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ARTICLE

Maple syrup urine disease in the Austronesian aboriginal tribe Paiwan of Taiwan: a novel DBT (E2) gene 4.7 kb founder deletion caused by a nonhomologous recombination between LINE-1 and Alu and the carrier-frequency determination

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Maple syrup urine disease (MSUD) is an autosomal recessive inborn error disorder derived from the accumulation of the branched-chain amino acids (BCAAs) leucine, isoleucine and valine. Either the E1α, E1β or DBT (E2) genes are responsible for this neurometabolic disease. Here, we report the identification and characterization of a novel E2 gene 4.7 kb deletion as a rare nonhomologous recombination of the long interspersed nuclear elements 1 (LINE-1) in intron 10 and the Alu in the 3′ UTR of the E2 gene from three classic MSUD patients of the Austronesian aboriginal tribe Paiwan in Taiwan. The E2 gene 4.7 kb deletion accounted for five out of six alleles in the three unrelated Paiwanese MSUD patients, indicating a founder effect. Carrier-frequency study revealed one deleted heterozygote out of 101 normal Paiwanese. As the nine Taiwanese Austronesian aboriginal tribes share a common origin, this E2 4.7 kb deletion may be preserved in some of the other Austronesian aboriginal tribes of Taiwan. This is the first comprehensive genetics study of MSUD in the Austronesian tribal groups as well as in Taiwan.
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Introduction

Maple syrup urine disease (MSUD; types Ia MIM#248600, Ib MIM#248611 and II MIM# 248610), a metabolic disease that was first described by Menkes *et al*,[1] is an autosomal recessive inborn error disorder resulting from the defective activity of mitochondrial branched-chain α-ketoacid dehydrogenase (BCKAD), which catabolizes the oxidative decarboxylation of branched-chain α-ketoacids (BCKAs) from the branched-chain amino acids (BCAAs) leucine, isoleucine and valine.[2] Mutations in any of the E1α or E1β or DBT (dihydrolipoamide branched chain transacylase, E2) components of the BCKAD complex can result in classic, intermediate or intermittent MSUD.[3] Clinically, MSUD patients manifest with poor feeding, lethargy, seizures, developmental delay, psychomotor retardation, coma or death. Early diagnosis is essential to decrease morbidity and mortality. Molecular analysis of MSUD may be beneficial for early genetic counseling.

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MSUD appears in all ethnic groups with a general incidence of less than 1 in 185 000 newborn infants in the general population.[2] Two founder mutations of MSUD in specific populations have been reported: An E1α Y393N mutation in the Old Order Mennonite population at a frequency of 1/176[4] and an E1β R183P mutation in Ashkenazi Jews at a frequency of 1/113.[5] Here, we report a novel E2 gene 4.7 kb founder deletion caused by an unusual nonhomologous recombination between the long interspersed nuclear elements 1(LINE-1) and Alu in three classic MSUD patients of the aboriginal tribe Paiwan. We also present the molecular characterization of the deletion breakpoints as well as the carrier frequency of this deletion in the tribe Paiwan, one of the nine Austronesian aboriginal tribes in Taiwan.

Materials and methods

Mutation analysis of the MSUD patients

Three unrelated classic form MSUD cases, one boy (patient 1) and two girls (patients 2 and 3), were documented in terms of clinical symptoms and abnormal elevation of plasma BCAAs. All patients belonged to the aboriginal tribe Paiwan of Taiwan. Genomic DNA was extracted from peripheral blood by the salting-out method. The intronic PCR primers were derived from *Homo sapiens* chromosome 19 working draft sequence segment (Gene Bank GI: 12741466) and *Homo sapiens* chromosome 6 working draft sequence segment (Gene Bank GI:12731894) to amplify all exons of E1α and E1β genes, respectively for sequence analysis (primer sequences are available by request). The E2 gene mutation analysis of genomic DNA was performed as previously described.[6] The amplicons were sequenced bidirectionally with the ABI PRIAM 3100 genetic analyzer (Applied Biosystems, Foster City, CA, USA). The primer set DelF (5′-TTCTGCAGGTCTGCTGGAGT-3′) and DelB (5′-TGAGATGGCGTCTTGCTCTG-3′) were used for amplification of the deleted allele. The PCR was annealed at 64°C for 40 s and extended for 50 s at 72°C.

