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Dental Anomalies and Muscle Segment Homeobox1 Gene Polymorphism in Non-syndromic Cleft Lip with or without Palate Children

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KEYWORDS Cleft lip, Cleft palate, Hypodontia, Mutation, Polymerase chain reaction

ABSTRACT

This study aims to determine the prevalence of dental anomalies and *MSX*1 gene 799G>T polymorphism and its association with non-syndromic cleft lip with or without palate (NSCL±P) attending Hospital Universiti Sains Malaysia. Clinical and radiological assessments on 37 NSCL±P patients and 80 non-cleft children were done to detect dental anomalies. The buccal cells were collected and polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) was used to identify polymorphism. NSCL±P was higher among males (54%) and mostly unilateral cleft lip and palate (51.3%). The prevalence of dental anomalies in morphology in NSCL±P was 18.9% (95% CI: 5.7%, 32.2%) and non-cleft was 6.3% (95% CI: 0.8%, 11.7%). Hypodontia in NSCL±P was 75% (95% CI: 61.2%, 90.2%) and non-cleft was 7.5% (95% CI: 1.6%, 13.4%). There was a significant association between NSCL±P and anomalies in morphology (*P*= 0.04; OR=3.5)) and number (*P*< 0.01; OR= 40). There was an absence of rare 799G>T polymorphism in all NSCL±P and non-cleft children indicating that all samples contain common 799G polymorphism. In conclusion, the prevalence of dental anomalies in morphology and number was significantly higher in NSCL±P compared to non-cleft children. However, it was not significantly associated with *MSX1* 799G>T polymorphism.

INTRODUCTION

Non-syndromic cleft lip with or without cleft palate (NSCL±P) is one of the most common congenital disabilities worldwide that affects the lip and palate [1] and affects facial appearance and teeth development, function and aesthetics [2]. The most common dental anomaly in cleft children is hypodontia [3] while the peg-shaped tooth is described as the most common morphological anomaly [4,5]. The co-occurrence of NSCL±P and hypodontia were reported frequently in humans, which may parallel to the sharing or similar developmental mechanism and genetic risk [5]. These anomalies take place during various stages of teeth development.

Many studies have reported that NSCL±P was due to genetic and/or environmental factors [6]. Various studies showed that tooth agenesis observed in NSCL±P was associated with the mutation of muscle segment homeobox1 (*MSX1)* gene [7]. 799G>T is one of the variations for the *MSX1* gene [7].

In Malaysia, there is no known study done on the prevalence and association between dental anomalies and MSX1 799G>T polymorphism in NSCL±P and non-cleft children. Therefore, the study was conducted to investigate the prevalence of dental anomalies among NSCL±P children and to screen the mutational of MSX1 799G>T polymorphism among NSCL±P and non-cleft children. Understanding these anomalies would help in planning for a better management of NSCL±P and non-cleft children since presence of various dental anomalies would lead to various dental issues such as occlusal vertical dimension [8] aesthetic and malalignment of teeth [9]. Thus, early prevention of complications due to dental anomalies can be part of the prevention strategies

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and genetic counselling during the management of CL±P children in the multidisciplinary clinic.

MATERIALS AND METHODS

This study was approved by the Human Research Ethics Committee of the Universiti Sains Malaysia (Reference No: USM/JEPeM/140357) and informed consent was obtained prior to the data collection. A comparative cross-sectional study was conducted on the registered NSCL±P children aged 7-13 years old from the Combined Multidisciplinary Clinic, Hospital Universiti Sains Malaysia (Hospital USM) while non-cleft children without a history of cleft from the outpatient dental clinic as a comparative group. The patients who had cleft palate only were excluded from the study. The sample size was calculated by single proportion formula and 51 NSCL±P children were selected by using simple random sampling whereby 103 non-cleft children in the comparison group were selected using systematic random sampling. The information on socio-demographic status and types of NSCL±P were obtained. Clinical oral examination was done to check for dental anomalies in terms of tooth morphology; peg-shaped, fused, geminated, dens evaginatus (DE) and dens invaginatus (DI) teeth and number; supernumerary or hypodontia. The orthopantomogram (OPG) was then taken to confirm the presence of dental anomalies radiographically.

