WNT signaling pathway genes expression profile in isolated hypodontia

YAMAN KASHOURA

PhD Thesis

Department of Orthodontics

Prof. Dr. ULAŞ ÖZ

2021-NICOSIA

TURKISH REPUBLIC OF NORTHERN CYPRUS
NEAR EAST UNIVERSITY
INSTITUTE OF GRADUATE STUDIES

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THESIS APPROVAL
Directorate of Institute of Health Sciences

This study was accepted by our jury as a doctoral thesis in the orthodontics program.

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AKNOWLEDGEMENT

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LIST OF ABBREVIATIONS

RNA……………………………………………………….…………… Ribonucleic acid

DNA…………………………………………………….. Deoxyribonucleic acid

mRNA………………………………………………… Messenger ribonucleic acid

tRNA………………………………………….…………. Transfer ribonucleic acid

rRNA……………………………………….…………. Ribosomal ribonucleic acid

cDNA ………………………………………………….. Complimentary DNA

A………………………………………………………....…………. Adenine

G………………………………………………….....………………….. Guanine

C………………………………………………….....……………………Cytosine

T………………………………………………….……....……………Theymine

PCR……………………………………………….… Polymerization chain reaction

RT- PCR…………………….……… Reverse Transcription- Polymerization chain reaction

qPCR ……………....…..… Real time-PCR or quantitative polymerization chain reaction

C_T………………………………..…………………….………… Threshold cycle

T_m………………………………………………….……Melting temperature

BMP …………………………………………………..... bone morphogenetic protein

WNT ………………………….…………………………... wingless-related integration site

SHH …………………………….……….…....……….…… Sonic hedgehog

FGF ……………………………………………….…… fibroblast growth factor

EDA………………………………………………..….……….. Ectodysplasin A

TGFβ …………………………………………...……... Transforming growth factor beta

ATP …………………………………..……………………… Adenine triphosphate

DSL ………………………………………………………………….. Dishevelled

LRP ……………………………………….………..… lipoprotein receptor-related proteins

PCP …………………………………….………...…………… Planar cell polarity

Dspp ⋯⋯⋯⋯⋯⋯⋯⋯⋯⋯⋯⋯⋯⋯⋯⋯⋯⋯⋯⋯ Dentin sialophosphoprotein

GAPDH ……………………………...… glyceraldehyde 3-phosphate dehydrogenase

GSK3……………………………….…………………Glycogen synthase kinase-3

APC………………………………………………...……Adenomatous polyposis coli

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İzole Hipodontide WNT Sinyal Yolu Genlerinin Ekspresyon Profili

Öğrencinin Adı: Yaman KASHOURA

Danışman: Prof. Dr. Ulaş Öz

Bölüm: Ortodonti Ana Bilim Dalı

Özet

Amaç: Bu çalışmanın amacı, hipodontinin gelişimi sırasında PAX9, WNT10a ve AXIN2 genlerinin potansiyel rolünün ve ilişkisinin araştırılmasıdır.

Hazırlık/Örnekleme: Her iki cinsiyetten toplam 40 katılımcı iki eşit olarak gruba ayrılmıştır. Çalışma grubu, izole hipodontili sağlıklı akraba olmayan bireylerden ve kontrol grubu ise tam dentisyonu olan sağlıklı bireylerden oluşmuştur. Çalışma grubunun yaş ortalaması 17 $\pm$7 , kontrol grubunun ise 16.5 $\pm$6 'dır.

Gereç ve Yöntemler: RNA, tükürük örneklerinden izole edilmiş ve cDNA buna göre sentezlenmiştir. PAX9, WNT10a, AXIN2 ve GAPDH primerleri ana karışıma eklenmiş ve QPCR gerçekleştirilmiştir. Hedef genlerimizin ekspresyonu referans gene normalize edildiğinden gen ekspresyonu $2^{-\Delta\Delta Ct}$ yöntemine göre hesaplanmıştır.

Bulgular: Sonuçlarımıza göre PAX9'un (p=0.018) çalışma grubunda anlamlı olarak daha düşük ifade edildiğini göstermiştir. WNT10a, çalışma grubunda (p=0.005) anlamlı şekilde yüksek regülasyon gösterirken, AXIN2, gruplar arasında istatistiksel anlamlılık göstermemiştir (p=0.69).

Sonuç: Çalısmamızın bulguları, WNT10a'nın aşırı ekspresyonunun hipodonti gelişiminde olası bir rolünügöstermektedir.

Anahtar Kelimeler: Hipodonti, WNT10a, PAX9, AXIN2, MSX1, Gen ekspresyonu

WNT signaling pathway genes expression profile in isolated hypodontia

Name of the student: Yaman KASHOURA

Supervisors: Prof. Dr. Ulaş ÖZ

Department: Department of Orthodontics

Abstract

Objective: The aim of this study is to investigate the potential role and the association of *PAX9*, *WNT10a*, and *AXIN2* gene' expressions during the occurrence of hypodontia.

Setting/Sample Population: We recruited 20 healthy unrelated individuals with isolated hypodontia and another 20 healthy unrelated individuals in the control group consisted of with full dentition. The mean age for the study group was 17 ±7 and it was 16.5 ±6 for the control group.

Materials and Methods: RNA was isolated from saliva samples, and cDNA was synthesized accordingly. *PAX9*, *WNT10a*, *AXIN2*, or *GAPDH* primers were added to the mastermix and QPCR was performed. Gene expression was calculated according to the $2^\wedge\text{-}\Delta\Delta Ct$ method as the expression of our target genes was normalized to the reference gene.

Results: Our results showed a significant lower expression of *PAX9* in the study group (p=0.018). Whilst *WNT10a* was significantly up-regulated in the study group (p=0.005), *AXIN2* showed no statistical significance between groups (p=0.69).

Conclusion: Our results indicate the potential role of the overexpression of *WNT10a* in the development of hypodontia.

Keywords: Hypodontia, WNT10a, PAX9, AXIN2, MSX1, Gene expression.

1. Introduction

Assessing patients prior to orthodontic treatment planning is mandatory to determine the any congenital missing permanent teeth since they may lead to complications such as malocclusion, periodontal damage, deficient alveolar bone growth, difficulties in speech, and abnormal skeletal relationship, leading to a rather costly and challenging multidisciplinary treatment (Rakhshan, 2015). Abnormalities in the size, structure, and number of teeth result from disturbances during the morpho-differentiation stage of development and these abnormalities cause complications in treatment planning (Altug-Atac and Erdem, 2007). Hypodontia is one of the most common heritable developmental anomalies in humans (Dhanrajani, 2002; Forgie et al., 2005; Thind et al., 2005). The etiology of tooth agenesis is multifactorial and it can be caused by environmental factors such as trauma, chemotherapy and radiation therapy, and in the majority of cases, it has a genetic basis (Nasman et al., 1997). Tooth agenesis can be isolated/non-syndromic or associated with developmental syndromes. It can also be classified according to the number of teeth missing as hypodontia represents the congenital absence of one to six teeth, whereas oligodontia represents the absence of more than six teeth, and anodontia describes the complete absence of the whole dentition (Rakhshan, 2015). However, the prevalence of hypodontia varies among different demographic and geographic populations, in terms of the type of dentition (primary or permanent dentition) as well as between genders (Brook, 2009; Flores-Mir, 2005; Bondemark and Tsiopa, 2007). The prevalence of hypodontia is between 0.03 to 10.1 percent in various populations (Muller et al.,1970). In primary dentition, the prevalence is between 0.5 and 0.9 percent, whilst oligodontia is rare, with an estimated prevalence of 0.25 percent (Thompson and Popovich, 1974; Salama and Abdel-Megid, 1994; Johannsdottir et al., 1997; Rolling and Poulsen, 2001). Whereas the prevalence of hypodontia in different racial groups had been reported as follows: 1.5-3 percent in Caucasians, 6-9.2 percent in Asians, and 7.7 percent in African-Americans (Graber, 1978 ; Lynham, 1990 ; Nordgarden et al., 2002 ; Cho et al., 2004 ; Larmour et al., 2005).

