

A Research and Study Approach on PAX9 Gene Mutations Associated with Tooth Agenesis and Canine Tooth Impaction

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Abstract

Tooth agenesis is one of the most common congenital abnormalities in humans. It is a result of stopped tooth development and is genetically inherited. Paired box 9, (*PAX9*), is a gene that is responsible for congenital tooth agenesis and has been identified using knockout mouse models. Understanding the effects of *PAX9* gene and how it expressed in human teeth is important because it plays critical roles in fetal development, related to missing permanent molars and even cancer growth. *PAX9* mutations occur from the range of single nucleotide substitutions, by changing one amino acid, premature termination occurs and abolishes protein function to haploinsufficiency. A pattern of occurrence and malformations of the teeth needs to be identified for proper genotype-phenotype associations. This includes identifying the specific mutation and its loci present on the exon from the *PAX9* gene in order to be edited using methods like CRISPR to prevent future tooth agenesis. The proposed experiment is a small step in the scientific path to develop a mechanism for tooth regeneration.

The Phenotype

Tooth agenesis is a common congenital abnormality in humans, and human dentition is a complex process that requires many biochemical interactions between tissue components. The condition is seen to affect between 3% and 10% of the U.S. Population, with more appearances in women than men (Cleveland Clinic, 2022). Congenital conditions mean that one is born with the specific trait. Tooth agenesis is the medical term for missing teeth. It affects both primary teeth (often referred to as baby teeth) and permanent teeth (also known as adult teeth). Tooth agenesis most often affects permanent teeth. Since all baby teeth develop by the age of three, dentists should note where the absence is located. Usually, all permanent teeth are present between ages of 12-14 and dentists should be aware of inappropriate tooth development (NORD, 2019). Dental agenesis is found in individuals who lack certain teeth because they do not develop. Tooth agenesis is problematic in overall oral health because it makes the physical deformation of food, or chewing, harder. In extreme cases, speaking can also become more difficult with many teeth absent, as well as cause jaw deformation and inadequate bone growth (Cleveland Clinic, 2022). Treatments for these conditions can require dentures, dental implants, orthodontic care (such as braces), or dental bridges. The reason why I am interested in this topic is because I still have two baby molar teeth on my bottom dentures, and when they are gone, there will be no permanent teeth to replace them. This is seen to be a genetic problem, as my mother faced the same issue with her teeth around my age, which she needed implants for in her mid-30s.

Tooth, or dental, agenesis is found in three different types: anodontia, hypodontia and oligodontia. Each category is dependent on how many teeth an individual is missing. Anodontia is the complete absence of teeth, hypodontia is the absence of one to six teeth, and oligodontia is the absence of six or more teeth. These instances can occur in any location in the mouth, but the teeth that are most commonly absent are usually either the lower second premolars, the upper second premolars and the upper lateral incisors (Cleveland Clinic, 2022). The lower second premolars are the teeth that are in front of the molars on the bottom jaw. The upper second premolars are in front of the molars on the top. Upper lateral incisors are the small teeth that can be on either side of the top two front teeth. Non-syndromic hypodontia is the most common form of congenital tooth absence, mostly seen in the permanent dentition (Chhabra et al., 2013).

Aside from having missing teeth, some common symptoms include small, peg-shaped teeth, gaps and spaces between teeth, as well

as large, rectangular teeth that have abnormally large pulp chambers (Cleveland Clinic, 2022). Tooth agenesis can be associated with some genetic disorders like ectodermal dysplasia, where the skin, sweat glands, hair, nails, teeth and mucous membranes develop abnormally (Cleveland Clinic, 2022). A cleft lip or cleft palate is frequently seen in combination with this disease as well. Tooth agenesis is caused by the abnormality of a band of epithelial tissue which is present in a typically developing tooth. Tooth agenesis is genetically inherited and can be passed down from many generations, as there are no known prevention methods.