Heterozygote detection of the E2 4.7 kb deletion and carrier-frequency determination in the Paiwan population

The heterozygote of the E2 gene 4.7 kb deletion was detected with genomic DNA by duplex PCR with primers DelF, DelB, Ex11F (5′-CATTTCTAGGCCATTCCCCG-3′) and Ex11B (5′-TGCTGGCACAGCTAGGGTTT-3′), as previously described. The carrier-frequency study by duplex PCR was carried out with the approval of the Ethical Committee of the Taichung Veterans General Hospital. The geographic range included in our study was the mountainous area in Taitung County, one of the indigenous habitats of the tribe Paiwan in Southeastern Taiwan. A total of 242 blood samples were collected. The subjects of this study were unrelated individuals whose parents were both Paiwanese. A total of 101 unrelated normal Paiwanese including 67 female and 34 male subjects were enrolled. Informed consent was obtained from all participants and all personal identifiers were stripped from the samples for the carrier analysis.

Results

MSUD E1α, E1β and E2 gene mutation analysis

Molecular analysis revealed no mutation in either the E1α or the E1β genes of all the three Paiwanese MSUD patients. Patient 2 carried a heterozygous 2-bp (AT) deletion (c.88-89delAT) in exon 2 of the E2 gene, causing a frame shift downstream of residue (−26) in the mitochondrial targeting presequence[7] (Figure 1a). No further E2 sequence variation was found in this patient. For patients 1 and patient 3, exon 11 of the E2 gene could not be amplified from genomic DNA with the primer set 11f–E2-3′,[6]

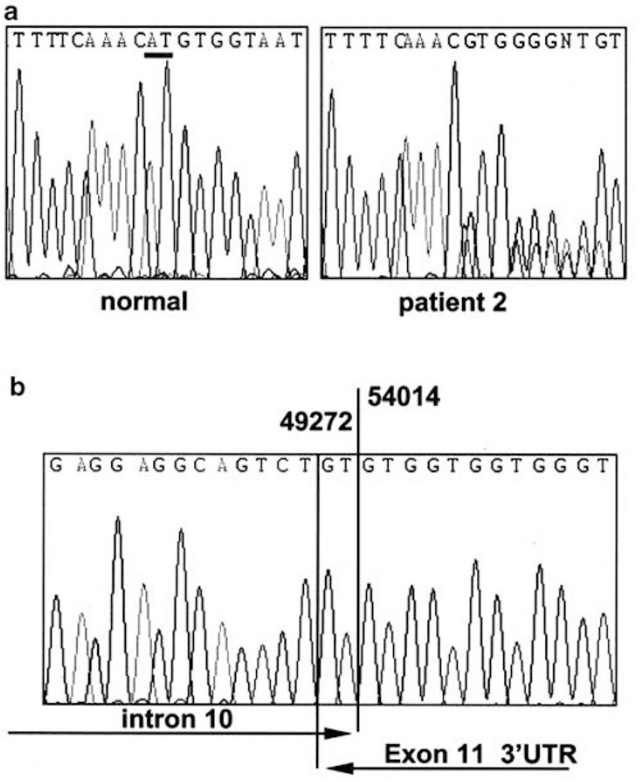

Figure 1 Sequencing trace of the MSUD patients. (**a**) Partial exon 2 sequences of normal control and patient 2. A 2-bp AT heterozygous deletion (c.88-89delAT) was identified in exon 2 of patient 2. (**b**) A primer set DelF–DelB based on the PCR walking results was used to amplify the deletion junction of the E2 gene of patients 1 and 3. The junction sequence revealed a 49273–54013 (GeneBank NT_028050, GI: 22041703) deletion flanking parts of intron 10 and the 3′ UTR of exon 11.

although it was amplified successfully in patient 2 and normal controls. In attempting to achieve a successful exon 11 amplification, several genomic DNA PCR primer sets extending 2 kb beyond both coding region ends of exon 11 (Gene Bank GI: 22041703) were tried; however, negative PCR results were still obtained. We therefore postulated that there might be a homozygous deletion starting from somewhere around intron 10 of the E2 gene in patients 1 and 3.

