Identification of Novel Candidate Genes Associated with Non-Syndromic Tooth Agenesis in Mongolian Families

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Identification of Novel Candidate Genes Associated with Non-Syndromic Tooth Agenesis in Mongolian Families

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Tooth agenesis is a prevalent developmental anomaly characterized by the absence of teeth. The identification of numerous genes linked to the etiology of tooth agenesis contributes to enhancing understanding of tooth development. However, there is still a need fully elucidate novel causal genes and their associated signaling pathways.

This study aimed to identify genetic variants associated with non-syndromic TA in nine families from Mongolia using whole-exome sequencing (WES) and bioinformatic analysis. The study enrolled 41 participants, including three inherited and six non-inherited families. WES analysis was performed on 14 saliva samples from individuals with non-syndromic TA.

The potential candidate genes were identified through variant filtering and segregation analysis. The filtered variants were then analyzed in silico mutation impact analysis. WES

analysis identified 21 variants associated with TA, and 5 of these variants met all filtering criteria. These variants were located in the exon region of MAST4, ITGA6, PITX2, CACNA1S, and CDON genes.

The variant in PITX2 was found in eight participants from both inherited and non-inherited families, while the MAST4 variant was identified in six participants from inherited families. Additionally, the CDON variant was identified in five participants from both inherited and non-inherited families. Gene set enrichment analysis revealed the significance of five candidate genes associated with functional terms related to focal adhesion and the calcium channel complex.

The study has identified novel candidate gene variants associated with non-syndromic TA in Mongolian families. Furthermore, the results emphasize the significance of genes related to the Wnt signaling pathway, focal adhesion and calcium channel complexes in the genetic mechanisms of tooth development.

Keywords: Tooth agenesis, Genetic variants, Whole-exome sequencing, Bioinformatic analysis, In silico mutation, Mongolian population

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I. INTRODUCTION

Tooth agenesis (TA) is a common developmental anomaly that results in the congenital absence of one or more permanent teeth in humans. It affects approximately 200 million individuals worldwide, and its incidence varies by geography, population, and race [1, 2]. TA can lead to several complications, including oral symptoms, masticatory dysfunction, physiological issues, speech impairments, aesthetic concerns, and financial burdens, which can significantly affect the quality of life [3, 4].

Tooth development begins with sequential and reciprocal interactions between embryonic tissues, such as oral epithelium and ectomesenchyme [5]. This process is regulated by various signaling pathways, including BMP, FGF, SHH, TNF, and WNT, which are essential for the

tooth bud, cap, and bell stages [6, 7]. Disruptions in these signaling pathways during tooth development may cause TA [8].

TA can be categorized based on the number of missing permanent teeth, typically excluding third molars. Hypodontia refers to the absence of fewer than six permanent teeth, while oligodontia is defined as the absence of six or more permanent teeth. Anodontia describes the complete absence of all permanent teeth [9] . The worldwide prevalence of third molar agenesis is estimated to range from 5.32% to 56% [10]. Therefore, it is typically excluded from dental numbering due to their common absence. In the majority of cases (83%) involving TA only one or two permanent teeth are affected, while the occurrence of the absence of six permanent teeth is exceptionally rare (0.14%) [11].

TA can also be classified as non-syndromic or syndromic, depending on the involvement of other organs or tissues. Non-syndromic TA, also known as isolated TA, is characterized by dental abnormalities without other tissue symptoms. On the other hand, syndromic TA involves missing teeth and other developmental abnormalities such as nails, hair, skin and sweat glands. Some examples of syndromic TA include cleft lip/plate [12], ectodermal dysplasia [13], Axenfeld-Rieger syndrome, and Witkop syndrome [14-16]. Non-syndromic TA occurs more frequently than syndromic TA.

The etiology of TA can be attributed to genetic or environmental factors [17-20]. However, genetic factors play a more significant role in the pathogenesis of TA [21, 22]. Identifying the genetic factors associated with TA can improve diagnosis and treatment. Recent studies have identified several genes associated with TA, including MSX1 [23], PAX9 [19], BMP4 [24], AXIN2 [25], EDA [26], EDAR [27], EDARADD [28], WNT10A [4], WNT10B [29], LRP6 [30], PITX2 [31], FGFR2 [3], and CACNA1S [3]. Current studies employing WES and bioinformatic analysis have explored TA. Researchers suggest that EDA, EDAR, WNT10A, and PCNT are linked to syndromic TA. Conversely, studies have indicated that genes such as PAX9, MSX1, LRP6, WNT10A, WNT10B, AXIN2, PITX2, and BMP4 are associated with

non-syndromic TA [32-34]. These genes exhibit autosomal-dominant, autosomal-recessive, or X-linked mechanisms of inheritance [35].

The highest genetic risk for non-syndromic TA is attributed to mutated genes that encode components within the canonical Wnt/β-catenin signaling pathway. The Wnt signaling pathway consists of several key components, including extracellular secreted glycoproteins (comprising 19 Wnt ligands in humans), receptors with seven transmembrane spans (Frizzled and LRP5/6), cytoplasmic proteins (DVL, APC, AXIN, GSK3 β , β -catenin), nuclear transcription factors (TCF/LEF), and various other molecules associated with Wnt (such as DKK1, KREMEN 1, ANTXR1 and MSX1) [33].

The initial connection between TA and Wnt pathway became evident when a mutation in the AXIN2 gene was discovered in a family with oligodontia. AXIN2 is responsible for producing a protein that inhibits the Wnt/β-catenin signaling pathway and is prominently expressed in enamel knot and mesenchymal odontoblasts during tooth development [18]. WNT10A, belonging to the Wnt family present in the dental epithelium, interacts with the Frizzled transmembrane receptor and LRP6. This interaction subsequently triggers the activation of Wnt/β-catenin pathway, which leads to abnormal tooth development [33].

MSX1 and PAX9 are among the first genes associated with TA, and their protein products act as transcription factors essential for the tooth germ's development from mesenchymal cells [36, 37]. Mutations in the MSX1 gene, which encodes a transcriptional repressor involved in both the Wnt and BMP4 pathways, have been consistently identified as the cause of non-syndromic TA [38]. Recently, it has been shown that PITX2 contributes to TA and is involved in the early stages of tooth development, including the formation of tooth germs and bud morphology [39].

In recent years, the next-generation sequencing (NGS) approach, specifically WES platforms, such as Illumina, has been extensively used for identifying biomarkers of genetic disease diagnosis [40]. Although only 2% of the human genome consists of exons, 85% of the

genetic variations responsible for highly penetrant diseases reside in this small genome region [31]. WES has improved diagnostic accuracy, shortened the diagnostic process, and is more cost-effective than traditional methods. This approach identifies genetic variations that may contribute to the development of TA. Bioinformatic analysis can then be used to analyze the WES data [41]. It can help identify potential candidate genes and variants that may contribute to the condition, especially in a limited number of participants. Furthermore, it can help predict the functional impact of genetic variants and provide insights into the pathogenesis of the condition.

The study aims to investigate the genetic variants of non-syndromic TA in Mongolian families through the utilization of WES and bioinformatic analysis.

II. MATERIALS AND METHODS

1. Ethics approval

This study was approved by Institutional Review Boards at Ministry of Health in Mongolia (IRB No. 285, Approved on 7th September 2022). This clinical study was conducted in accordance with Helsinki Declaration. All enrolled participants were informed about consent and ascertained. Nine participants were juvenile's, written informed consent was obtained from their guardians.

2. Subjects

A total of 41 individuals from nine Mongolian families were enrolled in the study. Among these individuals, 15 were identified with non-syndromic TA. However, in Family 1, one participant (I-2) who had non-syndromic TA was unable to provide saliva samples for analysis. Consequently, 14 participants who had both clinical data and saliva samples available underwent WES analysis. Participants with TA were identified through clinical examination and panoramic radiograph. It was confirmed that the missing permanent teeth were not due to extraction or injuries. The general clinical examination confirmed that all study participants exhibited non-syndromic TA and had normal hair, skin, sweat glands, facial features and nails.

3. Radiographic assessments

Panoramic radiographs were obtained from 14 participants.

4. Sample collection

Saliva samples were collected from each participant using the Oragene DNA Self-Collection Kit (DNA Genotek Inc., Ottawa, Canada). A 2 ml saliva was then collected and subsequently mixed with a DNA-preserving solution in a tube, following the guidelines set forth by the manufacturer.

5. Control group

The study utilized exome sequencing data from a randomized subsample of 100 healthy individuals (Koreans), obtained from the Ansan-Ansung population consisting of 3703 individuals. These subsamples were provided by the Korea BioBank, Center for Genome Science, National Institute of Health, Korea Centers for Disease Control and Prevention. The individuals selected from the reference population were chosen randomly, regardless of sex and age, and had no history of significant diseases.

6. Whole-exome sequencing

DNA samples were processed for WES using the SureSelectXT Human All Exon V5 kit and Novaseq 6000 platform. The DNA quality was assessed through 1% agarose gel electrophoresis and PicoGreen® dsDNA Assay. The library was prepared by fragmenting genomic DNA, ligating sequencing adapters, and amplifying the adapter-ligated DNA using PCR. A hybridization buffer was then prepared by mixing SureSelect hyb #1, #2, #3 and #4 reagents, and the amplified DNA fragments was concentrated and SureSelect blocks #1, #2, and #3 reagents were added. The DNA blocking agent mixture and hybridization buffer were incubated, and a RNase block was added to the SureSelect oligo capture library, which was then incubated. After adding the hybridization buffer and DNA blocking agent mix to the capture library, the mixture was incubated at 65℃ for 24 hours. The captured library was then washed with SureSelect binding buffer and eluted with nuclease-free water. Finally, the library was amplified and tagged with index tags. The libraries were pooled in equimolar amounts and subjected to sequencing using the Illumina Novaseq 6000 system following the protocol for 2x100 sequencing.

