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A translation re-initiation variant in KLHL24 also causes epidermolysis bullosa simplex and dilated cardiomyopathy via intermediate filament degradation

Vermeer, Mathilde C S C; Al-Shinnag, Mohammad; Silljé, Herman H W; Gaytan, Antonio Esquivel; Murrell, Dedee F; McGaughran, Julie; Melbourne, Wei; Cowan, Timothy; van den Akker, Peter C; van Spaendonck-Zwarts, Karin Y

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










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rs4327152 and 0.593 cM between c.650_653delCTGT and rs4327152 (Figure 1b). Based on these data, the age of the c.796C>T mutation was predicted to be 126 generations, which corresponds to the Shang Dynasty in Chinese history. By the same token, the ages of the c.522dupT and c.650_653delCTGT mutations were 86 generations and 106 generations, respectively; these correspond to the Han and Zhou Dynasties, respectively. It was not possible to estimate the age of c.455G>T owing to the small sample size and lack of recombination data.

To date, NPPK has not been reported from any countries outside East Asia except Finland.⁶ This remarkable geographical distribution also provides support for the founder effect with regional divergence in patients with NPPK. One of the frequent Chinese mutations, c.650_653delCTGT, has not been reported in Japanese, Korean or other Asian populations. Intriguingly, two prevalent Japanese SERPINB7 mutations,^{7,8} c.218_219del2ins12 and c.830C>T, and the Finnish founder mutation,⁶ c.1136G>A, were completely absent from our cohort, reflecting different founder events. The data that support the findings of this study are available from the corresponding author, upon reasonable request.

In conclusion, we confirmed three new founder mutations in SERPINB7 in the Chinese population. Moreover, this is the first study to explore the time of origin of several founder mutations. Our findings provide recommendations for genetic counselling for patients with NPPK. The data that support the findings of this study are available from the corresponding author, upon reasonable request. Additionally, mutation screening of these four founder mutations in SERPINB7 can serve as a cost-effective diagnostic strategy in Chinese patients with a suspected diagnosis of NPPK.

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Juan Liu ^{1,2,3}, Zhiming Chen ², Linghan Hu ², Zhongya Song ², Ran Mo ², Lemuel Shui-Lun Tsang ⁴, Yihe Liu ², Xin Huang ², Zhuoqing Gong ⁵, Ruiyu Xiang ², Zhimiao Lin ⁶ and Yong Yang ^{2,3}

¹Department of Dermatology, the First Affiliated Hospital of Nanjing Medical University, Nanjing, China; ²Genetic Skin Disease Center, Jiangsu Key Laboratory of Molecular Biology for Skin Diseases and STIs, Institute of Dermatology, Chinese Academy of Medical Sciences and Peking Union Medical College, Nanjing, China; ³Center for Global Health, School of Public Health, Nanjing Medical University, Nanjing, China; ⁴College of Medicine, University of Tennessee Health Science Center, Memphis, TN, USA; ⁵Department of Dermatology, Peking University First Hospital, Beijing, China; and ⁶Dermatology Hospital, Southern Medical University, Guangzhou, China

Correspondence: Yong Yang.

Email: yyang@pumcdern.cams.cn

J.L. and Z.C. contributed equally to this work.

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A translation re-initiation variant in *KLHL24* also causes epidermolysis bullosa simplex and dilated cardiomyopathy via intermediate filament degradation

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DEAR EDITOR, KLHL24 is a ubiquitin ligase that mediates keratin^{1,2} and desmin³ degradation in the skin and heart, respectively. Previously, five start-codon variants in KLHL24 caused gain-of-function, due to absence of 28 N-terminal amino acids (KLHL24-ΔN28) via loss of the translation-initiation codon.^{1,2,4} Currently, 48 patients with heterozygous start-codon variants leading to epidermolysis bullosa simplex (EBS) with high risk for dilated cardiomyopathy (DCM) have been reported.^{3,5} Here, we publish a four-generation Australian

