO) conditions. Results represent the mean $\pm$ SD for Hsp70 (n=3) and for Hsp40 (n=4) independent experiments. Statistical significance was set at $p \leq 0.05$.

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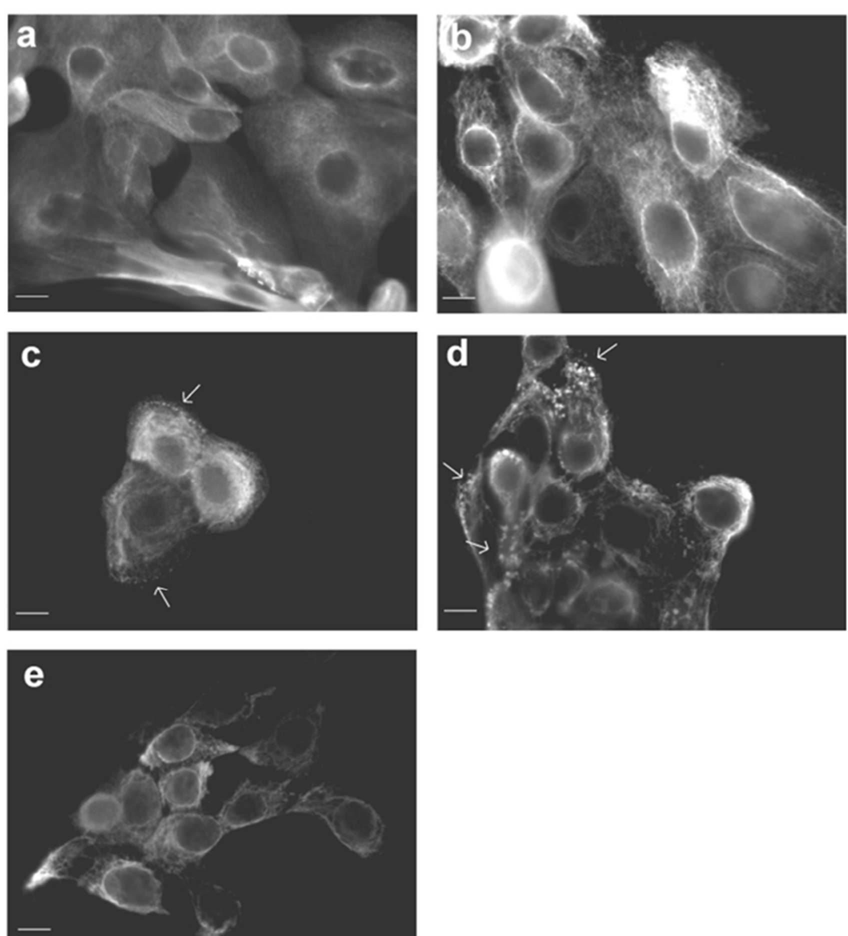

Fig. 1 Reduction of keratin aggregates in EBS21 derived keratinocytes by TMAO. Intermediate filaments were stained with an anti-K5 antibody in unstressed (a) and heat-stressed CTRL keratinocytes (b), unstressed (c) and heat-stressed EBS21 keratinocytes (d), and TMAO pretreated heat-stressed EBS21 keratinocytes (e). Keratin aggregates are marked with arrows. Scale bars=10μm.
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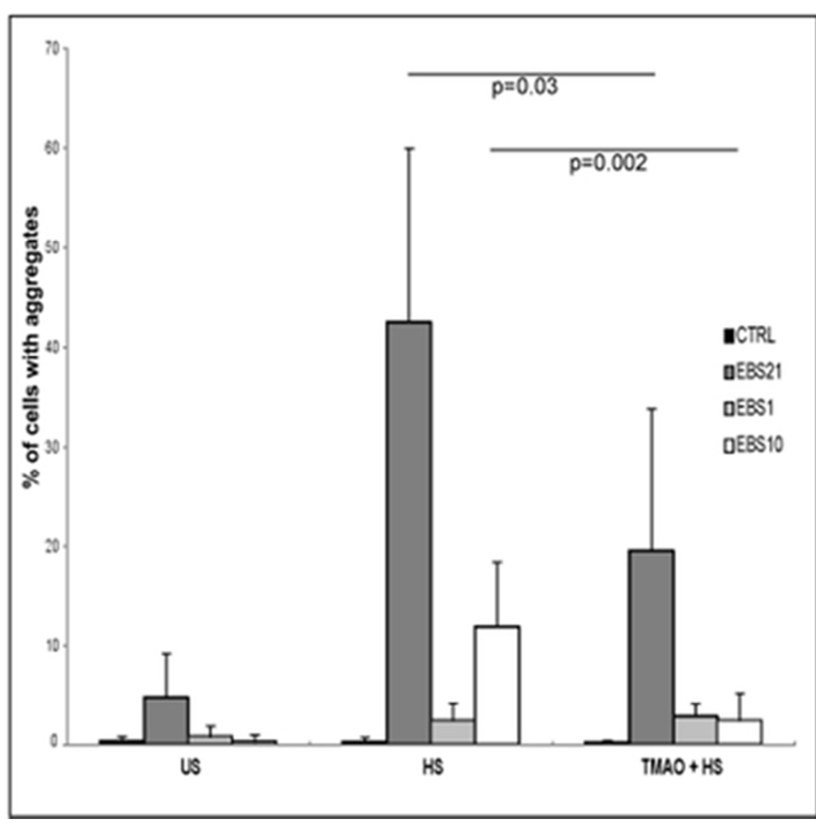