7. Family-level statistical analysis of WES data and variant filtering

The study grouped patients from the same family into one subgroup to enhance the effects of a family inheritance. If not, each family's sole patients are placed in the opposite grouping. The WES data were used to identify variants with only protein-coding transcripts being taken into account.

Fisher's exact test was used to compare the allele frequencies of each mutation between the control and each subgroup. To qualify risky variants of TA, variants with association P value ($p < 0.05$) and Odds ratio (ORs > 1) were considered statistically significant (Table 2). The threshold of OR was set as > 1, which just implies that the variant ratio was higher in the patient subgroup than in control cohort. Then, the significant associations of the variant were evaluated with p-values.

After statistical analysis, the study subsequently collected variants that can be associated with TA by disrupting protein function. To reduce the systematic differences between the sample subgroups, variants on TA-related genes filtered using public databases of gene-phenotype relationships. Additionally, variants related to TA were filtered using associated gene lists from Open Targets (OT) [42] (EFO_0005410, tooth agenesis), Gene Ontology (GO) [43] (GO:0042476, odontogenesis), and Human Phenotype Ontology (HPO) [44] (HP:0000677, oligodontia). This step helps narrow the range of variants to the specific area of interest, reducing the noise signal from the other variants.

Variants with annotation of HIGH impact in SnpEff [45] and variants with MODERATE impact (Table 1) but are predicted to damage its function with the results of computational prediction tools (PolyPhen2 [46] and SIFT [47]) or lower minor allele frequency in the Asian population (ASN MAF [48]) than 0.1 were filtered. Figure 1 flow chart provides a step-by-step visualization of the variant filtration process, providing clarity and transparency regarding the analytical pipeline.

Table 1. Impact category of effects in SnpEff.

Putative Impact	Effects(Sequence Ontology term)
HIGH	chromosome_number_variation
HIGH	exon_loss_variant
HIGH	frameshift_variant
HIGH	rare_amino_acid_variant
HIGH	splice_acceptor_variant
HIGH	splice_donor_variant
HIGH	start_lost
HIGH	stop_gained
HIGH	stop_lost
HIGH	transcript_ablation
MODERATE	3_prime_UTR_truncation+exon_loss
MODERATE	5_prime_UTR_truncation+exon_loss_variant
MODERATE	coding_sequence_variant
MODERATE	disruptive_inframe_deletion
MODERATE	disruptive_inframe_insertion
MODERATE	inframe_deletion
MODERATE	inframe_insertion
MODERATE	missense_variant
MODERATE	regulatory_region_ablation
MODERATE	splice_region_variant
MODERATE	TFBS_ablation
LOW	5_prime_UTR_premature start_codon_gain_variant
LOW	initiator_codon_variant
LOW	splice_region_variant
LOW	start_retained

LOW	stop_retained_variant
LOW	synonymous_variant
MODIFIER	3_prime_UTR_variant
MODIFIER	5_prime_UTR_variant
MODIFIER	coding_sequence_variant
MODIFIER	conserved_intergenic_variant
MODIFIER	conserved_intron_variant
MODIFIER	downstream_gene_variant
MODIFIER	exon_variant
MODIFIER	feature_elongation
MODIFIER	feature_truncation
MODIFIER	gene_variant
MODIFIER	intergenic_region
MODIFIER	intragenic_variant
MODIFIER	intron_variant
MODIFIER	mature_miRNA_variant
MODIFIER	miRNA
MODIFIER	NMD_transcript_variant
MODIFIER	non_coding_transcript_exon_variant
MODIFIER	non_coding_transcript_variant
MODIFIER	regulatory_region_amplification
MODIFIER	regulatory_region_variant
MODIFIER	TF_binding_site_variant
MODIFIER	TFBS_amplification
MODIFIER	transcript_amplification
MODIFIER	transcript_variant
MODIFIER	upstream_gene_variant

Table 2. The threshold of Odds ratio (ORs).

Subgroup	CHROM	ID	REF	ALT	Effect	Impact	Gene_Name	OR	Fisher_p
Family 1	2	.	G	GA	frameshift_variant	HIGH	ITGA6	99	0.00171572
	5	rs201910335	C	CGCT	disruptive_inframe_insertion	MODERATE	MAST4	64	0.00395857
Family 2	5	rs201910335	C	CGCT	disruptive_inframe_insertion	MODERATE	MAST4	32	0.00941702
	4	rs2278782	G	A	stop_gained	HIGH	PITX2	6.33333333	0.04701823
Family 3	1	rs3850625	G	A	missense_variant	MODERATE	CACNA1S	27.5714286	0.00154489
	5	rs201910335	C	CGCT	disruptive_inframe_insertion	MODERATE	MAST4	32	0.00941702
Non-Fam	4	rs2278782	G	A	stop_gained	HIGH	PITX2	4.52380952	0.02661926
	11	rs12274923	G	A	missense_variant	MODERATE	CDON	4.5	0.03387269

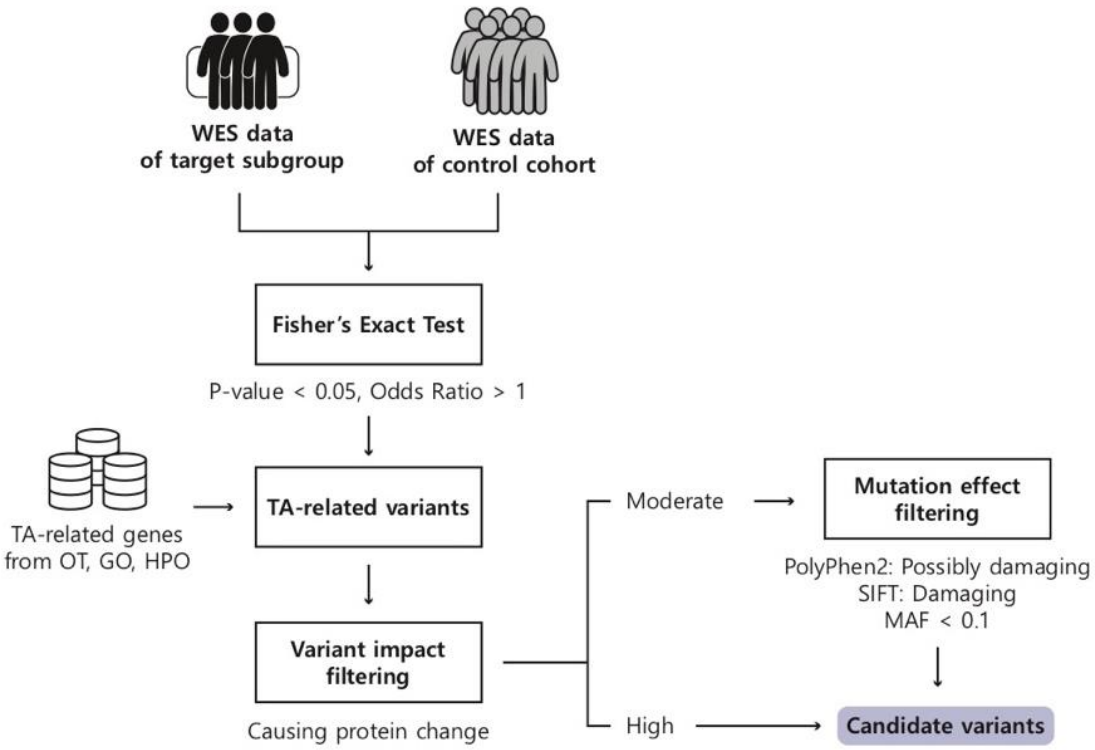


Figure 1. Flow chart detailing the variant filtration pipeline.

8. In silico mutation analysis

Sorting Intolerant From Tolerant (SIFT) [47] and Polymorphism Phenotyping v2 (PolyPhen2) [46] to detect mutations that may be responsible for TA. All possible non-redundant protein sequences in Ensembl database were analyzed for the two missense variants. SIFT determines the sequence conservation across multiple species, assuming mutations in highly conserved regions to be intolerable. PolyPhen2 predicts mutational impact by utilizing sequence co-evolution and protein structure. Table 6 summarizes the candidate genes with identified variants and their mutation impact analysis.