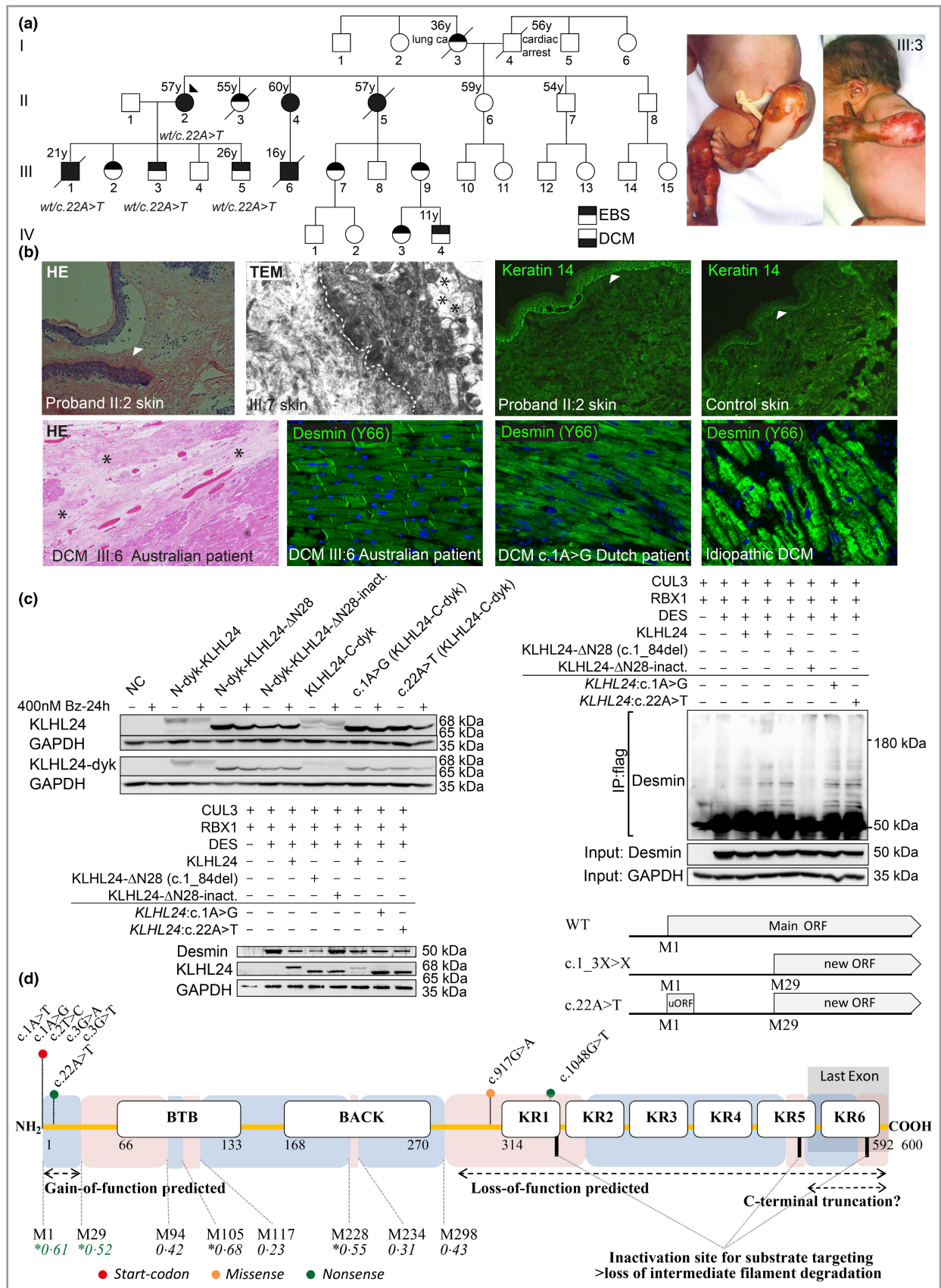


Figure 1 (a) Pedigree of an Australian family with epidermolysis bullosa simplex (EBS) and/or dilated cardiomyopathy (DCM), with the aplasia cutis congenita depicted on the right. The arrowhead indicates the proband and co-segregation confirmation of variant KLHL24:c.22A>T is illustrated below family members. γ , years. (b) Analysis of *ex vivo* skin and heart. Top left to right: haematoxylin and eosin staining (HE) of patient skin ($\times 20$), transmission electron microscopy of patient skin (*, cytolysis) ($\sim \times 13\ 500$) and immunofluorescence stainings of keratin-14 ($\times 20$). Arrowheads/dotted line, basement membrane. Bottom left to right: HE of a postmortem heart (*, fibrosis) ($\times 10$) and immunofluorescence stainings of cardiac desmin ($\times 20$). (c) Western blot analysis of transfected HEK293A cells. Top left: the analysis of different KLHL24 clone transfections. Bz, Bortezomib; N, N-terminal; C, C-terminal; dyk, FLAG-tagged; KLHL24- Δ N28-inactivated: mutagenesis at E355A, E535K and Y584C; NC, negative control; antibodies KLHL24-dyk (M2) and KLHL24 (ab104089); KLHL24 = 68 kDa; KLHL24- Δ N28 = 65 kDa. Bottom left: the functional desmin levels (50 kDa) after different co-transfections (desmin: KLHL24>1:1 transfection ratio). Right: immunoprecipitated desmin levels (> 50 kDa) after different co-transfections (IP desmin-dyk M2; desmin: KLHL24>5:1 transfection ratio). (d) Schematic representation of KLHL24, including the clinically reported variants, protein domains [BTB, BACK and Kelch Repeats (KR)], in-frame start-codons (M1, M29..) with predicted translation-initiation sites, based on the Kozak sequence (unlikely: 0.5 > x > *0.5:likely), with the confirmed active translation-initiation sites in green; (u)ORF, (upstream) open reading frame.





