HHS Public Access

Author manuscript

Clin Oral Investig. Author manuscript; available in PMC 2019 April 10.

Published in final edited form as:

Clin Oral Investig. 2019 March ; 23(3): 1481–1487. doi:10.1007/s00784-018-2577-9.

Candidate gene sequencing reveals mutations causing hypoplastic amelogenesis imperfecta

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Abstract

Objective—Amelogenesis imperfecta (AI) is a rare hereditary disorder affecting the quality and quantity of the tooth enamel. The purpose of this study was to identify the genetic etiology of hypoplastic AI families based on the candidate gene approach. Materials and methods We recruited three Turkish families with hypoplastic AI and performed a candidate gene screening based on the characteristic clinical feature to find the pathogenic genetic etiology.

Results—The candidate gene sequencing of the *LAMB3* gene for family 1 revealed a heterozygous nonsense mutation in the last exon [c.3431C > A, p.(Ser1144*)]. *FAM20A* gene sequencing for families 2 and 3 identified a homozygous deletion [c.34_35delCT, p.(Leu12Alafs*67)] and a homozygous deletion-insertion (c.1109 + 3_1109 + 7delinsTGGTC) mutation, respectively.

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study. The study protocol was reviewed and approved by the Institutional Review Board at Seoul National University Dental Hospital (CRI05003G) and at the University of Istanbul (IRB00009905).

Conclusion—The candidate gene approach can be successfully used to identify the genetic etiology of the AI in some cases with characteristic clinical features.

Clinical relevance—Identification of the genetic etiology of the AI will help both the family members and dentist understand the nature of the disorder. Characteristic clinical feature can suggest possible genetic causes.

Keywords

Amelogenesis imperfecta; Candidate gene sequencing; *LAMB3*; *FAM20A*; Deletion-insertion mutation; Nonsense mutation

Introduction

The formation of tooth enamel (amelogenesis) is under strict control of ectomesenchyme interactions [1]. Once enamel forming cells (ameloblasts) are differentiated, the enamel matrix starts to be secreted on the initial dentin matrix which is secreted by dentin forming cells (odontoblasts). The secreted enamel matrix begins to calcify, and further maturation of the calcified enamel matrix results in sound enamel which is the hardest tissue in the human body [2].

Genetic and local (environmental) factors affecting any of the stages of amelogenesis can cause various types of enamel defects: hypoplastic, hypocalcified, and hypomatured [3]. Amelogenesis imperfecta (AI) is a collective term for a rare hereditary condition that mainly affects tooth enamel. AI can occur as an isolated form or as a syndromic manifestation.

AI is heterogeneous in clinical phenotype as well as in genetic etiology. To date, there are more than a dozen genes involved in the non-syndromic AI [4–19]. Additionally, several genes are known to cause the syndromic form of AI such as *FAM20A* in enamel renal syndrome and *CNNM4* in Jalili syndrome [20–22].

Given the fact that there is an increasing number of candidate genes causing AI and the cost of next generation sequencing is decreasing, whole-exome sequencing seems to be a comprehensive way to identify the genetic etiology of unknown AI cases. However, candidate gene sequencing for several or a single gene could be a valuable way to find a genetic cause for certain cases based on the clinical characteristics and hereditary pattern, because it could provide a fast and economic diagnosis.

In this study, we recruited three AI families and performed mutation analyses using the candidate gene approach based on their characteristic clinical feature and pedigree analysis. Mutational analyses successfully identified underlying mutations in *LAMB3* and *FAM20A*.

Materials and methods

Enrollment of human subjects

Three Turkish families with hypoplastic AI were recruited for this genetic study. The study protocol was reviewed and approved by the Institutional Review Board at Seoul National University Dental Hospital, the University of Istanbul, and the University of Michigan.

Clinical and radiological examinations were performed, and saliva or blood samples were collected with the understanding and written consent of each participant according to the Declaration of Helsinki.

Polymerase chain reaction (PCR) and sequencing

DNA was isolated from the peripheral whole blood or saliva of the participating family members with the NucleoSpin genomic DNA purification kit (Macherey-Nagel GmbH & Co., Düren, Germany). The primers and conditions for the Sanger sequencing of the exons and exon-intron boundaries of *LAMB3* and *FAM20A* were described previously [23, 24]. PCR amplifications were done with the HiPi DNA polymerase premix (Elpis Biotech, Daejeon, Korea), and PCR amplification products were purified with a PCR Purification Kit and protocol (Elpis Biotech). DNA sequencing was performed at a DNA sequencing center (Macrogen, Seoul, Korea).