34 Fig. S1 Quantification of keratin aggregates in EBS keratinocyte cell lines. Graph depicts the percentage of
35 keratinocytes showing keratin aggregates in CTRL, EBS21, EBS1 and EBS10 unstressed (US), heat-stressed
36 (HS) or TMAO pretreated keratinocytes with heat-stress (TMAO+HS). Results represent the mean + SD of
37 three independent experiments (500 cells were counted for each experiment). Statistical significance was
38 set at $p \leq 0.05$.
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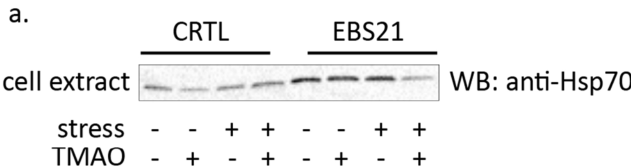

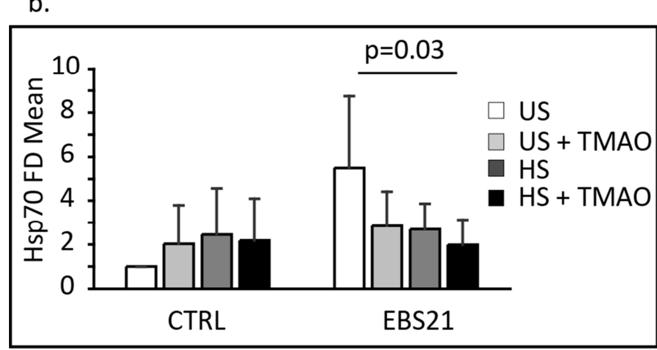

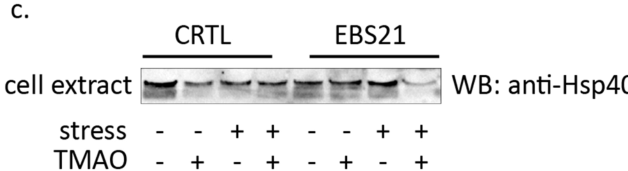

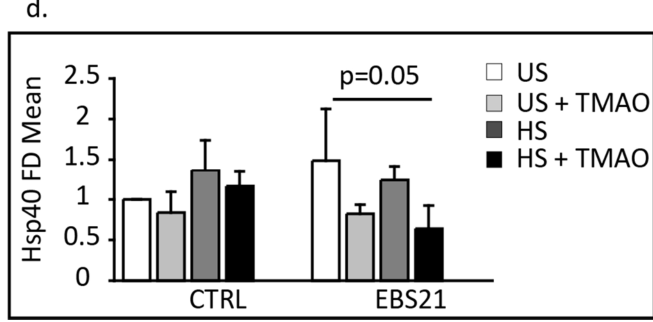

Fig. 2 TMAO reduced Hsp70 and Hsp40 protein levels in heat-stressed EBS21 derived keratinocytes. A representative western blot (WB) revealed with anti-Hsp70 (a) and anti-Hsp40 (c) antibodies comparing cell extracts from CTRL and EBS21 derived keratinocytes (-/+) stress and/or (-/+) TMAO as indicated. Graphs show Hsp70 (b) and Hsp40 (d) fold difference (FD) compared to unstressed CTRL, for CTRL and EBS21 keratinocytes in unstressed (US), unstressed and pretreated with TMAO (US+TMAO), heat-stressed (HS) and heat-stressed and pretreated (HS+TMAO) conditions. Results represent the mean + SD for Hsp70 (n=3) and for Hsp40 (n=4) independent experiments. Statistical significance was set at $p \leq 0.05$.
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Supporting Information

Additional supporting data may be found in the supplementary information of this article.

