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ORIGINAL ARTICLE

Exome sequencing identified a missense mutation of *EPS8L3* in Marie Unna hereditary hypotrichosis

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ABSTRACT

Background Marie Unna hereditary hypotrichosis (MUHH) is an autosomal dominant disorder characterised by coarse, wiry, twisted hair developed in early childhood and subsequent progressive hair loss. MUHH is a genetically heterogeneous disorder. No gene in 1p21.1–1q21.3 region responsible for MUHH has been identified.

Methods Exome sequencing was performed on two affected subjects, who had normal vertex hair and modest alopecia, and one unaffected individual from a four-generation MUHH family of which our previous linkage study mapped the MUHH locus on chromosome 1p21.1–1q21.3.

Results We identified a missense mutation in *EPS8L3* (NM_024526.3: exon2: c.22G->A:p.Ala8Thr) within 1p21.1–1q21.3. Sanger sequencing confirmed the cosegregation of this mutation with the disease phenotype in the family by demonstrating the presence of the heterozygous mutation in all the eight affected and absence in all the seven unaffected individuals. This mutation was found to be absent in 676 unrelated healthy controls and 781 patients of other disease from another unpublished project of our group.

Conclusions Taken together, our results suggest that *EPS8L3* is a causative gene for MUHH, which was helpful for advancing us on understanding of the pathogenesis of MUHH. Our study also has further demonstrated the effectiveness of combining exome sequencing with linkage information for identifying Mendelian disease genes.

INTRODUCTION

Hereditary hypotrichosis is a kind of inherited hair disorder which can grossly be characterised into two major groups according to the absence (non-syndromic hereditary hypotrichosis) or presence (syndromic hereditary hypotrichosis) of extra-cutaneous features. Alopecia universalis congenita/alopecia alopecia congenita with papular lesions (AUC/APL), hereditary hyportrichosis simplex (HHS), localised autosomal recessive hypotrichosis and Marie Unna hereditary hypotrichosis (MUHH; OMIM 146550/612841) are different forms of non-syndromic hereditary hypotrichosis. AUC/APL is characterised by early-onset, complete hair loss including eyebrows, eyelashes, armpit hair, pubic hair and body hair, and/or small erythematous papules of head and neck. Individuals with HHS typically show normal hair at birth, but hair loss and thinning of the hair shaft start without characteristic hair shaft anomalies during early childhood and progress with age. The affected individuals of HHS are affected scalp hair and/or body hair. The individuals with localised autosomal recessive hypotrichosis have hypotrichosis simplex with shortened length of the hair shaft, skin fragility/ectodermal dysplasia and woolly hair with palmoplantar keratoderma and cardiomyopathy syndrome, and associated with absence or scarcity of eyebrows, eyelashes and pubic hair.

MUHH is a rare autosomal dominant congenital hair disorder which was first described by the German dermatologist Marie Unna.[1] MUHH is distinguished from other forms of hypotrichosis by the presence of a twisting hair; affected individuals of this disease have normal, sparse or absent hair at birth, then develop to coarse, twisted and wiry hair during childhood and progress during puberty to an almost complete alopecia. Eyebrows, eyelashes and body hair are also markedly diminished or absent. No other ectodermal abnormalities are observed. So far, two linkage loci for MUHH have been mapped to chromosome 8p21[2] and 1p21.1–1q21.3.[3] After that, an international collaboration discovered that MUHH is caused by heterozygous mutations in the second 5′-untranslated region of the *HR* gene (*U2HR*) recently.[4] The MUHH pedigree in this study was reported previously by Yan *et al*[5] and Yang *et al*.[3] The former study found that affected individuals had little or no scalp hair at birth, wiry and irregular hair on the scalp that had been difficult to manage in childhood, and their forehead and parietal hair were bald or sparse in puberty. Eyebrows and eyelashes had always been thin. Axillary and pubic hair failed to develop. But the affected individuals have modest scarring alopecia and normal vertex hair. The sequent study had identified a locus for MUHH on chromosome 1p21.1–1q21.3 in this family.