Impacted canine tooth surgery is dental procedure where the canine tooth doesn't erupt properly, causing it to erupt through the gum tissue (Jacobs, J.O., 2017). This process is like the orthodontic procedure I am currently going through. When a canine tooth is stuck and unable to erupt into intended position, this is when it is considered impacted. Canine teeth are critical strong biting teeth that help in chewing and masticating food. They are also responsible for leading teeth into proper bite alignment and aesthetically shaping a smile (Jacobs, J.O., 2017). Common reasons for canine teeth impaction can include insufficient space, alignment problems, genetics, and or early loss or removal of primary teeth (Jacobs, J.O., 2017). Canine teeth also have a narrow pathway in which to erupt to because of one of the following reasons: other front teeth are already fully established, canine teeth are usually larger than other teeth, or canine teeth have longer roots, which develop more slowly (Jacobs, J.O., 2017).

When looking at the types of canine tooth impaction, the maxillary canine teeth are most impacted, with impaction present on one side only. Another type is palatal impactions, where the tooth points inward towards the top of the mouth, and the outward part is either pointed toward the cheek or stuck in the roots of adjacent teeth (Jacobs, J.O., 2017). Impacted canine teeth are present in 2% of the population, and women experience the condition twice as often as men (Jacobs, J.O., 2017). The most common treatment for impacted canine teeth is exposure and bracketing, which consists of a surgical procedure and orthodontic care.

The Genotype

Genetic disorders, like that of tooth agenesis, is determined by the genes inherited from the father and the mother, one gene from each parent. Depending on the gene that is involved, genetic inheritance can follow different ways of inheriting the specific gene. People can inherit tooth agenesis in four ways: autosomal dominant, autosomal recessive, X-linked dominant and X-linked recessive (Cleveland Clinic, 2022). In most cases, the mutation is inherited as an autosomal dominant with incomplete penetrance (Fauzi et al., 2017).

Several different genes have been associated with hypo/oligodontia and anodontia which include *EDA*, *EDRA* and *EDARADD* genes (NORD, 2019). The genes *EDA*, *EDAR*, *EDARADD* and *WNT10B* are associated with tooth formation. Many other genes are also involved in hypo/oligodontia, which include *MSX1*, *PAX9*, *IRF6*, *GREM2*, *AXIN2*, *LRP6*, *SMOC2*, *LTPB3*, *PITX2*, and *WNT10B* (NORD, 2019). This study approach will focus on the gene *PAX9* (or Paired box 9) which plays important roles in the development of organogenesis and has been associated with missing permanent molars.

Paired box 9 is a common transcription factor involved in the development of human dentition. Mutations in the *PAX9* gene can cause changes in the number, position, or morphology of the teeth (Bonczek, 2017). Furthermore, these mutations can occur from a single nucleotide substitution: by changing one amino acid to premature termination and diminishing protein function to haploinsufficiency (Fauzi et al., 2017). There are 50 different mutations that are associated with various types of dental agenesis and inherited teeth defects located in both intron and exon regions (Bonczek, 2017). Most of these mutations are either characterized as missense mutations or insertions/deletions which lead to amino acid substitution in the paired domain region of the protein. In humans, *PAX9* is found on the long arm of chromosome 14 (14q13.3), containing 5 exons (Bonczek, 2017). Exon 1 is representative of the 5'-untranslated region and exons 2 to 5 consist of 1026 bases, including the stop codon, which encode 341 amino acids (Bonczek, 2017).

In my future experiment, exon 2, a 627-nucleotide sequence located on chromosome 14 of the *PAX9* gene will be used. The exon is almost entirely made up of untranslated regions, excluding the end of the sequence where the initiation codon and the first base of the second triplet are located (Bonczek, 2017). Exon 2 is a highly conserved area which contains paired-domain regions and have the most mutations out of all exons (Fauzi et al., 2017). Two mutations that affect the start of translation were found on the exon. These mutations are associated with haploinsufficiency of the *PAX9* gene. Haploinsufficiency is when one copy of a gene is inactivated or deleted, and the other copy that is functional is not able to return the gene to normal function. More specifically, the ribosome on the gene was unable to initiate translation of the mRNA that was affected (Bonczek, 2017). These mutations in the genotype result in non-syndromic oligodontia, holding similar patterns of missing teeth throughout family members. These individuals were observed to lack first or second premolars (baby teeth) and all permanent molars (Bonczek, 2017). In specific scenarios, one of the canines was missing or some teeth were described as "peg shaped" (Bonczek, 2017). Haploinsufficiency of the *PAX9* is a proven cause of tooth agenesis (Bonczek, 2017).