Splicing assay

A genomic fragment (988 bp) of the *FAM20A* gene including exons 6 and 7 was amplified with the Pfu DNA Polymerase (Elpis biotech) and cloned into the pTop Blunt V2 vector (Enzynomics, Seoul, Korea). Wild type and mutant sequences were subsequently subcloned into the pSPL3 vector after double digestion with *EcoRI* and *XhoI* restriction endonucleases. Cloned vectors were transfected into COS-7 cells, and total RNA was isolated after 36 h. Reverse transcriptase-PCR was performed using the vector primers (SD6: 5’-TCTGAGTC ACCTGGACAACC-3′; SA2: 5’-ATCTCAGTGGTATT TGTGAGC-3′). The amplification bands were excised from an agarose gel and characterized by sequencing.

Results

Family 1

The proband was a 10-year-old female in a non-consanguineous nuclear family (Fig. 1a). The proband had irregular hypoplastic pits and grooves on all permanent teeth (Fig. 1b–d). Her remaining deciduous molars exhibited dentin on occlusal surfaces due to the loss of the thin enamel by presumably attrition. Panoramic radiograph revealed similarly affected developing permanent teeth (Fig. 1e). Her father had many prosthetics, but he reported his teeth were not as bad as the teeth of his daughter. Careful examination of the father showed several hypoplastic pits and grooves on the remaining permanent teeth (Fig. 1f–h).

LAMB3 mutational analysis of the proband identified a heterozygous mutation (NM_000228.2; c.3431C > A) caused by a transversion change from cytosine to adenine (Suppl. Fig. 1). This mutation would introduce a premature stop codon [NP_000219.2; p.(Ser1144*)] in the last exon. This nonsense mutation being located in the last exon would escape from the nonsense mediated decay system (NMDS) translating into a truncated LAMB3 mutant protein. The father also had this mutation.

Family 2

The proband was an 8-year-old female who was a second child from a consanguineous marriage (Fig. 2a). Her enamel was smooth hypoplastic, and the gingival tissue was

generally hyperplastic (Fig. 2b–d). The left side deciduous molars (both maxillary and mandibular) were ankylosed causing a posterior open bite on the left side. Panoramic radiograph showed signs of eruption disturbances for all second permanent molars (Fig. 2e).

Mutation analysis of FAM20A identified a homozygous 2-bp deletion mutation (NM_017565.3; c.34_35delCT) in the proband (Suppl. Fig. 2). This mutation would introduce a translation codon frameshift changing leucine at amino acid position 12 to alanine with a novel 66-amino-acid sequence [NP_060035.2; p.(Leu12Alafs*67)]. This mutant transcript with an early termination codon would be degraded by NMDS, and the proband would have no functional FAM20A protein at all.

Family 3

The proband was a first child, a 17-year-old female, from a consanguineous family (Fig. 3a). She had generalized gingival hyperplasia and a bilateral posterior open bite (Fig. 3b–d). Her teeth had generally hypoplastic enamel and black pigmentations. Rough enamel surfaces will make it difficult to maintain good oral hygiene. She still had several deciduous teeth (the right maxillary deciduous canine, left maxillary deciduous second molar, and left mandibular deciduous second molar), and multiple permanent teeth could not erupt into the oral cavity (Fig. 3e). The third child, a 7-year-old male, also had a similar clinical phenotype except for the retained deciduous teeth (Fig. 3f). A panoramic radiograph (Fig. 3g) showed severe pulpal calcification and root malformations (especially in the molars).

FAM20A mutational analysis of the proband revealed a homozygous 5-bp deletion and insertion in the donor site of intron 7 (NM_017565.3; c.1109 + 3_1109 + 7delinsTGGTC) changing the donor site sequence from GTGAGTT to GTTGGTC (Suppl. Fig. 3). The affected brother also had a homozygous mutation. A splicing assay of the wild type vector showed a single strong band with normal splicing, while the mutant vector resulted in abnormal splicing products (Fig. 4). The longest product had an additional intron and the cloning vector sequence after exon 7. The splicing product with a similar length had the GTTG sequence after exon 7. The strongest splicing product of the mutant vector had an exon 7 deletion, and some splicing products had both exon 6 and 7 deletions. All these mutant splicing products would result in a frameshift and introduce an early termination codon causing these transcripts to be degraded by the NMDS.

