Human Mesiodens Is Associated with a Synonymous Variant in the *ACVR2A* Gene

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앞으로 이러한 성취의 기쁨과 감사의 마음을 가까운 사람들과 저를 필요로 하는 분들께, 사랑과 도움으로 돌려드리겠습니다. 다시 한번 감사드립니다.

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Human Mesiodens Is Associated with a Synonymous Variant in the *ACVR2A* Gene

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Supernumerary teeth mean additional teeth in addition to the normal number of teeth. Mesiodens are supernumerary teeth located arround the maxillary central incisors in the median region of the palate. The etiology of mesiodens has not been clearly identified. Studies on the genes responsible for human supernumerary teeth have been conducted, but no studies have identified the genes responsible for nonsyndromic and nonfamilial human mesiodens. The purpose of this study is to identify possible causative genes associated with nonsyndromic and nonfamilial human mesiodens.

Among the patients who visited Yonsei university dental hospital, 96 cases and 83 controls

were selected as subjects. Subjects collected their own saliva samples and DNA was extracted from saliva samples. The targeted panel consisted of 101 target genes related to tooth development. Targeted sequencing of this panel was initially performed on a discovery set (39 cases and 27 controls); association tests were performed after genotyping of 9 selected variants in a validation set (57 cases and 56 controls).

The association test confirmed that among these 9 variants, *ACVR2A* (rs1128919), a synonymous variant, was identified as being significant. Moreover, in silico analysis was performed and demonstrated the instability of ACVR2A mRNA with the G allele being *ACVR2A* (rs1128919).

This study suggests that human mesiodens is associated with *ACVR2A* (rs1128919), a synonymous variant. Through the research conducted in this study, there are implications of a decrease in the expression of ACVR2A which is associated with the formation of human mesiodens.

Keywords: human mesiodens, synonymous variant, *ACVR2A* (rs1128919)

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I. Introduction

Supernumerary teeth are teeth that appear in addition to the normal dentition (Garvey, et al., 1999). Mesiodens are the most common supernumerary teeth located between the maxillary central incisors. They occur more frequently in males than in females (Gündüz, et al., 2008). The etiology of mesiodens has not been clearly identified.

Some syndromes are associated with multiple supernumerary teeth. Those that occur in familial adenomatous polyposis such as Gardner syndrome are associated with mutations in the *APC* gene, and supernumerary teeth in cleidocranial dysplasia are associated with

mutations in the *RUNX2* gene (Galiatsatos and Foulkes, 2006; Groden, et al., 1991; Mundlos, et al., 1997; Wang, et al., 2009). In Nance-Horan syndrome, Robinow syndrome, and Tricho-Rhino-Phalangeal syndrome, supernumerary teeth are associated with mutations in *NHS*, *ROR2*, and *TRPS1*, respectively (Burdon, et al., 2003; Kantaputra, et al., 2008; Mazzeu, et al., 2007; Momeni, et al., 2000; van Bokhoven, et al., 2000).

A recent study of familial multiple supernumerary teeth without other syndromes has suggested an association with the *PDGFRB* gene (Bae, et al., 2017). However, that study focused on familial supernumerary premolars, not mesiodens (Bae, et al., 2017). In another recent study, Kim et al. showed that cooperative interactions among genes related to the BMP, SHH, and WNT signaling pathways are involved in the formation of nonsyndromic and nonfamilial human mesiodens (Kim, et al., 2017). However, specific genes were not identified. Lei, et al. (Lei, et al., 2011) investigated the potential involvement of the *PAX6* gene, which is related to supernumerary teeth in the upper incisor region of mice and rats (Kriangkrai, et al., 2006; Quinn, et al., 1997), with nonsyndromic and nonfamilial human mesiodens in 20 Chinese children, but found no explanatory associations.

No previous studies have identified possible causative genes for nonsyndromic and nonfamilial human mesiodens. The purpose of this study was to identify possible causative genes associated with human mesiodens.

II. Materials and methods

1. Subject population

The subjects were Korean patients who visited Yonsei university dental hospital. Subjects with mesiodens (cases) and subjects without mesiodens (controls) were enrolled separately. Having a parent or sibling with supernumerary teeth was an exclusion criterion. None of the subjects had specific systemic or genetic disorders. Mesiodens was diagnosed by oral and radiographic examination with panoramic and periapical radiographs. Controls were randomly enrolled. The absence of mesiodens was diagnosed by oral and radiographic examination with panoramic radiographs.

This study was approved by the institutional review board of Yonsei university dental hospital (Yonsei IRB No. 2-2014-0036). This study met the guidelines of the Declaration of Helsinki. All subjects, or subjects' parents or guardians provided informed consent.