9. Gene set enrichment analysis

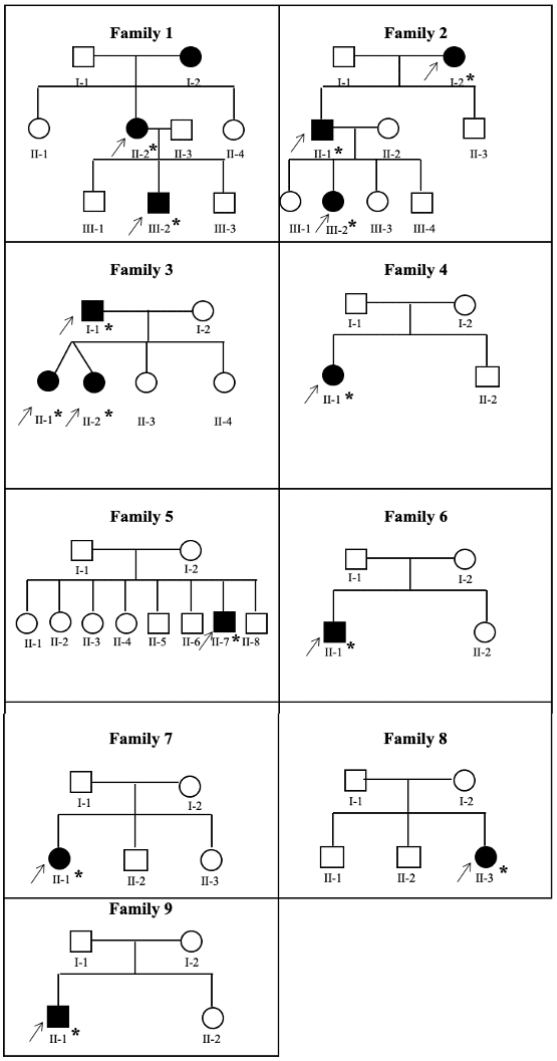
The g: Profiler [49] was used to perform gene set enrichment analysis (GSEA) for the candidate genes. The functional terms were derived from various data sources, including Gene Ontology [50] (GO molecular function, GO cellular component, and GO biological process), as well as biological pathways such as KEGG [51] and Reactome [52]). To increase the functional association of each gene, the gene sets were expanded by adding neighbor genes in the protein-protein interaction (PPI) network. The STRING (v11.5) [53] database was used for this process, with a confidence level of 700. The resulting network showed the relationship among the expanded gene sets, while the plot displays the function terms significantly enriched in the candidate genes at $p_{adj} < 0.01$.

III. RESULTS

1. Study subjects

The present study included nine Mongolian families, three of which were inherited families, as shown in Figure 2 a. WES analysis was conducted on a total of 14 samples, consisting of eight females and six males, with ages ranging from 9 to 69 years (Figure 2 b and Table 3). All 14 individuals were clinically diagnosed with non-syndromic TA, which was further confirmed through intra-oral examination and panoramic radiograph.

A



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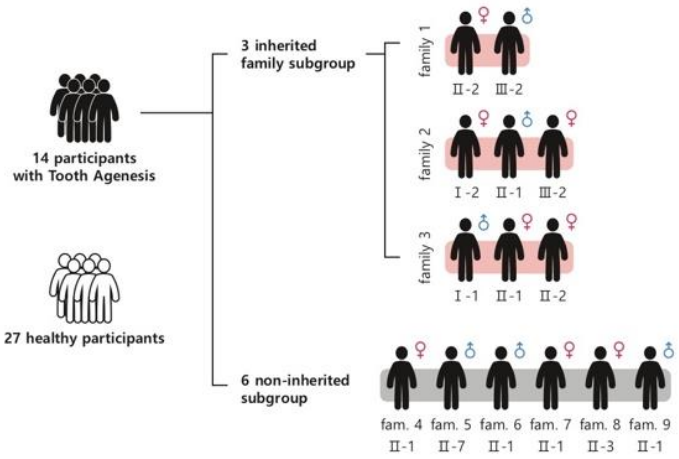


Figure 2. Pedigrees of the nine families and subgroup assignment of family members in the study. (**A**) Squares indicate males, and circles indicate females. Filled symbols represent individuals diagnosed with non-syndromic TA, while empty circles indicate unaffected subjects. Arrows indicate probands of each family. The asterisks represent subjects who underwent WES analysis. (**B**) Classification assignment of family members in subgroups.

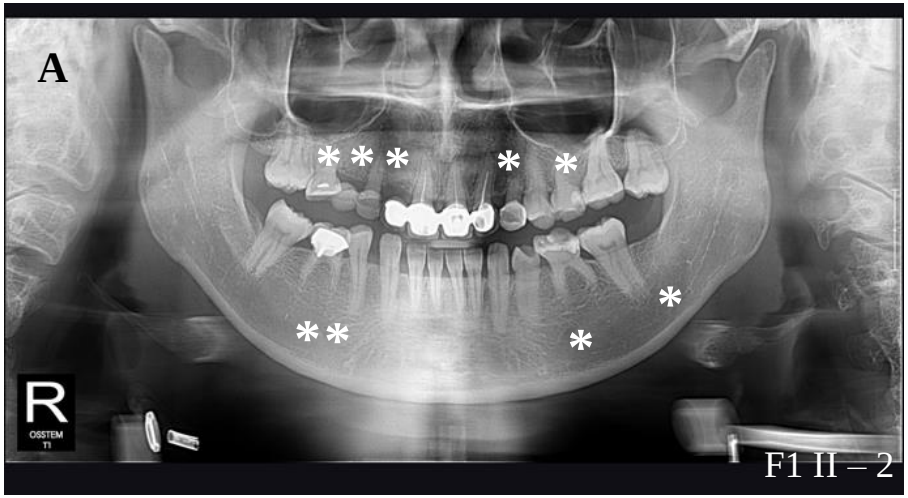
Table 3. The age and gender of participants.

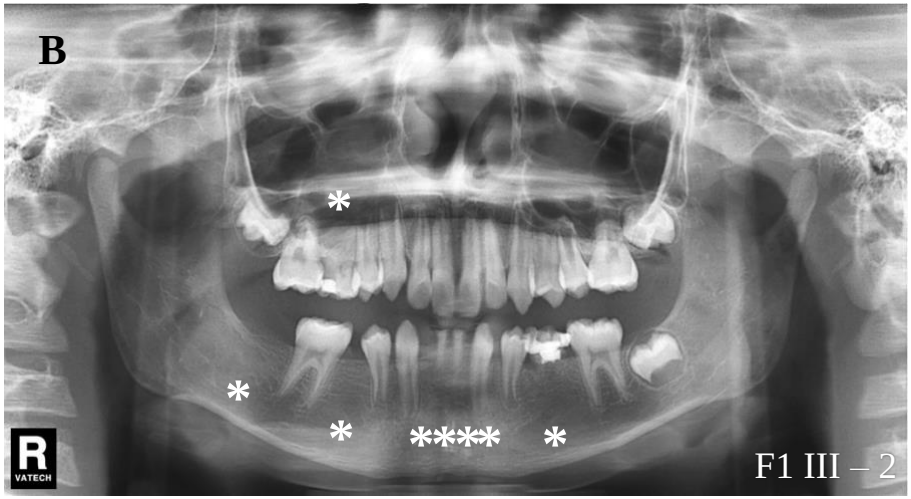
Family	Participant number	Age	Sex
1	II-2	30	F
1	III-2	12	M
2	I-2	69	F
2	II-1	42	M
2	III-2	15	F
3	I-1	46	M
3	II-1	15	F
3	II-2	15	F
4	II-1	9	F
5	II-7	12	M
6	II-1	13	M
7	II-1	19	F
8	II-3	9	F
9	II-1	14	M

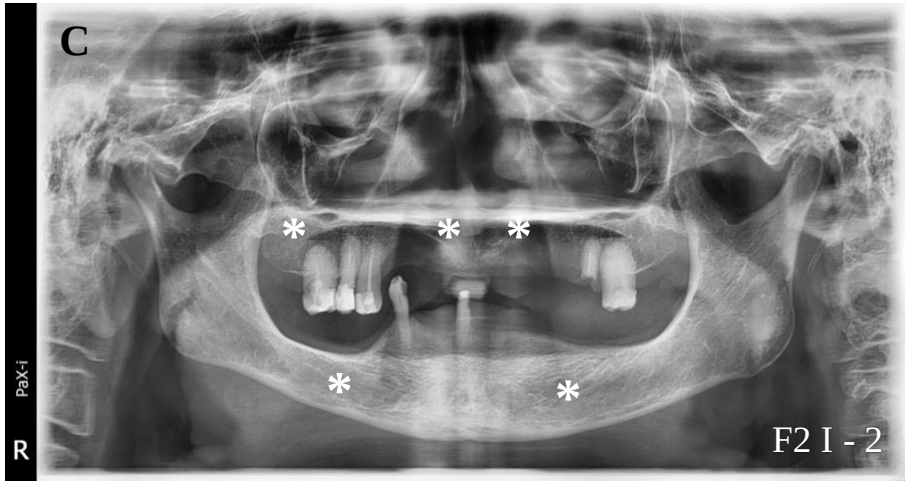
2. Clinical findings of families with non-syndromic TA