Discussion

Laminin-332 (laminin-5) is a basement membrane component and formed by three subunits encoded by *LAMA3*, *LAMB3*, and *LAMC2* [25]. Recessive mutations in these genes cause junctional epidermolysis bullosa (JEB), a syndrome featuring skin fragility with enamel hypoplasia [26]. A person with a single allele defect is a carrier of JEB and usually does not have a clinical phenotype. However, some heterozygous mutations have been shown to cause enamel malformations with characteristic hypoplastic pits and grooves [10, 23, 27–29]. These mutations are frameshift or nonsense mutations that can escape from the NMDS. Expressed mutant proteins with a truncated C-terminus seem to interfere with the proper functioning of the laminin-332 basement membrane beneath ameloblasts by a dominant negative mechanism.

The mutation identified in family 1 (NM_000228.2; c.3431C > A) has been reported before in another family [23]. The nucleotide after the mutation position is G, and this change (c. 3432A > G) is a single nucleotide polymorphism (rs1049607) with a 0.463 minor allele frequency. Because family 1 in this study and another family in a previous study share the same mutation sequence (TAG at the 1144 codon position) and ethnic background, it is possible they have inherited it from a common ancestor.

Fam20A, acting as a homodimer, is a pseudokinase that forms a functional complex with Fam20C, and this complex is involved in extracellular protein phosphorylation [30, 31]. Recessive mutations in *FAM20A* were identified, for the first time, in a family with amelogenesis imperfecta and gingival hyperplasia (AI and gingival fibromatosis syndrome; OMIM #614253) [20, 24]. Soon after, it was found that *FAM20A* recessive mutations cause renal calcification as well (Enamel-Renal syndrome; OMIM #204690) [21, 32]. Analysis of a *Fam20a* knockout mouse revealed ectopic calcification in multiple tissues with severe calcification in the kidney [33]. This finding was strengthened by a new family with pulmonary calcification as well [34].

Both homozygous *FAM20A* mutations identified in this study (c.34_35delCT in the family 1 and c.1109 + 3_1109 + 7delinsTGGTC in the family 2) would result in a frameshift introducing an early termination codon and therefore be degraded by the NMDS. All affected individuals in both families showed a very similar clinical phenotype. Especially severe intrapulpal calcification was seen in family 3 as evidence of ectopic calcification (Fig. 3g). The mutation in family 2 has also been reported before in a family with the same genetic background; therefore, it could be a mutational hotspot or an example of the identical by descent [24].

In this study, we used the candidate gene approach based on unique clinical phenotypes. In family 1, characteristic irregular hypoplastic pits and grooves suggested *LAMB3* as a prime candidate gene. In families 2 and 3, consanguinity in the pedigree analysis and the clinical features such as generalized hypoplastic enamel, gingival hyperplasia, and eruption failures in the molar region suggested *FAM20A*. A similar characteristic phenotype can be seen in ENAM mutations as horizontal hypoplastic grooves and in *AMELX* mutations in affected females as vertical irregular hypoplastic grooves due to a lyonization (random X-inactivation) pattern [35, 36].

In conclusion, we recruited three AI families and performed mutational analysis with the candidate gene approach based upon the clinical phenotype and inheritance patterns. Mutational analysis successfully identified mutations in *LAMB3* and *FAM20A* that cause characteristic enamel malformations with or without other clinical symptoms. The candidate gene approach can be a valuable tool to identify the genetic etiology of AI families and further strengthen genotype-phenotype correlations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank the participants in this study for their cooperation.

Funding This work was supported by grants by the National Research Foundation of Korea (NRF) grant funded by the Korea government (NRF-2017R1A2A2A05069281) and NIDCR/NIH grant DE015846.

