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Detection and comparison of two types of ATP2C1 gene mutations in Chinese patients with Hailey–Hailey disease

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Abstract The gene ATP2C1 is identified as the defective gene in Hailey–Hailey disease (HHD). The nonsense and missense are two common types of mutations and have ,respectively, been detected in many HHD patients. The aims of our study were to identify the pathogenic ATP2C1 abnormality in Chinese HHD patients, and to compare nonsense and missense mutations in vivo to provide further understanding of the molecular and the physiological basis of HHD. The nucleotide sequencing of the ATP2C1 gene was performed in HHD patients, unaffected family members and 100 unrelated individuals. Meanwhile, we detected and analyzed the clinical manifestations, the expression of ATP2C1 mRNA and hSPCA1 protein in the two types of mutations. Three heterozygous mutations were identified, including a previously reported nonsense mutation (R799X), two novel missense mutations (D644G) and (R417K). The results of comparisons between two types of mutations showed that the common clinical features, the similarly low-level expressions of ATP2C1 mRNA and hSPCA1 protein, but the ATP2C1 mRNA expression of nonsense mutation was lower than missense mutation and even less than half the level of normal people. Our findings expand the known spectrum of ATP2C1 mutations in HHD. We supported the haploinsufficiency theory as prevalent mechanism in both types of mutations, and believed that the differences of ATP2C1 mRNA expressions in peripheral blood may relate with the type of mutation and reflect the state of illness of patients.

Introduction

Hailey–Hailey disease (HHD, OMIM 169600) is a rare autosomal-dominant disorder of keratinocyte cohesion in the suprabasal layers which is also known as benign familial chronic pemphigus. This disease usually presents after puberty, mostly in the third or fourth decade of life. It is characterized by recurrent eruption of vesicles, painful erosions, and scaly erythematous plaques involving the neck, groin, axillae, anal and other intertriginous regions. Histopathology shows suprabasal keratinocyte acantholysis with mild dyskeratosis [1]. Clinically, the lesions are often exacerbated by heat, sweating, trauma, infection, and UVB exposure. The severe erosions will limit physical activity and cause considerable pain, even lead to affective disorder severely influencing the quality of life [9].

Molecular studies revealed that HHD is caused by mutations in the ATP2C1 gene encoding the human secretory pathway calcium ATPase 1 (hSPCA1) [7] located in the Golgi membranes [12]. The hSPCA1 protein serves to pump Ca^{2+} or Mn^{2+} actively across Golgi membranes, thus contributing to control of the intracellular homeostasis of these cations and maintain desmosomal integrity [8, 15].

In this study, we screened the ATP2C1 gene in two families with familial HHD (FHHD), and a patient with sporadic HHD (SHHD), meanwhile realized the molecular diagnosis for the asymptomatic individuals in these HHD pedigrees. Furthermore, we made use of newly acquired

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and prior obtained data to compare nonsense and missense mutations in vivo to provide further contributions to the understanding of the molecular and physiological basis of HHD.

Materials and methods

Patients

In FHHD-A, the proband (FHHD-A-II-1) was a 68-year-old male with HHD who had a 30-year history of severe recurrent vesicles and erythematous plaques mixed with areas of dry, crusted erosions in the axilla, groin, waist and navel (Fig. 1a). Family investigation showed a four-generation pedigree with HHD, consisting of 8 symptomatic patients and 5 asymptomatic individuals (FHHD-A-III-1, III-3, IV-1, IV-2, IV-3) among the total 20 family members (Fig. 1c). In FHHD-B, it was a three-generation pedigree

consisting of 3 symptomatic patients and 3 asymptomatic individuals (FHHD-B-II-5, III-1, III-2, III-3) among the total 11 family members (Fig. 1d), the proband (FHHD-B-II-3) was 36-year-old female. The sporadic patient (SHHD-1) was 70-year-old male. All patients showed the typical clinical features of HHD. Further more, we made full use of the data from the previous study [10], and requested some patients with identified mutation Q865X or G645V to join our study (Table 1). Histology of each HHD patient showed the acantholysis above basal cell layer (with 'dilapidated brick wall' appearance).