The Molecular Function of the Gene Products and a Mouse Model

Since the underlying problem of tooth agenesis is an absence of teeth, a potential scientific study could aim to find a therapeutic solution for tooth regeneration. Mouse models aim to clarify the genetic factors, as well as molecular and pathological mechanisms that underlie different conditions of tooth agenesis. *PAX9* is a gene that is responsible for congenital tooth agenesis and has been identified using knockout mouse models. Mice who lack *PAX9* show impaired development of organs, musculature, and the skeleton, including the absence or abnormal development of teeth. Developing a treatment method using cell-based tissue engineering is common in diseases that need regeneration. But a genetic abnormality like tooth agenesis can't follow the standard tissue engineering approach. This reason is because USAG-1 is inhibited in the earliest stages of tooth development. Investigations of a single-gene knockout in mice showed the loss of function of *USAG-1* (Murashima-Suginami et al., 2021). *USAG-1* is a bifunctional protein that counteracts two signaling molecules needed for tooth development, BMP and Wnt. There is a strong connection between *USAG-1* and the potential "cure" for congenital agenesis, but it is unknown if inhibiting *USAG-1* at a specific location will be enough to regenerate teeth.

USAG-1 mice with a 106-bp deletion in exon 1 were formed using CRISPR-Cas. A monoclonal anti-*USAG-1* antibody was therefore used in the study for the purpose of local arrest and recovery of a tooth developing (Murashima-Suginami et al., 2021).

To test rather or not the inhibition of *USAG-1* will cure congenital tooth agenesis, five mice were purified with the *USAG-1* monoclonal antibodies using a bioactive human *USAG-1* recombinant protein from *Escherichia coli* as an antigen and *USAG-1* mice (Murashima-Suginami et al., 2021). In addition, other tagged *USAG-1* recombinant proteins were taken from *E. coli*. The reactivity of each of the monoclonal antibodies with the *USAG-1* was tested through immunoprecipitation. All antibodies showed binding affinity between the mouse and human *USAG-1* recombinant proteins. Due to these results, researchers were able to further develop their research by asking if *USAG-1* relates to the BMP and Wnt signaling pathways when determining how many teeth are present. BMP signaling was found to be an essential factor in determining the number of teeth in mice (Murashima-Suginami et al., 2021). Furthermore, a neutralizing antibody was able to regenerate a tooth.

Another study looked at *PAX9* mouse models with a consistent cleft palate phenotype to test small-molecule Wnt agonist therapies (Jia et al., 2017). Absence of the *PAX9* gene was seen to alter the expression of Wnt pathway genes. Small-molecule Wnt agonists were injected into the veins of a pregnant *PAX9* mouse which restored Wnt signaling and led to the growth of palatal shelves, as well as cell proliferation and osteogenesis (Jia et al., 2017). This experiment was deemed successful and can be a possible therapy route for human cleft palates that result from a single-gene disorder.

There are 66 *PAX9* variations that are associated with non-syndromic tooth agenesis - most are missense and frameshift variants (Liu et al., 2022). Nucleotide substitution is a common example of a missense mutation. Many frameshift mutations occur in exon 2 and 4 which involve insertion and deletion mutations. The deletion of 8 nucleotides and insertion of 288 foreign nucleotides within exon 2 led to a disrupting event of the C-terminal DNA binding domain of the *PAX9* paired domain region (Fauzi et al., 2017). In addition, an oligodontia patient that had missing molars, premolars and incisors showed insertion of a single guanine nucleotide inside the exon 2 of the *PAX9* paired-domain region, which led to a frameshift mutation between the N- and C-terminal of the DNA binding domains because of extension of several guanine series (Fauzi et al., 2017). Most of the *PAX9* frameshift, deletion and missense termination mutations cause hypodontia in both primary and permanent dentitions, whereas missense substitution mutations only affect the permanent dentition (Chhabra et al., 2013).

PAX9 inactivation using Wnt1-Cre mice causes cleft secondary palate and tooth agenesis and shows that the *PAX9* expressing mesenchymal cells of the nose, palate, and teeth come from the neural crest cells (Kist et al., 2007). In this specific study, the *PAX9* allele is useful in studying the *PAX9* function in specific tissues of adult mice. Mice that are deficient in *PAX9* die shortly after birth.