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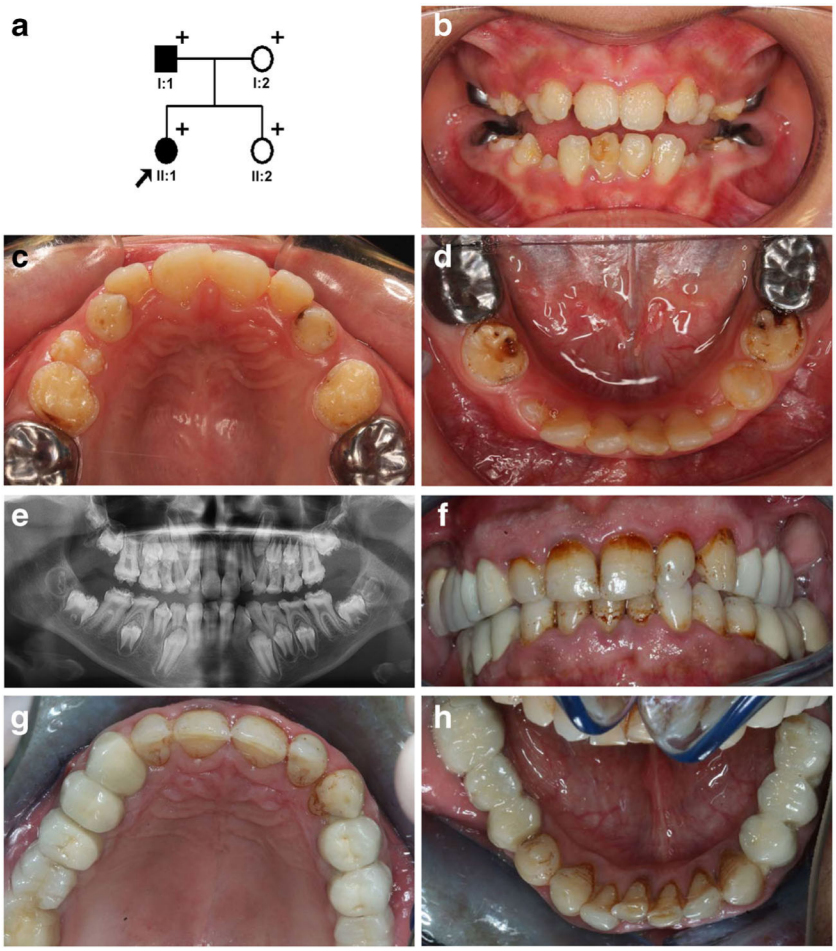

Fig. 1.

Pedigree and clinical photos of family 1. **a** Pedigree of family 1. Family members who participated in this study are indicated with a plus (+) symbol. An arrow indicates the proband, and black symbols indicate the affected individuals. **b–d** Frontal, maxillary, and mandibular clinical photos of the proband at age 11. **e** Panoramic radiograph of the proband at age 10. **f–h** Frontal, maxillary, and mandibular clinical photos of the father of the proband at age 40