family where 14 members suffer(ed) from EBS and five from DCM. Next-generation sequencing and segregation analysis identified a novel heterozygous variant in KLHL24:c.22A>T [p.(Arg8*)] in the proband and several other affected members (Figure 1a). All EBS-affected members had aplasia cutis congenita, mild skin fragility and variably developed hypopigmentation, dystrophic toenails, hypoplastic teeth and frequent dental caries, but no alopecia or oral defects. Skin biopsies revealed basal intraepidermal blister formation, but normal keratin-14 staining (Figure 1b). Following the sudden cardiac death of patient III:6 at 16 years of age, where post-mortem evaluation showed an enlarged heart with fibrosis and a reduction in desmin staining (Figure 1b), four other EBS-affected members were diagnosed with DCM. Patient III:1, diagnosed at age 16 years, had chronic dyspnoea from a young age, limited exercise tolerance, palpitations and an ejection fraction of 31%. He received heart failure medication, but declined an implantable cardiac defibrillator (ICD) and died suddenly at 21 years of age. The proband II:2, diagnosed with DCM at age 46 years, carries an ICD and has a current ejection fraction of 25%, while patients II:4 and II:5 were diagnosed at 49 and 44 years of age, respectively. The latter died from end-stage heart failure at age 57 years of age. The data that support the findings of this study are available from the corresponding author upon reasonable request.

Variant KLHL24:c.22A>T was absent from controls in the Genome Aggregation Population Database and had not been reported in ClinVar or HGMD variation databases. While predicted to result in loss-of-function, we hypothesized that this nonsense variant also causes N-terminal truncation (KLHL24- Δ N28) via translation re-initiation, resulting in gain-of-function and excessive degradation of intermediate filaments. To prove this, we cloned start-codon variant c.1A>G and variant c.22A>T into the pcDNA3.1(+)-C-DYK backbone containing the wildtype KLHL24 sequence. These plasmids encode different KLHL24 wildtype and variant proteins, fused to a C-terminal FLAG-tag. After overexpression in HEK293A cells, we compared the abundance and weight of the resulting KLHL24 protein with our validated wildtype and KLHL24- Δ N28 mutants.³ Transfection experiments confirmed that variant c.22A>T results in a protein, equal in abundance and weight as the protein pathology of the previously

reported start-codon variants (Figure 1c). These results imply that KLHL24:c.22A>T generates a nonfunctional upstream open reading frame (uORF; Met1_Arg8),⁶ causing skipping of translation of Leu9_Glu28, due to the stop-codon at position 8. Translation re-initiating takes place at Met29, leading to protein levels similar to the validated KLHL24- Δ N28 mutant. To prove that the c.22A>T mutant is functionally as active as the c.1A>G and KLHL24- Δ N28 mutants, we cloned DES and KRT14¹ into the pcDNA3.1(+)-N-DYK backbone. Co-transfections were performed using either DES or KRT14, with different combinations of ubiquitin-ligase factors RBX1, CUL3 and KLHL24 clones. Desmin levels were indeed excessively reduced in combination with KLHL24:c.22A>T, comparable with the c.1A>G and KLHL24- Δ N28 mutant (Figure 1c). Immunoprecipitation studies substantiated that the KLHL24:c.22A>T/RBX1/CUL3-ubiquitin-ligase complex leads to equally excessive ubiquitination levels of desmin as the KLHL24:c.1A>G/RBX1/CUL3-ubiquitin-ligase complex (Figure 1c). Meanwhile, (ubiquitinated) keratin-14 levels remained unaltered in all transfection conditions.