Tooth formation is a complex process which requires the activation of several cellular pathways in a specific temporal (time dependent) and spatial (location specific) pattern. Some of the key proteins involved in tooth development include fibroblast growth factor (FGF), sonic

hedgehog (SHH), transforming growth factor beta (TGFβ), bone morphogenetic protein (BMP), wingless-related integration site (WNT), and ectodysplasin A (EDA) (Kratochwil et al., 2002; Ockeloen et al., 2016).

The WNT signaling pathway, which was identified by Nusse and Varmus in 1982, is made up of specific proteins that are work together to regulate key cellular processes such as embryonic development, cell proliferation, differentiation and cell migration (Tamura and Nemoto, 2016). β-catenin levels are regulated by WNT signals, as when a cell receives WNT signal, β-catenin binds to the transcription factor of the TCF family, which regulates the expression of WNT target genes.

AXIN2 (also known as "Conductin" or "Axil") also has binding sites for β-catenin, GSK3B, APC, and disheveled. The expression of *AXIN2* is tissue- and stage-specific and it serves as a negative-feedback of the WNT signaling pathway.

PAX9 is a member of the paired box (PAX) family and it plays an important role in tooth development during embryogenesis. *PAX9* is expressed in the mesenchyme during early tooth development and is highly expressed at the initiation of the bud and cap stages of tooth formation (Matalova et al., 2008). Therefore, proper *PAX9* regulation is essential for normal tooth development.

The objective of this study was to investigate the association the between the gene expression of *PAX9*, *WNT10a*, and *AXIN2* in buccal cells contained in patient saliva and the occurrence of hypodontia.

2. General Information

2.1 Biology of cell

Human bodies are formed by cells that reproduce, grow, and are capable of information processing, response to stimulus, and carrying out a vast pattern of chemical reactions. Cells consist of small molecules such as amino acids, simple sugars, and ions (e.g., calcium). The concentration of these molecules is controlled by proteins inserted into the cellular membrane. Adenine triphosphate ATP is the most-known small molecule that forms the cell as they store energy that is required when cells split for instance. They also consist of small molecules known as monomers that can be joined to form larger molecules called polymers. There are three types of polymers that are produced in cells and are frequently known as macromolecules: proteins, polysaccharides, and amino acids (Lodish et al., 2008).

Generally, proteins may assist cell shape, structure and/or they may play a role in executing a cellular task(s). The complex three-dimensional structure and function of proteins are determined as a chain of amino acids is formed (Bairoch, 2000).

Proteins can serve as a structural component of the cell (structural proteins). They can also act as sensors that change the shape of cell when ion concentrations, temperature, or any changes in the properties of a cell. Proteins can be utilized as enzymes that accelerates the chemical reactions more rapidly in than they ought to do without the help of these protein catalysts. They also bind to individual genes, causing them to express or not (regulatory proteins) (Pickel et al., 2013).

Molecular genetics mechanisms

Deoxyribonucleic acid (DNA) carries information that is required to produce cells and the tissues of organism; this information is reserved in hereditary units called genes that assure the continuity from a generation to a generation. The process in which that information is transmitted known as transcription. However, the reserved information in DNA is duplicated into ribonucleic acid (RNA), which has three definite tasks in protein synthesis:

- Messenger RNA (mRNA) conducts the formation stored within the DNA in which the order of amino acids is specified in the course of protein synthesis.
- Transfer RNA (tRNA) carries the information which is interpreted from the mRNA by a process known as translation where a stepwise assembly of amino acid into protein occurs.
- Ribosomal RNA (rRNA) aids the process in which translation occurs.

When the correct amino acid sequence is brought by the tRNA, they are joined together via peptide bonds to form proteins (Alberts, 2008; Brenner et al., 1961).

The double-helix complex of DNA was suggested by Watson and Crick (1953). DNA consists of two polynucleotide strands that intertwine together and are joined by hydrogen bonds between nucleotide bases to make up the double-helix structure. Two sugar-phosphate backbones are located on the outermost of the helix while nucleotide bases are on the inside of the helix. However, the direction of the two strands is antiparallel, which means that the 5' to 3' direction between strands are opposite. (Figure 2.1) Moreover, Adenine (A) and Thymine (T) are paired together through two hydrogen bonds while Guanine (G) and Cytosine (C) are paired together by three hydrogen bonds, these nucleotide base-pairs are often called Watson-Crick pairs.
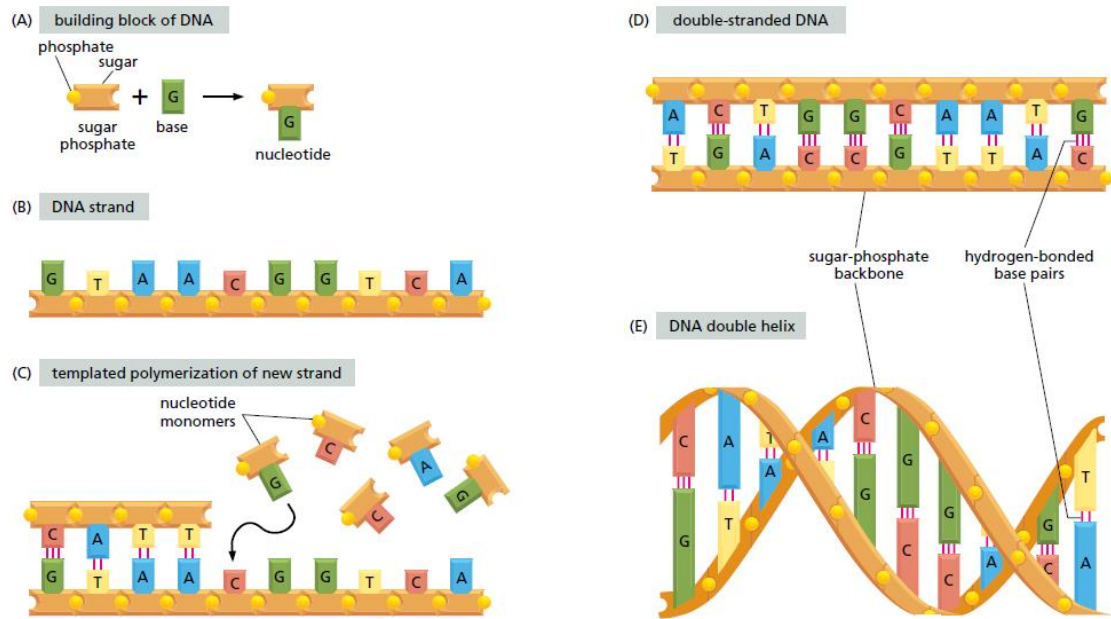
Figure 2.1. The DNA double-Helix (Alberts, 2008).

The way DNA guides RNA synthesis in which protein synthesis is directed was also discovered by Crick (1970) and it is known as central dogma. (Figure 2.2) However, this simplified representation did not consider the importance of proteins in nucleic acids synthesis. As discussed earlier, proteins are generally essential for the regulation of the expression of genes.
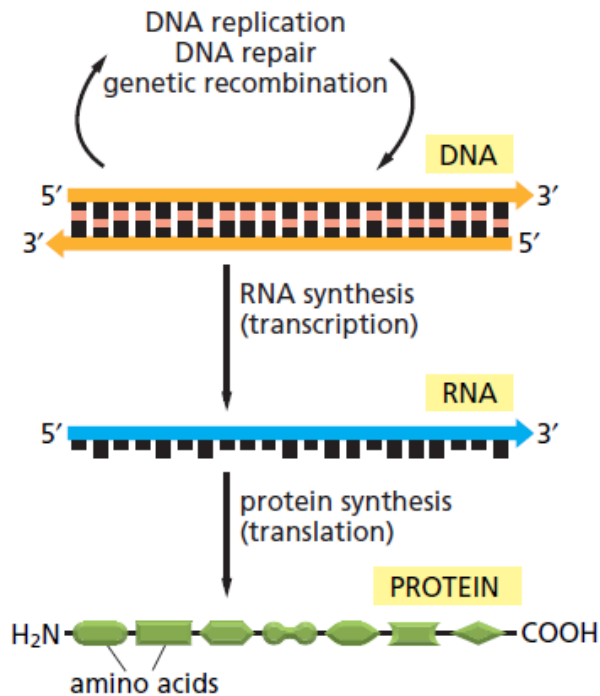
Figure 2.2. The pathway from DNA to protein (Alberts, 2008).