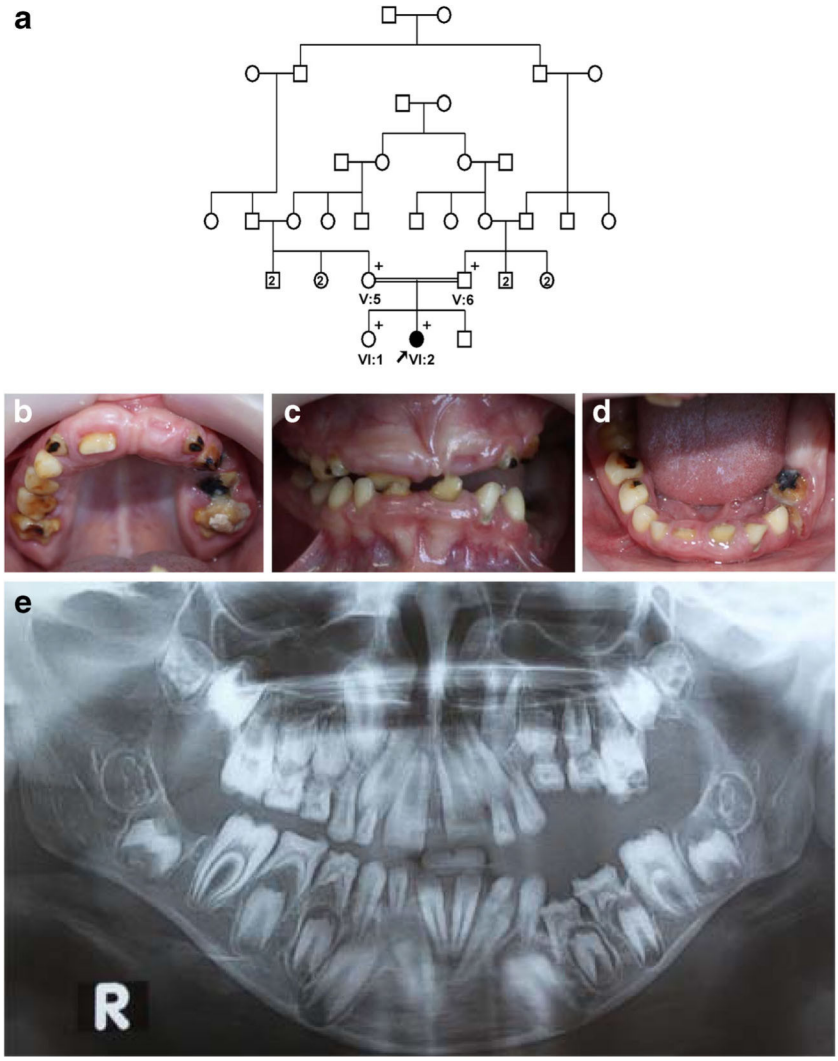

Fig. 2.

Pedigree and clinical photos of family 2. **a** Pedigree of family 2. Family members who participated in this study are indicated with a plus (+) symbol. An arrow indicates the proband, and the black symbol indicates the affected individual. Numbers in the subject symbol indicate the number of siblings. **b**–**d** Maxillary, frontal, and mandibular clinical photos of the proband at age 8. **e** Panoramic radiograph of the proband at age 8

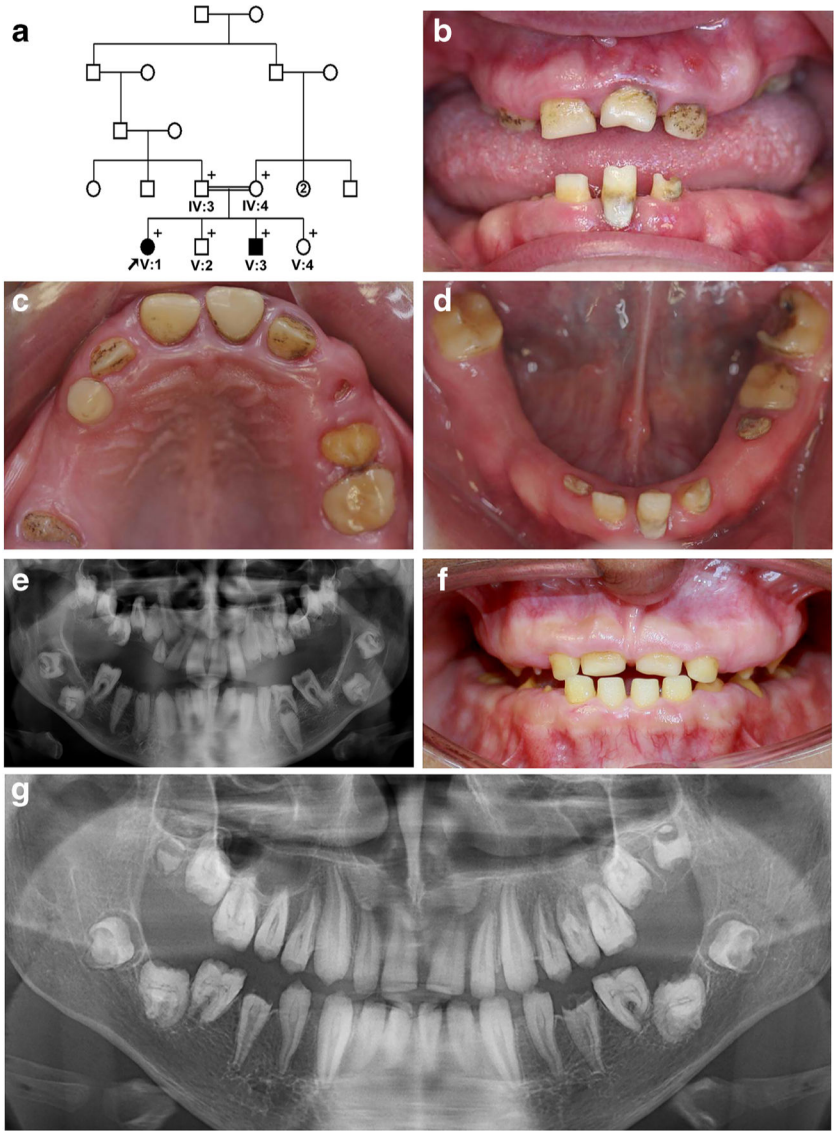

Fig. 3.