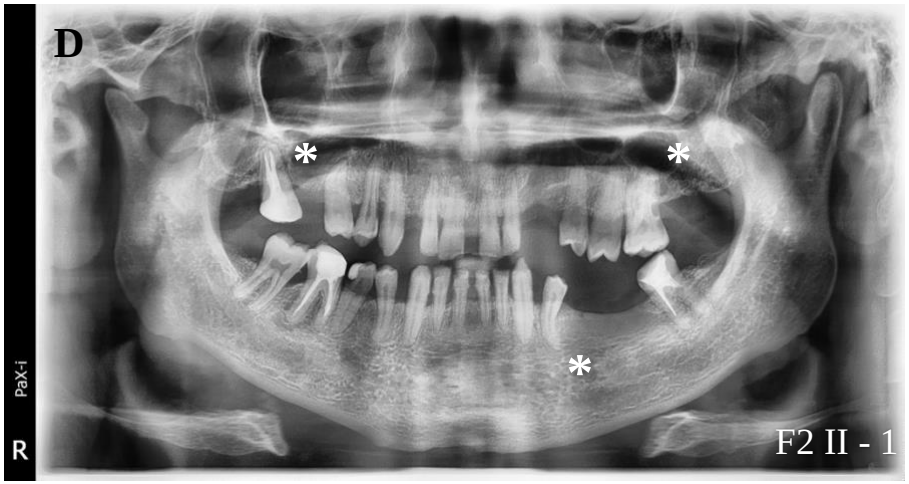
Family 1 consisted of a 30-year-old mother and a 12-year-old son as participants, with the mother (F1 II-2) missing nine permanent teeth (Figure 3 A) based on clinical and panoramic radiographs. The middle son (F1 III-2) was also found to be missing eight permanent teeth (Figure 3 B). Family 2 included a 69-year-old grandmother, a 42-year-old son, and a 15-year-old granddaughter as participants, where the grandmother (F2 I-2) had a total of five missing permanent teeth (Figure 3 C), her son (F2 II-1) was missing three permanent teeth (Figure 3 D), and her granddaughter (F2 III-2) was missing a significant number of teeth, specifically twenty-six permanent teeth (Figure 3 E). In Family 3, the participants were a 46-year-old father and his 15-year-old dizygotic twin daughters. The father (F3 I-1) was missing two permanent teeth (Figure 3 F), and one twin daughter (F3 II-1) missing six permanent teeth (Figure 3 G) while the other twin (F3 II-2) was missing four permanent teeth (Figure 3 H).

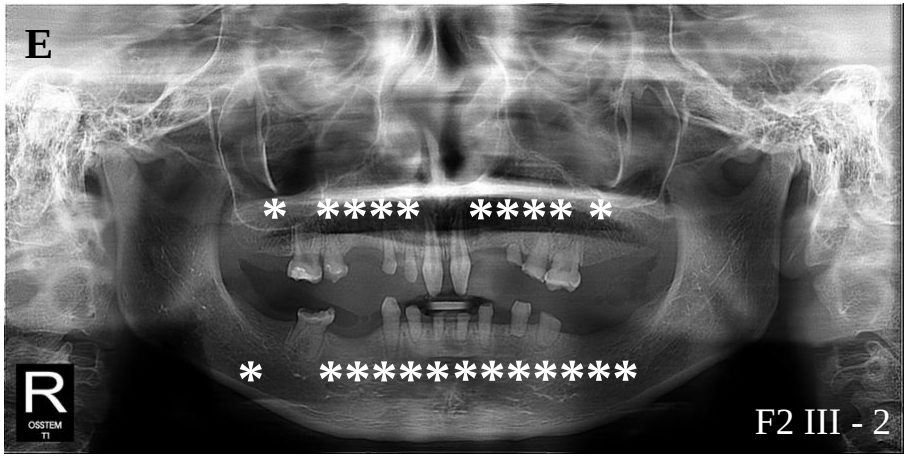
The remaining six families did not exhibit inherited patterns, and none of the family members of the participants showed any signs of TA. Family 4 included an 8-year-old girl (F4 II-1) was found to be missing seven permanent teeth (Figure 3 I), while in Family 5, an 11-year-old male (F5 II-7) was missing six permanent teeth (Figure 3 J). Family 6 had a 12-year-old boy (F6 II-1) who was missing twelve permanent teeth (Figure 3 K) and in Family 7, an 18-year-old girl (F7 II-1) was missing seven permanent teeth (Figure 3 L). Family 8 had an 8-year-old girl (F8 II-3) missing six permanent teeth (Figure 3 M); in Family 9, a 13-year-old boy (F9 II-1) was diagnosed with congenital agenesis and was missing a total of eleven permanent teeth (Figure 3 N). The participants who exhibit missing teeth, as indicated in the chart, are listed in Table 4.