Experiment For the Future

The locus of the mutation in *PAX9* gene causing the tooth agenesis can potentially be edited by scientists using methods like CRISPR to prevent future tooth agenesis. Further efforts in detecting the gene loci that contributes to dental agenesis should be made. In addition, molecular genetic techniques can help scientists identify the genetic factors that cause tooth agenesis as well as the mechanisms that can lead to this condition. In addition, the genetic and pathogenetic mechanisms involved in syndromic and non-syndromic hypodontia to further understand tooth agenesis. A pattern of occurrence and malformations of the teeth need to be identified. Thus, gene mapping is a useful technique for families who are at risk of developing different forms of tooth agenesis, such as hypodontia, oligodontia, or anodontia. Using this, scientists can show the connections between a specific gene mutation and a form of tooth agenesis. The goal of this study is to analyze the genotypic-phenotypic relationships in the *PAX9* gene as well as identify the different variants that are related to non-syndromic tooth agenesis. This study will aim to reveal that *PAX9* haploinsufficiency or a loss of function of the *PAX9* gene will lead to tooth agenesis in patients.

First, the study will need a series of tooth agenesis patients, being diagnosed with non-syndromic hypodontia. These diagnoses will require clinical examination by a dental professional and panoramic or periapical radiographs. This information can identify the location of the missing teeth, as well as the name of the tooth itself. Clinical consent and interviews will be required for the study, especially to ask the individuals about their family history regarding tooth agenesis. This way, each patient will have a pedigree dating back, if possible, three to four generations. The experiment then can be categorized as genetically inherited through autosomal dominant, autosomal recessive, X-linked dominant or X-linked recessive means. The teeth of the patient and their family members should be measured and compared to those of healthy patients in the same age category. It should be indicated whether the teeth are primary or permanent teeth. X-Ray pictures of the affected areas in the dentures of the patients could also be useful for the experiment. Medical history should be obtained to identify any health conditions associated with hair, nails or sweat glands, as they can be related to tooth agenesis.

The genomic DNA of all the participants can be extracted from a peripheral blood sample using a BioTek DNA Whole-blood Mini Kit following the manufacturer's instructions (Liu et al., 2022). The entire exome should be sequenced to search for pathogenic variants. Afterwards, DNA isolation will be performed using a DNA purification technique. For sequencing, exon 2 of *PAX9* will be amplified by a polymerase chain reaction (PCR). Specific forward, reverse and semi-nested forward primers should be designed from exon 2 of the gene. Information on the PCR conditions include having annealing temperatures that range from 55 degrees Celsius to 64 degrees Celsius with 32-34 cycles

for each forward-reverse primer. PCR products can be purified by 2% gel electrophoresis and Nucleo-Spin Extract Kits (Liu et al., 2022). Once the strands of the PCR products are sequenced, they can be analyzed with a DNA sequencer and compared to specific entries on official websites such as NCBI or OMIM.org. Restriction-enzyme analysis is also necessary, using the forward and reverse primers for the 5' terminal amplicon of exon 2 (Liu et al., 2022). This will help with amplifying the region of the mutation. Once the fragment is amplified, the products, which are digestion products, can be run through a 2% gel electrophoresis. Sequencing of the gene reveals information on the specific nucleotide transition location of the affected individuals. In other words, the information which will be obtained is how the form of tooth agenesis is genetically inherited, as well as how the specific location of the mutation relates to the phenotype of the patient. The mutation can be identified as a missense, frameshift, or silent mutation based on its location.