KLHL24 contains nine exons (main ORF: exons 3–9) (Figure 1d). Two homozygous variants previously led to loss-of-function,⁷ implying that homozygous nonsense-inducing variants after Met298 will result in loss-of-function, causing hypertrophic cardiomyopathy without skin fragility. Upstream of position 299, KLHL24 contains eight methionines that could serve as potential start-codons. Here, we report the first variant downstream of the start-codon results in gain-of-function. This is remarkable and our analyses indicate that this is the result of translation re-initiation, as the resulting KLHL24 protein has the same size as a variant that lacks the first translation-initiation codon. Re-initiation of translation after a short uORF⁶ is not uncommon and it is likely that other nonsense-inducing variants between nucleotides c.4_84, can also result in the truncated protein KLHL24- Δ N28. Of note, we detected similar amounts of desmin degradation by the KLHL24:c.22A>T/RBX1/CUL3 and KLHL24:c.1A>G/RBX1/CUL3-ubiquitin-ligase complex, but could not detect KLHL24-mediated degradation of keratin-14 in transfected HEK293A cells. This is in line with follow-up studies to the initial study on KLHL24-related EBS from Lin et al.^{2–4} Although, further studies to elucidate the KLHL24-induced activity

towards keratin-14 are necessary, this may be related to ageing.⁸ To conclude, this study shows that gain-of-function variants in KLHL24 causing EBS and DCM do not originate only in the start-codon and suggest that any nonsense-inducing variant affecting nucleotides c.4_84 will likely cause the same effect on protein level and a similar potential lethal phenotype.

Mathilde C.S.C. Vermeer ,¹ Mohammad Al-Shinnag,^{2,3} Herman H.W. Silljé,¹ Antonio Esquivel Gaytan,¹ Dedee F. Murrell ,^{4,5} Julie McGaughran,^{2,3} Wei Melbourne,⁴ Timothy Cowan,^{4,5} Peter C. van den Akker,⁶ Karin Y. van Spaendonck-Zwarts,^{2,3} Peter van der Meer ¹ and Maria C. Bolling ⁷

¹Departments of Cardiology, (Center for Blistering Diseases), University of Groningen, University Medical Center Groningen, Groningen, the Netherlands;

²Genetic Health Queensland, Royal Brisbane and Women's Hospital, Brisbane, Australia; ³Faculty of Medicine, University of Queensland, Brisbane, Australia; ⁴Department of Dermatology, St George Hospital, Kogarah, NSW, Australia; ⁵Faculty of Medicine, University of New South Wales, Sydney, Australia; ⁶Department of Genetics (Center for Blistering Diseases), University of Groningen, University Medical Center Groningen, Groningen, the Netherlands; and ⁷Department of Dermatology (Center for Blistering Diseases), University of Groningen, University Medical Center Groningen, Groningen, the Netherlands

Correspondence: Maria C. Bolling.

Email: m.c.bolling@umcg.nl

M.C.S.C.V., M.A.-S. and H.H.W.S. contributed equally.

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Conflicts of interest: The authors declare they have no conflicts of interest.

Data availability: The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Plasma cells and acute rejection of a near-total face transplant: an incidental finding or an evolving plasma-cell-mediated rejection?

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DEAR EDITOR, Plasma-cell-rich acute rejection (PCAR) is a rare type of rejection observed thus far only in kidney and liver transplants. PCAR is considered to be a subtype of T-cell-mediated rejection or antibody-mediated rejection. Despite aggressive immunosuppression, PCAR is difficult to treat, is associated with poor graft outcomes, and has a high likelihood of ultimate rejection.^{1–3}

The first face transplant performed in the USA has the longest follow-up of any face transplant patient, at 11 years. The patient historically battled 17 prior biopsy-proven episodes of Banff grades II–IV acute rejection that were all managed with immunotherapy modifications. The most recent episode of rejection demonstrated increased plasma cells on histopathology. Patient consent is on file for use of photos and information and this study has Institutional Review Board approval.

The patient presented in early May 2019 with multiple ulcers and facial bleeding that had worsened over the past month, raising concern for infection, rejection or trauma. On 3 May 2019, biopsies from areas of ulceration and erythema demonstrated a superficial perivascular and perifollicular lymphocytic inflammatory infiltrate with associated demodex folliculitis. On immunohistochemistry, a cluster of B cells and plasma cells were appreciated surrounding deep blood vessels and follicles. Decreased CD4/CD8 (1 : 1) and kappa/lambda (1 : 1) ratios were demonstrated by immunohistochemistry analysis. B-cell