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Two Null Variants Q2417X and R3409X in *FLG* Gene are Relevant to Ichthyosis Vulgaris in a Chinese Family

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ABSTRACT Ichthyosis vulgaris (IV) is one of the most ordinary hereditary keratinising illness, characterised by scaly, dry skin, and palmoplantar hyperlinearity. The function loss of filaggrin (*FLG*) gene mutations is considered as the molecular aetiology for IV. In this study the pathogenic variants of a Chinese IV family were explored by exome sequencing and PCR sequencing. Two null heterozygous variants in *FLG* coexisted in the pedigree, including c.7249C>T (Q2417X) and c.10225C>T (R3409X). The Q2417X variant was only found in the proband and their healthy mother, whereas the R3409X variant was observed in the other affected family members. The proband with Q2417X showed a much more severe phenotype than the patients with R3409X did, but their mother with Q2417X presented no clinic symptoms. The results strongly support that IV is a semidominant keratinising disorder that involved hereditary and environmental risk aspects and expanded the *FLG* variants spectrum in Asian populations.

INTRODUCTION

Ichthyosis vulgaris (IV; OMIM 146700) is one of the most ordinary genetic cornification dermatosis in humans, and its incidence is from 1:250 to 1:5300 (Samdani et al. 2010). The disease exhibits dry, scaly skin, and palmoplantar hyperlinearity and may or may not keratosis pilaris and atopy (Smith et al. 2006; Gruber et al. 2007). The IV symptoms are most outstanding in cold or in winter, and can alleviate or disappear after adolescence in most patients. Previous reports reveal that 2.5 to 27.0 percent of atopic dermatitis (AD) patients possess similiar clinical manifestations to IV patients (Bremmer et al. 2008). AD dermatosis frequently presents chronic, pruritic, recurrent and inflammatory features (Bieber 2008; Sandilands et al. 2009). It shares an intricate pathogenesis with IV, involving immunological, hereditary, and environmental factors (On et al. 2017). *FLG* gene (NM_002016) on 1q21 is currently determined to be a primary disease-causing gene of these diseases (Smith et al. 2006; Akiyama 2010). It encodes a keratin filament-associated protein (filaggrin), which is critical for the generation of periostracum in the course of epidermal differentiation (Sandilands et al. 2007). Periostracum is responsible for preventing the access of sensitizins, pathogens, and toxic chemicals and decreasing water loss through the epidermis (O'regan et al. 2009; Osawa et al. 2011). The function loss of *FLG* variants was identified as the molecular aetiology of impaired skin barrier in AD and IV patients (Smith et al. 2006). These variants were reported to be transmitted in a semidominant mode with heterozygotes showing a mild or asymptomatic sign with approximately ninety percent penetrance and compound heterozygotes and homozygotes presenting a serious symptom (Smith et al. 2006). The *FLG* gene is composed of three exons. Exon 1 (15bp) is 5'-noncoding region and exon 2 (159bp) comprises a start codon. Amazingly, exon 3 is an extraordinarily enormous segment with 12.7-14.7kb and encodes the majority of the multimeric profilaggrin involved in 10 to 12 tandem repetitive fragments with the filaggrin of 37-kDa (Presland et al. 1992). Because the *FLG* is a large molecular gene with highly repetitive segments, it is very hard to perform routine and successful sequencing of *FLG*. Therefore, it remains challenging to conventionally identify the *FLG* variants of IV and AD. However, it is a strong and cost-efficient strat-

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egy to use whole exome sequencing (WES) to explore the causative mutations of the diseases. In the current study, by combining WES with PCR sequencing, the findings displayed two null variants (Q2417X and R3409X) in *FLG*, associated with the Dong Chinese pedigree with IV, and further confirmed the possible existence of additional modifiers in IV.

Objective

This study aimed to clarify the clinical phenotype of a Chinese family affected with IV, and to elucidate its molecular aetiology by combining exome sequencing with PCR sequencing.

METHODOLOGY

Clinical Material

A Dong Chinese pedigree affected by ichthyosis vulgaris was recruited from Hunan province in China. Medical information of the family was fully recorded. Eleven blood samples (four affected and seven unaffected individuals) were collected for further genetic analysis. The diagnosis of individuals with IV was conducted clinically by experienced dermatologists and immunologists according to clinical manifestation of early onset, dry skin, alterable scaling on the trunk and limbs, hyperlinearity of palmoplantar dermatoglyph and a positive peedigree history.

Ethics Statement

Each participant of the study signed informed consent following the Helsinki Declaration. This work was authorised by the medical ethical council of Hunan University of Medicine. Collection procedure of blood samples was in accordance with the ethical principle of human experimentation.