Data S1. Material and methods

Table S1.Clinical status and mutations found in three EBS cases

Figure S1. Quantification of keratin aggregates in EBS keratinocyte lines

Additional references

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Supporting information

Data S1: Materials and methods

Immortalized keratinocytes establishment

We established four new primary and stable keratinocyte cell lines from one healthy control (CTRL) and from one severely affected generalized EBS-DM patient (EBS Dowling-Meara) (EBS21), with a p.Arg125Ser substitution in K14 that we previously genetically defined (s7). The two others EBS cell lines (EBS1 and EBS10) have a p.Ile377Thr and a p.Ile412Phe substitution respectively (Table S1). The ethic committee from the Hôpital Sainte-Justine and from the Centre intégré de santé et de services sociaux du Saguenay–Lac-Saint-Jean approved the study and the four individuals gave their informed consent.

Primary cultures were established from non-blistered skin using standard protocols. Cells were maintained in standard keratinocyte tissue culture medium containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 25% Ham's F12 medium, 10% fetal calf serum, 0.4 µg/ml hydrocortisone, 10^{-10} M cholera toxin, 5 µg/ml human transferrin, 1.3 ng/ml thyronine, 24.3 µg/ml adenine, 5 µg/ml bovine insulin, 10 ng/ml human recombinant epidermal growth factor and cultured on 3T3 fibroblast feeder cells. Primary keratinocyte lines were immortalized by co-plating with mitomycin C-treated PT67 retroviral producer cells producing a recombinant retrovirus expressing E6 and E7 (kindly provided by Dr. Baki Akgul, Institute of Virology, University of Cologne, Cologne, Germany). Three to four days later, producer cells were selectively removed and mitomycin C-treated 3T3 feeder cells were added to the culture, a day prior to selection. Transductants were selected with G418 (70-100 µg/ml for 5 days). When cells became independent, they were routinely cultured in the standard medium described above. To limit the impact of further genetic variations, immortalized EBS21, EBS1 and EBS10 cells were evaluated for the presence of the characteristic R125S, I377T or I412F (K14) mutation (data not shown) and all experiments were carried out on cells with almost identical passages between control and EBS (5-8 passages after immortalisation).

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Keratinocyte lines heat-shock and pretreatment with TMAO and 4-PBA

Immortalized EBS21 and CTRL keratinocytes were cultured in 24-well plates on 13mm coverslips in duplicates, and then were subjected to thermal stress as previously described with some modifications (5). Briefly, cells were heat-shocked at 43°C for 35 min followed by a recovery period of 15 min. Dose-response curves were established for each molecule to determine the optimal dose and time of incubation. Three assays per molecule were performed as follow, keratinocytes were pretreated with one of the two chemical molecules trimethylamine N-oxide (TMAO) (150mM, Sigma-Aldrich) or sodium 4-phenylbutyrate (4-PBA) (10mM, Calbiochem), dissolved in culture medium and subsequently cultured for 24 h prior to thermal stress. Cells were then processed for immunofluorescence staining. As a difference was observed for TMAO, replication assays for TMAO were done with immortalized EBS1 and EBS10 cell lines.

Immunofluorescence

Coverslips were washed with PBS, then fixed in 4% paraformaldehyde in PBS for 15 min and permeabilized for 4 min at room temperature with Triton stabilization buffer (PBS, 0.2% Triton X-100). Unspecific binding epitopes were blocked with 5% normal goat serum, 5% bovine serum albumin (BSA) in PBS for 30 min. Subsequently, cells were incubated for 90 min with an anti-K5 antibody diluted in a blocking solution (1% BSA in PBS) (Clone XM26, 1:65 dilution; Cedarlane, ON, Canada). Then, cells were incubated for 45 min with a Cy3-conjugated goat anti-mouse antibody (1:800 dilution; Cedarlane, ON, Canada). Following PBS washes, coverslips were mounted on glass slides using Dako fluorescent mounting medium (Dako). Slides were examined under an inverted fluorescence microscope (AxioObserver, Zeiss, Germany) and 500 cells were counted for all the conditions in each experiment in the duplicate coverslips. Pictures of representative fields were taken using the AxioVision software (Rel. 4.8, Zeiss, Germany). Data analysis was performed using Student's t-test. Results are expressed as mean of cells with aggregates ± SD. Statistical significance was assumed for $P \leq 0.05$.

