

PAX9 Polymorphism in Non-Syndromic Hypodontia in the Malaysian Population

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Abstract

Non-syndromic hypodontia is the developmental absence of more than one tooth that appears as an independent congenital oral trait. Genetic mutations responsible for tooth agenesis have been identified in paired box 9 (PAX9) and muscle segment homeobox 1 (MSX1), genes encoding transcription factors that play crucial roles during odontogenesis. This study aimed to determine the role of PAX9 and MSX1 in non-syndromic hypodontia. Data collection started from April 2016 to June 2017. Thirty-three non-syndromic hypodontia patients volunteered in this study. Clinical examination and panoramic radiography were performed on a cohort of 31 unrelated Malaysian patients with non-syndromic hypodontia and 50 healthy controls. Unstimulated saliva samples were collected for genetic screening purposes following a series of DNA extraction, amplification via PCR, purification, and sequencing processes. Genetic assessment of PAX9 and MSX1 showed no mutations in all exons. Instead, two single nucleotide polymorphisms, G>C, rs4904210 (Ala240Pro) and C>T, rs12881240 (His239) were identified in exon 3 of PAX9. The SNPs were missense substitutions and synonymous codons (silent substitutions) found in both the case and control groups. However, rs12881240 association between hypodontia phenotype was not established due to unclear evidence of His239 affects the risk of occurrence of non-syndromic hypodontia. In contrast, rs4904210 may contribute to non-syndromic hypodontia due to the significant differences between case and control in the general population. This study suggests that differences in genetic variants may be attributed to ethnic diversity in the population, which causes variations in phenotype patterns and distributions.

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Introduction

Hypodontia is the most common form of tooth anomaly affecting people worldwide. One to six teeth fail to erupt in the oral cavity and remain invisible on radiographs.¹ The prevalence of hypodontia ranges from 2.6% to 11.3%, while in the Malaysian population, the prevalence is between 2.8 % and 3.2 %, which falls within the lower range.²⁻⁵ There are two forms of hypodontia: non-syndromic and syndromic. Non-syndromic hypodontia is an isolated oral trait that

appears independently. Meanwhile, syndromic hypodontia is associated with diseases such as cleft lip and palate, Rieger syndrome, Witkop tooth-nail syndrome, Down syndrome, and ectodermal dysplasia.^{6,7} Non-syndromic hypodontia is further divided into sporadic or familial. Some studies have found that familial non-syndromic hypodontia is inherited via an autosomal dominant, autosomal recessive, and sex-linked pattern of inheritance.^{8,9} Hypodontia is more prevalent in the permanent dentition, but may also occur in primary dentition at lower rates.³

Knowledge of the etiological basis of congenitally missing teeth remains weak even though much progress has been made in understanding the developmental basis of tooth formation. However, hypodontia resulting from disturbances during odontogenesis involves mutations in several genes such as *PAX9*, *MSX1*,

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EDA, *WNT10A*, and *AXIN2*.¹⁰ Many studies report that genetic mutations occurring predominantly in *PAX9* and *MSX1* are responsible for non-syndromic hypodontia.¹¹⁻¹³ *PAX9* and *MSX1* are genes encoding transcription factors that play essential roles during odontogenesis. Studies conducted on mouse animal models have revealed that *PAX9* and *MSX1* gene knockout resulted in an arrest in tooth development at the bud stage and caused other craniofacial abnormalities, including cleft palate.¹⁴ Studies in humans determined that mutations in *PAX9* cause missing molars, while mutation of *MSX1* is related to missing incisors and premolars.^{9,15}

Previous studies conducted by Mani *et al.* (2014) focused on the epidemiology of hypodontia in Malaysia, specifically in the paediatric population.⁵ However, there are no published reports regarding genetic involvement in hypodontia in the Malaysian population yet. Thus, this study aimed to determine the potential role of *PAX9* and *MSX1* in non-syndromic hypodontia.

