Supplementary information

Deficiency of the human cysteine protease inhibitor cystatin M/E causes hypotrichosis and dry skin

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Supplementary Material & methods

Infinium_CytoSNP_850K genotyping array analysis

DNA amplification, tagging and hybridization were performed according to the manufacturer's protocol (Illumina). The array slides were scanned on an iScan Reader (Illumina). Data analysis was performed using Nexus Copy Number 5.0 (BioDiscovery, El Segundo). The HapMap control set provided by the manufacturer was used as a control. 50-200 ng of DNA was used. Standard settings for SNP arrays in Nexus were adjusted: a cutoff value of 0.15, homozygous frequency threshold of 0.95 and minimum loss-of-heterozygosity (region with LOH) length of 5000 kb were set (SNP-FASST Rank Segmentation). QC measurement in Nexus was used as a measure of the array profile quality. Samples with QC<0.13 were further analyzed. UCSC built Hg19 (Human February 2009, Genome Reference Consortium GRCh37 Assembly) was used to analyze the data.

Exome sequencing

Exome sequencing was performed on genomic DNA extracted from the blood of the index patient (PIII:2). Exome enrichment (Agilent SureSelectXT Human All Exon 50Mb), exome sequencing (Illumina HiSeq), read alignment (BWA) and variant calling (GATK) were done at BGI-Europe (Denmark). Variant annotation, selection, and prioritizing were done by the department of Human genetics, Radboudumc with an in-house developed software tool. The 'full' dataset was analyzed for deletions, insertions, nonsense and canonical splice site mutations, and missense mutations (PhyloP of base >3.5). Only those variants that are likely to be causative (by literature, expression profile, etc.) were selected for further studies.

Biophysical measurements

Prior to measurements, patients and healthy volunteers acclimatized for at least 15 minutes in a temperature-controlled room with the body site to be assessed, the right mid-ventral forearm,

uncovered. The biophysical techniques included: (*i*) trans epidermal water loss measurement for the indirect assessment of the skin barrier function (Aquaflux AF200, Biox), (*ii*) capacitance for the indirect measurement of SC hydration (Epsilon E100, Biox), and (*iii*) skin redness and pigmentation (a* and b* values measured by spectrophotometer 2600d, Konica Minolta). Three measurements per body site were performed. In addition, morphological imaging with *in vivo* reflectance confocal microscopy (RCM, Vivascope 1500 system, Caliber) was performed. At least four horizontal maps of 4 mm x 4 mm (Vivablock) were made at the level of the SC/stratum granulosum, stratum spinosum, the dermal epidermal junction and dermis. Two vertical mappings (Vivastack) were made by capturing a series of images of 0.5 mm x 0.5 mm starting from the skin surface up to 150 µm in dept with steps of 3 µm. Thickness of the SC and living epidermis were measured as previously described.[1]

RNA isolation and qPCR analysis

The epidermis of the skin biopsies was separated from the dermis by dispase (Roche) treatment for 2 hours at 4°C. Epidermal equivalents were directly placed in lysis buffer and stored at -80°C. RNA isolation, cDNA synthesis and qPCR analysis was performed as described earlier.[2] Target gene expression was normalized to the expression of the house keeping gene, human acidic ribosomal phosphoprotein P0 (*RPLP0*). The ΔΔCt method was used to calculate relative mRNA expression levels.[3]

Morphological and immunohistochemical analysis

Skin biopsies (3 mm) and reconstructed epidermal equivalents were fixed in a 4% buffered formalin solution (Baker Mallinckrodt, Deventer, The Netherlands) for 4 hours and subsequently embedded in paraffin. 6 µm paraffin sections were stained with hematoxylin and eosin or with antibodies using an indirect immunoperoxidase technique (Vectastain, Vector Laboratories).

Protein extraction from epidermal equivalents

Proteins were extracted from the 3D epidermal equivalents using a buffer containing 20 mM Tris-HCl (pH 8), 0.1 M NaCl, 1 mM EDTA, 2 mM dithiothreitol (DTT), followed by three cycles of freeze/thawing of the epidermis in extraction buffer and a mild sonification of the lysate for 1 minute at 4°C. For enzymology experiments, the extracts were centrifugated for 10 minutes and 15.000 × *g* at 4°C. The supernatants that harbor the soluble epidermal proteins were stored at -80°C. For Western blotting, sonificated lysates were stored at -80°C.