For the past decades, the genes underlying Mendelian diseases have been identified through positional cloning, a process of meiotic mapping, physical mapping and candidate-gene sequencing. Recently, exome sequencing (also known as targeted exome capture) was demonstrated to be a cheaper but efficient strategy to selectively

sequence the whole genome coding regions. It has been widely used to identify genes for rare monogenic diseases and can also provide molecular identification of Mendelian diseases when the clinical diagnosis is uncertain.[6–9] Here, we identified a novel causative gene for MUHH by combining exome sequencing with the linkage information from our previous study.[3]

MATERIALS AND METHODS

Clinical sample

A four-generation MUHH family consisting of 21 individuals was first reported by Yan *et al*[5] from Anhui province in China (figure 1A). There were nine affected individuals including four males and five female subjects. After obtaining written informed consent from all the participants, EDTA anticoagulated venous blood samples were collected from eight patients and seven controls. The clinical features, histological characteristics, light microscopic examination and disease history supported the diagnosis of MUHH.[5] Sanger sequencing was performed to exclude *U2HR* mutation in this family. After that, two affected individuals (II3 and III10) and one unaffected individual (III2) were selected for exome sequencing. An additional 12 members (six cases and six controls) were evaluated in mutation validation analyses.

In addition, the unpublished exome sequencing data of ethnically and geographically matched 1457 subjects including 676 unrelated controls and 781 unrelated patients of other disease were used to filter variants. All the 1457 subjects were excluded MUHH by at least two dermatologist examinations.

Genomic DNA was extracted from peripheral blood lymphocytes by standard procedures using FlexiGene DNA kits (Qiagen, California, USA). This study was approved by Anhui Medical Institutional Review Board and conducted according to the Declaration of Helsinki Principles.

Exome capture library preparation and capture

Exome capture was carried out using Agilent SureSelect Human All Exon Kit (in solution), guided by the manufacturer's protocols. In brief, the qualified genomic DNA samples were randomly fragmented to 200–500 bp in size, followed by end-repair, A-tailing and paired-end index adapter ligation. Ligation-mediated PCR amplified extracted DNA purified by the Agencourt AMPure SPRI beads. Final libraries were validated by Bioanalyzer analysis (Agilent) and quantitative PCR. Each captured library was loaded on Hiseq 2000 platform and paired-end sequencing was performed, with read lengths of 90 bp, providing at least 50 average depth for each sample. Raw image files were processed by Illumina pipeline (V.1.3.4) for base calling with default parameters.

Next-generation sequencing data analysis

SOAPaligner 2.20[10 11] was used to align the reads to human reference hg19 with parameters set to '-a -b -D -o -v 2 -r 1 -t -n 4'. SOAPsnp (V.1.03)[12] was used to call consensus genotypes with Phred-like quality of at least 20 and at least four coverage depth was considered as the high-confident genotype. SNP (single nucleotide polymorphisms) were extracted from consensus