Genomic DNA was extracted from Buccal cells from the cheek cells according to the protocol of the manufacturer (GeneAll Biotechnology Co. Ltd, Korea). Firstly, the swab was placed firmly in sterilized 1.5 ml microcentrifuge tube. Then, 400 µl of 1 x PBS was added to the tube then vortexed and spun shortly. 20 µl of Proteinase K and 400 µl of buffer BL were then added to the tube containing the sample. After incubating, at 56^{o}C for one hour, 400 µl of ethanol was added and mixed by vortexing. Subsequently, the mixture was transferred to a spin vacuum (SV) column, then 600 µl of buffer BW and 700 µl of buffer TW were added and centrifuged for 1 min at 8000 rpm. Finally, 50 µl AE buffer was added and incubated at a room temperature for 5 min then was centrifuged at 13000 rpm for 1 min, and extracted genomic DNA was kept at -20^{o}C freezer. The polymerase Chain Reaction (PCR) was carried out by amplifying the genomic DNA using one set of primer for the exon 2 of the *MSX1* gene (Fw: 5´GGCTGATCATGCTCCAATGC3´and Rv: 5'CAGGAAACAGCTATGACCCTGGAAGGGGCCAGAG GCTC3´ (Reverse mutagenesis primer). The amplification was performed in a total volume of 50 µl, containing 50 ng of genomic DNA, Dream Taq PCR Master Mix (2X), 50% DMSO and Nuclease free water. PCR was performed by utilizing a thermal cycler machine (Bio-Rad, USA). The PCR condition were as follows: initial denaturation for 10 minutes at 95^{o}C continued by 35 cycles of denaturing at 95^{o}C for 90 seconds, annealing at 60.3^{o}C for 90 seconds, extension at 72^{o}C for 90 seconds and final extension at 72^{o}C for 5 minutes. The PCR product in size 448 base pairs (bp) was then electrophoresed on 1% agarose gel to confirm the presence of the amplicon. PCR products were purified by using a commercial PCR purification kit (Qiagen, Germany). The 448bp products of *MSX1* obtained were digested with FastDigest DdeI (HpyF3I) restriction endonuclease (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. DNA sequencing was performed to validate the genotyping *MSX1* and PCR Restriction Fragment Length Polymorphism (RFLP) assay. Purified PCR products were sequenced using the same primers as that described in the PCR amplification and compared with the reference sequence available in NCBI GenBank (AF426432) database using nucleotide BLAST (BLASTn) program.

SPSS version 22.0 statistical software was used for data entry and data analysis. The prevalence of dental anomalies and mutation were calculated at 95% confidence interval (CI). Simple logistic regression followed by multiple logistic regression controlling for gender were done to determine the association between the study factor (NSCL±P versus non-cleft) and categorical outcome (dental anomalies and *MSX1* polymorphism). The Receiver Operating Characteristic (ROC) curves for the area under the curve and the classification table for the sensitivity, specificity and correctly classified were also obtained in order to evaluate the model fitness. The *P* value was set as significant at $P<0.05$.

RESULTS

A total of 37 NSCL±P and 80 non-cleft children participated in this study. Males outnumbered females in unilateral cleft lip and palate (UCLP), 68.4%; females outnumbered males in bilateral cleft lip and palate (BCLP) and cleft lip (CL), 55.6% and 66.7% respectively. It was found that UCLP was the most common type of cleft (51.3%) and mostly presented on the left side (32.4%).

The prevalence of morphological anomalies among NSCL±P children was 18.9% (95% CI: 5.7%, 32.2%) compared to non-cleft children which was 6.3% (95% CI: 0.8%, 11.7%), and for numerical anomalies

was 75.0% (95% CI: 61.2%, 90.2%) among NSCL±P and 7.5% (1.6%, 13.4%) among non-cleft children. As shown in Table 1 all the morphological anomalies were contributed by peg-shaped teeth that occurred 33.3% in BCLP, 11.1% in CL and only 1.3% in non-cleft children. DE was only found among non-cleft children (3.8%) and UCLP (5.5%). There were no other types of morphological anomalies and supernumerary teeth detected among the children in this study. Table 2 shows the results of multiple logistic regression. The morphological and numerical anomalies of the teeth (outcome variable) were significantly associated with NSCL±P children; *P*= 0.044 and *P* <0.001 respectively after controlling for gender. The risk for having anomalies in morphology was 3.5 times and in number of teeth was 40 times more in NSCL±P children than non-cleft children.

