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

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Running title: A heterozygous mutation in *LAMB3* causes hypoplastic AI

Key Words

Amelogenesis, Amelogenesis Imperfecta, *LAMB3*, Laminin-5

Abstract

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.

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 genotypes¹. The impact of AI on affected individuals and their families is considerable². To date, mutations in 7 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 co-segregating abnormalities³⁻⁹.

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- β 3 (*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 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, Ontario, Canada) or via venous blood samples using conventional techniques. Screening of genes known to cause dominant AI (*ENAM* and *FAM83H*) was performed as previously described¹⁰. Whole exome sequencing was performed on a HiSeq2000 using the Nimblegen v2 chip by Otogenetics Corporation (Norcross, USA). Resulting data were analysed and annotated using the DNAnexus (<https://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 Fig.1A) presented with irregular hypoplastic AI and an autosomal dominant family history spanning four generations. Affected family members had experienced many symptomatic (e.g. frequent pain) and aesthetic problems with their teeth (Fig.1B). Some individuals had received extensive restorative dental care and required multiple dental extractions. Review of the medical

histories revealed no other health problems co-segregating 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 hypothesized 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 Fig.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 more than 50 bp from an exon; known polymorphisms (variants listed in versions of dbSNP up to and including dbSNP129 or those with a minor allele frequency greater than 1%); 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 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 re-tested 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 minor allele frequency (MAF) of greater than 0.1% 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 approximately 1/14,000 in the genetically heterogeneous US population¹¹. 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 3bp in-frame deletion in the *HOMER* gene (p.D547del; rs148005528) was excluded as it occurs within a run of eight aspartic acid residues, and four other 3bp deletion SNPs occur within in 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 1q32 (Fig.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 widely¹². This variant was therefore considered a strong candidate for the 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 JEB¹³. Sequencing of the *LAMB3* variant using primers F:5'-GTTGGGAGTCTTGGGGAGTC-3' and R:5'-AAGCATTCCAACCCAATCTG-3' in all available family members revealed perfect segregation with the disease phenotype (Fig.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 identification of disease-causing gene mutations. Here we show that by subjecting the more distantly related affected individuals within a pedigree to whole exome sequencing, the requirement for locus mapping is circumvented. Using this method, we were able to identify a novel heterozygous frameshift mutation in *LAMB3*, encoding a subunit of Laminin-5, as a cause of dominant hypoplastic AI.

Recessive mutations in *LAMB3* are already known to cause JEB, characterized by intense skin blistering arising within the lamina lucida of the basement membrane, caused by a failure of hemidesmosome formation between the epidermis and lamina densa¹⁴. Laminins are a family of network-forming trimeric proteins that are major constituents of the basal lamina, an extracellular matrix layer of the basement membrane. Laminin-5 consists of 3 polypeptides, alpha-3, beta-3, and gamma-2, encoded by the genes *LAMA3* (MIM 600805), *LAMB3*, and *LAMC2* (MIM 150292), respectively. Recessive mutations in these genes, and also in *COL17A1* (MIM 113811), cause Herlitz (severe) and non-Herlitz (milder) JEB (MIM 226700)¹⁵, with *LAMB3* mutations accounting for around 80% of cases¹⁶. Digenic JEB has also been noted, in which compound heterozygous changes in *COL17A1* combine with a heterozygous *LAMB3* variant to cause JEB, though any two of the mutations described do not suffice to cause the disease¹³.

Most JEB cases also have enamel defects, including those due to recessive *LAMB3* mutations, though these defects vary in severity¹². Furthermore, some

heterozygous carriers of *COL17A1* and *LAMA3* mutations display an AI phenotype, though it is not common¹⁷⁻¹⁹. No such cases have been reported for *LAMB3* heterozygotes. Additionally, *Col-17* and *Lama3* knockout mice have defects in amelogenesis, with further examination revealing defects in Tomes' processes causing disturbed enamel matrix secretion and malformed enamel rods^{20,21}. While a *Lamb3* mouse model exists, recessive mutants died within 24 hours of birth and no examination of teeth was performed²².

The mutation identified in this study is a premature termination codon in the final exon of *LAMB3*. Nonsense mutations in the last exon of a gene are not thought to cause nonsense-mediated decay (NMD) since an exon/exon junction upstream of the mutation is required to bind NMD initiation factors²³. It therefore seems likely that a mutated protein will be expressed, with the C-terminal 40 amino acids of this 1172 amino acid protein replaced by 27 incorrect amino acids. The C-terminus of *LAMB3* primarily consists of an α -helical domain responsible for Laminin-5 subunit assembly²⁴ (Fig.1D). The phenotype observed may therefore be a result of gain of function due to the mutant protein partially inhibiting the assembly of the heterotrimeric Laminin-5 complex. Interestingly 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 knock-out 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 mineralisation²⁶. 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 frame-shift 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.