Materials and methods

Ethical approval and clinical examinations

The research protocol was approved by the International Islamic University Malaysia Research Ethics Committee (IREC) (ID 572).

This study targeted patients treated at the Polyclinic of Kulliyah of Dentistry, International Islamic University Malaysia within one year (April 2016 to April 2017). Thirty-one sporadic unrelated hypodontia patients and 50 healthy controls with normal dentition volunteered to be in the study. The inclusion criteria in this study were congenital absence of one to six teeth. The diagnosis was verified with a panoramic x-ray which functionally confirmed no mineralization of the crown, no embedded tooth within the alveolar bone (delayed tooth eruption), and no evidence of tooth extraction or oral surgery. All clinical procedures were carried out by two dentists for calibration purposes (κ -value = 0.775, $p < 0.05$) and to avoid bias. Unstimulated saliva samples were collected from each patient for genetic assessment.

Genetic assessment

DNA was extracted using the QIAamp DNA Mini Kit (Qiagen) according to the

manufacturer's instructions. Two *PAX9* exons were studied, which are exon 2 and exon 3. For *MSX1*, the studied exons were exon 1 and exon 2. Later, the DNA was amplified using polymerase chain reaction (PCR). The primers for the respective genes are listed in Table 1.

Gene	Exon	Primers	Expected size (bp)
<i>PAX9</i>	ex2F	5'-CCA GCC TTC GGG GAG GTG AA-3'	640
	ex2R	5'-GAC GCT GCA CAT CCA CAC G-3'	
	ex3F	5'-GTG GGT CAG AGA ATT TGG AA-3'	589
<i>MSX1</i>	ex3R	5'-CAC GAA GGA TCT GGC TCG T-3'	
	ex1F	5'-CTG GCC TCG CCT TAT TAG C-3'	766
	ex1R	5'-GCC TGG GTT CTG GCT ACT C-3'	
	ex2F	5'-ACT TGG CGG CAC TCA ATA TC-3'	698
	ex2R	5'-CAG GGA GCA AAG AGG TGA AA-3'	

Table 1. List of *PAX9* and *MSX1* primers for PCR.

PCR was prepared according to the First Base REDiant 2X Master Mix protocols provided by the manufacturer. A 50 µl reaction volume consisted of 10 µM forward and reverse primers, 2X REDiant master mix, DNA samples (0.1-100µg), and nuclease-free water.

For *PAX9*, 30 cycles of PCR were performed under the following conditions: denaturation at 94 °C for 30 s, 30 s of annealing at 58 °C and 59 °C for exon 2 and exon 3, respectively, extension at 72 °C for 1 min and a final extension at 72 °C for 10 min. Meanwhile, for *MSX1*, the protocol was 30 cycles of denaturation at 95 °C for 30 s, annealing at 57 °C for both exons 1 and exon 2 for 30 s, extension at 72 °C for 1 min and a final extension at 72 °C for 10 min. A Bio-Rad Thermal Cycler T100 was used for PCR. After amplification, the PCR products were run on 1% agarose gel electrophoresis. The PCR mix for *PAX9* methodology was modified from Pereira *et al.* (2006), while the PCR mix for *MSX1* was modified from Xuan *et al.* (2008).^{7,16}

Before sequencing, PCR products were purified for sequencing using the Geneaid Gel/PCR DNA Fragment Extraction kit. This kit was used to recover or concentrate the DNA fragments from the agarose gel. All the samples were sent for sequencing services by First Base Laboratories.

Statistical analysis

All sequences were compared to the BLAST (Basic Local Alignment Search Tool) databank (<https://blast.ncbi.nlm.nih.gov/>) and SNP bank (<http://www.ncbi.nlm.nih.gov/snp/>). Meanwhile, chromatograms were visualized using Sequence Scanner software to detect possible polymorphisms in the sequences. The non-parametric Chi-square test was carried out using IBM SPSS software to analyze polymorphism comparisons between case and control groups. Significance levels, the alpha threshold was set at 5% (0.05) throughout the study.

