Whole-exome sequencing, without prior linkage, identifies a mutation in LAMB3 as a cause of dominant hypoplastic amelogenesis imperfecta

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The conventional approach to identifying the defective gene in a family with an inherited disease is to find the disease locus through family studies. However, the rapid development and decreasing cost of next generation sequencing facilitates a more direct approach. Here, we report the identification of a frameshift mutation in LAMB3 as a cause of dominant hypoplastic amelogenesis imperfecta (AI). Whole-exome sequencing of three affected family members and subsequent filtering of shared variants, without prior genetic linkage, sufficed to identify the pathogenic variant. Simultaneous analysis of multiple family members confirms segregation, enhancing the power to filter the genetic variation found and leading to rapid identification of the pathogenic variant. LAMB3 encodes a subunit of Laminin-5, one of a family of basement membrane proteins with essential functions in cell growth, movement and adhesion. Homozygous LAMB3 mutations cause junctional epidermolysis bullosa (JEB) and enamel defects are seen in JEB cases. However, to our knowledge, this is the first report of dominant AI due to a LAMB3 mutation in the absence of JEB.

Keywords: amelogenesis; amelogenesis imperfecta; LAMB3; laminin-5

INTRODUCTION

Amelogenesis imperfecta (AI) is the collective term given to a genetically heterogeneous group of inherited defects of tooth enamel biomineralisation. Typically all teeth of the primary and secondary dentitions are affected, with marked variations in clinical phenotypes that are influenced by the underlying genotypes. The impact of AI on affected individuals and their families is considerable. To date, mutations in seven genes (AMELX, MIM 30039; ENAM, MIM 606585; KLK4, MIM 603767; MMP20, MIM 604629; FAM83H, MIM 611927; WDR72, MIM 613214 and C4orf26, MIM 614829) have been shown to cause either autosomal dominant, autosomal recessive or X-linked AI in the absence of other cosegregating abnormalities.

Methods for identifying the defective gene in any given disorder generally involve identification of a disease locus followed by candidate gene sequencing. More recently next generation sequencing (NGS) has led to a hypothesis-null method of mutation identification through complete sequencing of exons at a locus in a single affected individual. With this approach a more manageable number of variants are highlighted after bioinformatic filtering than would be expected if examining the entire exome. These are then prioritised for segregation analysis, determination of frequency in control samples, analysis of evolutionary conservation and likely impact on protein function in order to determine the pathogenic variant.

Here, we report a further simplification of this approach, leading to the identification of a frameshift mutation in LAMININ-β3 (LAMB3; MIM 150310) in a family segregating non-syndromic dominant hypoplastic AI. Whole-exome NGS of three affected individuals, without prior genetic linkage, proved sufficient to identify the causative change.

PATIENTS AND METHODS

Members of the family AI-17, originating from Ireland, were recruited in accordance with the principles of the declaration of Helsinki, with informed consent and ethical approval. DNA samples were obtained from family members using either Oragene DNA sample collection kits (DNA Genotek, Kanata, ON, Canada) or via venous blood samples using conventional techniques. Screening of genes known to cause dominant AI (ENAM and FAM83H) was performed as previously described. Whole-exome sequencing was performed on a HiSeq2000 using the Nimblegen v2 chip by Otogenetics Corporation (Norcross, GA, USA). Resulting data were analysed and annotated using the DNASexus (https://www.dnanexus.com/) data storage and analysis facility. Additional bioinformatic analysis of variants was performed using PolyPhen2 (http://genetics.bwh.harvard.edu/pph2/). The presence of potential variants was confirmed and segregation checked by PCR and Sanger sequencing using standard protocols. A multi-point Lod score was generated using Superlink Online (http://bioinfo.cs technion.ac.il/superlink-online/).

RESULTS

The proband of family AI-17 (arrowed in Figure 1a) presented with irregular hypoplastic AI and an autosomal dominant family history spanning four generations. Affected family members had experienced many symptomatic (eg, frequent pain) and aesthetic problems with their teeth (Figure 1b). Some individuals had received extensive
restorative dental care and required multiple dental extractions. Review of the medical histories revealed no other health problems cosegregating with AI. In particular, no fragility, blistering or other skin problems were noted.