Determination of the E2 4.7 kb deletion and the carrier study

Primers DelF and DelB flanking the deletion junction were defined from patients 1 and 3 after several rounds of the PCR walking approach (data not shown). The sequences of amplicon DelF–DelB revealed a 4741 bp genomic DNA deletion spanning position 49273 to position 54013 (*Homo sapiens* chromosome 1 working draft sequence segment, Gene Bank-NT_028050, GI: 22041703) (Figure 1b) or the (IVS10 -4140–1del;c.1296-1896del) mutation (Gene Bank NT_028050, GI: 22041703; *Homo sapiens* dihydrolipoamide branched-chain transacylase mRNA, Gene Bank GI:4503264), which includes the 3′ half of intron 10, the entire coding region of the terminal exon 11 and the 5′ part of the 3′ UTR of the E2 gene (Figure 2a).

The composition of the repeated elements in the E2 gene was analyzed with REPEATMASKER (http://repeatmasker.-genome.washington.edu/cgi-bin/RepeatMasker) to determine if repeated elements were involved in this deletion event. We found that Alus comprised 21.4% and LINES comprised 21.6% of the E2 gene. A centromerically oriented 6.4 kb long interspersed nuclear elements 1 (LINE-1) occupied about 60% of intron 10. The 3′ UTR sequences of this LINE-1 revealed that it belonged to the L1PA7 family. In the 3′ UTR of the E2 gene, four Alu elements, Alu Sx, Alu Jo, Alu Sx and Alu Jo, are arranged in that order (Figure 2a). The 5′ breakpoint of this E2 4.7 kb deletion occurred in ORF1 of the L1PA7, while the 3′ breakpoint was located in the right arm of the first Alu Sx in the E2 3′ UTR (data not shown).

Duplex PCR was used to detect the heterozygous deletion status of the alleles. The primer set Ex11F–Ex11B served as the internal control to amplify exon 11 and DelF–DelB was used to amplify the deletion junction fragment. For normal alleles, the primer set Ex11F–Ex11B produces a 244-bp amplicon of exon 11, and Ex11F–DelB generates a 750-bp PCR fragment. The primer combinations of DelF–DelB and DelF–Ex11B were amplified unsuccessfully because of the nearly 5 kb distance between the forward and backward primers on the normal allele. For the deleted allele, a 388-bp fragment from the primer pair DelF–DelB was generated (Figure 2a). Patients 1 and 3 generated only a 388-bp fragment from duplex PCR analysis, indicating that both of them were E2 gene 4.7 kb deletion homozygotes. Patient 2, an AT 2-bp deleted heterozygote, showed a heterozygous E2 gene 4.7 kb deletion (Figure 2b).