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Conflict of Interest

The authors declare no conflict of interest.

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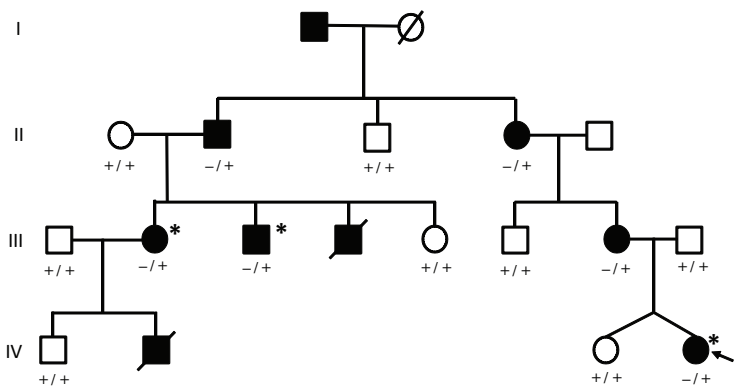
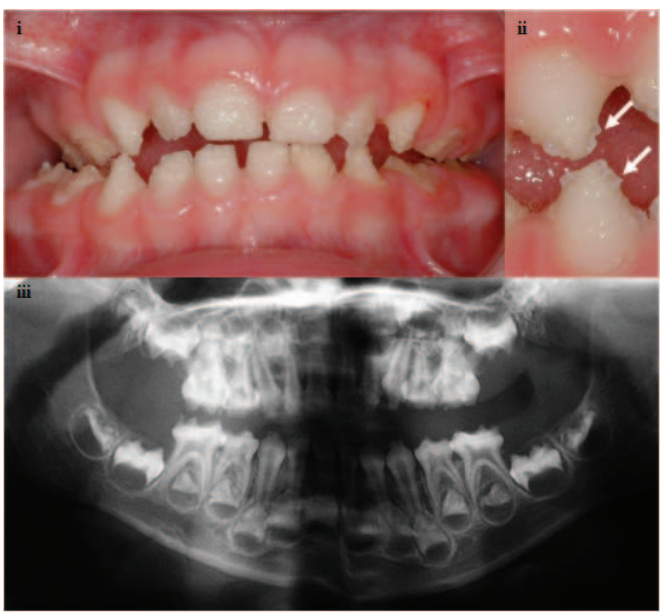
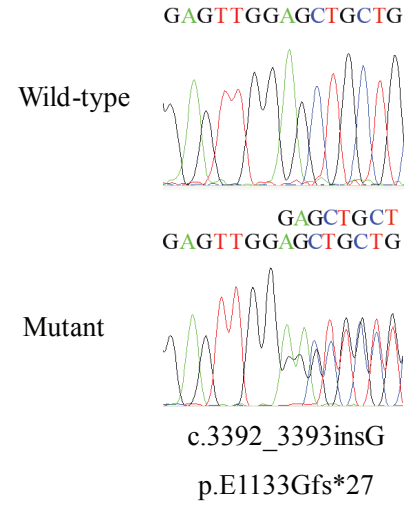
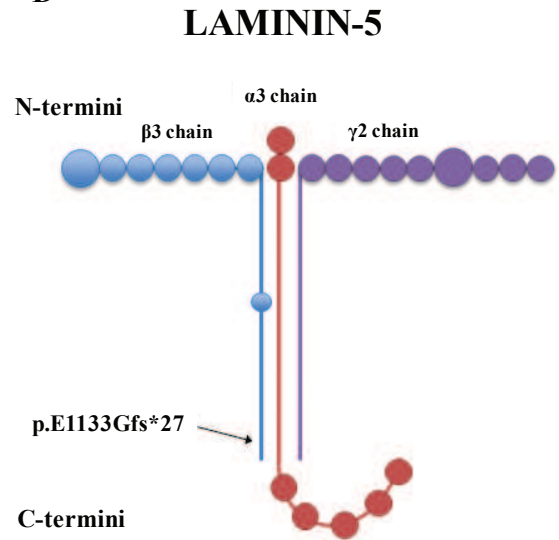
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Figure Legend

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).

A**B****C****D**

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

Table 1 – The list of variants after filtration. 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.