Table 1 Distribution of dental anomalies between NSCL±P and non-cleft children (n =117)

	Non-cleft (n=80)	**NSCL±P (n=37)**		
Variables		**UCLP (n=19)**	**BCLP (n=9)**	**CL (n=9)**
	Freq (%)	**Freq (%)**	**Freq (%)**	**Freq (%)**
Morphology				
Normal	75 (93.7)	16 (84.2)	6(66.7)	8 (88.9)
Peg shaped	1 (1.3)	2 (10.5)	3 (33.3)	1 (11.1)
Dens evaginatus	4 (5.0)	1(5.3)	-	-
Number				
Normal	74 (92.5)	4 (21.1)	2 (22.2)	3 (33.3)
Hypodontia	6 (7.5)	15 (78.9)	7 (77.8)	6 (66.7)
supernumarary	-	-	-	-

Table 2 Association between NSCL±P and dental anomalies (morphology, number) by using Multiple Logistic Regression

Variable	**NSCL±P Freq (%)**	**Non- cleft Freq (%)**	**Adjusted OR (95%CI)**	**LR statistic (df)**	***p*-value**
Morphology[a]					
Normal	30 (28.6)	75 (71.4)	1.00		
Abnormal	7 (58.3)	5 (41.7)	3.5 (1.03, 11.97)	4.102(1)	0.004
Gender[b]					
Male	54.1	40 (50.0)	1.00	0.031(1)	0.86
Female	45.9	40 (50.0)	1.12 (0.33, 3.78)		
Hypodontia[c]					
Normal	9 (10.8)	74 (89.2)	1.00	40.2 (1)	<0.001
Abnormal	28 (82.4)	6 (17.6)	39.9 (12.77, 124.58)		
Gender[d]					
Male	54.1	40 (50.0)	1.00	0.04 (1)	0.52
Female	45.9	40 (50.0)	1.5 (0.46, 4.51)		

The PCR amplified partial part of *MSX1* gene selected in this study as was shown in Figure 1(A). The size of PCR product was 448bp. After obtaining the PCR products of *MSX1* gene, it was digested by using the specific restriction enzyme Dde1. Figure 1(B) shows the 448bp PCR products were incompletely digested only on two restriction sites into three specific bands of 220, 150bp and negative control band. Thirty NSCL±P samples and sixty samples of non-cleft children were sequenced to identify *MSX1* gene 799G>T (Figure 2(A)). The sequencing results were analyzed and compared with the reference sequence available in NCBI GenBank (AF426432) database using the nucleotide BLAST (BLASTn) program (Figure 2(B)). However, the results have shown an absence of rare 799G>T polymorphism in all NSCL±P and non-cleft children indicating that all samples contain common 799G polymorphism.

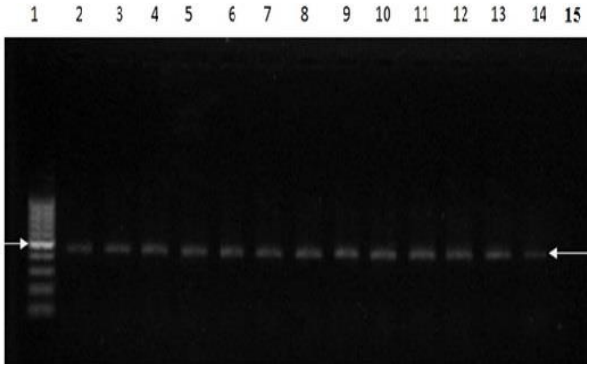

Figure 1(A) Image of agarose gel electrophoresis (1%) of PCR products of amplified muscle segment homeobox1 (MSX1) gene (448bp). Lane 1, 100bp ladder. Lane 2-14, PCR product 448bp. Lane 15, negative control.