Results

No mutations were observed in both *PAX9* and *MSX1* in any of the samples. Instead, two polymorphisms were detected in exon 3 of *PAX9*, which are rs12881240 and rs4904210. A synonymous His239codon (rs12881240; NG_013357.1:g13980C>T) was found in 9 cases, whereas missense substitution Ala240Pro (rs4904210; NG_013357.1:g.13981G>C) was detected in 10 cases. Examples of non-syndromic hypodontia can be referred in Fig. 1A) and 1B).

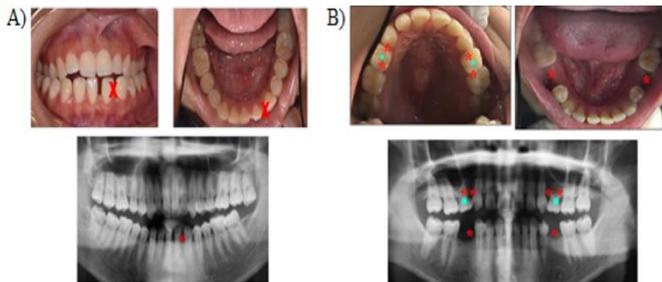


Figure 1. A) Patient missing one tooth (32). B) Patient with six missing teeth (from left; 15, 14, 12, 22, 24, 25, 35, 44, 45), the red mark indicates missing teeth and green mark indicates retained primary teeth.

Allele distributions of rs12881240 and rs4904210 are tabulated in Table 2. The allele distribution of rs12881240 showed a significant difference ($p=0.008$) between case and control groups, while rs4904210 exhibited no significant differences ($p=0.086$) were present between the case and control groups.

		Case		Control		p-value	
		n	proportion	n	proportion		
a. Allele distribution	rs12881240	C	22	71	7	70	0.008*
		T	9	29	3	30	
	rs4904210	G	20	64.5	5	50	0.086
		C	11	35.5	5	50	
b. Genotype distribution	rs12881240	CC	18	58.1	7	70	0.160
		CT	13	41.9	3	30	
		TT	0	0	0	0	
	rs4904210	GG	7	22.6	1	10	0.019*
		GC	17	54.8	5	50	
		CC	7	22.6	4	40	

Table 2. Difference analysis of *PAX9* exon 3 on allele and genotype distribution in case and control groups.