The known dominant AI genes ENAM and FAM83H were screened and excluded by PCR and Sanger sequencing in affected individual III:2. We then hypothesised that whole-exome sequencing of three distantly related, affected family members could allow the pathogenic mutation to be identified, without any prior mapping of a locus. Exonic DNA from individuals III:2, III:3 and IV:4 (indicated with * in Figure 1a) was therefore captured using a Nimblegen v2 exome reagent and clonally sequenced on a HiSeq2000 sequencer. The data generated are summarised in Supplementary Table 1.

The variants identified in each sample were filtered to exclude those 450 bp from an exon; known polymorphisms (variants listed in versions of dbSNP up to and including dbSNP129 or those with a minor allele frequency (MAF) 41%); and any variants, which did not pass standard Genome Analysis Toolkit variant filters. Total variants remaining after each of these stages of filtering are presented in Supplementary Table 2. Next, homozygous changes were excluded in light of the clear dominant inheritance in the family, and the remaining variants in each sample were compared to identify changes shared by all the three affected individuals sequenced. At this stage, any variant not within an exon or consensus-splice sequence was excluded. In addition, two of the remaining variants were retested using Sanger sequencing and found to be absent, and were therefore assumed to have been artefacts of NGS. This left twelve variants to take forward for further analyses (Table 1).

Of these, eight were present in dbSNP with a MAF of 40.1% and were therefore considered unlikely to be the cause of this rare dominant disease, given that the frequency of all forms of AI is estimated to be only 1/14000 in the genetically heterogeneous US population.11 The MAF for a ninth variant was unknown but its presence in both the Watson and Venter genomes suggests it is a benign SNP present in the normal population. One further variant was predicted to be non-pathogenic by bioinformatic prediction software PolyPhen2. A 3 bp in-frame deletion in the HOMEZ gene (p.D547del; rs148005528) was excluded as it occurs within a run of eight aspartic acid residues, and four other 3 bp deletion SNPs occur within this run, each removing a single aspartic acid residue, suggesting that the deletion of a single residue is non-pathogenic.

This left only a single variant, a frameshift in the LAMB3 gene (NM_000228—c.3392_3393insG; p.E1133Gfs*27) on chromosome 1q22 (Figure 1c), as the potential cause of the disease phenotype. Homozygous LAMB3 mutations cause junctional epidermolysis bullosa (JEB) and enamel developmental defects are a universal finding in JEB, although severity and clinical expression vary widely.12 This variant was therefore considered a strong candidate for the

Figure 1 (a) Pedigree of family AI-17 illustrating segregation of the wild-type (+) and variant (−) alleles for the LAMB3 variant with the disease phenotype. The proband is marked with an arrow. Individuals who were whole-exome sequenced are marked with an asterisk. (b) Clinical phenotype observed in the proband at 3 years of age. A generalised, irregular hypoplastic AI is observed with small islands of enamel evident on close inspection (arrows). These are absent from where the teeth occlude indicating that the clinical phenotype has probably been modified by post-eruptive changes. The panoramic radiograph of the proband confirms that the enamel is hypoplastic on formation. (c) Electropherogram showing the LAMB3 mutation and wild-type sequence. (d) Location of the LAMB3 change with respect to the Laminin-5 heterotrimer (based on Nakano et al).28
pathogenic mutation in this family. Further investigation of the exome sequences of affected members of AI-17 confirmed that no variants were present in the collagen type XVII, alpha 1 gene (COL17A1), mutations in which can act in concert with LAMB3 mutations to cause digenic JEB.\textsuperscript{13} Sequencing of the LAMB3 variant using primers F:5'-GGTTGAGCTTTGGAGAGTC-3' and R:5'-AAGCATTCAACCCAATCTG-3' in all available family members revealed perfect segregation with the disease phenotype (Figure 1a). Furthermore, the variant was excluded from 180 Caucasian control individuals. A Lod score of 2.3 was achieved using the LAMB3 variant as a genetic marker, assuming 100% penetrance and an allele frequency of 0.0001.

**DISCUSSION**

Current methods of gene identification generally require the mapping of a locus prior to Sanger sequencing or NGS. However, the wealth of data generated by NGS allow a range of techniques to be performed on just a single data set, leading to a shift in the methodological process of identifying disease-causing gene mutations. Here, we show how, using primers F:5'-GGTTGAGCTTTGGAGAGTC-3' and R:5'-AAGCATTCAACCCAATCTG-3', and additional 3-bp deletion SNPs in a simple repeat containing additional 3-bp deletion SNPs, present in Venter and Watson genomes.