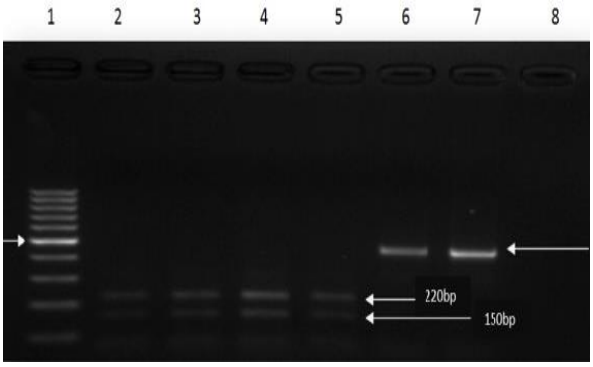

Figure 1(B) Image of agarose gel electrophoresis (1.5%). Lane 1, 100bp ladder. Lane 6-7, PCR product of amplified *MSX1* gene 448bp. Lane 2-5, digested PCR product by Dde1 220bp and 150bp. Lane 8, negative control.

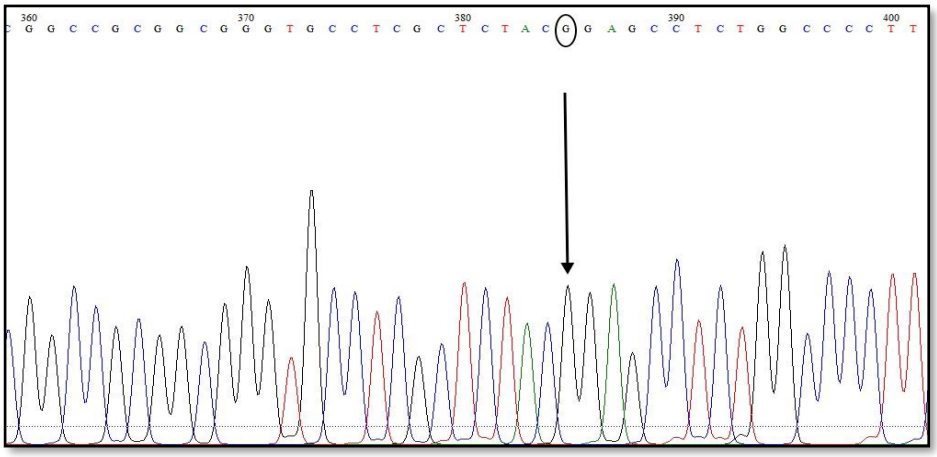

Figure 2(A) Image of DNA sequencing of PCR products for *MSX1* exon 2. Arrow shows the location of the G799 in *MSX1* gene.

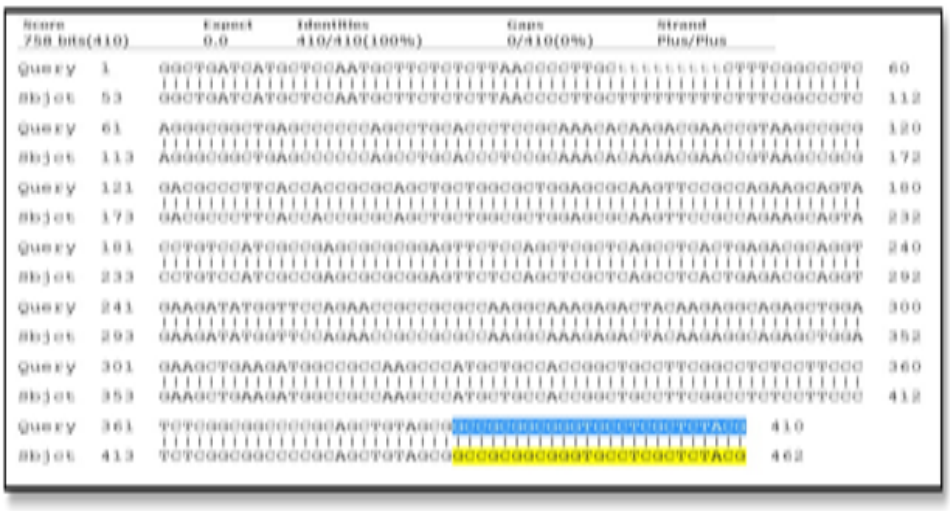

Figure 2(B) BLASTn result obtained from National Center of Biotechnology Information program (NCBI) for genotype (GG) at position 799 in *MSX1* exon 2.