TOPO TA clone sequencing can also be used to confirm the status of possible frameshift variants (Liu et al., 2022). This provides a highly efficient cloning reaction for the insertion of Taq polymerase-amplified PCR products in a plasmid vector for sequencing. The full-length coding sequence of the wild-type *PAX9* should be subcloned into the expression vector using enhanced green fluorescent protein to synthesize the plasmid. After the construction of plasmid, cell culture and transfection can be next (Liu et al., 2022). Transfection is a method that is used to insert foreign nucleic acids into eukaryotic cells. Modifying the host cell's genetic components can help in studying gene function, regulation and protein function. The proteins should be harvested after transfection and used in a Western blot analysis (Liu et al., 2022). Western blot testing can determine if the variant proteins affected the expression in the genome. In summary, western blot procedure relies on three key steps. First, the separation of proteins due to size in a gel electrophoresis. After the transfer of the separated proteins, the identification of the target proteins can be identified based on the matched antibodies. Polyvinylidene difluoride membranes is a popular technique that is used for the detection and characterization of proteins. If this is combined with immunodetection such as using mouse antibodies, the behavior of the *PAX9* gene can be identified. This information can tell scientists the specific variants of the *PAX9* gene, as well as through gene sequencing data. After transfection is complete, reverse transcription can be done using the DNA obtained from the beginning. Cell lysates can also be collected from transfection, from the purification of the cellular DNA (Liu et al., 2022).

To further test the harmful variants/mutations identified in the *PAX9* gene, the use of bioinformatics is very important. In addition, examining the mRNA expression level of the *PAX9* variants (Liu et al., 2022). Protein variants also greatly affect the stability of the mRNA. The location of the variants can be identified and if they are conserved areas of the gene. A mutation tester can identify if the gene is causing the abnormalities. In addition, a pattern in the non-syndromic patients of where their specific tooth loss is in their dentures, their specific variant of the *PAX9* gene, and variant domains can be noted. Paired domain is a conserved function domain, and the protein *PAX9* is seen to regulate downstream of DNA through paired domain, which affects tooth development (Liu et al., 2022).

Once the specific mutation of the individuals is identified, the experiment can be advanced to a mouse model. The specific actions should be done to pregnant female mice, as the offspring will be examined after birth. The specific mutation, whether it be a deletion, insertion, or substitution, in exon 2 of the *PAX9* gene can be obtained from the human experiments can be inserted into USAG-1 pregnant mice using the CRISPR-Cas system. This technique allows scientists to edit the parts of the genome by altering the sections of the DNA sequence in mice. Cas9 binds to the target DNA and cuts it, shutting the targeted gene off. Knocking out genes in these lab mice will allow scientists to observe the traits and behaviors that are affected. This procedure is a preparation to obtain a PA-tagged mouse USAG-1 recombinant protein from human cells (Murashima-Suginami et al., 2021). The USAG-1 neutralizing antibody needs to be administered in the pregnant mice. Any supernumerary teeth should also be noted. From here, the use of a monoclonal anti-USAG can attempt to recover tooth development in mice. The USAG-1 mice can be immunized with human USAG-1 protein. The lymphocytes retrieved from the lymph nodes can be fused with myeloma cells in the presence of a 50% polyethylene glycol solution

and left to grow in a GIT medium containing HAT (Murashima-Suginami et al., 2021). Hybridomas will be a result of this, and scientists will need to identify the ones that secrete the anti-USAG-1 monoclonal antibodies. The culture will be loaded into a column and the bound antibody will be eluted by the elution buffer (Murashima-Suginami et al., 2021). As a result of the offspring's humoral passive immunity that is naturally acquired, the antibodies are passed from mother to fetus via placenta. The mother will pass the antibodies to her offspring, which will eventually obtain its own full immune system. The teeth of the offspring of the pregnant female mice should be investigated thoroughly at about 5 weeks as tooth development and organogenesis occur. The heads of the offspring will need to be dissected, cleaned, and preserved before the examination. Results can determine if USAG-1 can help rescue congenital tooth agenesis during early tooth development and return reformation of the tooth structure.

Conclusion

Tooth agenesis can affect daily life by leading to improper mastication and aesthetic problems, which can cause expensive dental bills. Embryonic tooth development requires complex signaling cascades and many expressions of several genes. Any alteration in such processes or improper development can lead to tooth agenesis. Several number of mutations in the *PAX9* gene are expressed in tooth agenesis, leading to hypodontia or oligodontia. *PAX9* gene mutations show how conditions can range from single nucleotide substitutions to changes in amino acid and premature termination to haploinsufficiency. More studies regarding how many mutations in development are responsible for causing improper tooth development are needed. Understanding the genetic mutations that cause tooth agenesis is important for geneticists, genetic counseling of family members, dental patients, and dentists because it can prevent future tooth agenesis diagnoses.

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