Mutational screens and RFLP analysis

After informed consent was obtained, blood samples were collected from HHD patients and unaffected family members, and 100 unrelated healthy people. Genomic DNA was extracted from peripheral blood leukocytes using TianGen DNA kit. All 28 exons and their flanking intron sequences
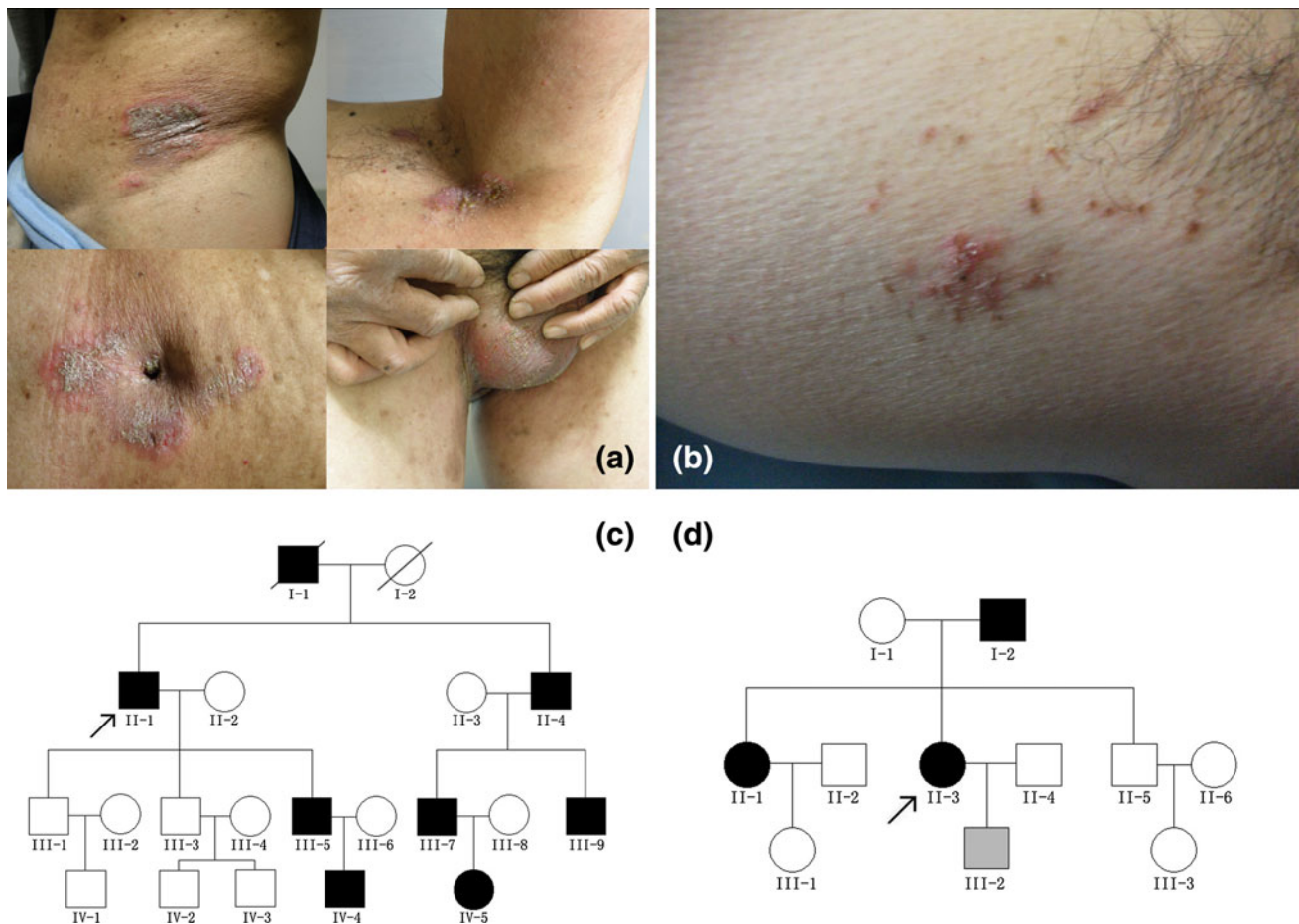
Fig. 1 **a** The clinical manifestation of the proband with erythematous plaques, vesicopustules and dry crusted erosions in the axilla, groin, waist and navel. **c** Pedigrees of the FHHD-A family. **d** Pedigrees of the FHHD-B family. Males are denoted by *squares* and females by *circles*. The affected members are symbolized by *solid symbols* and unaffected members by *open symbols*. The member (FHHD-B-III-2) who carried the mutation without symptoms is symbolized by the *gray symbol*. The proband is marked with an *arrow*. The deceased family members are indicated by "/". **b** Clinical features of the patient FHHD-III-5