2.2 Polymerization chain reaction

The double-helix structure of the DNA was suggested by James D. Watson and Francis Crick (1953) which was a huge step in the molecular biology. More considerable developments were made in the beginning of 1960's in which synthetic oligonucleotides were adopted as a template for DNA polymerase and DNA replication using two primers was first introduced (Khorana et al., 1976).

Polymerization chain reaction (PCR) is an in vitro technique in which DNA sequence is replicated and amplified to billion-fold amplitude (Mullis and Faloona, 1987).

Essential components of standard PCR

1. Taq/other thermo stable polymerase:

In 1986, Henry Erlich announces the use of Taq polymerase in PCR since it could sustain its activity under high temperatures which shortened the PCR process by eliminating the need for manually adding DNA polymerase at every cycle of the reaction because of its inability to tolerate the rapid heating and cooling (Saiki et al., 1988).

2. Template DNA:

DNA template (0.1-1ng total) is usually required in a reaction mixture of total 50μl. Templates that have amounts higher than this, result in nonspecific PCR products. Moreover, DNA must be purified since traces of phenol EDTA used in the isolation process inhibit Taq polymerase activity. This is usually prevented by washing the DNA pellet with 70% ethanol (Singh et al., 2014).

3. Primers:

Primer designing is extremely important for amplification of a target gene. However, while designing primers several factors should be kept in mind:

A. Primer length

Primers are usually 18 to 24 bases pair long. However, annealing temperature for primers is determined by the length of those primers (Chuang et al., 2013).

B. GC content

GC content is also important to determine the melting temperature of a sequence. A melting temperature of 56-62 is generally required for oligos that are 20 base pair long and have 50% GC content (Dieffebach, 1993).

C. Melting temperature (T_m)

Guanine and Cytosine content as well as primer length must be selected to set the T_m for the pair of primers in the range of 5 degrees within each other (Li, 2007).

D. Primer complementarity

Primers should not be complementary to other primers nor to itself to prevent any intra or inter primer homology from occurring which results in primer dimers (Vallone and Butler, 2004).

E. Estimating the melting and annealing temperatures of primer

The T_m of primers that are less than 25 nucleotides can be calculated using the following formula: $Tm= 4 (G + C) + 2 (A + T)$

F. Restriction site integration

At the 5' end of primers, the addiction of 3-6 nucleotides provides a restriction to the amplified sequence 3-6 nucleotides are added at the 5' end of the primer (Chuang et al., 2008).

4. dNTPs

They are used to boost the incorporation of nucleotides in the newly produced strand when used in the accurate concentration which is usually 200μM (Singh et al., 2014).

5. $MgCl_2$

They facilitate the nucleophilic attack that is retarded by the four negative charges of the dNTP and bond formation between nucleotides hence polymerization (Singh et al., 2014).

6. Thermal Cycler

Thermal cycle is an instrument that is used to change temperatures rapidly for denaturation, annealing, and extension.

The principle and the procedure of PCR

The concept of PCR is based on the separation of DNA into two strands at high denaturing temperature around 95 degrees due to breakage at the Adenine-Thyrosie and Cytosine-Guanine bonds. Through annealing temperature near 50-65 degrees primers bind to the 3' end of the separated single strand. At the extension temperature of 72 degrees, Taq

polymerase prolongs the new strand by the addition of dNTPs and double stranded molecules restructures. Several repetitions of this process, generates billions of copies of the targeted DNA sequence (Figure 2.3) (Muller, 2001).
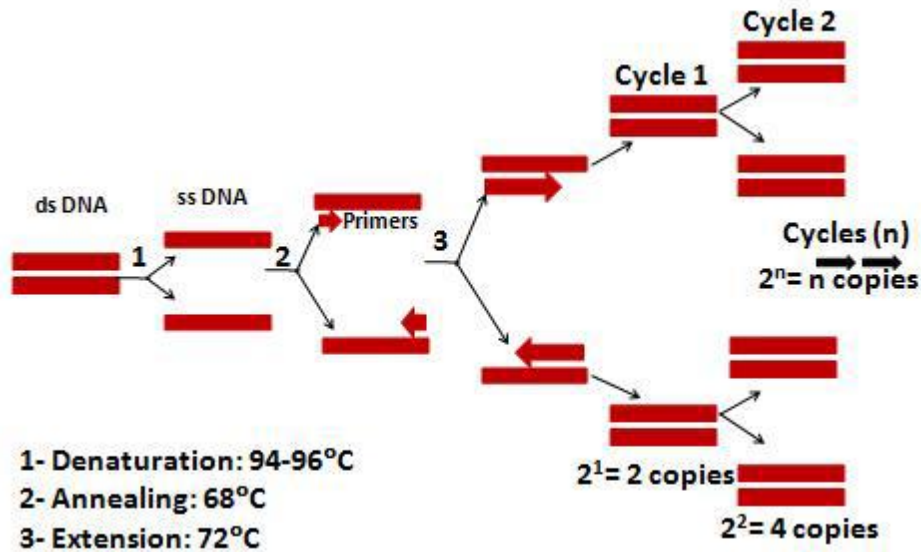
Figure 2.3. Concept of PCR (Singh et al., 2014).

Types of PCR

1. Standard PCR variants

The development of modifications on the previously discussed PCR led to variants in the PCR such as Allele specific PCR which enables a point-blank detection of point mutation in DNA (Ugozzoli and Wallace, 1991), asymmetric PCR which is used to replicate one strand only from the targeted DNA via unequal primer concentrations (Innes et al., 1988), and nested PCR which is invented to reduce the amplification of non-specific PCR products by using two sets of primers (Haff, 1994).

2. Reverse Transcription- Polymerization chain reaction (RT-PCR)

This method allows quantitative measuring of the expression levels of RNA by synthesizing complimentary DNA (cDNA) for isolated RNA by the aid of reverse transcriptase, amplification of cDNA is then performed using standard PCR (Temin and Mizutani, 1970).

3. Real time-PCR or quantitative PCR (qPCR)

Higuchi et al proposed the qPCR which enabled the detection of fluorescent reporter dye such as SYBR Green I to measure the amplification of DNA at each cycle. Threshold cycle (C_T) is the point at which the fluorescence has reached to a point that it can be measured during amplification. The main advantage of qPCR is that it allows amplification and detection in one step as it does not demand further amplification processing of the sample (Higuchi and Dollinger, 1992).

4. RT-PCR/qPCR combined

This technique is used when quantitative detection of RNA expression is to be measured, where both reverse transcription and quantitative PCR are combined and this method is known as qRT-PCR/ or RT-qPCR (Taylor et al., 2010).

2.3 The biology of tooth development

During embryogenesis, continuous cell and tissue interactions are responsible for cell organization into tissues and organs. The development of teeth is a particularly an ideal example for studies that is concerned about regulation of organogenesis, morphogenesis, and cell differentiation (Thesleff et al., 1996).