**Table 1 The list of variants after filtration**

| Variant | Gene | Protein change | dbsNP ID | Comment |
|---------|------|----------------|----------|---------|
| Chr1:204192659 G>A | PLEKHA6 (NM_014935) | Missense p.A1029V | rs145148095 | Allele Freq. >0.1% |
| Chr1:209788740 insC | LAMB3 (NM_000282) | Frameshift p.E1133Gfs*27 | None | Causes JEB, enamel hypoplasia often observed |
| Chr2:17962993 delAGTT | GEN1 (NM_001130009) | Frameshift | rs149936944 | Allele Freq. >0.1% |
| Chr3:43095112 G>A | FAM198A (NM_001129908) | Missense p.E464K | rs184462308 | Allele Freq. >0.1% |
| Chr5:96124300 C>T | ERAP1 (NM_001040458) | Missense p.R538K | rs142305981 | PolyPhen2 = 0 |
| Chr5:102888040 C>T | NUDT12 (NM_031438) | Missense p.A386T | rs139189967 | Allele Freq. >0.1% |
| Chr5:132556523 C>T | FSTL4 (NM_015082) | Missense p.D459N | rs140221506 | Allele Freq. >0.1% |
| Chr8:41791414 C>T | KAT6A (NM_001099412) | Missense p.A1442T | rs113195648 | Allele Freq. >0.1% |
| Chr9:119976883 G>A | ASTN2 (NM_014010) | Missense p.S257T | rs139148246 | Allele Freq. >0.1% |
| Chr12:122359397 insGAGGAGGAGGAGAAA | WDR66 (NM_001178003) | Insertion | rs67558673 | Allele Freq. >0.1% |
| Chr14:23744800 delCAT | HOMEZ (NM_020834) | Deletion p.547del | rs148005528 | Within a simple repeat containing additional 3-bp deletion SNPs |
| Chr17:6928019 insCAG | BCL6B (NM_181844) | Insertion | rs66978918 | Present in Venter and Watson genomes |

All twelve variants remaining after filtration were individually assessed for pathogenicity and excluded or highlighted as a good candidate based on the entry in the comments column.
there is no evidence in the literature that parents of JEB children with LAMB3 mutations, themselves heterozygous carriers of LAMB3 mutations, have AI. However, many of the mutations described in JEB cases are nonsense mutations near the amino-terminus, which are more likely than the mutation described herein to cause a complete knockout of the gene. It therefore seems reasonable to speculate that the mutation described herein is unusual in its effect, acting as a dominant gain of function mutation to cause AI, while the majority of LAMB3 mutations are knock-outs, causing no phenotype in carriers while manifesting as JEB with enamel defects in homozygotes. Dental examinations in carrier parents of JEB cases due to a range of LAMB3 mutations would further test this hypothesis.

Amelogenesis involves three ameloblast stages; pre-secretory, secretory and maturation. In mice, Laminin-5 expression is observed mainly within secretory ameloblasts, particularly in Tomes’ processes, and in the enamel matrix, highlighting its requirement in the secretory stage of amelogenesis.25,26 This correlates with the hypoplastic AI phenotype due to a reduced enamel matrix volume observed in the family investigated. Furthermore, Laminin-5 has been shown to be located on the apical and basal edge of secretory ameloblasts, suggesting it is required to anchor ameloblasts to the enamel matrix.25,27 A defect in Laminin-5 is likely to lead to failure of transport across the cell-enamel matrix junction. Additionally, correct anchoring and arrangement of ameloblasts is required for crystal rod orientation, structure and mineralisation.26 Loss of Laminin-5 function is therefore likely to interrupt the secretory stage of amelogenesis, leading to a reduction in the volume of enamel matrix and impaired crystal rod growth.

In summary, we have identified a frameshift mutation at the extreme C-terminus of LAMB3, an integral member of the Laminin-5 heterotrimer, as a cause of dominant hypoplastic AI. The AI phenotype observed is consistent with a failure in the secretory stage of amelogenesis due to poor ameloblast adhesion to the secreted enamel matrix. In addition, we have shown that whole-exome sequencing of three distantly related family members can suffice to identify the causative mutation in a dominant family.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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