Table 1 Summary of profiles of the patients/families

Family/Patient	Mutation	Sex	Age of onset (years)	Involved skin sites	Course of disease	Current status
FHHD-A-II-1	R799X	M	28	Axilla, groin, waist and navel	Flare up on occasion	Severe symptoms
FHHD-A-II-4	R799X	M	31	Axilla, groin, perianal	Remission after age 60	Moderate symptoms
FHHD-A-III-5	R799X	M	24	Only in axilla	Flare up by quitting therapy	Mild symptoms
FHHD-A-III-7	R799X	M	26	Axilla, groin, waist and shoulder	Flare up on occasion	Moderate symptoms
FHHD-A-III-9	R799X	M	25	Axilla, groin, perianal	Continual	Severe symptoms
FHHD-A-IV-4	R799X	M	19	Axilla, groin	Continual	Severe symptoms
FHHD-A-IV-5	R799X	F	22	Axilla, submamma, groin	Continual	Severe symptoms
FHHD-B-II-1	R417K	F	29	Axilla, submamma, popliteal fossa	Continual	Moderate symptoms
FHHD-B-II-3	R417K	F	26	Axilla, groin, popliteal fossa	Flare up on occasion	Severe symptoms
FHHD-B-III-2	R417K	M	Unknown	None	Until now disease free	None symptoms
SHHD-1	D644G	M	27	Axilla, groin, waist and perianal	Flare up by quitting therapy	Severe symptoms
FHHD-C-II-1	Q865X	M	24	Axilla, groin	Continual	Moderate symptoms
FHHD-C-III-1	Q865X	F	30	Axilla, groin, submamma	Flare up on occasion	Mild symptoms
FHHD-C-III-3	Q865X	M	25	Axilla, groin	Continual	Severe symptoms
SHHD-2	G645V	M	29	Axilla, groin, popliteal fossa	Flare up on occasion	Moderate symptoms

of ATP2C1 gene were amplified. The primers were designed according to the previously published information [2] and our previous study [10]. Amplification conditions were for 30 s at 94°C followed by 32 cycles of 30 s at 94°C, 45 s at 55°C and 45 s at 72°C, and a final extension for 5 min at 72°C. The purified PCR products were sequenced using dye terminator chemistry the BigDye terminator v3.1. The sequencing reactions were run on Applied Biosystems 3730XL DNA analyzers. Sequence comparisons and analysis were performed using Chromas Version 2.0.

The mutation D644G created a novel restriction site (5′-GAGG-3′) for the *Mnl*I restriction endonuclease. The 108 bp sequences were amplified by PCR, using a pair of primers 5′-CGCTACAGAAGAACGGTTCAG-3′, 5′-CTGTACCAGTCTGGCCCATC-3′. Then 5 μl of PCR products were digested with 1 U *Mnl*I (FastDigest, Fermentas) at 37°C for 2 h. Finally, the resulting digestion products were separated on a 4.0% agarose gel.

RT-PCR and real-time PCR

The expression of ATP2C1 mRNA was analyzed using RT-PCR analysis standardized by coamplification with the housekeeping gene GAPDH as an internal control. Total RNA isolated from the peripheral blood was reverse transcribed into cDNA (RevertAid[TM] First Strand cDNA Synthesis kit, Fermentas) and used for PCR with primers specific for ATP2C1 and GAPDH. The ATP2C1 primer sequences were 5′-GGAGCATACACTTGCCCGAGA-3′ (forward primer) and 5′-CACCTGTCAAGCTGGACT CATCA-3′ (reverse primer), giving a 131 bp PCR product.

For GAPDH, the forward primer was 5′-GCACCGT CAAGGCTGAGAAC-3′ and the reverse primer was 5′-TGGTGAAGACGCCAGTGGA-3′, giving a 138 bp PCR product. After amplification for 32 cycles (conditions ibid.), the PCR products were separated on 1.5% agarose gel and visualized by UV transillumination.

Meanwhile, ATP2C1 gene expression was quantified by real-time PCR using the Step One real-time PCR system (Applied Biosystems). The cDNA was added to a SYBR Premix Ex Taq[TM] II (DRR081S, TaKaRa) containing the forward and reverse primers of ATP2C1. The cDNA was amplified by 40 cycles of denaturation at 95°C for 5 s, annealing at 60°C for 31 s. During the first cycle, the 95°C step was extended to 35 s, and on the final cycle, the 60°C step was extended to 1 min to check the melting curve. We used three unrelated healthy people as control. The GAPDH gene was amplified in the same reaction to serve as the reference gene. The data were representative of three separate experiments.