The development of teeth starts as the dental epithelium thickens and forms the dental lamina. Cells that line within thickened epithelium band begin to proliferate and to invaginate to a position that it forms the dental placodes. Following this crucial step, further invagination forms the bud, cap, and bell stages of tooth development (Bei, 2009). (Figure 2.4)
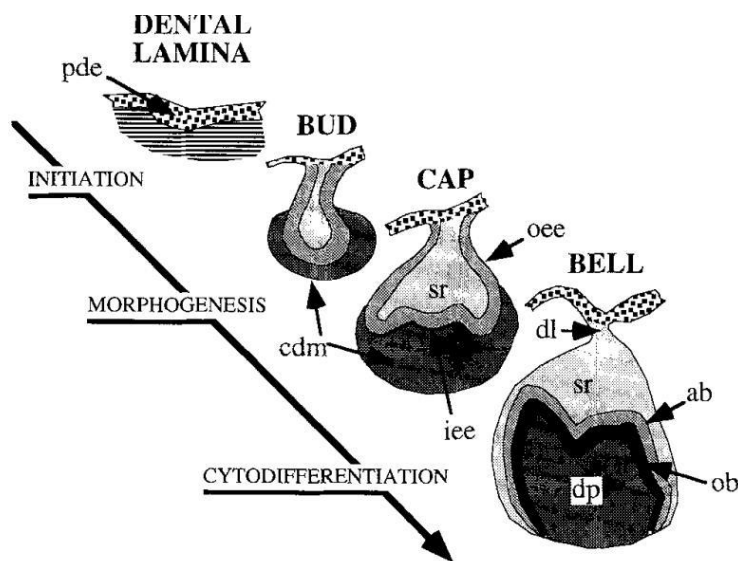
Figure 2.4. Schematic presentation of tooth morphogenesis. Initiation of tooth development is characterized by thickening of the presumptive dental epithelium and subsequent condensation of neural crest-derived mesenchymal cells around the epithelial bud. As a result of epithelial morphogenesis, the shape of the tooth crown is determined during the cap and bell stages. Ameloblasts (ab), condensed dental mesenchyme (cdm), dental lamina (dl), dental papilla (dp), inner enamel epithelium (iee), odontoblasts (ob) , outer enamel epithelium (oee), presumptive dental epithelium (pde), stellate reticulum (sr) (Thesleff et al, 1996).

With the development of in situ hybridization mRNA expression analysis became available as is growth and transcription factors in the embryonic tissues. Several studies, revealed that several genes are expressed in the dental mesenchyme during its condensation (Thesleff et al, 1996). (Figure 2.5)
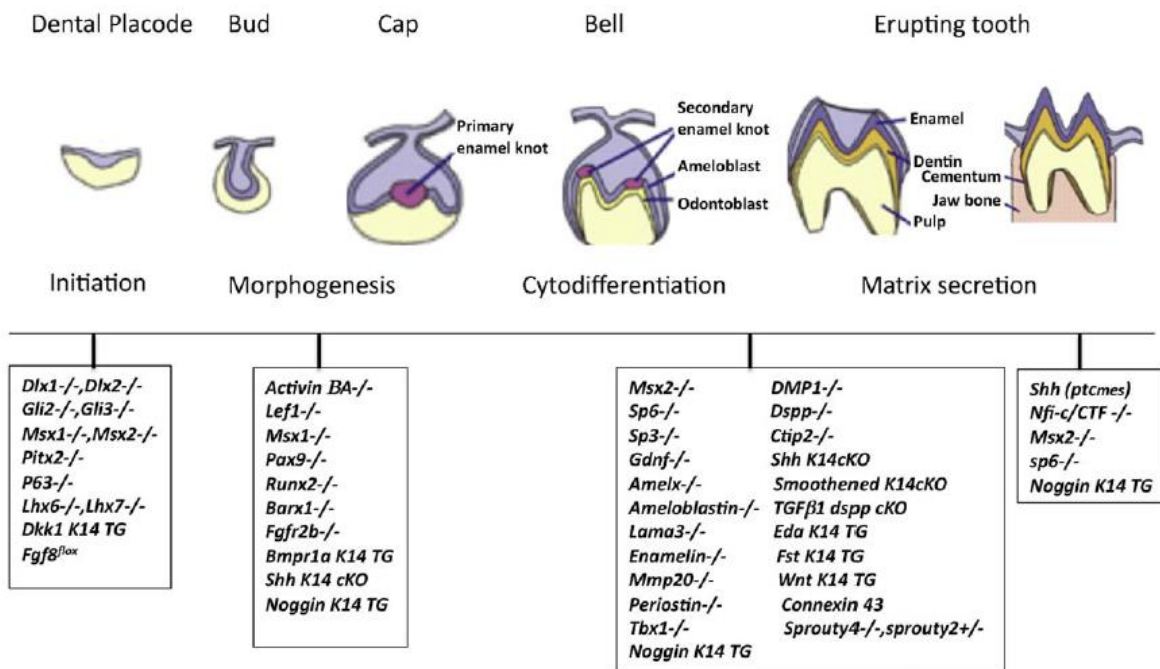
Figure 2.5. Genes responsible for tooth agenesis (Bei, 2009).

The competition between Fibroblast growth factor (FGF) 8 and Bone morphogenic protein (BMP) 4 regulates the process of tooth specification. Beyond the bud stage, restriction of homeobox transcription factor *PITX2* expression by these factors is essential for the development of teeth. For the invagination of the dental lamina to form tooth buds, homeobox genes *MSX1* and *MSX2* expression is important. Tooth bud starts to fold at its base during late bud stage in response to mesenchymal signals. The enamel knot then appears at the cap stage and it is believed that it regulates the shape of teeth. Studies on mice revealed that lack of the transcription factors *PITX1*, *MSX1*, and *PAX9* leads to failure in these processes which are targets of the intercellular WNT, FGF, and BMP pathways (Amand et al., 2000; Neubüser et al., 1997). Those genes function and are expressed during tooth development in several signaling pathways such as BMP (Bone morphogenesis protein), SSH, HH (hedgehog), FGF

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(Fibroblast growth factor), and WNT ligands to mediate the epithelial-mesenchymal interactions. However, disturbances in the expression of genes that are involved in these signaling pathways leads to severe abnormalities of tooth development, such as complete arrest of tooth development at early stages (Theslef, 2003; Fleischmannova et al., 2008) During the bell stage, TNF family member ectodysplasin (EDA) signal through its receptor in order to trigger the NF-KP pathway leading to the formation of multiple cusps. Dental papilla cells adjacent to the epithelium diversify into outer enamel epithelium, stellate reticulum, stratum intermedium, while preameloblasts and the enamel-secreting ameloblasts are formed by the differentiation of the inner enamel epithelium (Liu et al, 2008).

2.4 Role of WNT signaling pathway in tooth development

Two distinct WNT signaling pathway had been identified (canonical and noncanonical) and two types of proteins were identified for each pathway.

Canonical WNT Signaling pathway:

It is β -catenin-dependent pathway that is initiated then the WNT proteins binds to the receptors of the seven-transmembrane domain-spanning frizzled (Fz) family as well as to the co-receptors lipoprotein receptor-related proteins (LRP). Expression of certain target genes occur when β-catenin enters the nucleus and binds to the transcription factor as a result of accumulation of β-catenin due to the activation of Dishevelled (DSL) which inhibits GSK-3B. (Figure 2.6) (Tamura and Nemoto, 2016; Clevers, 2006).
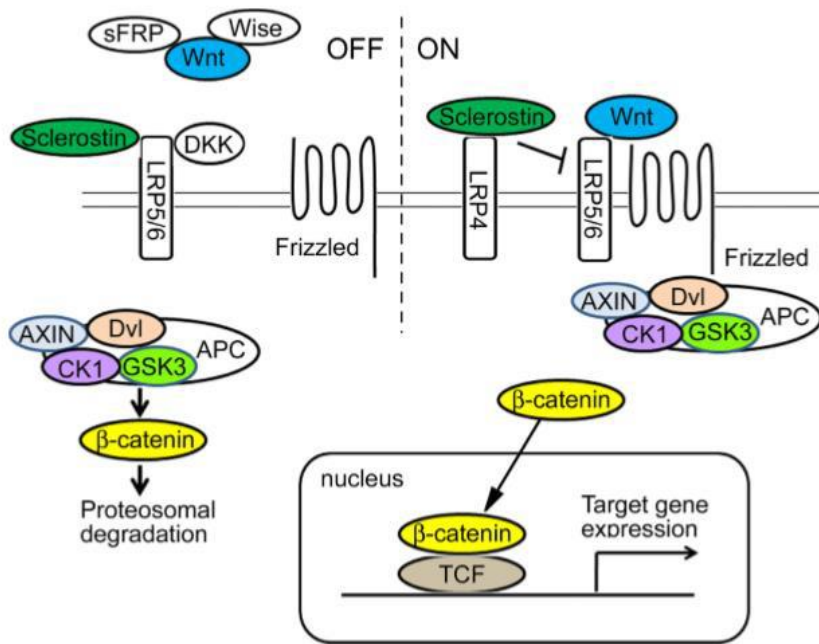
Figure 2.6. Illustrated scheme of canonical wnt signaling pathway (Tamura and Nemoto, 2016).