qRT-PCR

KRT16 and *KRT17* mRNA expression by quantitative real-time PCR (qRT-PCR) was done in triplicate with negative controls with or without TMAO pre-treatment for the CTRL, EBS21, EBS1 and EBS10 cell lines with no heat stress applied to the cells. Briefly, total RNA was extracted from confluent cell layers using the RNeasy plus Mini Kit (Qiagen, ON, Canada) according to manufacturer's recommendations. qScript™ cDNA SuperMix (Quanta Biosciences, MD, USA) with oligodT(20) primers were used for cDNA synthesis. The RT-PCR reaction was performed using TaqMan technology in the Rotor-Gene 6000 (Corbett Research, Sydney, Australia) with 0.67ng of cDNA, TaqMan primers (Applied Biosystems, ON, Canada) and PerfecTa qPCR ThoughMix (Quanta Biosciences, MD, USA) for a final volume of 20µl. Each sample was done in triplicates with a negative control. Quantification of gene expression was performed using the two standard curves method and significant differences of expression were assessed using the Student's t-test ($P \leq 0.05$). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene.

Cell lysates preparation and Western Blot

Cell lysates in each experimental condition were prepared as described elsewhere (4). Cells were washed twice in cold PBS and scraped after addition of lysis buffer (20mM Tris pH 7.5, 0.5% (w/v) Triton X-100, 0.5% (w/v) deoxycholate, 10mM EDTA, 30mM sodium pyrophosphate, 150mM NaCl, 0.5mM sodium orthovanadate, 1mM phenylmethylsulfonyl fluoride (PMSF), 0.5ng/ml aprotinin, 0.5ng/ml leupeptin and 0.64mM benzamidine). Cell suspensions were incubated on ice for 30 min, with periodic vortexing. Cell lysates were then centrifuged at 15 000g_{max} at 4°C for 30 min. Supernatants were collected and subjected to Bradford assay to determine protein concentration using a bovine serum albumin standard curve. Ten to fifteen micrograms (for Hsp70) and 30-40 micrograms (for Hsp40) of proteins after addition of Laemmli sample buffer were heated at 95°C for 5 min and were loaded on Stain-free™ gel and then transferred to TransBlot®Turbo™PVDF membranes (Bio-Rad, Canada). Western blot were performed in TBST-5% powder milk using anti-Hsp70 (clone C92F3A-5, dilution 1/10 000, Assay designs, ON, Canada) or anti-Hsp40 (clone EPR1530, dilution

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1/1000, Abcam, ON, Canada). Proteins were revealed by chemiluminescence and quantified with Image Lab™ software, which is included with the ChemiDoc™ MP imaging system (Bio-Rad, Canada). Quantification of the Hsp70 and Hsp40 protein levels was performed according to a published protocol using Image Lab™ software (s8). In each experiment, the fold difference was determined as described in Taylor SC and Posch A (2014) (s9), using untreated CTRL as the reference. The mean of fold difference values and its standard deviation (SD) were calculated. Statistical analysis was performed using the Student's t-test ($P \leq 0.05$).

Table S1 Clinical status and mutations found in three EBS cases

Case	Sex	EBS subtype	*KRT14* mutation	K14 mutation	Exon	Domain	Reference
EBS21	Male	EBS Dowling-Meara	373C>A	R125S	1	1A	(s7)
EBS1	Male	EBS Localized	1130T>C	I377T	6	2B	(s10)
EBS10	Female	EBS Localized	1295A>T	I412F	6	2B	(s11)

Figure legend

Fig. S1 Quantification of keratin aggregates in EBS keratinocyte cell lines. Graph depicts the percentage of keratinocytes showing keratin aggregates in CTRL, EBS21, EBS1 and EBS10 unstressed (US), heat-stressed (HS) or TMAO pretreated keratinocytes with heat-stress (TMAO+HS). Results represent the mean ± SD of three independent experiments (500 cells were counted for each experiment). Statistical significance was set at $p \leq 0.05$.