Exome Sequencing

Genomic DNA (gDNA) of the participants was isolated from their peripheral blood leukocytes utlising DNA extraction kit in the light of the corporation's specifications (Tiangen Biotech Co. Ltd, Beijing, China). WES for the proband (bⅢ3) and their aunt (aⅡ2) (Fig. 1a) was performed by the SierraVast Biomedical Technology Co. Ltd-Shanghai, China. On the basis of the corporation's protocols, Over 1.5μg of gDNA for each sample was utilised to build the exome library. The eligible gDNA was fragmented at random utilising the Covaris technique. Subsequently, the DNA library was integrated and hybridised for exons enrichment utilising Agilent SureSelectXT Human All Exon V7 following corporation's specifications (Agilent Technologies Inc, Santa Clara, CA). And then enriched exome fragments with 150bp paired-end were sequenced in Illumina Novaseq sequencer (Illumina, CA). The sequence data were preserved in FASTQ format. The mean depth for the subjects was estimated to be over 100×. The raw data were counterpointed to the human reference genome sequence of Hg19/GRCh37. Next the researchers filtered the single nucleotide variants (SNVs) and insertions/deletions (indels) out in the areas of segmental repetitions and unassigned chromosomes. After eliminating the repetitive reads and proofreading the base quality scores, SNVs and indels were primarily called employing Haplotyper of Sentieon for the two samples. Afterwards, variant quality score recalibration and various filter ways were employed to gain the high-quality variant for indels and SNVs. Annotations of SNVs and indels used SnpEff software and online databases, including OMIM, GnomAD, ClinVar and HGMD. Subsequently, the researchers preferentially selected *FLG* as a candidate gene, which has been reported to be responsible for IV. The guidances of American College of Medical Genetics (ACMG) were employed to evaluate the pathogenicity of mutations (Xu et al. 2019).

Variant Validation

After WES, PCR sequencing was utilised to validate genetic variants. Primer sequences of underlying pathogenic variants in *FLG* were employed as described previously (Bremmer et al. 2008). Gene amplification was conducted by PCR. It's reaction system was 20 μL involving10 μL of 2×Taq Master Mix (Vazyme Biotech Co.Ltd, Nanjing, China), 40 ng of gDNA, and 2.0 μL of each primer. Thermocycling conditions were as follows: predenaturation at 96°C for 2 minutes, denaturation at 94°C for 10 seconds, annealing at 57°C to 62 °C for 30 seconds, new chain extension at 72°C for 1 minute, and the amplication procedure was repeated 35 times. The ultimate extension with 72°C

was run for 5 minutes. The temperature of annealing was different on the basis of each pair of primers. The amplified products were first cataphoresised on 1.5 percent agarose gels, followed by purification using standard protocols, and then sequenced with an ABI 3730XL machine (Applied Biosystems) (Xu et al. 2019). Lastly, mutation identification was conducted with accessible gDNA of the family individuals, and followed by genotyping in 500 ethnicity-matched unrelated normal controls with direct sequencing.

RESULTS

Clinical Features

A total of four cases were diagnosed with ichthyosis vulgaris in the family. The pedigree presented an autosomal dominant manner of heredity with incomplete penetrance (Fig. 1a). The proband was a 21-year-old male inborn with palmar hyperlinearity (Fig. 1b). At the age of 3 years, he presented plenty of diffuse superficial fine scales on his extremities and trunk skin, especially on his lower limbs. His skin lesions were aggravated in winter and relieved or even disappeared in summer. He developed distinct hyperkeratosis in his palmoplantar at the age of 6 years. In the Chinese family, there were considerable alterations in the extent of universalisation and in the intensity of scaling. At present, the proband displayed a severe IV phenotype with prominent scaling on the trunk and limbs and obvious palmoplantar skin keratinisation, especially in winter, and his skin surface was quite rough and diffuse without itchy red patches (Figs. 1c and 1d), whereas the other three patients with moderate IV presented fine, grey scaling mainly only on the outer surfaces of the limbs (Fig. 1e). Family medical history revealed that all affected patients were undergoing the similar clinical process but to varying degrees in their lives.

Exome Sequencing

A total of 9804.37Mb raw reads for each sample were generated, showing an average coverage of 148×. All heterozygous variants from the two affected patients submitted to WES were screened for quality and detected against the dbSNP137, HapMap8, 1000 Genomes, and the YH1 public variant database. After synonymous and known common variants were excluded, in the known pathogenic *FLG* gene (ENST00000368799) of IV, two null heterozygous variants c.7249C>T (chr1-152280113,