Immunohistochemical staining

After tissues were fixed in 20% formalin and embedded in paraffin, 3 mm tissue sections were cut and prepared for immunohistochemical staining. It was performed according to the standard technique with Streptavidin-Peroxidase System kit (ZhongShan Bio.). Rabbit polyclonal antibody raised against amino acids 720–919 of SPCA1 were used (sc-5548, Santa Cruz Biotechnology). The secondary antibody was goat anti-rabbit labeled HRP (SP-90001 ZhongShan Bio.). Primary antibodies were diluted 1:50, and incubated at 4°C overnight. The secondary antibody

was reacted for 1 h at 37°C. 3, 30-diamino-benzidine solution (DAB) was used as the brown chromogen, followed by counterstaining with Mayer's hematoxylin. All specimens were stained under precisely the same conditions. Negative control stained without the primary antibody showed the background non-specific staining, and the normal skin was used as the control.

Results

Mutational analysis of the ATP2C1 gene

In our study, the heterozygous mutation C→T at nucleotide 2395 in exon 26 of the ATP2C1 gene was identified in the proband of FHHD-A pedigree, resulted in a nonsense mutation R799X (Fig. 2a). Another heterozygous mutation G → A at nucleotide 1659 in exon 16, which caused a missense mutation R417K, was found in the proband of FHHD-B pedigree (Fig. 2c). These two mutations were also, respectively, found in the affected family members. In this study, we provided the genomic survey for the individuals (FHHD-A-III-1, III-3, IV-1, IV-2, IV-3, and FHHD-B-II-5, III-1, III-2, III-3) without clinical manifestations so far. We identified the R417K mutation in the patient FHHD-B-III-2 and excluded the possible of disease in others. The third mutation was the heterozygous mutation A→G at nucleotide 1931 in exon 22, and caused a missense mutation D644G in the sporadic case SHHD-1 (Fig. 2e). None of these mutations was found in the healthy family members nor in the 100 control individuals, indicating that they were not likely to be neutral polymorphism (Fig. 2b, d, f).

The mutation D644G created a novel restriction site (5′-GAGG-3′) for the *Mnl*I restriction endonuclease, by which the 108 bp PCR products with the D644G mutation were cleaved into two shorter fragments 72 and 36 bp, whereas only an undigested 108 bp band was observed in the PCR products of a normal control (Fig. 2g). Therefore, this mutation was confirmed by restriction fragment length polymorphism (RFLP) analysis.

Genotype–phenotype comparison

We compared two types of mutations from the aspects of the age of onset, clinical manifestations, course of disease, and so on (Table 1). We found that the patients with missense or nonsense mutation had been showing common clinical features such as erythema, erosions, and scales at intertriginous areas, and had not shown any additional unique features in other aspects. As same as other previous studies, it was hard to establish a relationship between the genotype and phenotype merely depending on clinical

observation and data collection. In an interesting case of FHHD-A-III-5 (Fig. 1b), mutation analysis of ATP2C1 gene was performed and identified a C > T transition at nucleotide 2395 in a 39-year-old male with hyperkeratotic papules and slight erosions only in his axillae. A previous report about the identical mutation R799X described a sporadic patient with similarly atypical features [13]. But considering the clinical manifestations of other patients in the FHHD-A, we believed that the atypical clinical features were mainly due to non-genetic factors.

ATP2C1 mRNA levels are decreased in Hailey–Hailey patients

We made comparisons of the ATP2C1 mRNA expression between the HHD patients and unrelated healthy people (Fig. 3a, b). The results showed that the expression of all HHD patients was obviously lower than the normal people. Meanwhile, we found that the expression level of patients with nonsense mutation was lower than the patients with missense mutation and less than half the level of normal people, especially in patients with typical symptoms. The expression of unaffected family members was identical to the normal people (data not shown). More interestingly, we found that the ATP2C1 expressions of some patients (FHHD-A-III-5, FHHD-B-III-2 and FHHD-C-III-1) were closer to the expression level of normal people compared to other patients.

The expression of hSPCA1 is reduced in epithelial tissue with HHD

The intensity of immunohistochemically stained epidermal cells consistently demonstrated the strong cytoplasmic expression of SPCA1 in normal skin compared to hardly any expression in the negative control (Fig. 3c). There was a significant reduction of staining intensity in all HHD epidermis specimens compared to the normal one. Furthermore, the acantholytic areas were stained weakerly compared to para-acantholytic areas in same specimens of HHD patients (Fig. 3d–h).