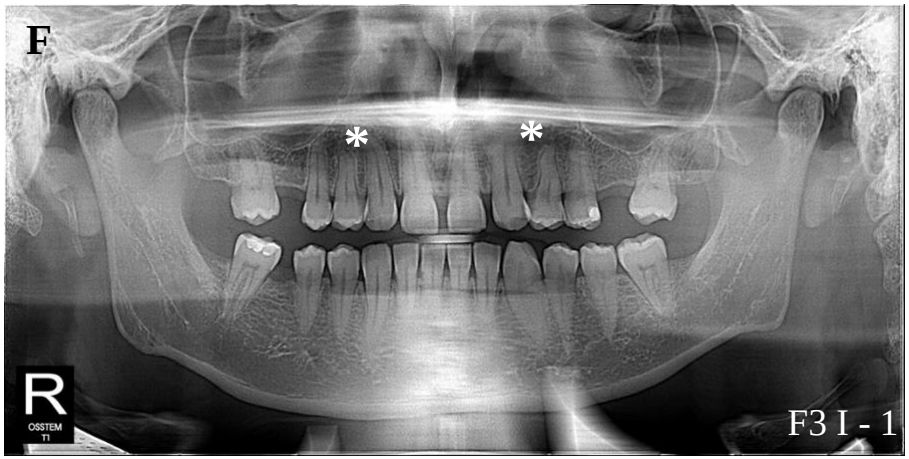


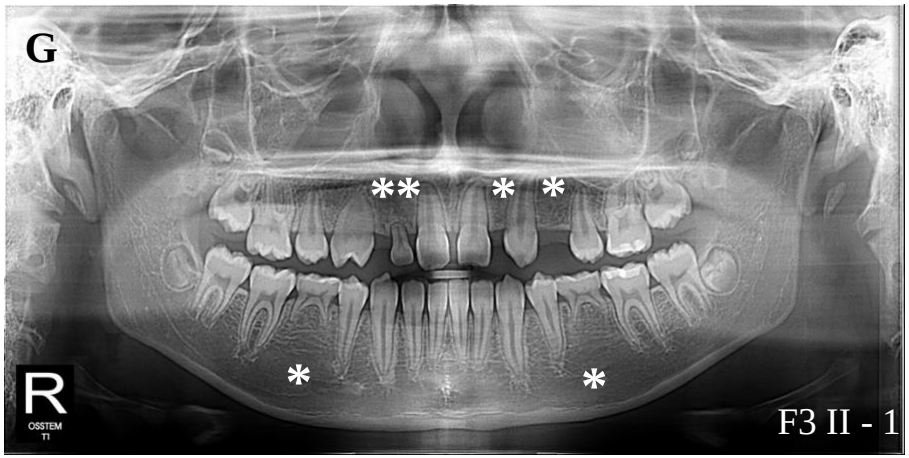


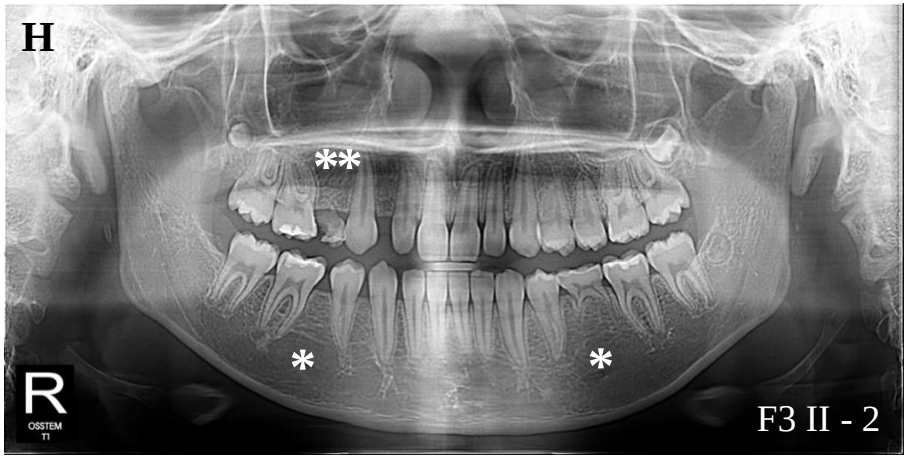


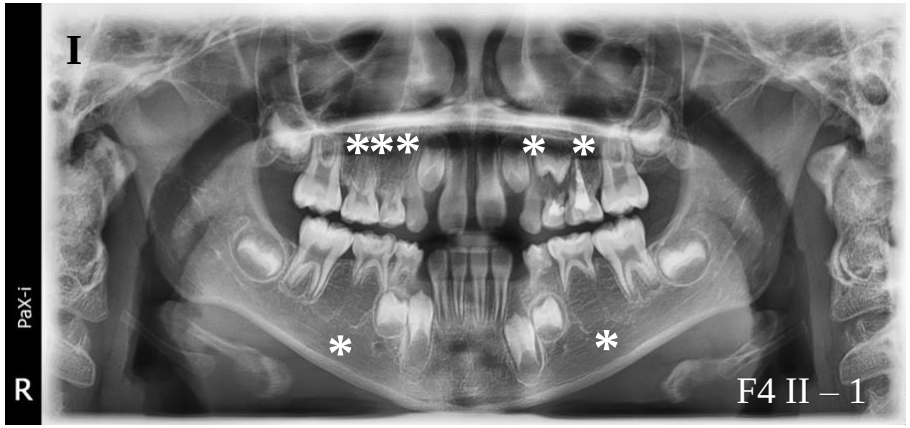


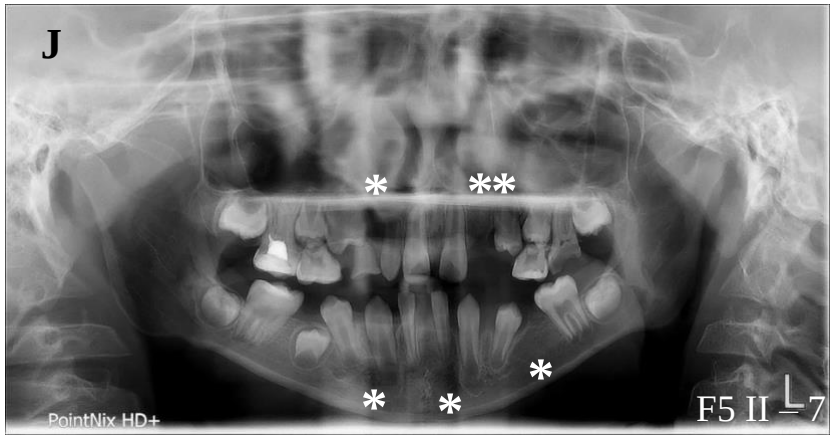


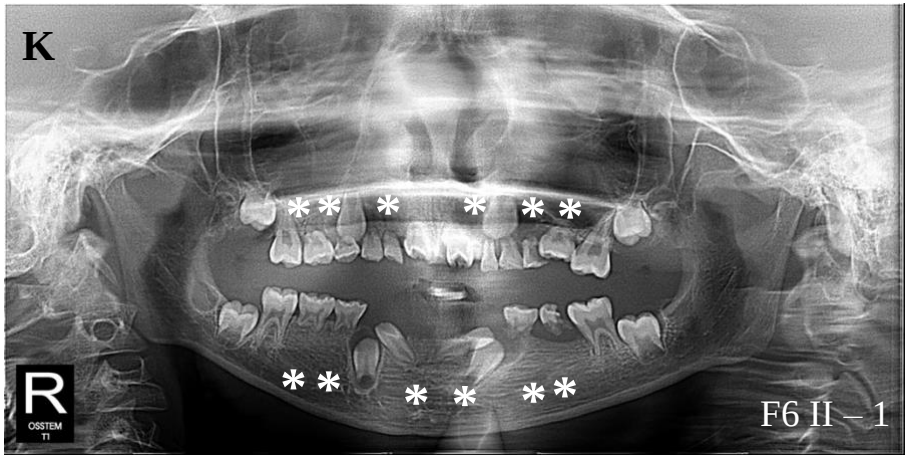


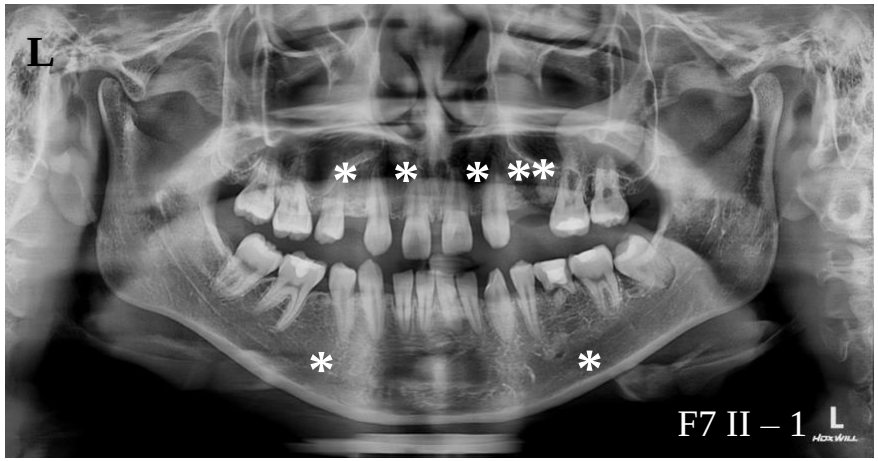












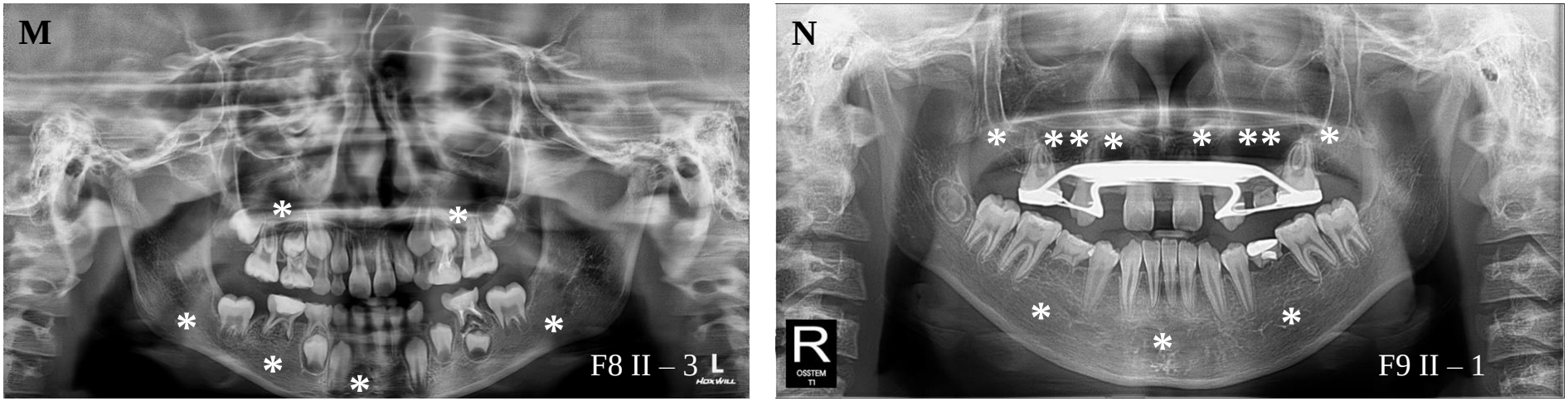


Figure 3. (**A** to **N**) Panoramic radiographs of 14 participants organized by their respective families illustrate the clinical findings of TA.

3. Candidate variants identified in WES analysis

WES was performed on all 14 participants who provided their consent. Total of five variants that met all the filtering criteria were identified through segregation analysis. Participants from inherited families with non-syndromic TA demonstrated autosomal dominant inheritance patterns, while those non-inherited families exhibited autosomal recessive inheritance (Table 4, 5). Among the Asian population, two rare missense variants were found: rs3850625 in CASNA1S with an Asian MAF of 0.0419 and rs12274923 in CDON with an Asian MAF of 0.0629. Additionally, a disruptive in-frame insertion in MAST4 (rs201910335) with an Asian MAF of 0.014 was also identified (Table 6).

Table 4. Phenotype (missing teeth) and genotypes of 5 variants from segregation analysis in 14 participants; minor risk allele in segregation analysis.

Family	Participants	Sex	TA Phenotype	Number of missing teeth	MAST4-C	ITGA-G	PITX2-G	CACNA1S-G	CDON-G
1	II-2	Female	Affected	9	**C/CGCT**	**G/GA**	**G/A**	G/G	**G/A**
1	III-2	Male	Affected	8	**C/CGCT**	**G/GA**	**G/A**	G/G	G/G
2	I-2	Female	Affected	5	C/C	G/G	**G/A**	G/G	G/G
2	II-1	Male	Affected	3	**C/CGCT**	G/G	**G/A**	G/G	**G/A**
2	III-2	Female	Affected	26	**C/CGCT**	G/G	**G/A**	G/G	**G/A**
3	I-1	Male	Affected	2	C/C	G/G	G/G	**G/A**	G/G
3	II-1	Female	Affected	6	**C/CGCT**	G/G	**G/A**	**G/A**	G/G
3	II-2	Female	Affected	4	**C/CGCT**	G/G	**G/A**	**G/A**	G/G
4	II-1	Female	Affected	7	C/C	G/G	**A/A**	G/G	**G/A**
5	II-7	Male	Affected	6	C/C	G/G	G/G	G/G	G/G
6	II-1	Male	Affected	12	C/C	G/G	G/G	G/G	G/G
7	II-1	Female	Affected	7	C/C	G/G	**G/A**	G/G	G/G
8	II-3	Female	Affected	6	C/C	G/G	**A/A**	G/G	**A/A**
9	II-1	Male	Affected	11	C/C	G/G	G/G	G/G	**G/A**

Table 5. A record of filtered variants. Gene, gene name; ID, variant id in SNP; CHR, chromosome; POS, base pair position; REF, reference allele; ALT, alternative allele.