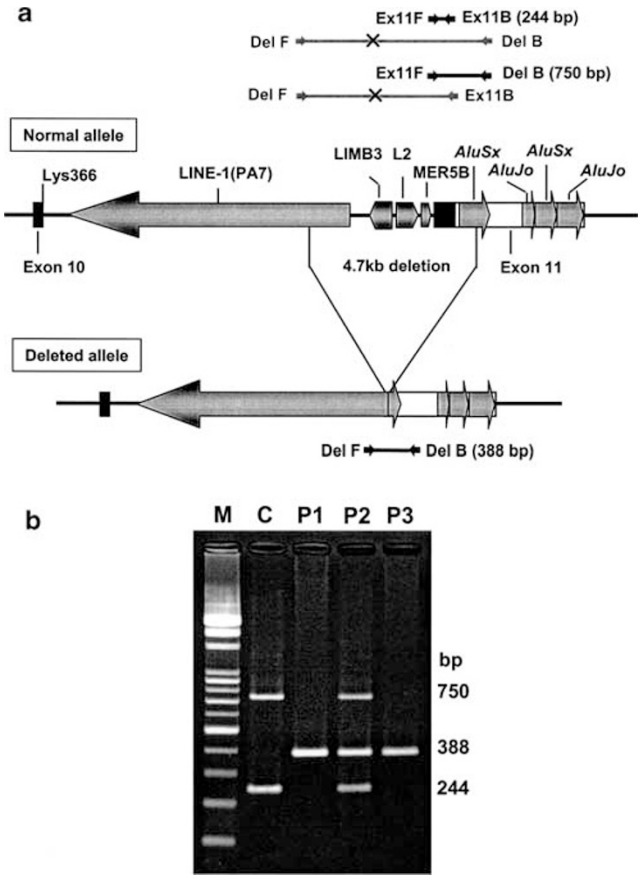

Figure 2 The repeated element composition of the E2 gene and the identification of the heterozygous 4.7 kb deletion by duplex PCR. (**a**) The 3′ E2 gene structure and the 4.7 kb deletion junction site. Repeated elements (Line-1, Alu, MER and LIMB) are indicated in gray. Black boxes represent coding exons, and the white box indicates the 3′UTR. Duplex PCR with primer sets Ex11F–Ex11B and DelF–DelB were used to identify the heterozygous 4.7 kb deletion. Lys366 is the last residue encoded by exon 10. The residue number follows the numbering systems of Fisher *et al*.[7] (**b**) Gel image of the duplex PCR of the three MSUD patients. The 244-bp band was generated by Ex11F–Ex11B and the 750-bp fragment was generated by Ex11F–DelB in normal control (**c**). The 388-bp duplex PCR products produced by primer set DelF–DelB in patients 1 and 3 represent a homozygous E2 4.7 kb deletion (P1 and P3). The presence of all three bands in patient 2 (P2) represents a heterozygous deletion carrier. A 100-bp DNA ladder was used as a marker (M).

The E2 4.7 kb deletion accounted for five out of six alleles in three Paiwanese MSUD patients revealing a founder effect of this deletion in the Paiwan population. Carrier-frequency study identified one E2 4.7 kb deleted heterozygote among 101 unrelated healthy aboriginal Paiwanese.