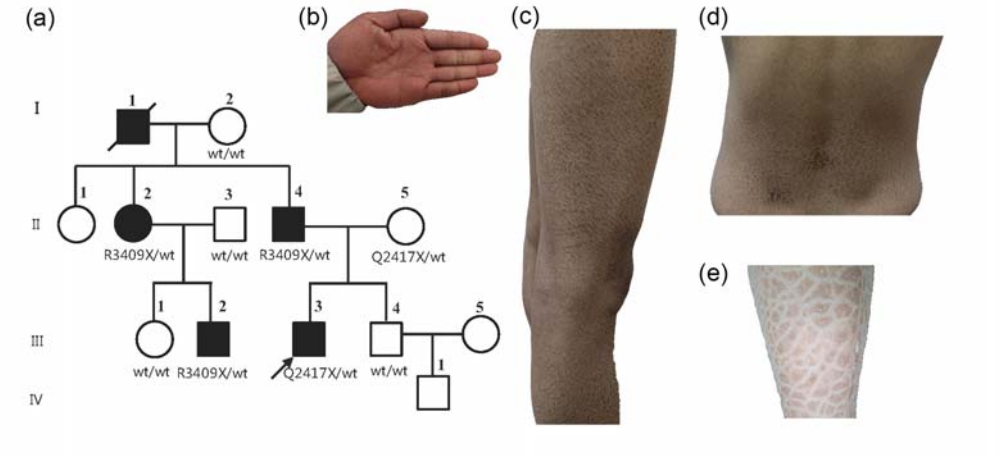

Fig. 1. Pedigree and photographs of IV family. (a) Affected subjects were demonstrated by solid circles (females) or squares (males). Healthy subjects were demonstrated by open symbols. The proband (bⅢ3) was indicated by a arrow. (b) Palmar hyperlinearity of the proband. (c) Diffuse superficial fine scales on the lower limbs of the proband. (d) Fine scales and xerosis on the back of the proband. (e) Gray scaling on the lower limbs of the proband's aunt (aⅡ2)

rs528722713) and 10225C>T (chr1-152277137, rs201356558) were identified in the proband and his aunt, respectively.

Identification of Pathogenic Mutations

The variant c.7249C>T (p.Q2417X) of the *FLG* was observed only in the proband (bⅢ3) and his normal mother (aⅡ5) (Fig. 2a), resulting in a premature stop codon at profilaggrin position 2417, but it was deficient in the other family subjects according to Sanger sequencing. The other c.10225C>T (p.R3409X) variant was identified in three affected patients (aⅡ 2, aⅡ4, bⅢ2) (Fig. 2b), causing a premature stop codon at profilaggrin position 3409, but it was not found in the other family members. Both variants were forecasted to be pathogenic in the light of ACMG. Moreover, by genotyping the researchers found that 2 (0.4%) and 0 were heterozygous in 500 ethnicity-matched unrelated normal controls for c.7249C>T and c.10225C>T, respectively. To the researchers' knowledge, the R3409X variant had not been previously reported in Asian patients.

DISCUSSION

Clinically, IV exhibits dry skin, excess scaling, keratosis pilaris, palmoplantar hyperlinearity and a powerful correlation with AD (Wells 1966; Oji et al. 2010). AD is a common chronic and recurrent inflammatory skin disease, which often shows the itchy red plaques in the flexible regions of the knees and elbows (Akdis et al. 2006). Histologically, these conditions result from a decrease in the quantity and dimension or full lack of epidermal keratohyalin granules (Sybert et al. 1985). These structures are linked to keratin filaments. During terminal epidermal differentiation, the huge profilaggrin molecules are segmentally sheared into 10 to 12 filaggrin proteins, ensuring suitable polymerisation of keratin filaments in the cytomatrix and production of lots of 'natural moisturising factors' in the epicuticula (Rawlings 2004; Presland 2009). The *FLG* variants in the epidermal differentiation compound have been confirmed as the genetic aetiology of AD and IV diseases (Smith et al. 2006; Akiyama 2010). Previous studies suggested that function loss of *FLG* variants is strikingly popular in Asians and Europeans (Palmer et al. 2006; Irvine et al. 2011). Nevertheless, a majority of *FLG* variants are observed only in different populations, namely the Africans, Europeans and Asians (Hsu et al. 2009). IV is transmitted from generation to generation in a semidominant mode with approximately ninety percent penetrance (Gruber et al. 2007; Oji et al. 2009), so the patients with either compound heterozygosity or homozygous are seriously affected, whereas the heterozygous individuals are only slightly affected or not (Smith et al. 2006). To date, more than 40 *FLG* mutations were found in IV subjects and the mutations involved only nonsense and frameshift variants that led to generate a truncated profilaggrin molecule impossibly formed efficient subunits of filaggrin (Sandilands et al. 2007), which revealed the *FLG* variants probably had a similar effect (Zhang et al. 2010). It still remains possible that other factors except for *FLG* gene variants can result in the ichthyosiform symptom