Gene	ID	CHR	POS	REF	ALT	Transcript ID	Protein change	Impact
MAST4	rs201910335	5	65892764	C	CGCT	ENST00000404260	Leu95dup	MODERATE
ITGA6	-	2	173337540	G	GA	ENST00000409532	Asp114fs	HIGH
PITX2	rs2278782	4	111542154	G	A	ENST00000557119	Gln193*	HIGH
CACNA1S	rs3850625	1	201016296	G	A	ENST00000367338	Arg1520Cys	MODERATE
CDON	rs12274923	11	125871715	G	A	ENST00000531738	Ala63Val	MODERATE

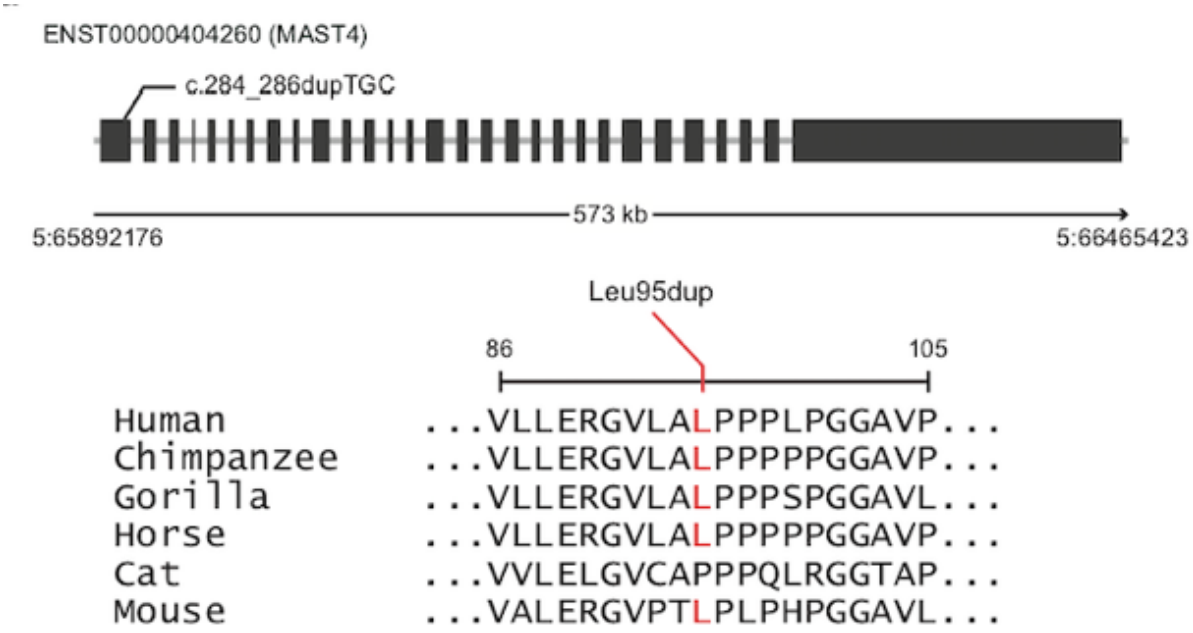
4. In silico mutation impact analysis of the variants

Two different computational tools were applied to analyze the impact of mutations in MAST4, CACNA1S, and CDON with filtered variants: SIFT [54] and PolyPhen2 [55]. For the two missense variants, canonical Ensembl sequences were analyzed (Table 6). The SIFT predicted a damaging effect on a missense variant in CACNA1S, while PolyPhen2 did not. In contrast, a variant in CDON was predicted to be possibly damaging by PolyPhen2 and had both damaging and tolerant prediction by SIFT. The variants were found to be located in evolutionarily conserved positions and might be under intense selective pressure. For instance, a missense variant in CACNA1S (located in exon 7, and arginine at the amino acid position 1520) and a variant in CDON (located in exon 10, and alanine at the amino acid position 63) were well-conserved in mammal orthologs (Figure 4 B, C). In the case of MAST4, the mutation has occurred at the site where sequences are well-conserved across mammal orthologs (Figure 4 A). To assess the gene tolerance to mutations concerning TA, Loss-of-Function observed/expected upper bound fraction (LOEUF) scores were measured. A lower LOEUF score indicates that a gene is more susceptible to damage by mutations and may have a more severe impact on affected individuals [56]. The findings revealed that MAST4 had a relatively low LOEUF score of 0.38 and that other genes also had LOEUF scores below 1(CACNA1S had a LOEUF score of 0.56 and CDON had a LOEUF score of 0.96), suggesting that the gene functions might be damaged by the variants (Table 6).

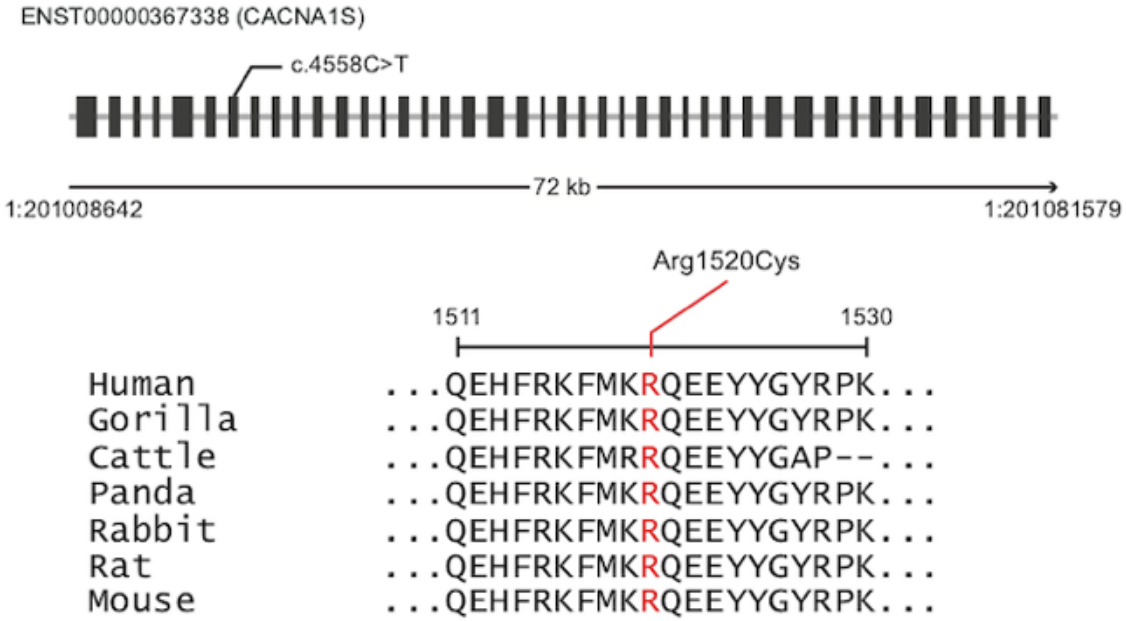
Table 6. Analysis of mutation impact on candidate genes; results of PolyPhen2 (B: benign, P: possibly damage) and SIFT (D: damage, T: tolerate). Effect; defining mutations; LOEUF, indicates the inverse score of loss-of-function intolerance; ASN MAF, alternative allele frequency in 1000Gp1 Asian descendent samples; MAF, alternative allele frequency in whole 1000Gp1 data.

Gene	UniProt ID	Effect	PolyPhen2	SIFT	LOEUF	MAF	ASN MAF
MAST4	O15021	disruptive_inframe_insertion	-	-	0.38	0.005814	0.01434
CACNA1S	Q13698	missense_variant	B	D, D	0.56	0.070971	0.041958
CDON	Q4KMG0	missense_variant	P, P, P	T, D, T	0.96	0.125916	0.062937