Discussion

In this study, we identified three heterozygous mutations in Chinese patients with HHD. The mutation 2395C > T (R799X) located in the luminal domain between transmembrane domains 7 and 8, which have been reported three times [2, 13, 11]. The ATP2C1 gene with this mutation was predicted to encode abnormal gene products of prematurely truncated mutant hSPCA1 proteins. Considering that this mutation had repeatedly been reported by different researchers in different race, we believe that 2395C > T
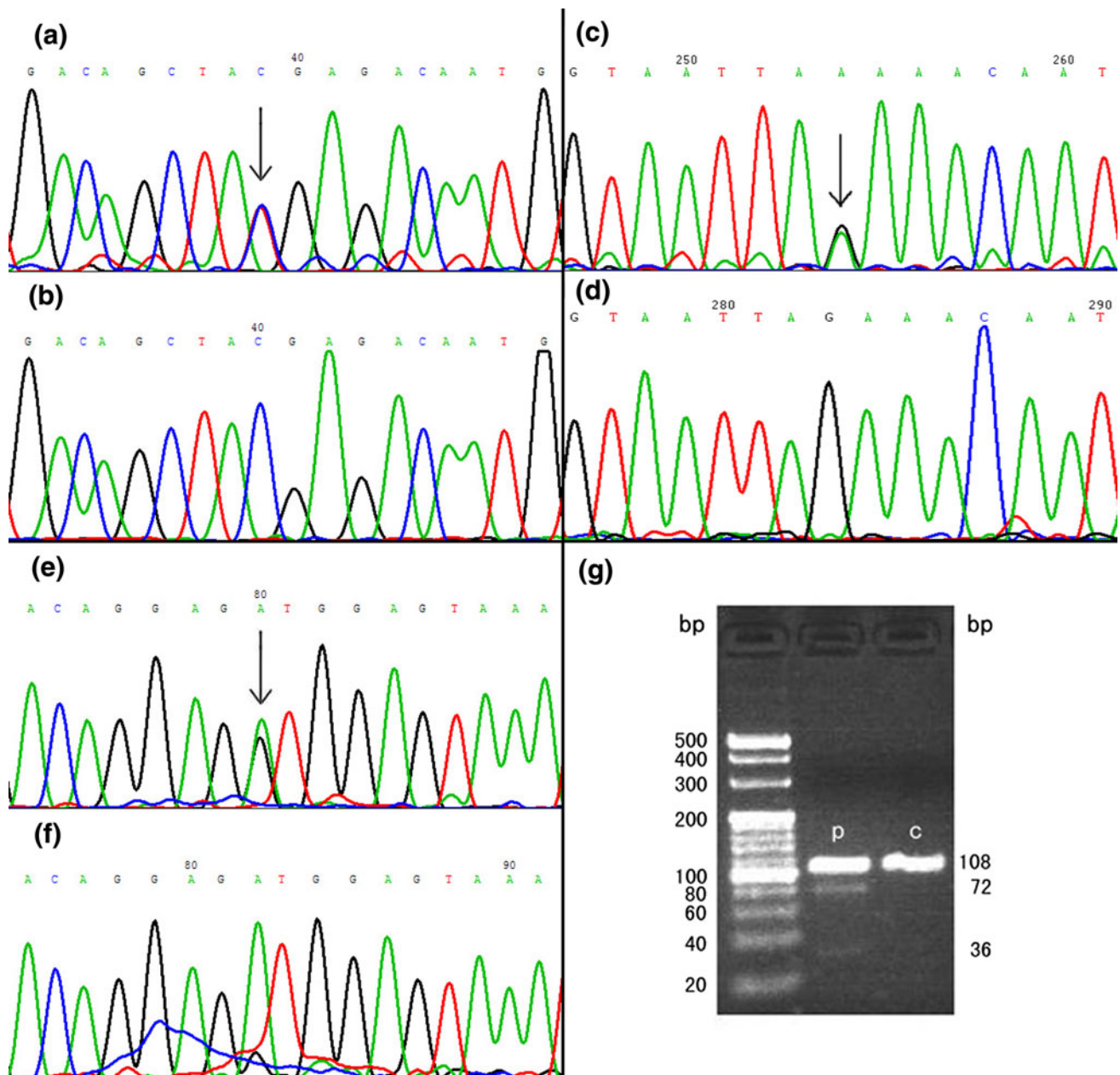
Fig. 2 **a** 2395C > T (R799X) heterozygous mutation in exon 26 of ATP2C1 gene in the proband of the FHHD-A pedigree; **c** 1659G > A (R417K) heterozygous mutation in exon 16 of ATP2C1 gene in the proband of the FHHD-B pedigree; **e** 1931A > G (D644G) mutation in exon 22 of ATP2C1 gene in sporadic case SHHD-1; **b, d, f** sequence of the normal persons; **g** The mutation D644G created a novel restriction site (5'-GAGG-3') for the *Mnl*I restriction endonuclease. The PCR products of the patient (P) were digested to 72 and 36 bp fragments, whereas, only an undigested 108 bp fragment is observed in a normal control (C)