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Additional references

s1 Hartl F U, Hayer-Hartl M. Protein folding - Molecular chaperones in the cytosol: from nascent chain to folded protein. Science 2002: 295: 1852-1858.

s2 Fujiwara M, Yamamoto H, Miyagi T, et al. Effects of the Chemical Chaperone 4-Phenylbutylate on the Function of the Serotonin Transporter (SERT) Expressed in COS-7 Cells. J Pharmacol Sci 2013: 122: 71-83.

s3 Yancey P H. Organic osmolytes as compatible, metabolic and counteracting cytoprotectants in high osmolarity and other stresses. J Exp Biol 2005: 208: 2819-2830.

s4 Gong B, Zhang L Y, Pang C P, et al. Trimethylamine N-oxide alleviates the severe aggregation and ER stress caused by G98R alpha A-crystallin. Mol Vis 2009: 15: 2829-2840.

s5 Burrows J A, Willis L K, Perlmutter D H. Chemical chaperones mediate increased secretion of mutant alpha 1-antitrypsin (alpha 1-AT) Z: A potential pharmacological strategy for prevention of liver injury and emphysema in alpha 1-AT deficiency. Proc Natl Acad Sci U S A 2000: 97: 1796-1801.

s6 Kerns M L, DePianto D, Dinkova-Kostova A T, *et al*. Reprogramming of keratin biosynthesis by sulforaphane restores skin integrity in epidermolysis bullosa simplex. P Natl Acad Sci USA 2007: 104: 14460-14465.

s7 Bchetnia M, Tremblay M L, Leclerc G, et al. Expression signature of epidermolysis bullosa simplex. Hum Genet 2012: 131: 393-406.

s8 Taylor S C, Berkelman T, Yadav G, Hammond M. A defined methodology for reliable quantification of Western blot data. Mol Biotechnol 2013: 55: 217-26.

s9 Taylor S C and PoschA. The Design of a Quantitative Western Blot Experiment. BioMed Research International 2014, Article ID 361590, 8 pages.

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s10 Rugg E L, Horn H M, Smith F J, et al. Epidermolysis bullosa simplex in Scotland caused by a spectrum of keratin mutations. J Invest Dermatol 2007: 127: 574-580.

s11 Bolling M C, Lemmink H H, Jansen G H L, et al. Mutations in KRT5 and KRT14 cause epidermolysis bullosa simplex in 75% of the patients. Brit J Dermatol 2011: 164: 637-644.

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Supporting Information

Additional supporting data may be found in the supplementary information of this article.

Data S1. Material and methods

Table S1.Clinical status and mutations found in three EBS cases

Figure S1. Quantification of keratin aggregates in EBS keratinocyte lines

Additional references

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Supporting information

Data S1: Materials and methods

Immortalized keratinocytes establishment

We established four new primary and stable keratinocyte cell lines from one healthy control (CTRL) and from one severely affected generalized EBS-DM patient (EBS Dowling-Meara) (EBS21), with a p.Arg125Ser substitution in K14 that we previously genetically defined (s7). The two others EBS cell lines (EBS1 and EBS10) have a p.Ile377Thr and a p.Ile412Phe substitution respectively (Table S1). The ethic committee from the Hôpital Sainte-Justine and from the Centre intégré de santé et de services sociaux du Saguenay–Lac-Saint-Jean approved the study and the four individuals gave their informed consent.

Primary cultures were established from non-blistered skin using standard protocols. Cells were maintained in standard keratinocyte tissue culture medium containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 25% Ham's F12 medium, 10% fetal calf serum, 0.4 µg/ml hydrocortisone, 10^{-10} M cholera toxin, 5 µg/ml human transferrin, 1.3 ng/ml thyronine, 24.3 µg/ml adenine, 5 µg/ml bovine insulin, 10 ng/ml human recombinant epidermal growth factor and cultured on 3T3 fibroblast feeder cells. Primary keratinocyte lines were immortalized by co-plating with mitomycin C-treated PT67 retroviral producer cells producing a recombinant retrovirus expressing E6 and E7 (kindly provided by Dr. Baki Akgul, Institute of Virology, University of Cologne, Cologne, Germany). Three to four days later, producer cells were selectively removed and mitomycin C-treated 3T3 feeder cells were added to the culture, a day prior to selection. Transductants were selected with G418 (70-100 µg/ml for 5 days). When cells became independent, they were routinely cultured in the standard medium described above. To limit the impact of further genetic variations, immortalized EBS21, EBS1 and EBS10 cells were evaluated for the presence of the characteristic R125S, I377T or I412F (K14) mutation (data not shown) and all experiments were carried out on cells with almost identical passages between control and EBS (5-8 passages after immortalisation).