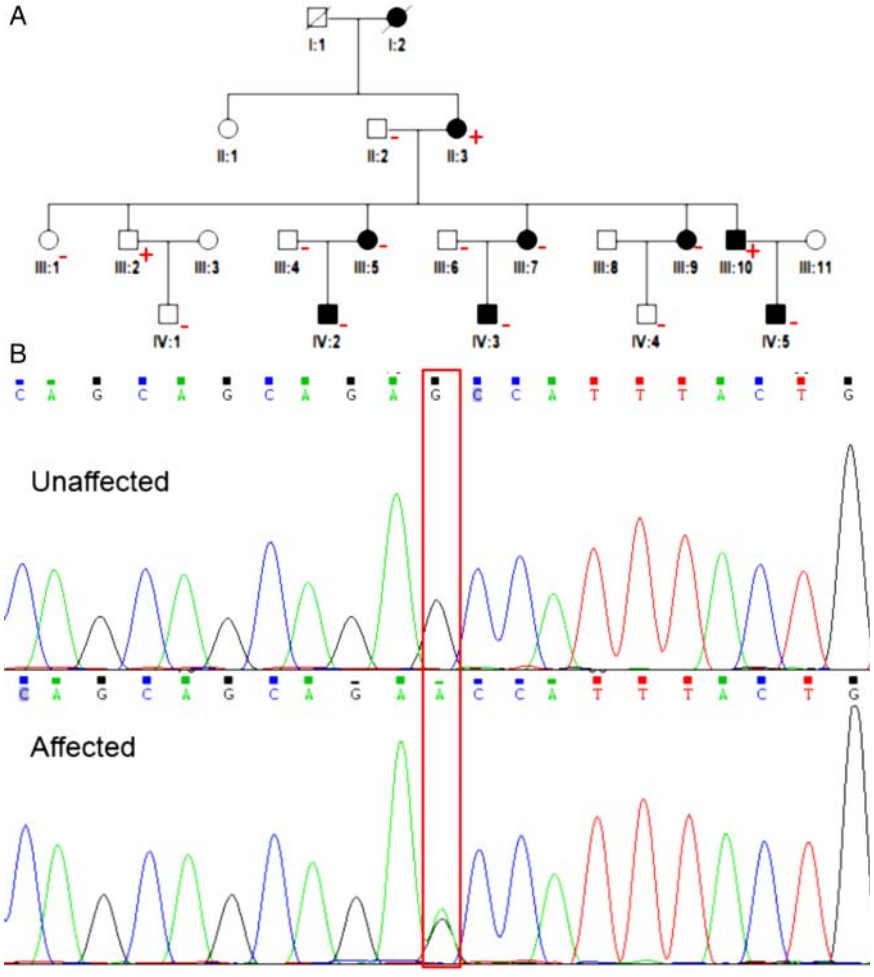

Figure 1 (A) The genealogical tree. '+' in pedigree indicates those who are subjected to exome sequencing, and '−' in pedigree indicates those who had undergone Sanger sequencing. (B) Chromatogram of the heterozygous c.22G>A variant resulting in the *EPS8L3* Ala8Thr substitution. This figure is only reproduced in colour in the online version.

genotypes files, and those achieved minimum quality score: Phred-like quality≥20, overall depth from 4 to 200, copy number estimate <2 and distance between two adjacent SNPs no less than 5.

For indels calling, BWA (V.0.5.8)[13] was used to gap align the sequence reads with default parameters to the human reference hg19. GATK IndelRealigner[14] was used to perform local realignment of the BWA-aligned reads and GATK IndelGentotyperV2 was used to call indels.

The variants were functionally annotated using an inhouse pipeline and categorised into missense, nonsense, read-through, splice-site mutations and coding indels, which are likely to be deleterious compared with synonymous and non-coding mutations. Based on these annotations, variants were filtered first for the non-synonymous (NS), splice acceptor and donor site (SS) mutations and coding indels (I), and then filtered against the most updated dbSNP database (V.135) and the control step by step. At last, the variants were shared between two cases but were neither presented in the public databases nor in the control individual of the family were considered to be the candidate variants.

To equally assess the functional importance of NS/SS/I mutations, the disease-causing variants were predicted by ANNOVAR[15 16] and PhastCons conservative score[17] analysis.

Sanger sequencing

Validation of the mutation was performed by Sanger sequencing in all the members of this family whose DNAs were available. PCR amplification was carried out in ABI 9700 Thermal Cycler using standard conditions. After the amplification, products were purified using a QIAquick PCR Purification Kit (Qiagen) and directly sequenced on ABI PRISM 3730 automated sequencer (Applied Biosystems). Sequence comparisons and analysis were performed by Phred-Phrap-Consed program, V.12.0.

RESULTS

We conducted exome sequencing in two affected individuals (II3 and III10) and one unaffected individual (III2) from one Chinese MUHH family where locus was mapped to 1p21.1–1q21.3.[3] A 5.85 Gb of average sequence was generated per individual as paired-end, 90-bp reads. After discarding the reads that had duplicated start sites and then mapping to the human reference genome (NCBI Build 37, hg19), 2.44 Gb mapped, targeted exome sequences with a mean depth of 64.83-fold were achieved. On average, 88.41% of the exome was covered at least 10-fold.

As mentioned in previous studies, we focused our analyses primarily on NS variants, SS and coding insertions/deletions (indels) that are more likely to be pathogenic mutations[8 18] (table 1). In addition, we predicted that variants underlying MUHH are rare and thus unlikely to be identified previously. We therefore selected the variants that were absent from the most updated dbSNP database (V.135) for further analysis. Assuming a dominant model, we found 91 novel NS/SS/indels that were shared by the two affected individuals but absent in the unaffected individual in this family. Of the 91 variants, only one heterozygous mutation in *EPS8L3* (NM_024526.3: exon2: c.22G->A:p.Ala8Thr) was found to be located within the linkage region on 1p21.1–1q21.3 established in our previous linkage study.[3] The mutation was predicted to be 'damaging' with 99.7% confidence by ANNOVAR program[15 16] and affect a conserved amino-acid residue by PhastCons software (PhastCons score great than 0.99),[17] respectively. Therefore, we selected *EPS8L3* as MUHH causative gene for further study.

Table 1 Summary of detected variants across three exomes

Variants	II3	III10	III2
Total number of variants (SNP+coding indels)	49 574+233	52 420+260	56 353+393
Synonymous-coding	8148	8369	8345
Missense	7139	7223	7148
Nonsense	57	67	59
Read-through	8	5	5
Splice site*	358	354	379
Intron	31 047	33 355	36 874
5′ UTRs†	745	826	975
3′ UTRs†	1507	1650	1865
Intergenic	565	571	703
Frameshift indels	120	131	227
Inframe indels	113	129	166

*Intronic SNPs within 4 bp of exon/intron boundary.
†Mutations in untranslated regions (UTRs) with 200 bp 5′ of initiation codon or 3′ of termination codon.