mutation must be a common disease-causing mutation. The novel mutation 1659G > A (R417K) located in the cytoplasmic domain of hSPCA1 was predicted to have a relationship with intracellular signal transduction. The other novel mutation 1931A > G (D644G) located in one of the two Mn^{2+}-binding sites (aspartic acid residues 644 and 648), which is conservative and important domain for vertebrate to transport Mn^{2+} from the cytosol to the lumen of the Golgi apparatus (searched in www.uniprot.org/uniprot/P98194). The possible effect of this mutation was that the change in the amino acid sequence would lead to abnormal protein folding, and damaging membrane trafficking of Mn^{2+}. In addition, we accessed to the previous reports about the two Mn^{2+}-binding sites and nearby region and found all the mutations (Q633X, M641R, G645R, G645V, D648Y, A651D, etc.) were detected in Asian (especially in Chinese) [7, 10, 16–18].
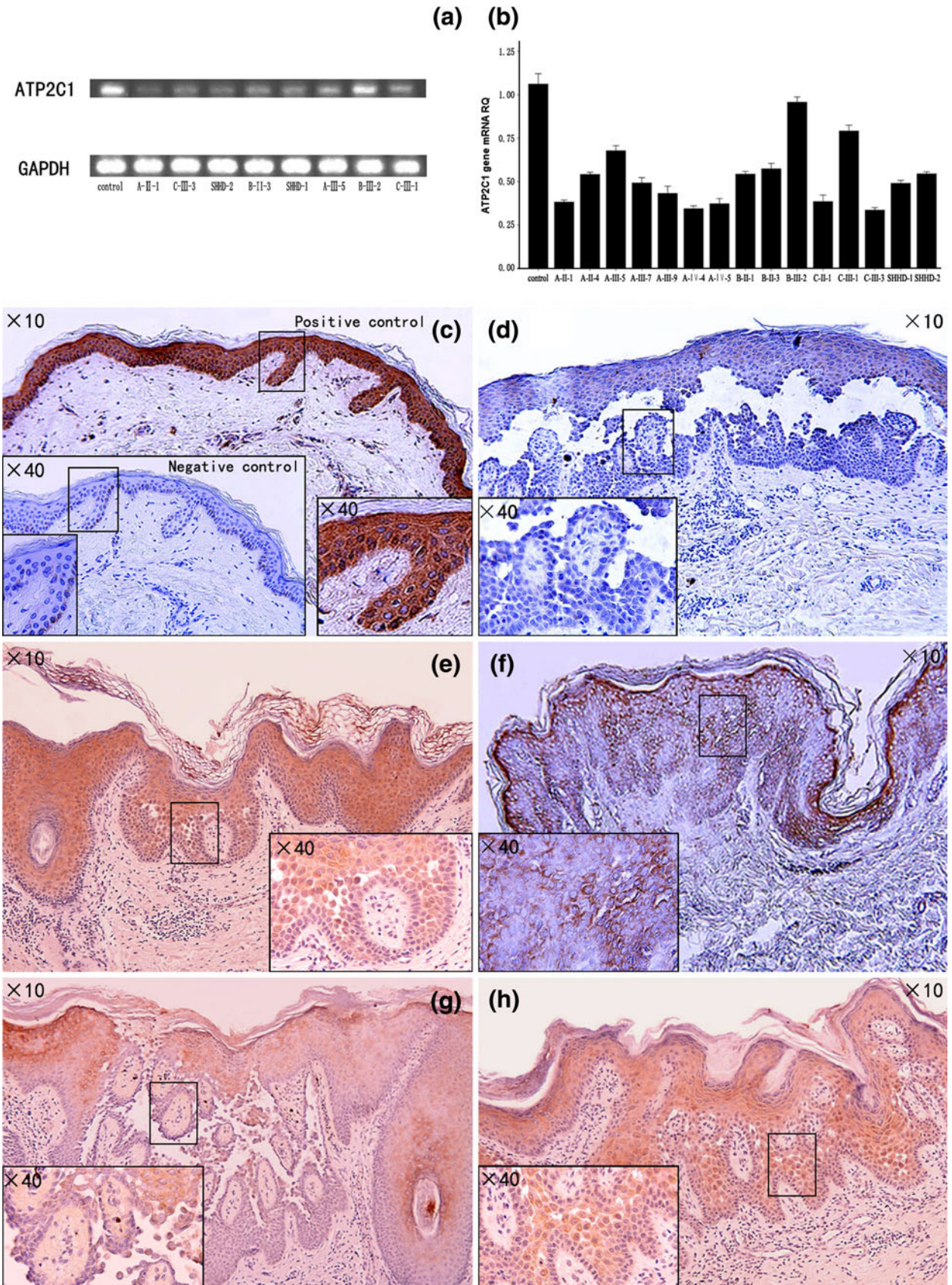
◄ **Fig. 3** The mRNA expression of ATP2C1 was assessed by RT-PCR (**a**) and real-time PCR (**b**). The mRNA expression of ATP2C1 obviously decreased in all the HHD patients compared to the normal people, meanwhile the expression of the HHD patients with nonsense mutation was lower than the patients with missense mutation and less than half the level of normal people. Each value of mRNA was normalized to the amount of GAPDH mRNA as an internal standard; The immunohistochemical staining of hSPCA1: normal skin (**c**), FHHD-A-II-1 (**d**), FHHD-B-II-3 (**e**), SHHD-1 (**f**), FHHD-C–III-3 (**g**), SHHD-2 (**h**), original magnification at ×10 (and ×40 in squares). **c** Normal skin showed a strong positive staining of epidermal cells and the negative control showed staining pattern without antibody to hSPCA1. **d–h** There was a significant reduction of staining intensity in all HHD epidermis specimens compared to the normal one, furthermore the staining intensity of the acantholytic areas were stained weakerly compared to para-acantholytic areas in the same specimen