Western blotting

Proteins were separated by SDS-PAGE using the NuPAGE electrophoresis system and pre-cast 12% Bis-Tris polyacrylamide gels under reducing conditions (Invitrogen). For immunoblotting the proteins were electroblotted onto polyvinylidene difluoride membrane (Invitrogen) for 60 minutes at 30 V. The membrane was blocked with Blotto (Thermo Scientific) and incubated o/n at 4°C with polyclonal goat anti-human cystatin M/E (clone AF1268, 1:250, R&D Systems) or mouse anti-human β-actin (clone AC-15, 1:100.000, Sigma). Detection of the proteins was established using rabbit anti-goat-HRP linked antibody (1:2000, Biovision) for cystatin M/E, and anti-mouse HRP-linked antibody (1:2000, Cell Signaling) for β-actin followed by chemiluminescence detection using SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo Scientific) and Image Lab software (Biorad)

Supplementary Figures

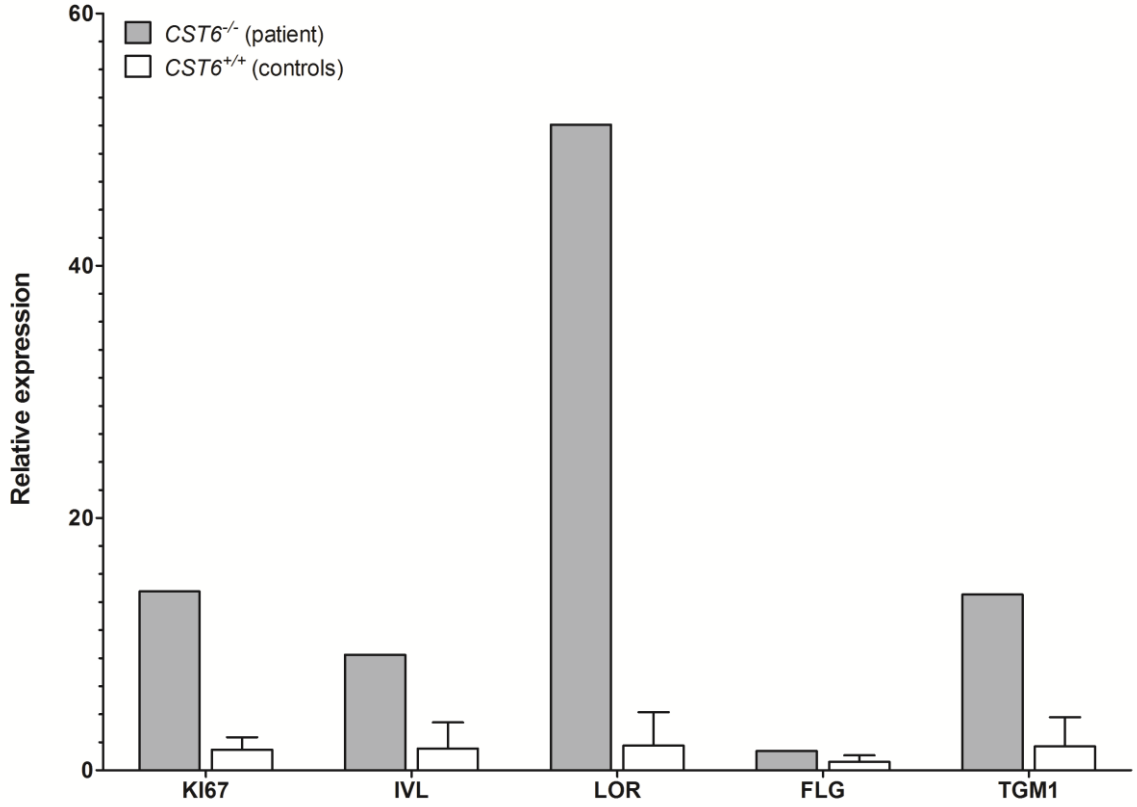

Figure S1. Gene expression. Relative mRNA expression levels in skin biopsies of the patient (grey bars) and healthy controls (*CST6* wild type, $CST6^{+/+}$, N=6, white bars). The patient showed induced expression of *KI67*, differentiation genes (*IVL*, *LOR* and *FLG*), and *TGM1*.

