Identification of a mutation in the Indian Hedgehog (IHH) gene causing brachydactyly type A1 and evidence for a third locus

T J Kirkpatrick, K-S Au, J M Mastrobattista, M E McCready, D E Bulman, H Northrup

MATERIALS AND METHODS

The finding of IHH mutations in BDA1 patients led us to examine this gene in the two BDA1 families we previously studied. We searched GenBank and extracted the genomic DNA sequence (NT_005289) using the cDNA sequence of IHH. IHH consists of three exons and spans approximately 5.5 kb of genomic DNA. The exon/intron boundaries of the three exons were identified and primers specifically targeting the splice sites and coding regions were designed using Oligo 4.1 Primer Analysis Software (Rychlik 1992) for polymerase chain reaction (PCR) amplification and sequencing. PCR primers used are as follows 5′ to 3′: 1F (CCAGGGCCCTGGCCCTCA), 2F (TCCTCCGGGCTGTTCTGCTC), 2R (TTCTGGGCTACCTGCTCC), 3F (CAAGGAGATTGTGTGAC), 3R (GGACCCGATCCAGGTCCC). We also used primer 1R as described by Gao et al. The three exons of IHH were then amplified by standard PCR and examined by electrophoresis on 3% MetaPhor agarose gels. Shrimp alkaline phosphatase (1 U) and exonuclease I (10 U) were used to remove excess dNTPs and primers from our samples. We then sequenced the exons using BigDye Terminator (v.1) reagent following the manufacturer’s protocol (Applied Biosystems, Foster City, CA). After purification through Sephadex G-50 Fine columns, the sequenced products were run on an ABI Prism 3100 automated sequencer and analysed with the ABI Sequencing Analysis 3.7 software. Any changes noted in the patients’ sequences were confirmed by sequencing in the reverse direction. The IHH gene was sequenced for all affected and unaffected people in the study.

RESULTS AND DISCUSSION

Our sequencing results identified a missense mutation in IHH in all affected members of family II, a family of Mexican descent (fig 1A). The missense mutation involved a 284A→G transition in exon 1 of these patients resulting in a Glu95→Gly mutation (fig 1B). Gao et al. reported a mutation of a different nucleotide (283G→A) in the same codon, resulting in a Glu95→Lys mutation. The recurrent mutation found within the same codon among unrelated patients of different ethnic backgrounds further supports the functional importance of Glu95 in the IHH peptide. The codon, Glu95, is conserved among hedgehog proteins and is suspected to play an important role in binding to the receptor for downstream cell cycle signalling. Gao et al. hypothesised that the novel mutations in IHH would cause BDA1 because of their position in the amino-terminal signalling domain. Amino acid changes in this domain of the closely related protein Sonic Hedgehog (SHH) have been shown to elicit a deleterious effect in SHH.
binding with its receptor Patched (PTC), also a receptor for IHH. Therefore, the three IHH mutations are also likely to disrupt binding of the IHH protein to PTC. If the missense mutations cause complete loss of binding, then BDA1 would be caused by haploinsufficiency of the wild type protein. The mutations may also result in partial loss or altered binding, the latter predicted to cause a gain of function effect. Our finding, together with that of Gao et al., suggests the carboxyl side chain of Glu95 is vital to IHH protein function. Functional assays will elucidate the effect of these mutations.

Before discovery of IHH mutations, Fukushima et al. reported a case with an apparently balanced 5q11.2 and 17q23 translocation in a girl with BDA1 and Klippel-Feil syndrome. Armour et al. conducted a genome-wide linkage study on a four generation BDA1 family of Canadian descent, with special attention to chromosomes 5 and 17. Their study identified linkage of BDA1 to an 11 cM region on chromosome 5p13.3-13.2. The region contains two notable candidate genes, cadherin-6 (CDH6) and natriuretic peptide receptor C (NPR3), whose proteins are suggested to have roles in osteoclast differentiation and skeletal development.

Before the mutations in IHH were published, we examined markers (D5S819, D5S1986, D5S477, D5S1506, and D5S663) for our two BDA1 families to determine if there was linkage to the 5p13.3-p13.2 region. In family I, the same haplotype from I.1 is transmitted to affected and unaffected subjects in the second (II.1, II.2, II.4, and II.5) and third generations (III.2 and III.3) (fig 2A). However, affected subject III.1 did not inherit any of the chromosome 5 markers from the affected grandparent, I.1. Taken together, our family I does not show linkage to the 5p13.3-p13.2 candidate BDA1 region.

Direct sequencing analysis detected no significant changes in the exons and splice sites of IHH in affected members of family I. We did detect a 753T→C transition resulting in a silent variant (Pro251→Pro) and producing a novel restriction enzyme Bsp1286I site. Testing of all family members showed that the Bsp1286I (T) allele segregated independently of BDA1 in the family (members II.5, II.6, III.2, and III.3), therefore excluding IHH as the disease causing gene (fig 2B). Our previous study using various genetic markers on several other chromosomes, and the chromosome 5 markers reported here, excluded non-paternity for II.6. Since the variant is within exon 3 and IHH is small (5.5 kb), it is unlikely that other changes in the introns of IHH are causing BDA1 in family I.

Exclusion of linkage to both IHH and chromosome 5p13.3-13.2 in our BDA1 family I points to the existence of yet a third locus for BDA1. SNP linkage analysis of the receptors of IHH, its target genes, or the downstream targets for CDH6 or NPR3 are future studies that could uncover the disease gene in our family I.

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Figure 2  (A) Haplotype analysis of microsatellite markers on chromosome 5p13.3-13.2 in the three generations (I, II, and III) of family I with brachydactyly type A1 (BDA1). Affected subjects are denoted by filled symbols. Markers are listed in order from pter to centromere (D5S819, D5S1986, DSS477, DSS1506, DSS663) next to the genotypes of I.1. Genotypes of the transmitted chromosome from I.1 is boxed and shown to transmit to both affected and unaffected descendants in generations II and III. (B) Results of BspI286I polymorphism analysis (M = DNA molecular weight marker V, Boehringer Mannheim). Exon 3, 753 T→C silent variant creates a novel BspI286I site. The upper singlet appears in all samples and corresponds to 339 bp. The doublet (bottom two bands) is of interest and corresponds to 145 bp (753T) and 125 bp (753C), respectively. Horizontal arrows on the left point to the digested DNA fragments in the gel with corresponding sizes.


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