Noncanonical Wnt pathway:

It is not β-catenin independent which means that it does not require β-catenin, or TCF transcription factor, but it required Fz and WNT ligands. However, those ligands collaborate with alternative WNT receptors such as tyrosine-kinase-like orphan receptor (Ror) 2 or receptor tyrosine-kinase Ryk. The noncanonical WNT pathway works by two methods:

A. Planar cell polarity (PCP) pathway, the cell polarity is controlled in this mechanism when cells orient themselves to a plane perpendicular to the apical axis.

B. The Wnt/Ca+2 pathway, in the intracellular Ca+2 is triggered by *WNT5a* in order to activate protein kinase C and Ca+2/calmodulin-dependent kinase II. (Tamura et al, 2016; Clevers, 2006).

The Wnt/β-catenin pathway in tooth development

Wnt/β-catenin signaling is progressively active in all regions during tooth development stages (figure 2.7), and it plays essential roles in these events:

Initiation Stage

During the initiation stage of mice tooth development, it has been noted that several WNT genes are expressed such as *WNT10b*, which is expressed specifically in the molar and incisor dental epithelial thickenings, *WNT4*, *WNT6*, and the Wnt receptor gene Fz6 expression is also detected in oral, facial, and dental epithelium. Several studies indicate that the role of WNT signaling pathway during the initiation stage of tooth development is localized to the epithelium of the dental lamina and forms dental. (Liu et al., 2008; Tamura and Nemoto al., 2016; Liu et al., 2010; Sarkar and Sharpe, 1999).

Bud Stage

Expression of the reported gene of WNT signaling pathway revealed that WNT signaling pathway is active in dental epithelial cells and in the underlying mesenchymal cells in the bud stage (Liu et al., 2008). Tooth development is arrested at early bud stage when WNT signaling is inhibited in dental epithelial and mesenchymal. It was found that the cutting out the β-catenin in oral mesenchyme leads to failure of advancement from the bud to the cap stage of tooth development (Liu et al., 2010; Sarkar and Sharpe, 1999).

Cap Stage

At the early cap stage, several genes expression were detected at the enamel knot such as *LEF1*, *WNT3*, *WNT6*, *WNT10b*, and *MFz6*. On the other hand, *WNT5a* and *MFrzb1* revealed a clear expression in the dental papilla mesenchyme (Liu et al., 2008; Liu et al., 2010; Sarkar and Sharpe,1999).

Bell Stage

In the development of secondary enamel knots, Wnt/β-catenin signaling was found to be active as it was found that reduction in size and the formation of blunter cusps was noted during

treatment of molar tooth germ explants with the Wnt inhibitors, and subsequent transplantation to renal capsules for additional development, implying that the size of molar tooth and its cusps development is regulated by Wnt pathway. Conforming with these findings, compulsory experimental in-vivo expression of *Dkk1* in oral and dental epithelia from the cap stage leads to the formation of blunted molar cusps. Moreover, mouse mutants that lacks *Sostdc1* tend to have enamel knots that are enlarged and extra cusps (Liu et al, 2010; Sarkar et al,1999).

Secretory Stage

In secretory odontoblasts, *WNT10a* was found to be expressed and co-localized with dentin sialophosphoprotein (Dspp), which is a tooth-specific noncollagenous matrix protein that regulates dentin mineralization. Further in-vitro data analysis illustrates that *WNT10a* is an upstream regulator for *Dspp* expression. Loss of ameloblasts and enamel reduction in post-natal incisors was observed when *WNT3* is overexpressed in dental epithelium. Therefore, it is suggested that inhibiting Wnt pathway have an important a role in enamel formation (Liu et al, 2010).
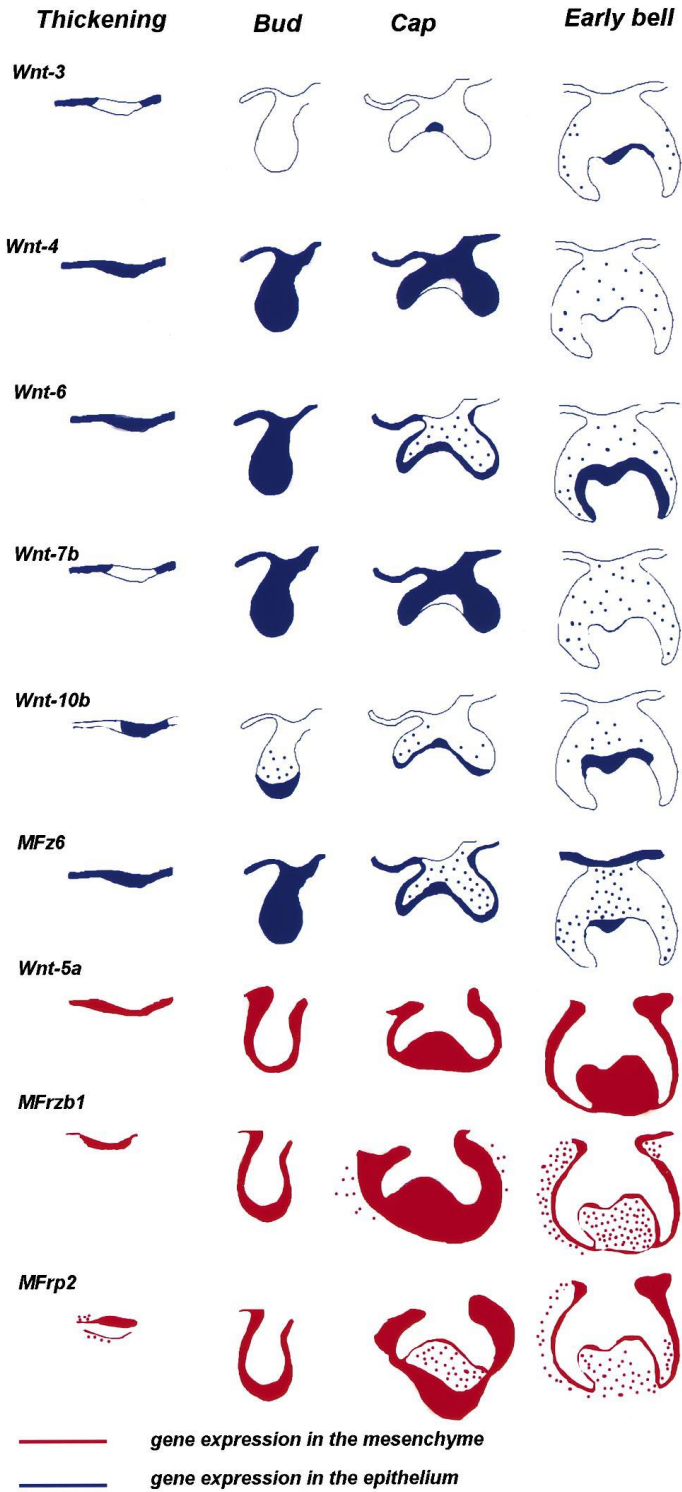
Figure 2.7. Diagrammatic representation of early tooth development. Expression of WNT pathway genes in epithelium shown in blue and in mesenchyme in red (Sarkar et al, 1999).

3. Material and methods

Ethical approval was granted by Near East University's ethics committee (YDU/2018/57-650). Written informed consent was obtained from all patients or, in case of minors, it was obtained from their parents.

A total of 40 healthy and unrelated individuals of both genders were divided equally into two groups. The experimental group consisted of individuals with hypodontia, while the controls were individuals with full dentition who were recruited and had undergone a thorough clinical and radiographic examination to confirm the missing tooth/teeth excluding the third molars. A brief medical and dental history was obtained and a physical examination assessing skin, hair, nails and any other signs that could suggest developmental syndromes was performed by the same clinician to eliminate the potential for the missing teeth to be associated with any syndrome.

The inclusion criteria for the experimental group of this study were healthy, unrelated individuals with hypodontia, and the lack of any signs of craniofacial syndromes. The control group consisted of individuals who were healthy, unrelated, had full permanent dentition and did not exhibit signs of craniofacial abnormalities. On the other hand, we excluded individuals who had revealed any signs of syndromes, as well as those with systematic diseases that may have affected the number of teeth present.