Keratinocyte lines heat-shock and pretreatment with TMAO and 4-PBA

Immortalized EBS21 and CTRL keratinocytes were cultured in 24-well plates on 13mm coverslips in duplicates, and then were subjected to thermal stress as previously described with some modifications (5). Briefly, cells were heat-shocked at 43°C for 35 min followed by a recovery period of 15 min. Dose-response curves were established for each molecule to determine the optimal dose and time of incubation. Three assays per molecule were performed as follow, keratinocytes were pretreated with one of the two chemical molecules trimethylamine N-oxide (TMAO) (150mM, Sigma-Aldrich) or sodium 4-phenylbutyrate (4-PBA) (10mM, Calbiochem), dissolved in culture medium and subsequently cultured for 24 h prior to thermal stress. Cells were then processed for immunofluorescence staining. As a difference was observed for TMAO, replication assays for TMAO were done with immortalized EBS1 and EBS10 cell lines.

Immunofluorescence

Coverslips were washed with PBS, then fixed in 4% paraformaldehyde in PBS for 15 min and permeabilized for 4 min at room temperature with Triton stabilization buffer (PBS, 0.2% Triton X-100). Unspecific binding epitopes were blocked with 5% normal goat serum, 5% bovine serum albumin (BSA) in PBS for 30 min. Subsequently, cells were incubated for 90 min with an anti-K5 antibody diluted in a blocking solution (1% BSA in PBS) (Clone XM26, 1:65 dilution; Cedarlane, ON, Canada). Then, cells were incubated for 45 min with a Cy3-conjugated goat anti-mouse antibody (1:800 dilution; Cedarlane, ON, Canada). Following PBS washes, coverslips were mounted on glass slides using Dako fluorescent mounting medium (Dako). Slides were examined under an inverted fluorescence microscope (AxioObserver, Zeiss, Germany) and 500 cells were counted for all the conditions in each experiment in the duplicate coverslips. Pictures of representative fields were taken using the AxioVision software (Rel. 4.8, Zeiss, Germany). Data analysis was performed using Student's t-test. Results are expressed as mean of cells with aggregates ± SD. Statistical significance was assumed for $P \leq 0.05$.

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qRT-PCR

KRT16 and *KRT17* mRNA expression by quantitative real-time PCR (qRT-PCR) was done in triplicate with negative controls with or without TMAO pre-treatment for the CTRL, EBS21, EBS1 and EBS10 cell lines with no heat stress applied to the cells. Briefly, total RNA was extracted from confluent cell layers using the RNeasy plus Mini Kit (Qiagen, ON, Canada) according to manufacturer's recommendations. qScript™ cDNA SuperMix (Quanta Biosciences, MD, USA) with oligodT(20) primers were used for cDNA synthesis. The RT-PCR reaction was performed using TaqMan technology in the Rotor-Gene 6000 (Corbett Research, Sydney, Australia) with 0.67ng of cDNA, TaqMan primers (Applied Biosystems, ON, Canada) and PerfecTa qPCR ThoughMix (Quanta Biosciences, MD, USA) for a final volume of 20µl. Each sample was done in triplicates with a negative control. Quantification of gene expression was performed using the two standard curves method and significant differences of expression were assessed using the Student's t-test ($P \leq 0.05$). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene.

Cell lysates preparation and Western Blot

Cell lysates in each experimental condition were prepared as described elsewhere (4). Cells were washed twice in cold PBS and scraped after addition of lysis buffer (20mM Tris pH 7.5, 0.5% (w/v) Triton X-100, 0.5% (w/v) deoxycholate, 10mM EDTA, 30mM sodium pyrophosphate, 150mM NaCl, 0.5mM sodium orthovanadate, 1mM phenylmethylsulfonyl fluoride (PMSF), 0.5ng/ml aprotinin, 0.5ng/ml leupeptin and 0.64mM benzamidine). Cell suspensions were incubated on ice for 30 min, with periodic vortexing. Cell lysates were then centrifuged at 15 000g_{max} at 4°C for 30 min. Supernatants were collected and subjected to Bradford assay to determine protein concentration using a bovine serum albumin standard curve. Ten to fifteen micrograms (for Hsp70) and 30-40 micrograms (for Hsp40) of proteins after addition of Laemmli sample buffer were heated at 95°C for 5 min and were loaded on Stain-free™ gel and then transferred to TransBlot®Turbo™PVDF membranes (Bio-Rad, Canada). Western blot were performed in TBST-5% powder milk using anti-Hsp70 (clone C92F3A-5, dilution 1/10 000, Assay designs, ON, Canada) or anti-Hsp40 (clone EPR1530, dilution