A



B



C

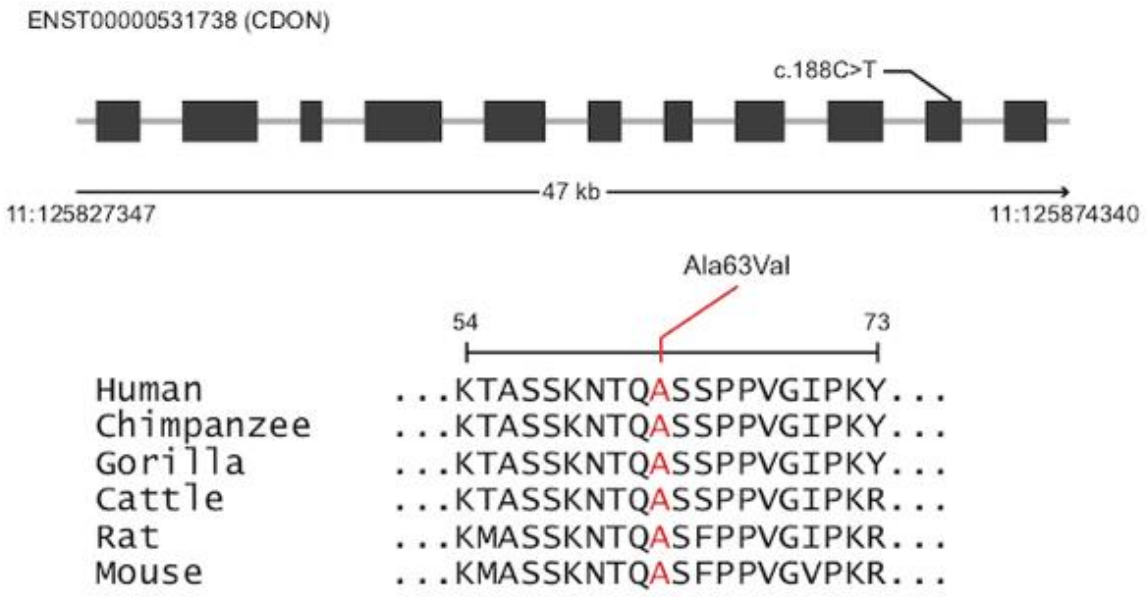
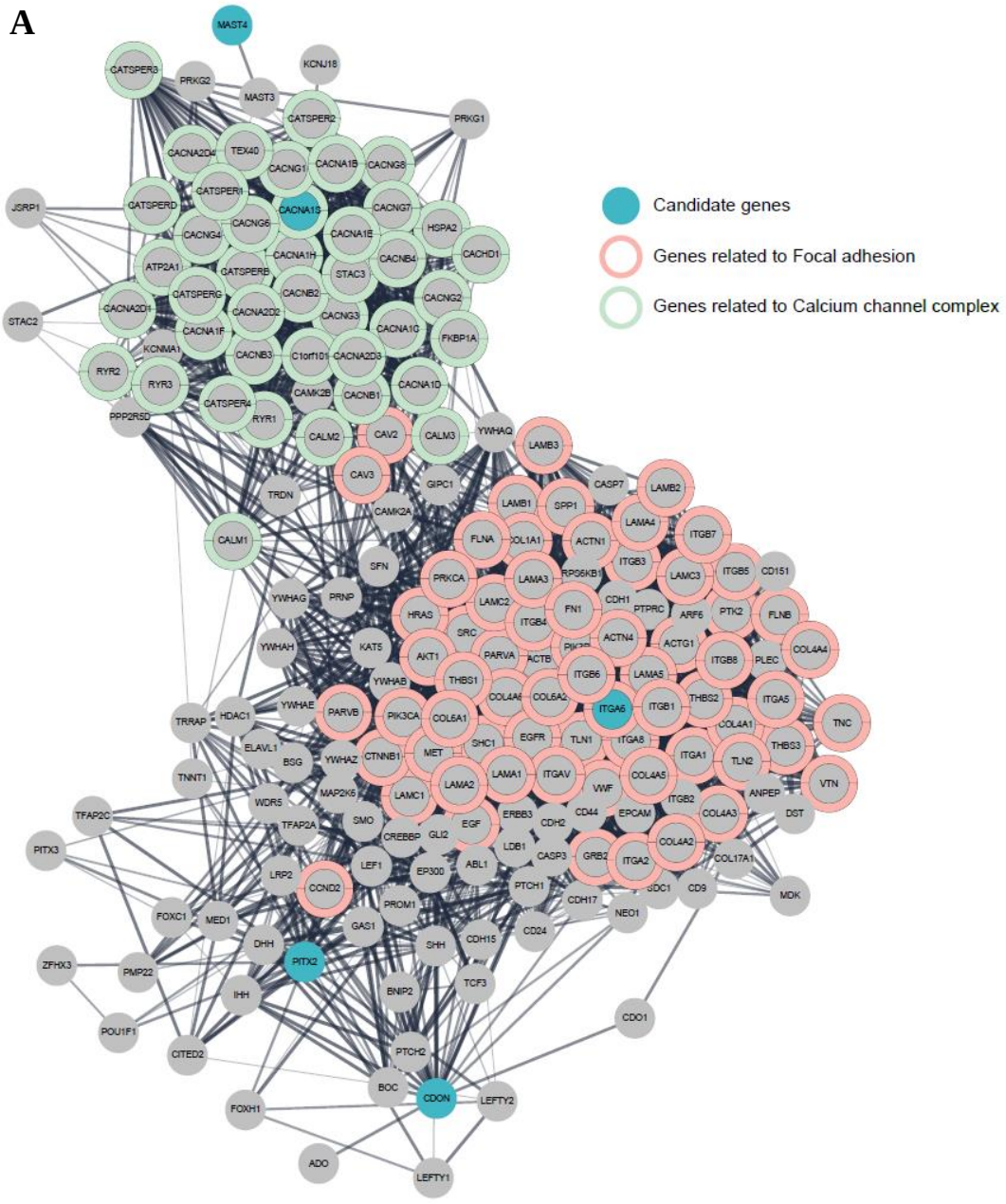


Figure 4. Mutation impact analysis of MAST4, CACNA1S, and CDON. (**A**) The amino acid sequence alignment of MAST4 in various mammalian orthologs revealed a mutated residue of Leu95dup, which is highlighted in red. (**B**) The amino acid sequence alignment of CACNA1S in various mammalian orthologs revealed a mutated residue of Arg1520Cys, which is highlighted in red. (**C**) The amino acid sequence alignment of CDON in various mammalian orthologs revealed a mutated residue of Ala63Val, which is highlighted in red.

5. Gene set enrichment analysis

Next, to comprehend the functional contribution of candidate genes in non-syndromic TA, an investigation of gene set enrichment analysis (GSEA) was performed (Table 7). This analysis uncovered three noteworthy categories, namely Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), and Reactome pathway (REAC). For the GSEA, the list of genes with neighbors in the PPI network was expanded. Using the five candidate variant genes, function terms related to focal adhesion and calcium channel complex were highly ranked and formed functional gene clusters in the PPI network (Figure 5).



B

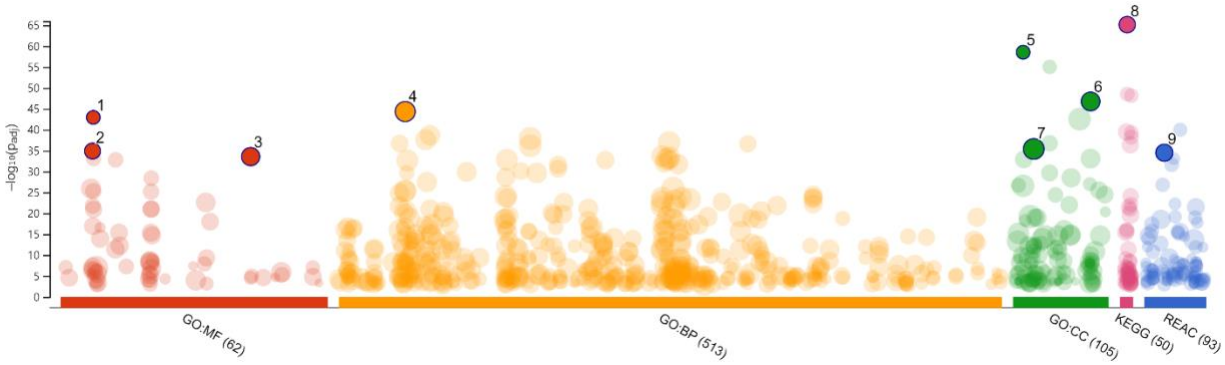


Figure 5. Gene set enrichment analysis result of candidate genes and its PPI network neighboring genes. (**A**) The PPI network of expanded gene set. Cyan-colored nodes are the five candidate genes. (**B**) Adjusted p-values of function terms as the result of GSEA. Highlighted dots are the high-ranked function terms related to the focal adhesion and calcium channel complex.

Table 7. List of highlighted function terms of the gene set enrichment analysis result plot.

	Source	Term	Term ID	Count	p-value
1	GO:MF	voltage-gated calcium channel activity	GO:0005245	28	1.10E-43
2	GO:MF	integrin binding	GO:0005178	35	1.23E-35
3	GO:MF	cell adhesion molecule binding	GO:0050839	52	2.66E-34
4	GO:BP	cell adhesion	GO:0007155	87	4.42E-45
5	GO:CC	voltage-gated calcium channel complex	GO:0005891	34	2.80E-59
6	GO:CC	plasma membrane protein complex	GO:0098797	66	1.69E-47
7	GO:CC	cell junction	GO:0030054	87	4.01E-36
8	KEGG	Focal adhesion	KEGG:04510	67	6.90E-66
9	REAC	Extracellular matrix organization	REAC: R-HSA-1474244	50	3.11E-35

IV. DISCUSSION

To the best of current knowledge, this is the first study to use a combination of WES and bioinformatic analysis to comprehensively investigate the genetic factors underlying functional mechanisms associated with TA in Mongolian families.

TA is a congenital disorder characterized by the absence of one or more teeth due to the failure to develop. The underlying mechanism of TA is complex and still needs to be fully understood [19]. In recent years, there has been a growing interest in utilizing WES and bioinformatic analysis to identify pathogenic variants in candidate genes associated with TA [36]. WES is a highly effective method for identifying genetic variants associated with genetic diseases, and genes involved in the developmental and differentiation of tooth germ cells have been discovered using this method [31].

Although new variants are still being discovered, the exact pathogenic mechanism underlying TA remains unclear. Previous studies on TA have primarily focused on genetic variants, with a limited investigation into the functional aspects such as protein function or molecular pathways. This study aimed to comprehend TA by investigating not only the genetic variants but also the underlying functional mechanisms involved in the process. To contribute to this understanding, a bioinformatic analysis was conducted. This encompassed comparing identified genetic variants with public databases and literature, as well as an assessment of the frequency of the variant in the general population (LOEUF analysis), a lower minor allele frequency in the Asian population (ASN MAF) and a prediction of the potential impact of the variant on protein function (SIFT and PolyPhen analyses).

In this study, impact variants in MAST4 were identified. A disruptive in-frame insertion in MAST4 with an Asian MAF of 0.014 indicates that this particular variant is rare with Asian population. The rarity of this insertion in the population underscores its potential significance, warranting further investigation into its functional implications and potential associations with

relevant phenotypic traits or diseases. Understanding the prevalence and impact of such variants contributes valuable insights to knowledge of genetic diversity and susceptibility within distinct populations. The result of variant impact analysis in the general population revealed that MAST4 showed a lower LOEUF score of 0.38 (Table 6) compared to CACNA1S and CDON, suggesting it could be related to the tooth damage and have a more severe impact on affected individuals.