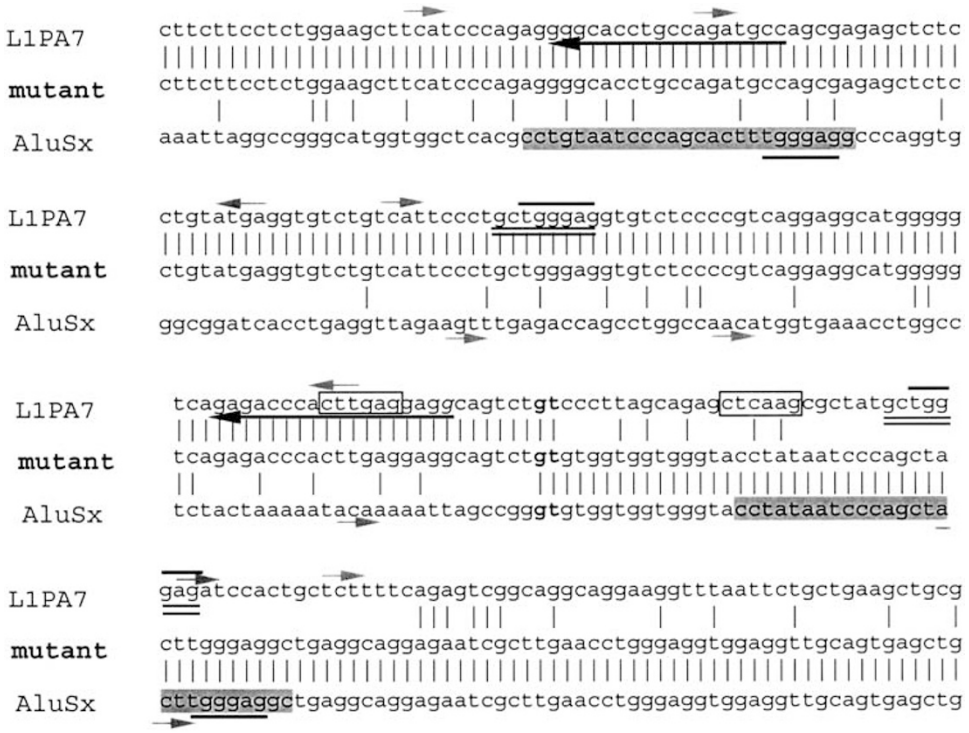

Figure 3 Nucleotide sequence alignment of the E2 4.7 kb deletion mutant and the normal allele. Vertical bars indicate nucleotide matches. Bold face gt represents the identical bases of the deletion junction. Sequences that are homologous to the forward or reverse topoisomerase I consensus binding site (5′-A/T-G/C-T/A-T-3′)[9] are indicated by gray arrows. Black arrows indicate topoisomerase II consensus sequences (5′-A/G-N-T/C-NNCNNG-T/C-NG-G/T-TN-T/C-N-T/C-3′).[10] Nucleotides inconsistent with the consensus sequences of topoisomerase II are indicated in italics. The 6-bp invert repeats flanking the 5′ breakpoint are boxed. The direct repeats flanking the 5′ breakpoint with the same sequences as *chi*-like elements are double underlined. The direct repeats in the 5′ and 3′ deletion junctions are marked with horizontal lines. Shaded letters represent the 26-bp Alu core sequence.

Characterization of the deletion

The sequences around the deletion junction showed two overlapped base pairs (gt) at the deletion junction (Figure 1b,3) and no significant homology between this L1PA7 and Alu Sx, indicating a nonhomologous recombination event (Figure 3). The recombination related elements appeared around the 5′or 3′ breakpoints (Figure 3) and are summarized in Table 1.

Discussion

Several different types of small mutations have previously been reported in the E2 gene.[6,7,12–14] However, large deletion of the E2 gene has only been reported once as a 15–20 kb genomic DNA loss resulting from the recombination between an intronic Alu in intron 6 and the coding sequences of the terminal exon 11.[15] Here we report another large fragment deletion in the E2 gene. This E2 4.7 kb genomic DNA deletion may lead to failure to splice intron 10 or to alternative splicing of intron 10 with several possible cryptic splice acceptor sites in the nondeleted intron 10 or nondeleted 3′UTR according to the analysis with BDGP: Splice Site Prediction by Neural Network (http://www.fruitfly.org/seq_tools/splice.html) (data not shown). The mutated E2 protein may have amino acids replaced or deleted after Lys366 (Figure 2a), thus it may lose the His391 active site[12] of the E2 catalytic domain resulting in the enzymatic inactivation of the E2 protein. The changed or missing amino acids after Lys366 may also destroy the structure of the E2 catalytic domain and disrupt the assembly of intermediated active trimers that interlock through carboxyl-terminal hydrophobic knobs to produce the native 24-meric core,[14] thus affecting the assembly of the correct 24-mers cubic structure.

Concerning the 3′ UTR deletion, a major class of regulatory *cis* elements comprising adenosine–uridine pentamers (AUUUA) termed AU-rich elements (AREs) are repeated once or several times within the 3′UTR and are often found within U-rich regions of mRNA. AREs that function as RNA-destabilizing elements and target mRNA for rapid degradation in the cytoplasm have been found in numerous mRNA species.[16] The deleted 3′UTR region of the E2 4.7 kb deletion is a U-rich area containing one ARE

Table 1 Recombination-related elements presenting around the 5′or 3′ breakpoints of E2 4.7 kb deletion and their possible effects

Elements	*Position*	*Possible effects*
Line-1	An inactive L1PA7 on 5′ breakpoint in intron 10 of E2 gene	Stimulate gene rearrangement.
		Formation of the homologous pairing with the LINE-1 at different locus
Alu	An integrated AluSx on 3′ breakpoint in 3′ UTR of E2 gene	Stimulate gene rearrangement
Alu core sequence[11]	14 bp downstream[a] and 95 bp upstream of the 3′ breakpoint	Stimulate recombination
Chi-like sequence[8]	55 bp upstream and 24 bp downstream of the 5′ breakpoint	Stimulate recombination
Direct repeats	55 bp upstream and 26 bp downstream of the 5′ breakpoint[b]	Slipped mispairing, may destroy the homology pairing between the LINE-1 s
	96 bp upstream and 31 bp downstream of the 3′ breakpoint[c]	Slipped mispairing
Invert repeats	Flanking the 5′ breakpoint symmetrically at a distance of 12 bp	Hairpin loop formation, may destroy the homology pairing between the LINE-1 s
Topoisomerase I[9]	Seven sites around the 5′ breakpoint	Strand breakage, may destroy the homology pairing between the LINE-1 s
	Four sites around the 3′ breakpoint	Strand breakage, may create recombigenic sites facilitating recombination
Topoisomerase II[10]	10 bp[d] and 99 bp upstream of the 5′ breakpoint.	Strand breakage, may destroy the homology pairing between the LINE-1 s
S/MARs	No significant MAR potential was predicted at the 5′ and 3′ breakpoint	

[a]Homologous to the Alu core sequences (22/26). [b]Part of the *chi*-like sequences. [c]Part of the Alu core elements. [d]1-bp mismatch with the consensus sequences.

pentamer (AUUUA) and one ARE hexamer (AUUUUA). The loss of these two AREs might produce a much more stable mutated E2 mRNA than normal E2 mRNA.