3.1 RNA Extraction and quantitative polymerization chain reaction

Saliva samples (4mL) were collected from each individual and prepared by mixing them with a 20mL phosphate buffer solution (1XPBS). They were then centrifuged at 3000 rpm for 5 minutes, the supernatant was discarded and 100 uL of 1XPBS was added to the pallet and mixed by pipetting followed by RNA extraction, which was performed according to the protocol given by Hybrid-R (Geneall, Lisbon, Portugal). Purified RNA was stored at -80 degrees for further use and RNA concentrations were measured on nanodrop (Thermo Fisher Scientific, USA). cDNA was synthesized according to the manufacturer's instructions provided with the

Wizscript cDNA synthesis kit (Wizbiosolutions, Seongnam, Korea). Subsequently, 100 ng of cDNA was used as a template for 20 µL of ready to use QPCR mastermix containing 0.5nmol of each primer (Intron sağlık ürünleri, Turkey) (Table 3.1). An Insta-Q 95 Plus thermocycler (Mumbai, India) was used, and the cycling conditions were as follows: 95°C for 5 minutes and 35 cycles at 95°C for 60 seconds, 58°C for 30 seconds, and 72°C for 45 seconds, followed by a final extension of 5 minutes at 72°C.

Primers	Sequences
AXIN2 **Exon7**	Forward: 5'CCTGGAGAGGGAGAAATGC 3' Reverse: 5' CATCACCGACTGGATCTC 3'
PAX9 **Exon 2**	Forward 5´-AGGCACCAAATGGTCTCCCAGCTGT-3´ Reverse 5´-GAAGCCGTGACAGAATGACTA-3´
WNT10a **Exon 2**	Forward 5' CCAACACCAATTCAGGGACC 3' Reverse 5' CTCTCGGAAACCTCTGCTGA 3'
GAPDH	Forward 5' CAAATTCCATGGCACCGTCAAG 3' Reverse 5' GCAAATGAGCCCCAGCCTTC 3'

Table 3.1. Primers' sequences.

The Delta-delta Ct method was applied to calculate the fold alterations in the expression of genes (Livak and Schmittgen, 2001). Therefore, a housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used to normalize the Ct values of our target genes. The normalized Ct values to GAPDH were expressed as Delta-Cts (ΔCt). Delta-delta Cts ($\Delta\Delta$Ct) was then used to express the normalized ΔCt values to PRE values. The formula: fold change = $2^{-\Delta\Delta Ct}$ was calculated for the delta-delta Cts ($\Delta\Delta$Ct). Thus, mRNA values were expressed as a

fold change from PRE (mean ±S. D). *GAPDH* expression did not show any differences at any of the post-intervention time-points. Since we used the 2^-ΔΔCt method to calculate the fold changes in gene expression, the higher values indicate higher gene expression.

3.1 Sample size calculation

G*Power version 3.0.10 (Franz Faul Universität) software was used to calculate the sample size that would be included in the study. The power of the study was set as 80%, level of significance was set at $\alpha = .05$ and size of effect was calculated as 0.85. The calculated sample size required for this study was 36. However, the total sample size was modified to 40 to reduce any missing data.

3.2 Statistical analysis

Descriptive statistics for all variables (arithmetic mean, median, standard deviation, maximum, and minimum) were calculated. The Shapiro-Wilks test for normality was performed and due to the violation of normal distribution for the qRT-PCR results, the non-parametric Mann–Whitney U test for independent groups was applied. Results are illustrated as boxplots showing the median values and interquartile range. Statistical significance was assumed at $p < 0.05$.", Graphpad Prism software (Demo Version 8.2.1 for Mac) was used for the data analysis.

4. Results

In this study, we included 40 patients and divided them equally into a study group and control group. The mean age for our experimental group was 17 ±7, while it was 16.5 ±6 for the control group, which was not significant (p-value=0.311). Also, the study group consisted of 20 individuals with a male to female ratio of 1.2:1, while, the control group consisted of 20 healthy unrelated individuals with a male to female ratio of 1.5:1. Moreover, the pattern of missing teeth in the experimental group was illustrated in Table 4.1.
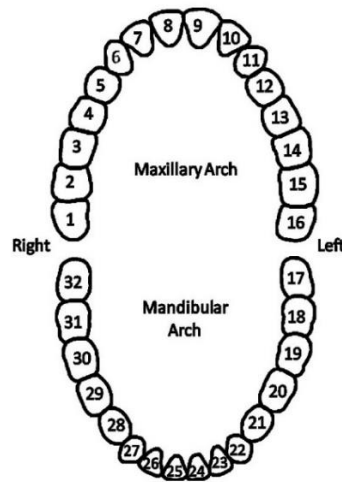
Pattern of missing teeth observed on a per patient basis (tooth numbers)	Males	Females
Missing only mandibular 2nd premolars (i.e., #20 and/or #29)	4	4
Missing only maxillary lateral incisors (I.e., #7 and/or #10)	1	1
Missing only maxillary 2nd premolars (i.e., #4 and /or #13)	4	3
Missing a combination of both mandibular 2nd premolars and maxillary incisors	1	0
Other patterns of missing teeth not described above	1	1
Total (n=20 patients total)	11	9

Table 4.1. The pattern of Hypodontia in the experimental group.

In both groups, we analyzed the gene expression of *WNT10a*, *PAX9*, and *AXIN2*, and a *GAPDH* mRNA expression was used to normalize our data of mRNA quantification values since it is consistently expressed in a wide variety of tissues and cells and the results are shown in Figure 4.
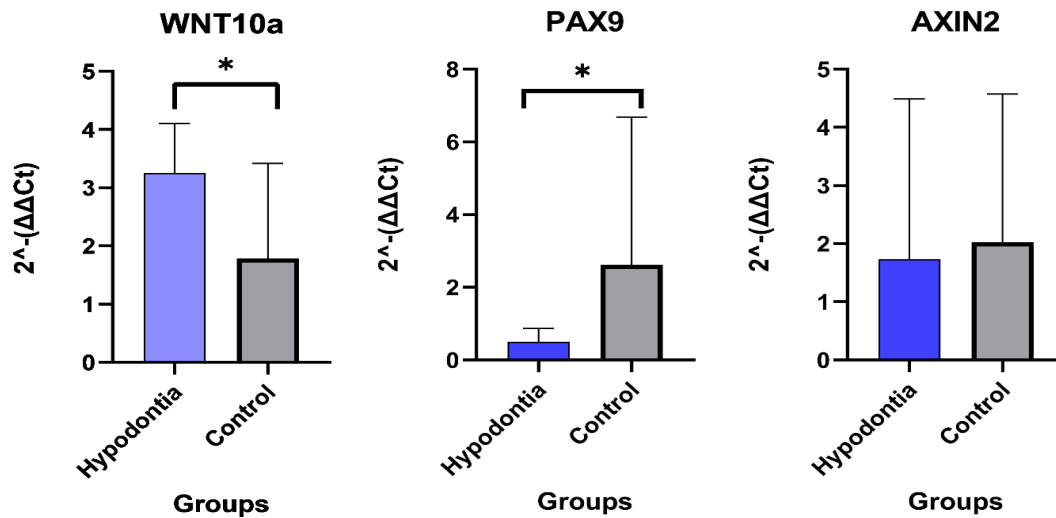
Figure 4. Real-time RT-PCR was used to determine the level of expression for WNT signaling pathway genes.