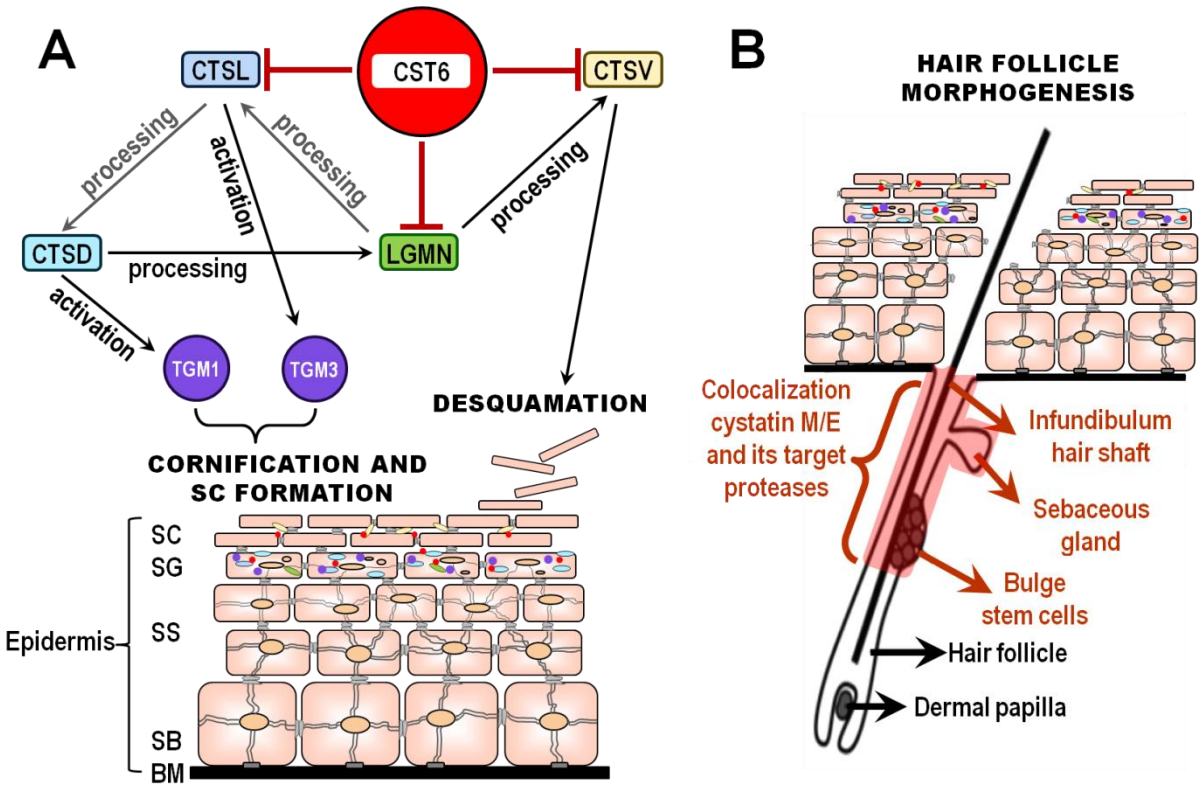

Figure S2. Proposed role of cystatin M/E in regulation of epidermal cornification, stratum corneum formation, desquamation and hair follicle maintenance. This scheme is compiled based on biochemical and cell biological data from human studies. Human cystatin M/E (CST6) is an inhibitor of legumain (LGMN), cathepsin L (CTSL) and cathepsin V (CTSV). (A) In the epidermis, CST6 is important in the cornification process by controlling CTSL activity, which is the elusive processing and activating enzyme for transglutaminase 3 (TGM3). CTSL also can process cathepsin D (CTSD), which in turn can activate TGM1. Inhibition of LGMN regulates the processing of (pro)-cathepsins. Inhibition of CTSV regulates desquamation, as CTSV is able to degrade (corneo)-desmosomal proteins like desmoglein-1, desmocollin-1, and corneodesmosin. Biochemical functions that are known from literature are depicted by grey (arrow) lines, black lines represent our previous investigations. BM, basal membrane; SB, stratum basale; SS, stratum spinosum; SG, stratum granulosum; SC, stratum corneum. (B) In the hair follicle, CST6 colocalizes with all target proteases in the distal part and infundibulum[4]. Co-localization is also found in the area around the bulge where the epithelial stem cells reside. CST6 is absent in the proximal part, the hair bulb. Colocalization with CTSL and LGMN, but not CSTV, is also found in the sebaceous glands[4].

Supplementary Tables

Table S1. Human protease/inhibitor disorders with skin and/or hair phenotypes

Disease (MIM#, gene locus)	Affected gene and mechanism	Clinical manifestations	Ref
Netherton syndrome (MIM 256500, 5q32)	*SPINK5* (MIM 605010). Autosomal recessive mutations in gene that encodes for serine protease **inhibitor** (LEKT1) results in increased protease activity in the SC, accelerated degradation of desmoglein-1, and overdesquamation of corneocytes.	Ichthyosis with variable erythroderma, hair shaft defects ('bamboo hair') and atopic features.	5