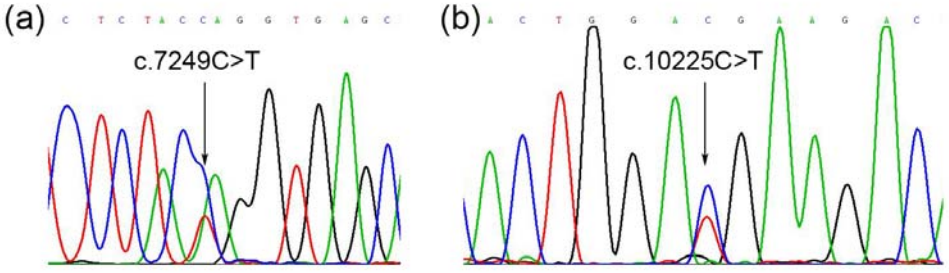

Fig. 2. Sequence chromatograms of the *FLG* gene. The arrow demonstrated the variant site. (a) The heterozygous c.7249C>T variant in the proband (bⅢ3) and his mother (aⅡ5). (b) The heterozygous c.10225C>T variant in the 3 affected patients (aⅡ2,aⅡ4,bⅢ2)

in the occurrence of AD and IV(Li et al. 2013; Hassani et al. 2018).

FLG is a particularly large and highly repetitive gene recognised generally to be very difficult to sequence for identification of causative variants. In this study, by combing exome sequencing with PCR sequencing for the proband and his family members, the result showed two null variants c.7249C>T (Q2417X) and c.10225C>T (R3409X) in *FLG* gene, which were major causes of IV patients from the Dong Chinese family. These variants each caused a truncated profilaggrin molecule unlikely to be generated by effective filaggrin subunits. Filaggrin was very significant for the terminational differentiation and shield role of the epicuticula. The both variants were also predicted to be pathogenic according to ACMG guidelines. The Q2417X was verified to cause simple IV, simple AD, and AD-associated IV in Asian patients (Chen et al. 2008; Hsu et al. 2009; Gong et al. 2019). In this study, the proband (bⅢ3) carried Q2417X variant from his mother and displayed serious IV symptom in his trunk and limbs without pruritic red patches, whereas his mother was completely absent for IV symptom. Subsequently, the researchers genotyped the variant Q2417X in 500 ethnicity-matched unrelated normal controls by direct sequencing, and found that 2 were heterozygous (0.4%), which was consistent with results reported by Hu et al. (2012). Therefore, the researchers speculated that some clinical symptoms in IV patients involved not only *FLG* variants but also environmental risk factors as well as variants of *FLG* modifier genes. The R3409X was identified to result in AD in African-Americans patients (Hu et al. 2012; Margolis et al. 2014). In the Dong Chinese family, the three affected members (aⅡ2,aⅡ4,bⅢ2) all carried the R3409X variant, whereas the variant was absent in 500 normal controls and the healthy family members. These patients showed moderate IV symptoms unrelated to AD in this study. Therefore, the researchers speculated that the variant in the *FLG* gene causing IV was probably associated with environmental risk factors, or the *FLG* gene interacted with other genes involved in IV (Akdis et al. 2006). The other gene variants within the chronic inflammation or epidermal differentiation complex may lead to the shortage of epidermal textural proteins at the epidermal shield, which may result in the ichthyosiform manifestation under the circumstance of AD (Li et al. 2013).

Until now, the Q2417X was reported three times to cause simple IV, simple AD, and AD-associated IV only within Asian patients (Chen et al. 2008; Hsu et al. 2009; Li et al. 2013), whereas it was absent in European and African-Americans patients. The R3409X was observed only once in African American patients with AD (Margolis et al. 2014; Margolis et al. 2019), whereas it was initially detected in Asian patients with IV in this study. These results revealed that there might be ethnic differences between *FLG* variants and clinical phenotype in particular ancestral populations.

CONCLUSION

In a word, a semi-dominant inheritance manner for IV was further validated in the Dong Chinese family by the Q2417X and R3409X in *FLG* gene. The findings strongly suggested that the other unknown genetic or environmental risk factors may be related to the occurrence of IV. Moreover, this study has vital significance for genetic counselling and molecular diagnosis for individuals with IV.

RECOMMENDATIONS

Collection of more cases is needed to thoroughly explain the relationship between *FLG* variants and IV clinical phenotype. The potential environmental risk factors, or other modifier genes interacting with the *FLG* gene causing semidominant inheritance of IV require further study.

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AUTHORS' CONTRIBUTIONS

Yongchun Long and Genyun Tang contributed equally to this study. The study design, program administrator, and drafting and revision of manuscript was completed by Haiou Jiang, while the experiment operation and data analysis was by

Yongchun Long and Genyun Tang. Lastly, data collection was by Yongchun Long, Xilu Yang, Wei Liu, Anli Shu and Hai Luo.

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