The QPCR analysis results of our genes of interest showed a statistical significance in the level of expression of *PAX9,* which was down-regulated in the hypodontia group by 0.1913-fold when compared to those of the control group (p=0.018). (Table 4.1) When *WN10a* expression was calculated, *WNT10a* was up-regulated in the hypodontia group by 1.824-fold more than the control group (p=0.005). (Table 4.2) *AXIN2* also showed a non-significant down-regulation in the hypodontia group by 0.8555-fold when compared to the control group (p=0.69). (Table 4.3)

Groups	Average Ct for Reference gene *GAPDH*	Average Ct for *PAX9*	ΔCt	ΔΔCt	2^-(ΔΔCt)
Hypodontia group	28.15	32.15	4	3.35	0.098073
	28.4	31.75	3.35	2.7	0.153893
	28.65	32.4	3.75	3.1	0.116629
	28.1	29.95	1.85	1.2	0.435275
	27.7	28.75	1.05	0.4	0.757858
	28.05	28.85	0.8	0.15	0.90125
	28.15	30	1.85	1.2	0.435275
	27.05	27.25	0.2	-0.45	1.36604
	28.35	30.45	2.1	1.45	0.366021
	28.25	30.2	1.95	1.3	0.406126
	28.5	31.35	2.85	2.2	0.217638
	28.75	30.1	1.35	0.7	0.615572
	28.25	29.45	1.2	0.55	0.68302
	28.6	30.9	2.3	1.65	0.31864
	27.9	28.2	0.3	-0.35	1.274561
	27.6	30	2.4	1.75	0.297302
	27.75	30.1	2.35	1.7	0.307786
	28.05	28.9	0.85	0.2	0.870551
	28.35	30.7	2.35	1.7	0.307786
	29.1	33.1	4	3.35	0.098073
Control Group	28.45	29.25	0.8	0.15	0.90125
	24.75	26.85	2.1	1.45	0.366021
	23.4	26.95	3.55	2.9	0.133972
	28.15	30.3	2.15	1.5	0.353553
	27.7	29.95	2.25	1.6	0.329877
	28.5	29.15	0.65	0	1
	23.95	25.45	1.5	0.85	0.554785
	28.25	30.1	1.85	1.2	0.435275
	28.8	26.45	-2.35	-3	8
	28.8	27.25	-1.55	-2.2	4.594793
	28.3	32.25	3.95	3.3	0.101532
	28.6	29.35	0.75	0.1	0.933033
	27.5	28.4	0.9	0.25	0.840896
	28.8	29.85	1.05	0.4	0.757858
	28.65	30.05	1.4	0.75	0.594604
	28.65	30.05	1.4	0.75	0.594604
	28.6	26.15	-2.45	-3.1	8.574188
	28.75	25.45	-3.3	-3.95	15.45498
	28.35	28.8	0.45	-0.2	1.148698
	28.1	26	-2.1	-2.75	6.727171

Average ΔCt Control = 0.65
Average ΔΔCt for experimental group = 1.19
Average ΔΔCt for control group = 0
P value = 0.0184 *

Table 4.2. Results of *PAX9* gene expression

Groups	Average Ct for Reference gene *GAPDH*	Average Ct for *WNT10a*	ΔCt	ΔΔCt	2^-(ΔΔCt)
Hypodontia group	28.15	24.15	-4	-1.7725	3.416455
	28.4	24.2	-4.2	-1.9725	3.924476
	28.65	24.5	-4.15	-1.9225	3.790794
	28.1	24.1	-4	-1.7725	3.416455
	27.7	24.55	-3.15	-0.9225	1.895397
	28.05	24.1	-3.95	-1.7225	3.300078
	28.15	24.2	-3.95	-1.7225	3.300078
	27.05	24.45	-2.6	-0.3725	1.294594
	28.35	24.35	-4	-1.7725	3.416455
	28.25	24.15	-4.1	-1.8725	3.661665
	28.5	24.45	-4.05	-1.8225	3.536936
	28.75	24.6	-4.15	-1.9225	3.790794
	28.25	24.25	-4	-1.7725	3.416455
	28.6	24.35	-4.25	-2.0225	4.062872
	27.9	24.05	-3.85	-1.6225	3.079081
	27.6	24.3	-3.3	-1.0725	2.103075
	27.75	24.25	-3.5	-1.2725	2.415798
	28.05	24.4	-3.65	-1.4225	2.680496
	28.35	24.35	-4	-1.7725	3.416455
	29.1	24.5	-4.6	-2.3725	5.178377
Control Group	28.45	23.85	-4.6	-2.3725	5.178377
	24.75	24.15	-0.6	1.6275	0.323649
	23.4	24.15	0.75	2.9775	0.126965
	28.15	24.5	-3.65	-1.4225	2.680496
	27.7	24.15	-3.55	-1.3225	2.500991
	28.5	26.8	-1.7	0.5275	0.693756
	23.95	23.65	-0.3	1.9275	0.262884
	28.25	28.2	-0.05	2.1775	0.221058
	28.8	24.55	-4.25	-2.0225	4.062872
	28.8	24.6	-4.2	-1.9725	3.924476
	28.3	24.3	-4	-1.7725	3.416455
	28.6	27.4	-1.2	1.0275	0.490559
	27.5	27.75	0.25	2.4775	0.179555
	28.8	25.95	-2.85	-0.6225	1.539541
	28.65	25.35	-3.3	-1.0725	2.103075
	28.65	24.4	-4.25	-2.0225	4.062872
	28.6	28.2	-0.4	1.8275	0.281752
	28.75	25.3	-3.45	-1.2225	2.333507
	28.35	26.7	-1.65	0.5775	0.670124
	28.1	26.55	-1.55	0.6775	0.625248

Average ΔCt Control = -2.2275
Average ΔΔCt for experimental group = -1.007
Average ΔΔCt for control group = 0.2135
P value = p=0.0056 *

Table 4.3. Results of *WNT10a* gene expression

Groups	Average Ct for Reference gene *GAPDH*	Average Ct for *AXIN2*	ΔCt	ΔΔCt	2^-(ΔΔCt)
Hypodontia group	28.15	27.85	-0.3	-0.3575	1.281204
	28.4	26.3	-2.1	-2.1575	4.461411
	28.65	31.6	2.95	2.8925	0.13467
	28.1	28.45	0.35	0.2925	0.816486
	27.7	26.6	-1.1	-1.1575	2.230705
	28.05	27.95	-0.1	-0.1575	1.115353
	28.15	26.65	-1.5	-1.5575	2.943433
	27.05	28.05	1	0.9425	0.52033
	28.35	28.6	0.25	0.1925	0.875088
	28.25	28.7	0.45	0.3925	0.761808
	28.5	28.3	-0.2	-0.2575	1.195405
	28.75	29.25	0.5	0.4425	0.735858
	28.25	24.65	-3.6	-3.6575	12.61878
	28.6	29.3	0.7	0.6425	0.640602
	27.9	29.05	1.15	1.0925	0.468948
	27.6	28.85	1.25	1.1925	0.437544
	27.75	27.6	-0.15	-0.2075	1.154686
	28.05	27.7	-0.35	-0.4075	1.326385
	28.35	29.6	1.25	1.1925	0.437544
	29.1	30	0.9	0.8425	0.557676
Control Group	28.45	28.5	0.05	-0.0075	1.005212
	24.75	26.05	1.3	1.2425	0.42264
	23.4	27.35	3.95	3.8925	0.067335
	28.15	26	-2.15	-2.2075	4.618742
	27.7	29.5	1.8	1.7425	0.298851
	28.5	27.35	-1.15	-1.2075	2.309371
	23.95	26.85	2.9	2.8425	0.139419
	28.25	24.8	-3.45	-3.5075	11.37268
	28.8	27.7	-1.1	-1.1575	2.230705
	28.8	30.95	2.15	2.0925	0.234474
	28.3	26.9	-1.4	-1.4575	2.74632
	28.6	30.1	1.5	1.4425	0.367929
	27.5	30.45	2.95	2.8925	0.13467
	28.8	27.85	-0.95	-1.0075	2.010424
	28.65	28.35	-0.3	-0.3575	1.281204
	28.65	27.55	-1.1	-1.1575	2.230705
	28.6	28.05	-0.55	-0.6075	1.523617
	28.75	27.05	-1.7	-1.7575	3.381117
	28.35	28.35	0	-0.0575	1.040661
	28.1	26.5	-1.6	-1.6575	3.154694

Average ΔCt Control = 0.0575
Average ΔΔCt for experimental group = 0.0457
Average ΔΔCt for control group = 0.001425
P value = 0.693

Table 4.4. Results of *AXIN2* gene expression

5. Discussion

Patients who suffer from tooth agenesis may face complications such as malocclusion, periodontal damage, lack of alveolar bone growth, and alterations in the skeletal relationships and unfortunately an unfavorable appearance leading to costly and challenging multidisciplinary treatment (Rakhshan, 2015). As mentioned above, tooth agenesis is a multifactorial trait and it has been documented that the WNT signaling pathway is found in the majority of isolated tooth agenesis cases. It has been identified that several genes are expressed during tooth development and mutations in those genes such as *PAX9*, *WNT10a*, *AXIN2*, and *MSX1* may lead to isolated or syndromic tooth agenesis (Nieminen, 2009). Generally, congenital missing permanent teeth tend to be the most distal teeth in their class including third molars, second pre-molars and/or lateral incisors and primary teeth are rarely missing. These teeth are most affected by mutations in *AXIN2* and *WNT10a* (Lammi et al., 2004; Arte et al., 2013; Arzoo et al., 2014; Yang et al., 2015).