1/1000, Abcam, ON, Canada). Proteins were revealed by chemiluminescence and quantified with Image Lab™ software, which is included with the ChemiDoc™ MP imaging system (Bio-Rad, Canada). Quantification of the Hsp70 and Hsp40 protein levels was performed according to a published protocol using Image Lab™ software (s8). In each experiment, the fold difference was determined as described in Taylor SC and Posch A (2014) (s9), using untreated CTRL as the reference. The mean of fold difference values and its standard deviation (SD) were calculated. Statistical analysis was performed using the Student's t-test ($P \leq 0.05$).

Table S1 Clinical status and mutations found in three EBS cases

Case	Sex	EBS subtype	*KRT14* mutation	K14 mutation	Exon	Domain	Reference
EBS21	Male	EBS Dowling-Meara	373C>A	R125S	1	1A	(s7)
EBS1	Male	EBS Localized	1130T>C	I377T	6	2B	(s10)
EBS10	Female	EBS Localized	1295A>T	I412F	6	2B	(s11)

Figure legend

Fig. S1 Quantification of keratin aggregates in EBS keratinocyte cell lines. Graph depicts the percentage of keratinocytes showing keratin aggregates in CTRL, EBS21, EBS1 and EBS10 unstressed (US), heat-stressed (HS) or TMAO pretreated keratinocytes with heat-stress (TMAO+HS). Results represent the mean $\pm$ SD of three independent experiments (500 cells were counted for each experiment). Statistical significance was set at $p \leq 0.05$.

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Additional references

s1 Hartl F U, Hayer-Hartl M. Protein folding - Molecular chaperones in the cytosol: from nascent chain to folded protein. Science 2002: 295: 1852-1858.

s2 Fujiwara M, Yamamoto H, Miyagi T, et al. Effects of the Chemical Chaperone 4-Phenylbutylate on the Function of the Serotonin Transporter (SERT) Expressed in COS-7 Cells. J Pharmacol Sci 2013: 122: 71-83.

s3 Yancey P H. Organic osmolytes as compatible, metabolic and counteracting cytoprotectants in high osmolarity and other stresses. J Exp Biol 2005: 208: 2819-2830.

s4 Gong B, Zhang L Y, Pang C P, et al. Trimethylamine N-oxide alleviates the severe aggregation and ER stress caused by G98R alpha A-crystallin. Mol Vis 2009: 15: 2829-2840.

s5 Burrows J A, Willis L K, Perlmutter D H. Chemical chaperones mediate increased secretion of mutant alpha 1-antitrypsin (alpha 1-AT) Z: A potential pharmacological strategy for prevention of liver injury and emphysema in alpha 1-AT deficiency. Proc Natl Acad Sci U S A 2000: 97: 1796-1801.

s6 Kerns M L, DePianto D, Dinkova-Kostova A T, *et al*. Reprogramming of keratin biosynthesis by sulforaphane restores skin integrity in epidermolysis bullosa simplex. P Natl Acad Sci USA 2007: 104: 14460-14465.

s7 Bchetnia M, Tremblay M L, Leclerc G, et al. Expression signature of epidermolysis bullosa simplex. Hum Genet 2012: 131: 393-406.

s8 Taylor S C, Berkelman T, Yadav G, Hammond M. A defined methodology for reliable quantification of Western blot data. Mol Biotechnol 2013: 55: 217-26.

s9 Taylor S C and PoschA. The Design of a Quantitative Western Blot Experiment. BioMed Research International 2014, Article ID 361590, 8 pages.

s10 Rugg E L, Horn H M, Smith F J, et al. Epidermolysis bullosa simplex in Scotland caused by a spectrum of keratin mutations. J Invest Dermatol 2007: 127: 574-580.

s11 Bolling M C, Lemmink H H, Jansen G H L, et al. Mutations in KRT5 and KRT14 cause epidermolysis bullosa simplex in 75% of the patients. Brit J Dermatol 2011: 164: 637-644.