Indeed, a disruptive variant (rs201910335) in the MAST4 gene was identified in 6 individuals from three inherited families with TA (Table 4). The MAST4 gene was first characterized in 2006 through bioinformatics analysis [57]. Studies have investigated its roles in crucial biological processes, including cell cycle progression, cell migration, and neuronal development [58]. The precise mechanism by which the MAST4 gene leads to TA is not fully understood up to this point. However, a recent study suggests that MAST4 is closely involved in amelogenesis process of mouse incisors and may serve as a critical regulator of Amelogenesis Imperfecta [59] by deactivating Wnt/β-catenin signaling pathway [60].

This result is particularly noteworthy and captures researchers attention because the Wnt signaling pathway is also a major pathway in TA [61, 33]. During tooth formation, Wnt signaling pathway is activated in the dental epithelium, which leads to the formation of a structure called the enamel knot. The enamel knot serves as a signaling center that helps direct the dental mesenchyme's growth and differentiation [33]. Mutations or alterations in the genes involved in Wnt signaling pathway, such as WNT10A, AXIN2, and LRP6, are known to be associated with TA. Mutations in WNT10A gene, which encodes a ligand for the canonical Wnt pathway, have been linked to TA [32]. The mutations in AXIN2, which is negative regulator of the gene canonical Wnt pathway, have been also been associated with TA, as well as other dental abnormalities [62]. In addition, variations in the LRP6 gene, which encodes a co-receptor for the canonical Wnt pathway, have been linked to TA [63].

These findings highlight the significance of Wnt and Wnt-associated signaling pathways in the genetic etiology of TA and may facilitate the identification of novel genes associated with this condition. The results suggest that the MAST4 gene's relationship with the Wnt pathway may significantly contribute to the development of TA. However, further research is necessary to comprehensively elucidate the underlying molecular mechanism through which the MAST4 and related pathways influence tooth development and contribute to the pathogenesis of TA.

A stop-gained mutation in PITX2 gene (rs2278782) was identified in 8 participants from both inherited and non-inherited families (Table 4). This finding is consistent with previous research linking PITX2 mutation to non-syndromic TA and dental anomalies [39, 64]. PITX2 is a transcription factor for proper tooth development by activating target genes through the Wnt signaling pathway, involving β-catenin [65]. Mutation on the PITX2 gene can disrupt the proper secretion of Wnt4, Wnt6, and Wnt10 from dental epithelium, resulting in a dysfunctional enamel knot leading to arrest of tooth development due to the absence of Wnt/β-catenin activity [66-68].

Variants in CDON and ITGA6 genes were also found, which are co-receptors for the SHH signaling pathway crucial for tooth development [69, 70]. The SHH signaling affects cell polarization in the early tooth bud, determining the number of teeth in the permanent dentition. It also interacts with other pathways, including Wnt, to ensure proper tooth development [71]. Therefore, these findings indicate a connection between the Wnt signaling pathway and TA.

Gene set enrichment analysis revealed the functional terms of five candidate genes associated with focal adhesion and calcium channel complex to be highly important, with two gene clusters linked by the candidate genes and their neighboring genes in the PPI network. Focal adhesions are specialized structures that allow cells to interact with extracellular matrix (ECM) through multiprotein complexes, mediating cell adhesion, migration, and signaling processes [72, 73] necessary for the formation of the tooth germ, enamel, dentin, and periodontal ligament [74-76]. Inhibiting focal adhesion kinase (FAK) can hinder the bud-to-

cap morphogenesis process, potentially leading to TA [77, 78]. Calcium signaling, regulated by voltage-gated channels (VDCC) [79] and important in tooth development [80], can be disrupted by mutations in calcium channel genes, such as CACNA1S, leading to dental malformations and abnormalities [81]. These findings indicate that disruptions in focal adhesion may affect tooth morphogenesis and differentiation, which may lead to TA. Furthermore, the significance of calcium signaling in tooth development suggests that calcium channelopathies can contribute to dental abnormalities.

The selection of a Korean control group from Ansan-Ansung population in this study is rooted in several key considerations. Firstly, Mongolia's current lacks of a comprehensive genetic databank presents a challenge in conducting comparative genetic studies aimed at identifying variants associated with non-syndromic TA within the Mongolian population. Secondly, despite Mongolia's absence of a dedicated databank, previous research has highlighted shared genetic similarities among East Asian populations, including both Koreans and Mongolians. Studies have consistently indicated commonalities in genetic inheritance patterns across ethnic groups in East Asia [82]. However, further studies are needed to explore specific genetic variations within the Mongolian population itself.

In light of the limitation of this study, specifically the inclusion of participants with varying degrees of TA in the same analysis, it would be beneficial for future investigations to stratify participants into distinct subgroups based on the severity of TA. This approach would allow for a more refined analysis and deeper understanding of the specific mutation types and genetic factors associated with each form of TA. Furthermore, WES did not consider the unaffected family members due to specific reasons such as logistical constraints, ethical considerations, and financial limitations, which led to their inability to participate in this study. Additionally, the relatively small sample size in this study may have limited the statistical power to detect smaller effects or associations accurately. While researchers have made efforts to address these limitations through bioinformatic analysis and functional analysis, larger and more

homogeneous cohorts are needed to further validate and expand upon these findings. Nonetheless, despite these limitations, this study provides valuable insights into the genetic basis of TA and identifies potential candidate genes associated with this condition.

The study has identified novel candidate genes and variants that contribute to the occurrence of non-syndromic TA in Mongolian families. Furthermore, the results emphasize the significance of Wnt signaling pathway genes, as well as focal adhesion and calcium channel complexes, in regulating the process of tooth development. The identification of novel pathogenic genes associated with TA can enhance the understanding of the disease's molecular mechanisms. This could lead to improved diagnosis, prevention, and treatment strategies. Moreover, these findings may guide clinical practice, inform clinical gene diagnosis, and facilitate the development of targeted treatment options for individuals affected by TA. In clinical practice, early detection of TA through biomarkers significantly enhances dental management and facilitates orthodontic and prosthetic treatments.

V. CONCLUSION

In conclusion, novel candidate genes and genetic variants contributing to non-syndromic TA in Mongolian families have been identified. The findings emphasize the importance of the Wnt signaling pathway and its associations in the molecular development of non-syndromic TA. Additionally, the research provides evidence for the involvement of focal adhesion and calcium channel complexes in the regulation of the tooth development process.

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ABSTRACT (KOREAN)

몽골인들에게서 비증후군 (non-syndromic) 치아결손와 관련된 유전자 변이 분석

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셈지드 데지드너러브

치아의 결손을 뜻하는 TA (tooth agenesis)는 흔하게 발견되는 발달이상이다. TA와 연관된 다수 유전자들의 발견은 치아 발달에 대한 이해도를 높이는 데에 기여하고 있다. 그러나 원인이 되는 유전자 및 관련된 신호 전달 경로는 여전히 명확히 밝혀지지 않았다. 본 연구는 WES (whole-exome sequencing)와 생물정보학 분석을 이용해 아홉 몽골 가족들에게서 비증후군 (non-syndromic) TA와 관련된 유전자 변이를 찾는 것을 목표로 하였다. TA가 유전성을 띈 세 가족과 유전성을 띄지 않은 여섯 가족을 포함한 총 41명의 지원자가 연구에

포함되었다. 그 중 비증후군 TA 환자 14명의 타액 샘플에 대해 WES 분석을 수행했다. 후보 유전자는 변이 필터링과 분리 분석을 통해 선별되었다. 필터링 된 변이들은 in silico 돌연변이 영향 분석을 진행했다.

우리는 WES 분석을 통해 TA와 연관된 21개의 변이를 발견하였고, 이중 5개가 모든 필터링 기준을 충족하였다. 해당 변이는 MAST4, ITGA6, PITX2, CACNA1S, CDON 유전자의 엑손 (exon) 영역에 위치했다. PITX2의 변이는 가족의 TA 유전성에 상관없이 8명의 환자에게서 발견된 반면, MAST4의 변이는 유전성을 띈 가족의 환자 6명에게서 발견되었다. 본 연구는 여러 가족 그룹에서 TA와 관련된 다양한 유전자 변이 후보를 확인하였고, 그 중 PITX2의 변이가 가장 일반적으로 발견되었다.

우리 연구 결과는 Wnt 신호 전달 경로와의 연관되어 있는 MAST4를 TA 발병에 대한 새로운 후보 유전자로 제시한다. 또한, 우리는 5가지 후보 유전자가 치아 발달에 필수적인 기능인 초점 부착 (focal adhesion), 칼슘 채널 복합체 (calcium channel complex)와 두드러지게 관련되어 있음을 발견하였다.

TA와 관련된 새로운 병원성 유전자의 식별은 질병을 일으키는 분자 메커니즘에 대한 이해를 향상시켜 더 나은 진단, 예방 및 치료를 가능케한다. 바이오마커를 기반으로 한 TA의 조기 발견은 치아 관리를 개선하고 교정 및 보철 치료를 용이하게 할 수 있다.

핵심 되는 말: 치아 결손, 유전자 변이, 전장엑솜시퀀싱, 생물정보학 분석, 인실리코 돌연변이 분석, 몽골 집단