Studies on families showed that isolated hypodontia is inherited as an autosomal dominant trait with variable expressions (da Silva et al., 2016), whereas another study showed that one family showed an autosomal recessive trait of inheritance (Stern, 2000). There has been controversy in the role of *MSX1* in isolated hypodontia. A study of a family showed that missense mutation of *MSX1* was detected in the affected family members with hypodontia (Silva et al., 2009). Also, a nonsense mutation was found in a Dutch family associated with hypodontia and various combinations of cleft lip and/or palate (Thesleff, 2000). On the other hand, *MSX1* was excluded from being responsible for development of hypodontia (van den Boogaard, 2000).

Mutations in *MSX1* has also described several syndromic forms of hypodontia such as Witkop syndrome, Wolf herschhom syndrome, and hypodontia associated with orofacial clefting (Jumlongras et al., 2001; Nieminen et al., 2003). *MSX1* was found to be one of the most important candidate genes that are responsible for non-syndromic cleft lip and palate as it had been proposed that mutations in *MSX1* contribute in 2% of all craniofacial anomalies (Jezewski et al., 2003).

However, in this study, QPCR had been used to analyze the expression WNT singling pathway genes such as *PAX9*, *AXIN2*, and *WNT10a* and our results showed that *PAX9* was significantly down-regulated in patients with hypodontia, which is in line with a previous study that had documented lower levels of expression of *PAX9* in a family with oligodontia (Šerý et al., 2015). Another study provided evidence that lower levels of *PAX9* expression have an effect on tooth morphogenesis and generate a non-syndromic form of oligodontia in mice (Kist et al., 2005).

Mutations in *PAX9* were also reported to affect the mesio-distal dimension of permanent teeth as Brook et al (2009) found that mutations in *PAX9* caused the greatest reduction of size of permanent canines in hypodontia. In early studies, mutations in *PAX9* were identified in the association with oligodontia in a family affected with congenital absence of most of their permanent molars and variable agenesis of second premolars as well as mandibular incisors (Stockton, 2000). However, mutations in *PAX9* did not show any increase in risk of syndromes or abnormalities other than oligodontia (Ruf et al., 2013). This concludes that epigenetic factors that affect or limit gene expression may lead to tooth agenesis (Kist et al., 2005).

Methylation of *MSX1* and *PAX9* have been associated with cancer development but yet have not been confirmed to be in relation tooth agenesis (Rauch et al., 2006; Dunwell et al., 2006).

Data acquired from mice backs up the association of *WNT10a*, *AXIN2*, and *PAX9* in tooth development. At the tooth initiation stage of development, the expression of *WNT10a* was greatly noted in the dental epithelium. Also, *WNT10a*, *PAX9*, and *MSX1* are required for normal tooth development beyond bud stage. During tooth development, *AXIN2* is expressed in the odontoblast, dental mesenchyme, and enamel knot (Klein et al., 2006; Chen et al., 2009).

AXIN2 mutations have also been found to be linked with various cancerous lesions such as ameloblastoma, ovarian cancer, prostate and skin cancer, and it has been investigated whether a direct link is present between tooth agenesis and any of the aforementioned tumors (Iavazzo et al., 2016). *AXIN2* was found to be expressed at lower levels in ameloblastoma tissues when compared to normal mucosal tissues (Wei et al., 2013). It was also found that lower *AXIN2*

expression is associated with a more aggressive prostate cancer (Hu et al., 2016). *AXIN2* is the most associated gene with tooth agenesis and is also frequently linked to ovarian cancer. A frame shift mutation was found in ovarian endometrioid AC (Wu et al., 2001). In terms of gene expression, Schmid et al. showed a spike overexpression of *AXIN2* in all serious cancer samples (Schmid et al., 2011). However, heterozygous mutations that cause in *AXIN2* were found to cause various degrees of hypodontia. *AXIN2* is a WNT signaling pathway inhibitor and the fact that the expression of *AXIN2* is stage-specific could be the reason behind the non-significance that was found between both groups.

WNT10a mutations was found to be linked to various types of syndromes ranging from ectodermal dysplasia, schop-schulz passargesyndrome, and odontoonychodermal dysplasia, to isolated hypodontia (Adaimy, 2007). Homozygous and heterozygous mutations in *WNT10a* may occur regardless to the great variability of the related clinical manifestations (Nawaz et al., 2009). Bohering et al (2009) announced that *WNT10a* hertozygotes revealed a gender-biased exhibition pattern as males tend to have significantly higher dental anomalies than females implicating the differences in *WNT10a* expression between genders. Moreover, Van Geel et al (2010) claimed the differences in the phenotype of variable ectodermal dysplasia between genders as males showed a significantly higher dental anomalies whereas females had more common hair and nail pathology. However, in the latest studies that were conducted on Dutch and French populations did not reveal any gender-influenced expression of hypodontia (Abdalla et al., 2014).

Mutations in *WNT10a* gene has been primarily found to be associated with ectodermal dysplasia but in our study, experimental group showed no signs of skin, nail, and hair abnormalities indicating the type of hypodontia included in our study. Ruiz-Heiland et al (2019) screened 20 individuals suffering from isolated oligodontia for mutations and revealed that 50% of those individuals had mutation in *WNT10a*. Van den Boogaard et al (2012) screened *WNT10A* mutations in 56% of their patients affected by isolated hypodontia. However, further studies noticed mutations in *WNT10A* in only 25% of 102 affected individuals (Arzoo et al., 2014).

In a study conducted on nine Thia patients six were found to have a rare congenital missing permanent canine associated with a peg-shaped maxillary lateral incisors with dens

invaginatus caused by the variant p.Gly213ser in *WNT10A* (Kantaputra et al. 2014). However, this variant was also found to cause tooth agenesis of bilateral maxillary lateral incisors but didn't affect the presence of the canines in one of the patients but most recent studies confirm that p.Gly213ser variant in *WNT10a* causes tooth agenesis (Mostowska et al. 2015; Yang et al. 2015).

The severity of tooth agenesis is in correlation with the number of defects in *WNT10a* alleles (Yang et al. 2015). As a tremendous study conducted on Sweden family affect with tooth agenesis reported that biallelic mutations in *WNT10a* were strongly associated with more missing teeth than monoallelic variants. The same study confirmed the association of *WNT10a* mutations with the absence of both mandibular and maxillary molars as well as mandibular central incisors (Bergendal et al., 2011).

Even though our study has shown a statistical significance in the expression of *WNT10a* target gene, further mutation analysis is required to determine the type of mutations in both groups if present and their effects on the function of genes. However, one of the major limitations of our study was the sample size included even though it was calculated fluctuations in the level of expression were seen between individuals in both groups indicating the need for larger cohorts to be included in this type of studies. Because of the restricted access to the laboratory since the beginning of the pandemic, we could not perform analysis on more genes nor mutation analysis was possible. Also, the fact that the pandemic limited our sample size, we could not recruit children, whom teeth are still developing; in order compare the level of expressions between them and between individuals whom teeth had already develop.

6. Conclusion

Based on the findings observed in our study, it could be suggested that there is a strong association between individuals with hypodontia and overexpression of *WNT10a* in the buccal cells contained in the patient's saliva, whilst *PAX9* has lower expression. On the other hand, AXIN2 was down-regulated in the experimental group but it was statistically insignificant. Thus, in the future mutation screening in a larger cohort is recommended to determine the prevalence of mutations and their impact on the molecular pathogenesis underlying hypodontia.

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