DISCUSSION

In this study, the NSCL±P children were recruited from the Combined Multidisciplinary Clinic Hospital USM. Due to the time limitation and difficulties in obtaining cooperation, only 37 NSCL±P children from the Combined Multidisciplinary Clinic and 80 non-cleft children from the outpatient dental clinic Hospital USM were recruited in this study. The mean age of the NSCL±P children was ranged between 9-11 years. UCLP (51.3%) was the most common type of the cleft, which was in agreement with other study reported that UCL/P represent 65.7% of individuals with cleft lip/ palate in Berlin [10].

In the present study, there was a significant association between NSCL±P children and morphological anomalies (*P*= 0.04) which was consistent with several previous studies [4,11,12]. The risk of having morphological anomalies of teeth in NSCL±P children was 3.5 times higher than in non-cleft children. A disturbance in the formation of the epithelium and mesenchyme during the process of odontogenesis can be manifested by atypical forms of dental development [13]. In terms of types of NSCL±P, BCLP in this current study was shown to have higher morphological anomalies (33.3%) compared to UCLP (15.8%) and only 11.1% in CL children. This result supports the fact that the increased proportion of dental anomalies was associated with the severity of the cleft [14].

A peg-shaped upper lateral incisor was observed as the second most common dental anomaly. The prevalence of peg-shaped tooth in BCLP (33.3%) was greater than UCLP (15.8%), which was in agreement with other reports in Turkey [11,15]. The previous study reported that the presence of peg-shaped tooth might associated with genetic or endocrinal disturbances such as pituitary dysfunctions [16,17].

DE teeth in UCLP children (5.5%) and in non-cleft children (3.8%) were higher than the previous study found in Chinese CLP patients (1.3%) and no DE was detected in non-cleft children [18]. Genetic factors that affect the morphogenesis and patterning of the tooth includes p63 gene and tumor necrosis factor signaling pathway. This is an essential regulator of epithelial morphogenesis of tooth germ which establishes tooth shape, particularly the typical cusp [19]. The finding was consistent with the previous study that showed the DE commonly presented at left UCLP [13]. In this study, there were no DI teeth found in our NSCL±P children, which was similar to another study [18]. However, the contrary finding in other studies found DI tooth in UCLP sides [11].

In terms of number, hypodontia had been shown as the most common dental anomalies in NSCL±P children, similar to previous studies of the different populations [14,20,21]. The risk of getting hypodontia is approximately 40 times among NSCL±P than non-cleft children. The discrepancy in the prevalence among different populations may be related to environmental, genetic factors and differences in methodological scales [22]. Missing teeth might be due to the destruction of the tooth germ of the permanent teeth, during cleft repair surgery that was done at the early age. The previous study found that the surgery for the closure of the hard palate in early infant is the most significant etiological factor for the missing of teeth outside the cleft area in the primary operated cleft patient. It was contrary with some published paper which based on the hypothesis that congenital factor causes the cleft that also responsible for hypodontia [23].

A significant association was found in this study between hypodontia and NSCL±P children (p<0.001), in agreement with previous studies [24,25]. However, findings from our study showed that, no rare polymorphism 799G>T was identified in NSCL±P and non-cleft children. Previous research has indicated that only 2% of *MSX*1 gene related to NSCL±P [26]. The presence of 799G>T is considered very rare. This finding is slightly similar to the one reported in Nepalese, where among 40 cleft patients, only one patient was identified with heterozygous 799G>T polymorphism which could be concluded that 799G>T is not considered as vital among NSCL±P [27]. Jyothish et. al [28] however concluded in their study that *MSX1* 799G>T can be considered as one of the genetic factors in the formation of NSCL±P.

CONCLUSIONS

The prevalence of dental anomalies in children with NSCL±P was higher than non-cleft children. There was a significant association between dental anomalies in number and morphology and NSCL±P children. Only wild homozygous genotype (GG) was identified in this study and *MSX1* 799G>T polymorphism was not significantly associated with cleft and hypodontia.

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DECLARATION OF INTEREST

Authors declare no conflict of interest.

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