Pedigree and clinical photos of family 3. **a** Pedigree of family 3. Family members who participated in this study are indicated with a plus (+) symbol. An arrow indicates the proband, and black symbols indicate the affected individuals. Number in the subject symbol indicates the number of siblings. **b**–**d** Frontal, maxillary, and mandibular clinical photos of the proband at age 17. **e** Panoramic radiograph of the proband at age 17. **f** Frontal clinical photo of the affected brother (V:3) of the proband at age 12. **g** Panoramic radiograph of the affected brother (V:3) of the proband at age 12

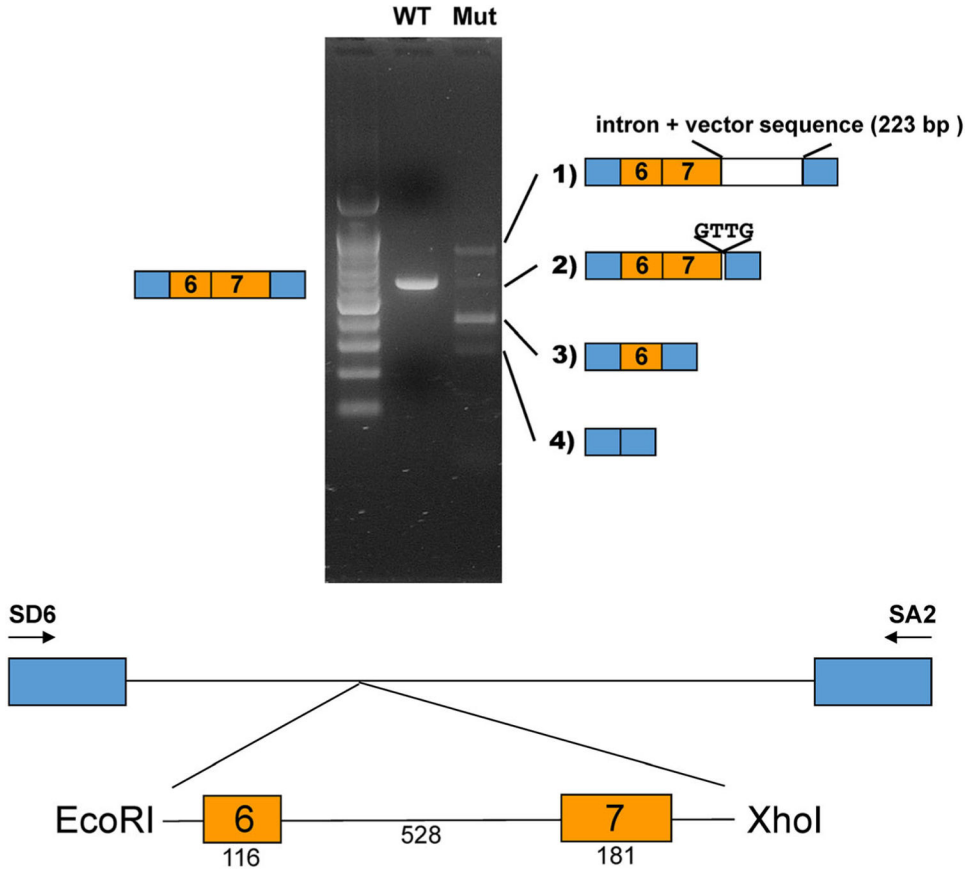

Fig. 4.

In vitro splicing assay. A genomic fragment that included exons 6 and 7 was cloned into the pSPL3 splicing vector using *EcoRI* and *XhoI* restriction endonucleases. Boxes in the diagram indicate the exons in the pSPL3 vector. The number of base pairs (bp) of the exons and intron is shown below the exon and the intron. RT-PCR with the vector primers (SD6 and SA2) revealed four weaker amplicons in the mutant vector compared to the single one in the wild type vector (WT, wild type; Mut, mutant). Sequencing identified abnormal splicing products in the Mut instead of in the normal splicing product in the WT