We then analysed the mutation in all the available affected and unaffected individuals (including three samples performed with exome sequencing) of this family by Sanger sequencing. All the eight affected individuals carried this heterozygous mutation in *EPS8L3* (NM_024526.3: exon2: c.22G->A:p.Ala8Thr) which was absent in the seven unaffected family members, suggesting complete cosegregation between the mutation and MUHH phenotype (figure 1B). Furthermore, this mutation was not detected in additional exome sequencing data of 676 unrelated, ethnically and geographically matched controls, as well as within the exome sequencing data of 781 unrelated, ethnically and geographically matched patients of other disease. All these results suggested that this mutation is a causal variant for MUHH, instead of a rare polymorphism.

DISCUSSION

In this study, we identified a missense mutation in *EPS8L3* (NM_024526.3: exon2: c.22G->A:p.Ala8Thr) which located within the linkage region 1p21.1–1q21.3 in this family.[3] This mutation was predicted to be damaging by the ANNOVAR program[15 16] and affect a conserved amino-acid residue by PhastCons software.[17] Subsequently, we confirmed that the mutation showed a complete cosegregation with the disease phenotype in the family, carried by all the eight affected and absent in all the seven unaffected individuals. We further confirmed the absence of the mutation in additional 676 controls and 781 subjects of other disease. Taken together, our findings have strongly implicated *EPS8L3* as a new gene for MUHH.

In mammals, the *EPS8* gene family contains *EPS8*, *EPS8L1*, *EPS8L2* and *EPS8L3*. These four proteins share collinear topology and contain a putative PTB (phosphotyrosine binding) domain, an SH3 domain and a C-terminal effector region. EPS8 is involved in the well-established pathway of epidermal growth factor receptor (EGFR), which is known to transmit extra-cellular mitogenic signals, such as EGF and transforming growth factor-α through activating a number of downstream signalling cascades like Rac and Rab5.[19] EGFR signalling is indispensable for the initiation of hair growth because the outer root sheath EGFR expression is downregulated with the completion of follicular growth.[20] Epithelial EGFR expression is important in delaying follicular differentiation that may serve to protect the hair follicle from immunological reactions.[21] Interesting, EGFR was found to control hair cycle progression and is crucial for hair follicle morphogenesis and development

in mouse.[20] In addition, defective and excessive transforming growth factor-α/EGFR signalling leads to abnormal hair morphogenesis and hairless phenotype, respectively.[22–24] Taken together, these data suggested that *EPS8L3* mutation might cause MUHH by resulting in hair follicle reduction through EGFR signalling pathway. Besides that, *EPS8* was found to be unregulated according to all bulge expression profile, suggesting *EPS8* might be a bulge molecular signature gene expressed in hairspheres. As the bulge region of the hair follicle serves as a repository for epithelial stem cells that can regenerate the follicle in each hair growth cycle and contribute to epidermis regeneration upon injury, this might suggest that *EPS8* plays a role in maintaining stem cells.[25 26] Of course, further study is warranted to determine whether this relationship between *EPS8* family and stem cells could contribute to MUHH or not.

In conclusion, our study identified *EPS8L3* as a disease gene for MUHH by combining exome sequencing with previously established linkage information in a large multi-generation MUHH family of Chinese population. Further biological studies of *EPS8L3* would shed new insights into the genetic aetiology and pathophysiology of MUHH. Our study has also demonstrated the effectiveness of exome sequencing, combined with linkage analysis, in discovering disease genes for Mendelian disorders.

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Contributors XZ, B-RG, L-QC, TJ, L-DS, J-JL and YC wrote the manuscript and revised it; J-CH, G-QS, Q-BL and XY performed exome capture and paired-end sequencing; TJ, Y-RL, JiW and JuW performed next-generation sequencing analysis and filtering; JZ, GC and X-FT performed PCR and Sanger sequencing; YL, H-YT, X-BZ, X-DZ and ML performed other data analysis; HC, P-GW and MG performed blood samples collection and selection; PL extracted Genomic DNA; X-JZ and SY conceived of this study and obtained financial support.

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Competing interests None.

Patient consent Obtained.

Ethics approval This study was approved by the Ethical Committee of Anhui Medical University and conducted according to the Declaration of Helsinki Principles.

Provenance and peer review Not commissioned; externally peer reviewed.

Data sharing statement 1457 subjects, including 676 unrelated controls and 781 unrelated patients without MUHH, used in our study are from an unpublished project of our group.

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