Note. Chi-square test, * $p<0.05$

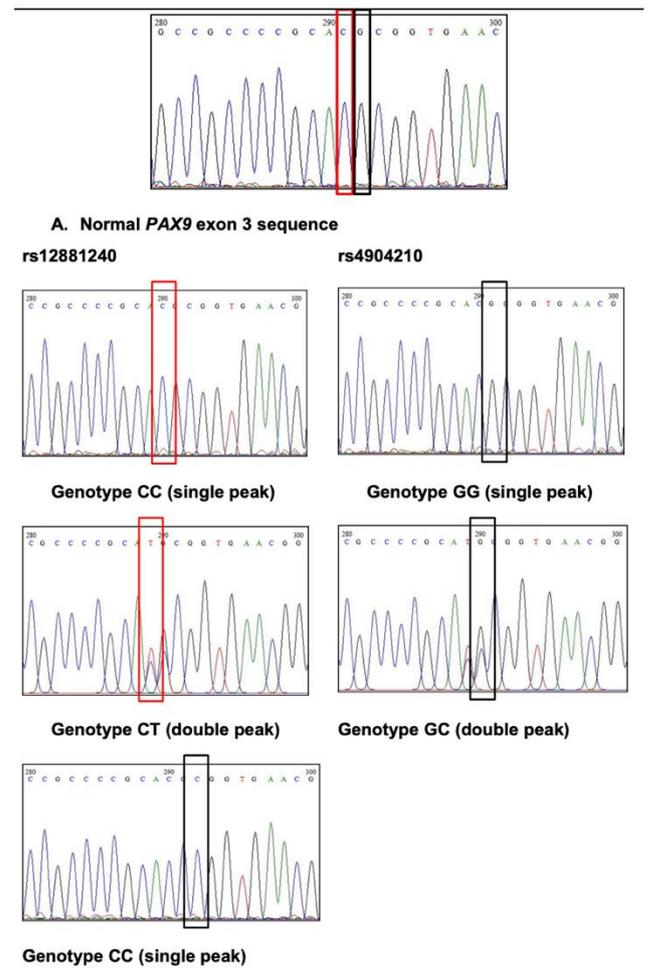


Table 3. Examples of chromatogram segment showing rs12881240 and rs4904210 polymorphism.

The three genotypic possibilities for rs12881240 are CC, CT, and TT; and GG, GC,

and CC for rs4904210, as tabulated in Table 2. rs12881240 displayed no significant association ($p=0.160$) with genotype distribution in case and control groups; meanwhile, rs4904210 presented a significant association ($p=0.019$) between case and control groups.

Examples of chromatogram segments showing the presence of two SNPs can be referred in Fig. 2. The sequences for each sample were compared against the typical sequence.

Discussion

Exclusion of PAX9 and MSX1 mutation association with non-syndromic hypodontia

PAX9 and *MSX1* are considered the primary candidate genes for non-syndromic hypodontia due to their active involvement in odontogenesis. In this study, the relationship between non-syndromic hypodontia and genetic basis could not be established due to the absence of mutation in both *PAX9* and *MSX1* genes. This finding is similar to those reported by several researchers.¹⁷⁻¹⁹ However, this was contrary to other studies reporting that mutations in *PAX9* and *MSX1* are responsible for non-syndromic hypodontia.²⁰⁻²⁶

The absence of *PAX9* and *MSX1* mutations in this study population may suggest the involvement of genes other than *PAX9* and *MSX1* in non-syndromic hypodontia, such as *AXIN2*, *WNT10A*, *EDA1*, and *EDARADD*. This involvement is due to the complex overlapping signaling cascade processes in odontogenesis, which involves several genes, molecules, and signaling pathways.²⁷⁻³¹ For example, mutations in *AXIN2* were reported to cause missing molars, premolars, lower incisors, and upper lateral incisors.³²⁻³⁵ Meanwhile, mutations in *WNT10A* have been reported to cause the elimination of the initiation codon, leading to no protein production or translation of alternative open reading frames, leading to the absence of lateral incisors and second premolars.^{19,36}

Additionally, a study reported that alteration of *BMP4*, which is required by the dental lamina to proliferate and differentiate during the bud and cap stages of tooth development, may contribute to non-syndromic hypodontia.³⁷ Another element to consider is epigenetic influences that reduce gene expression during odontogenesis, such as *PAX9*

post-transcriptional modulation and *MSX1* post-translation.¹⁷

It is also possible that mutations were present in introns or other regulatory regions. Non-syndromic hypodontia is caused by mutations in members of the paired-box and muscle segment homeobox family of genes.¹⁸ For example, *PAX1* expression in the mesenchyme is strong alongside *PAX9* during the early stages of tooth development. Furthermore, co-expression of *MSX2* with *MSX1* is strong at almost all epithelial-mesenchymal tissue interaction sites, even though *MSX2* expression is restricted to the tooth-forming region at the early cap stage in enamel knots.^{38,39}

Single nucleotide polymorphism His239 (rs12881240) and Ala240Pro (rs4904210)

The first SNP is rs12881240, a silent substitution/synonymous codon in His239 (g.13980C>T). This SNP occurs in the promoter region, outside of the DNA-paired binding domain. Since CAC and CAT both encode the amino acid histidine, *PAX9* protein function was not altered. Similar polymorphisms were reported in three case-control studies in Brazilian, Southwest Han Chinese, and Indian populations.^{15,40,41}