As there had been no report about the mutation in other race so far, it implied this nucleotide region might be an Asian unique mutated region.

Although, the precise generation mechanism of acantholysis was still unknown, it was suggested that the mutations in ATP2C1 would undermine the functions of hSPCA1 leading to abnormal intracellular signal transduction, and finally damage the cell-to-cell adhesion.

In previous researches, a spectrum of missense, frame-shift, splice-site, and nonsense mutations have since been reported in ATP2C1. Among them, 20% are nonsense mutations, 30% are frame shift mutations leading to premature termination codons (PTCs), 28% are missense mutations [14]. 50% of mutations leading to PTCs points to haploinsufficiency as the predominant mechanism for HHD, but other researchers believe that missense mutations may cause HHD through the dominant negative mechanism [3]. The haploinsufficiency theory predicts the effect of mutations is an absence or significant reduction in the level of mutant hSPCA1 protein expression as a consequence of nonsense-mediated mRNA decay [6]. However, the dominant negative mechanism presumes that the low expression level of mutant hSPCA1 protein is not the result of impaired mRNA levels, and suggests that the mutations introduce structural perturbations into hSPCA1. This could result in either abnormal protein folding or destabilization of the correctly folded protein, thus making it sensitive to endoplasmic reticulum-mediated quality control [4, 5]. In this study, we investigated and compared nonsense mutation and missense mutation in 3 pedigrees and 2 sporadic cases, 15 HHD patients in total. Interestingly, we found that the expression of ATP2C1 mRNA and hSPCA1 protein manifested consistently decline in all the patients. Therefore, our findings supported that the theory of haploinsufficiency was the predominant mechanism in both types of mutations, especially in patients with typical symptom.

There were no clear genotype–phenotype correlation obtained by clinical observation and data collection from these HHD patients, but we found the difference of ATP2C1 mRNA expression in peripheral blood between the two types of mutations. The expression level of HHD patients with nonsense mutation was lower than the patients with missense mutation and even less than half the level of normal people, though all of them had the typical clinical features. We believed that the different levels of ATP2C1 mRNA expression implied the discrimination between the two types of mutations. It was regretful that there were a few reports about the ATP2C1 mRNA expression in peripheral blood, so its physiological significance in HHD needed further studies. Meanwhile we found the expressions of some patients in this study (FHHD-A-III-5, FHHD-B-III-2 and FHHD-C-III-1) were closer to the normal people than the other patients. By carefully analyzing the clinical data, we speculated that the relatively high level of expression was due to these patients with mild symptom or without any symptom at present (such as FHHD-B-III-2 who inherited the mutation from the mother, are until now disease free). So, we believe that the level of ATP2C1 mRNA expression in peripheral blood might reflect pathogenetic condition of HHD patients.