Papillon-Lefèvre syndrome (MIM 245000, 11q14.1-q14.3)	*CTSC* (MIM 602365). Autosomal recessive mutations in this cysteine **protease** (cathepsin C) are associated with a reduced host response against bacteria in dental plaques; unknown role in epidermal differentiation and desquamation (intracellular degradation/processing proteins).	Hyperkeratosis of palms and soles, severe early onset periodontitis.	6
ARCI11 (MIM 610765, 11q24.3)	*ST14* (MIM 606797). Autosomal recessive mutations in the transmembrane serine **protease** (Matriptase). Involved in degradation of SC corneodesmosomes and profilaggrin-processing. Matriptase could be considered as a key enzyme in terminal epidermal differentiation.	Ichthyosis with hypotrichosis, eye abnormalities (photophobia and blepharitis), teeth abnormalities, hypohidrosis.	7
IFAP/Bresheck syndrome (MIM 308205, Xp22.12)	*MBTPS2* (MIM 300294). X-linked recessive mutations in this membrane-embedded zinc metallo**protease** are associated with disturbed cholesterol homeostasis and ER stress response	IFAP syndrome (ichthyosis follicularis with atrichia and photophobia) with or without additional features (e.g. corneal opacifications, mental retardation, skeletal malformations).	8
Peeling skin syndrome 4 (PSS4) (MIM 607936, 3q21.1)	*CSTA* (MIM 184600). Autosomal recessive mutations in the cysteine protease **inhibitor** cystatin A cause cell-cell adhesion defects in human keratinocytes	Exfoliative ichthyosis with peeling of nonerythematous skin on the palms and soles.	9
Peeling skin syndrome 5 (PSS5) (MIM 617115, 8q22.1)	*SERPINB8* (MIM 601697). Autosomal recessive mutations in this serine protease **inhibitor** cause cell-cell adhesion defects in human keratinocytes.	Exfoliative ichthyosis with superficial peeling of dorsal and palmar surfaces of the hands and feet.	10
keratolytic winter erythema (KWE) (MIM 148370, 8p23-p22)	*CTSB* (MIM 116810). Autosomal dominant tandem duplications in a non-coding genomic region containing an active enhancer element for the cysteine **protease** cathepsin B, which is involved in keratinocyte homeostasis.	Episodes of palmoplantar erythema and epidermal peeling.	11