The mechanism by the His239 synonymous codon affects non-syndromic hypodontia is uncertain and poorly understood. Despite no changes in amino acids, the substitution of C by T in the gene sequence replaces *Bst*UI and *Dr*allI with new restriction enzyme sites such as *Fat*I, *Cvi*II, *Sph*I, *Nsp*I, and *Nla*III.²⁶ However, these site changes did not affect the physiological properties of the *PAX9* protein. Therefore, it can be suggested that *PAX9* is highly selective and can only function normally when the correct histidine codon is present; changes in the amino acid codon may affect phenotype.

The second SNP is rs4904210, a missense substitution (g.13981G>C) causing a change in alanine (GCG) by proline (CCG). This SNP occurs in the coding region outside the DNA-paired binding domain. Related polymorphisms were reported in two case-control studies.^{15,40} and three familial studies. All of these studies reported rs12881240 as described above. Changes in the structure and function of *PAX9* protein and mRNA secondary structures caused by the substitution of alanine by proline may increase the occurrence of non-syndromic hypodontia phenotype.¹⁶ Replacement of alanine

caused *Cac8I*, *ActI*, and *BstUI* restriction enzyme sites to be replaced by *StyD4I*, *BssKI*, *ScrKI*, *NclI*, *MspI*, and *HpaII*. These changes, however, did not affect the properties of *PAX9* protein.

Generally, both SNPs were detected mainly in mild hypodontia, and the most common missing tooth was identified as a lateral incisor. Mutation of *PAX9* leads to the absence of maxillary lateral incisors, all posterior maxillary teeth, and also mandible molars.¹⁶ Considering that polymorphism in DNA sequence variants also causes minor alterations in *PAX9*, this could increase the risk of hypodontia.

rs12881240 Ala240Pro (g.1031G>C) was associated with agenesis of the third molar instead of the risk of hypodontia.¹⁶ The third molar was identified as the most common missing tooth, but its association with their hypodontia phenotypes could not be established. The Southwest Han Chinese population showing a significant difference in rs4904210 variants between their phenotype and control population also exhibited agenesis of third molars.⁴⁰ Since third molars were ruled out in this study, it was challenging to confirm the association of this SNP with agenesis of the third molar.

It is established that differences in mutations and polymorphism variants affect the pattern and distribution of missing teeth. However, the mechanisms are still poorly understood. The mechanism is most likely due to ethnic variation and population diversity that cause inconsistencies in phenotype patterns.^{1,3,41,42} This result is supported by findings described in the literature, where different populations or ethnicities have different *PAX9* mutations/ polymorphisms leading to variations in prevalence, pattern, and distribution of non-syndromic hypodontia.

Conclusions

In both target genes *PAX9* and *MSX1*, no mutations were detected within all exons. Instead, two single nucleotide polymorphisms (SNP) were identified in exon 3 of *PAX9*. The SNPs are silent substitutions or synonymous codons, C>T, rs12881240 (His239), and missense substitution of G>C, rs4904210 (Ala240Pro) were found in both the case and control groups. Although several studies attribute the risk of non-syndromic hypodontia to polymorphisms in *PAX9*, no association was established in variants of

rs4904210. Furthermore, no clear evidence was found that Ala240Pro contributes to or influences the risk of occurrence of non-syndromic hypodontia.

However, variants of rs12881240 may contribute to the non-syndromic hypodontia phenotype due to the significant differences between the case and the general control population. Thus, it can be concluded that in this population, variants of rs12881240 contributing to non-syndromic hypodontia and mild hypodontia is identified as the most common form of tooth agenesis, with the lateral incisor being the most common missing tooth.

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Declaration of Interest

The authors declare no conflict of interest in this study.

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