In summary, our study expands the mutations spectrum of HHD in Chinese patients and realizes the molecular diagnosis for the asymptomatic individuals in the HHD pedigrees. Our findings supported that the haploinsufficiency theory was the predominant mechanism in both types of the mutations, and revealed the levels of ATP2C1 mRNA expression may related to the type of mutation in HHD. Comparing the patients with different degrees of clinical symptoms, we believed that the ATP2C1 mRNA expression analysis in peripheral blood of HHD patients may provide important information for the prevention, treatment and prognosis of HHD in the clinical.

References

1. Burge SM (1992) Hailey–Hailey disease: the clinical features response to treatment and prognosis. Br J Dermatol 126:275–282
2. Chao SC, Tsai YM, Yang MH (2002) Mutation analysis of ATP2C1 gene in Taiwanese patients with Hailey–Hailey disease. Br J Dermatol 146:595–600
3. Cheng Y, Cheng YM, Zhao G et al (2011) A novel missense mutation of the ATP2C1 gene in a Chinese patient with Hailey–Hailey disease. Biochem Biophys Res Commun 406:420–422
4. Daiho T, Yamasaki K, Suzuki H et al (1999) Deletions or specific substitutions of a few residues in the NH$_2$-terminal region of sarcoplasmic reticulum Ca^{2+}-ATPase cause inactivation and

rapid degradation of the enzyme expressed in COS-1 cells. J Biol Chem 274:23910–23915

5. Fairclough RJ, Dode L, Vanoevelen J et al (2003) Effect of Hailey–Hailey disease mutations on the function of a new variant of human secretory pathway Ca^{2+}/Mn^{2+}—ATPase (hSPCA1). J Biol Chem 278:24721–24730

6. Fairclough RJ, Lonie L, van Baelen K et al (2004) Hailey–Hailey disease: identification of novel mutations in ATP2C1 and effect of missense mutation A528P on protein expression levels. J Invest Dermatol 123:67–71

7. Hu Z, Bonifas JM, Beech J et al (2000) Mutations in ATP2C1, encoding a calcium pump, cause Hailey–Hailey disease. Nat Genet 24:61–65

8. Kitajima Y (2002) Mechanisms of desmosome assembly and disassembly. Clin Exp Dermatol 27:684–690

9. Korner J, Rietschel M, Nothen MM et al (1993) Familial co-segregation of affective disorder and Hailey–Hailey disease. Br J Psychiatry 163:109–110

10. Li XL, Xiao SX, Peng ZH et al (2007) Two novel mutations of the ATP2C1 gene in Chinese patients with Hailey–Hailey disease. Arch Dermatol Research 299:209–211

11. Majore S, Biolcati G, Barboni L et al (2005) ATP2C1 gene mutation analysis in Italian patients with Hailey–Hailey disease. J Invest Dermatol 125:933–935

12. Martin J, Behne, Chia-Ling Tu, Ida Aronchik et al (2003) Human keratinocyte ATP2C1 localizes to the golgi and controls golgi Ca^{2+} Stores. J Invest Dermatol 121:688–694

13. Nemoto-Hasebe Ikue, Akiyama Masashi, Osawa Rinko et al (2008) Diagnosis of Hailey–Hailey disease facilitated by DNA testing: a novel mutation in ATP2C1. Acta Derm Venereol 88:399–400

14. Szigeti Re'ka, Kellermayer Richard (2006) Autosomal-dominant calcium ATPase disorders. J Invest Dermatol 126:2370–2376

15. Sudbrak R, Brown J, Dobson-Stone C et al (2000) Hailey–Hailey disease is caused by mutations in ATP2C1 encoding a novel Ca^{2+} pump. Hum Molec Genet 9(7):1131–1140

16. Yan XX, Tian HQ, Yu YX et al (2010) Mutation analysis of ATP2C1 gene in patients with Hailey–Hailey disease. Chin J Dermatol 43(6):393–395

17. Zhang F, Yan X, Jiang D et al (2007) Eight novel mutations of ATP2C1 identified in 17 Chinese families with Hailey–Hailey disease. Dermatology 215(4):277–283

18. Zhu YG, Yang S, Gao M et al (2006) Two novel mutations of the ATP2C1 gene in Chinese families with Hailey–Hailey disease. J Dermatol Sci 42:125–127