Table S2. qPCR primers

HUGO gene symbol	Description gene/protein	Forward primer (5' → 3')	Reverse primer (5' → 3')
FLG	Filaggrin	acttcactgagtttcttctgatggtatt	tccagacttgagggtctttttctg
IVL	Involucrin	acttatttcgggtccgctaggt	gagacatgtagagggacagagtcaag
KI67	Ki-67	aaaccaacaaagaggaacacaaatt	gtctggagcgcagggatattc
LOR	Loricrin	aggttaagacatgaaggatttgcaa	ggcaccgatgggcttagag
RPLP0	Ribosomal phosphoprotein P0	caccattgaaatcctgagtgatgt	tgaccagcccaaaggagaag
TGM1	Transglutaminase 1	cccccgcaatgagatctaca	atcctcatggtccacgtacaca

Table S3. Antibodies for immunohistochemistry

Target protein	Antibody clone, manufacturer	Dilution
Cystatin M/E (CST6)	AF1286, R&D	1 : 200
CD31	DAKO M0823	1 : 5
Elastica v Gieson	Merck 1.15974.0002	
Filaggrin (FLG)	NCL-filaggrin, Novocastra	1:200
Involucrin (IVL)	MON-150, generated by our group[12]	1:20
Ki67	DAKO, M7240	1 : 50
Loricrin (LOR)	PRB145P, Covance	1:2000
Transglutaminase-1 (TGM1)	H-87, Santa Cruz	1:100

Table S4. Clinical characteristics of the patients

Characteristics	Patient PII:2	Patient PIII:2
Age (years):	32	10
Sex:	Male (father)	Female (daughter)
Skin:	Dry skin (whole body) Itching Eczema	Extremely dry skin (face, hands, arms) No itching Eczema
Hair:	Scalp hair disappeared at 4 years Sparse body hair Slowly growing Facial hair (beard) falls out	Scalp hair disappeared at 4 years Sparse body hair Slowly growing
Eyes:	Normal eye brows Little eye lashes Photophobia (sunglasses required) Extremely dry eyes, no tears	Normal eye brows Little eye lashes (break off) Photophobia (mild) Blepharitis (continuous)
Other:	Hyperlinearity Abnormal sweating (only after extreme physical exercise) No teeth or nail abnormalities No palmoplantar keratoderma	Hyperlinearity Abnormal sweating (little) No teeth or nail abnormalities No collodion membrane No palmoplantar keratoderma

Table S5. Biophysical parameters

Mid-ventral forearm	Patient PII:2	Healthy controls
Transepidermal Water Loss	(n=1)	(n=27)[13]
TEWL [g/m2h]	13.52 ± 0.34[a]	10.4 ± 1.54
Spectrophotometer	(n=1)	(n=11)[14]
a* (Redness)	9.08 ± 0.18[a]	5.1 ± 0.6
b* (Pigmentation)	16.34 ± 0.16[a]	14.9 ± 1.5
RCM	(n=1)	(n=4)
Stratum Corneum Thickness (µm)	15.46 ± 0[b]	10.98 ± 1.58[c]
Epidermis (µm)	58.76 ± 4.38[b]	38.48 ± 4.39

Data are represented as the mean ± SD. Data are based on [a] three measurements within one person, [b] two measurements within one person, and [c] three measurement per person (= 12 measurements).

Table S6. K_i values of wild type human cystatin M/E and the mutated variant p.Gln121*

Cystatin M/E	K_i Cathepsin L	*Ki* Cathepsin V	K_i Cathepsin B	K_i Legumain
Wild type	1.78[h]	0.47[h]	>100[a]	0.25[h]
p.Gln121*	>100[b]	>100[c]	>100[d]	>100[e]
Denaturated	>100[f]	n.d.	n.d.	>100[g]

[a]$v_i/v_0 > 0.71$ at [I] = 300 nM, [b]$v_i/v_0 > 0.86$ at [I] = 300 nM, [c]$v_i/v_0 > 0.98$ at [I] = 300 nM, [d]$v_i/v_0 > 0.96$ at [I] = 300 nM, [e]$v_i/v_0 > 1.09$ at [I] = 300 nM, [f]$v_i/v_0 > 1.15$ at [I] = 300 nM, [g]$v_i/v_0 > 0.64$ at [I] = 300 nM, [h]K_i data wild type protein from Cheng *et al.*[15].

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