Alus and LINE-1 have been implicated in gene insertion and recombination, and accordingly are associated with several human diseases.[17–19] Nonhomologous recombination between LINE-1 and Alu causing gene deletion, like the E2 4.7 kb deletion we report here, has only been reported once in the dystrophin gene[20] and has never been documented in the MSUD E2 or other genes. The L1PA7 in intron 10 of the E2 gene contains multiple small deletions, insertions and point mutations that leave the ORF1 and ORF2 of L1PA7 truncated (data not shown), indicating that the L1PA7 had lost its transposable activity, and it was thus probably not an active LINE-1. The 3′ breakpoint of this 4.7 kb deletion was located on the right arm of the Alu Sx rather than the left arm, although the Alu recombination hotspots usually occur on the left arm due to the presence of the 26-bp core sequence.[21] There was a homologous Alu 26-bp core sequence (22/26) on the right arm of the Alu Sx in the recombining strand (Figure 3). We therefore inferred that Alu might play a role in the E2 4.7 kb deletion.

It is generally agreed that multiple mechanisms are involved in nonhomologous recombination.[19] Scaffold/matrix-attached regions (S/MARs) may also be closely related to breakage events and gene recombination.[17,19] We used MAR Finder (http://www.futuresoft.org/MAR-Wiz) to analyze the S/MARs potential with the 3′ part of the E2 gene over a 12 kb region encompassing exon 10 to the end of the 3′ UTR including the deletion region; however, no significant MAR potential was predicted at the 5′ or 3′ breakpoint sites (data not shown). The other specific elements including topoisomerase I and II recognition sites, *chi*-like sequences, direct repeats and inverted repeats that we characterized around the 5′ and 3′ breakpoints have also been implicated in gene recombination.[22] However, there is no evidence that these elements and sequence structures directly mediated this particular E2 nonhomologous recombination. We hypothesize that the possible cause of the Alu-LINE-1 associated nonhomologous recombination about this E2 4.7 kb deletion may be the 'homology-associated nonhomologous recombination'.[23]

In Taiwan, fewer than 10 MSUD cases without accompanying gene analysis have been reported since 1986,[24,25] although the incidence is much higher in the aboriginal tribes of Taiwan according to our data. The E2 4.7 kb deletion we describe here is not only the first MSUD genetic mutation identified in Taiwan but also a founder mutation in the Paiwan tribe. The aboriginal tribes of Taiwan, belonging to the Austronesian family, comprise 1.5% of the total population of Taiwan, and are divided into nine major groups including Amis, Puyuma, Yami, Atayal, Saisiyat, Bunun, Tsao, Rukai and Paiwan. As HLA class I genetic studies have shown, traits are highly homogenous within each tribe but different among tribes, and yet they have affinities to form a cluster together in the population dendrogram due to long-term isolation.[26] The

tribes regionally distributed throughout the rugged internal mountains and along the eastern coast have individually distinct and sometimes mutually unintelligible languages and different material cultures and social organizations. They have common ancient origins that are temporally and geographically consistent with genetic, linguistic and archaeological studies.[27]

In addition to the MSUD and the E2 4.7 kb deleted carriers identified in the tribe Paiwan, we also found the same E2 4.7 kb deletion in a carrier of the Puyuma tribe during the study period. This implies that this disease or deletion may also be preserved in some of the other Austronesian aboriginal tribes of Taiwan. We suggest that MSUD gene screening should be provided for the tribe Paiwan and the other Austronesian aboriginal tribes in Taiwan for the further investigation of the E2 gene 4.7 kb deletion status. We believe that this information may also be helpful for the study of MSUD among Austronesian families in other countries.

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