2. Sample collection and DNA extraction

Subjects collected their own saliva samples using an Oragene DNA self-collection kit 5G-600 (Genotek, Ottawa, Ontario, Canada). Genomic DNA was extracted from saliva samples using QuickGene-Mini80 (FUJIFILM, Tokyo, Japan). These procedures were performed according to the manufacturer's instructions.

3. Procedure

The total sample sizes were 96 cases and 83 controls. Candidate variants were selected based on targeted sequencing of a discovery set comprising the first 39 cases and 27 controls. Then, an additional 57 cases and 56 controls (the validation set) were collected. After targeted sequencing, genotyping for the selected variants was performed on the validation set and an association test was performed. This process is described in the flow chart in Figure 1. Sample sizes of each sample set are described in Table 1.

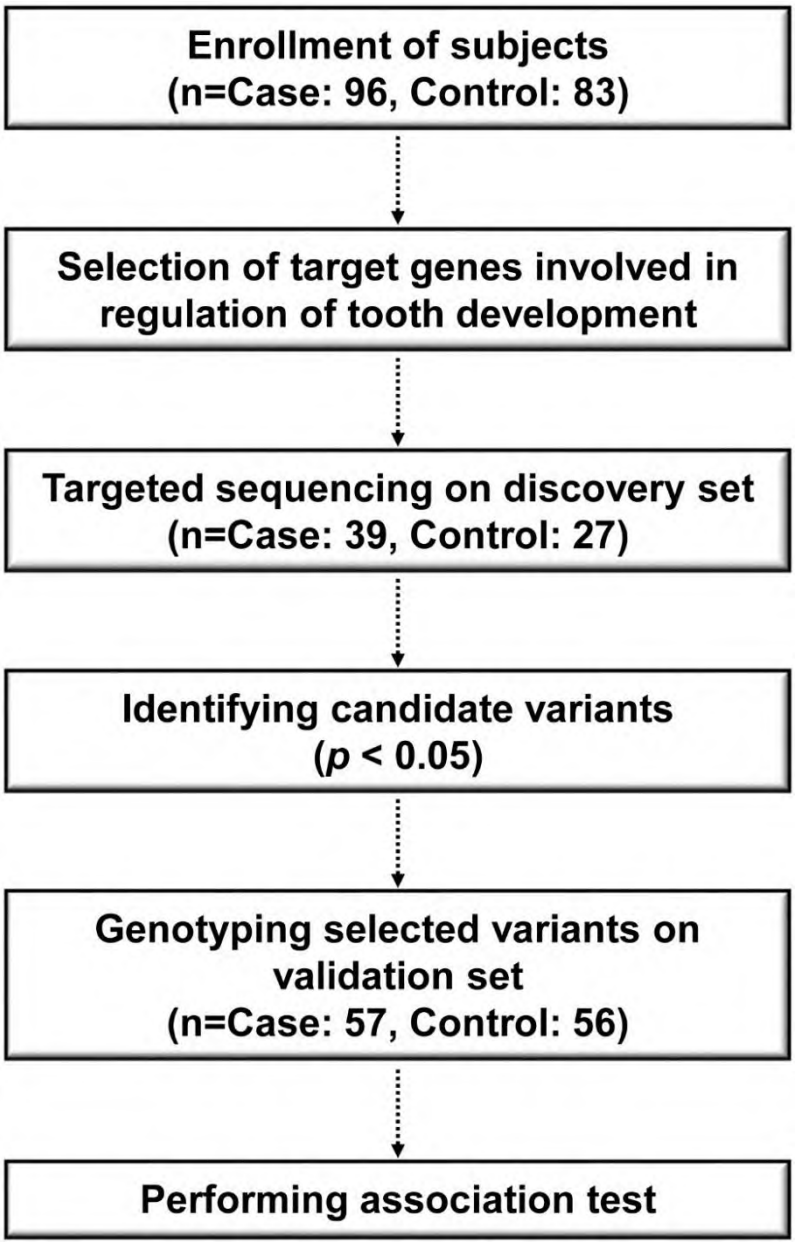

Figure 1. Flow chart of the process from enrollment of subjects to association testing.

Table 1. Sizes of each sample set.

	Case group	Control group	Total
Total saliva samples*	96	83	179
Discovery set**	39	27	66
Validation set†	57	56	113

*Total saliva samples include both the discovery set and validation set.

**The discovery set is the initial sample set used for next-generation sequencing.

†The validation set is the set collected after the discovery set for genotyping.

4. Identifying variants through targeted next-generation sequencing of the discovery set

First, 101 related target genes were selected. The existing targeted sequencing panel (Kim, et al., 2017), which is a set of genes and related genes that are important for tooth development, was used. Targeted next-generation sequencing (**NGS**) of the 101 target genes was performed for the discovery set (39 cases and 27 controls). Solution-based hybridization for DNA enrichment and sequencing was performed. Using an association test, variants that were associated in targeted NGS were sorted and prioritized; a total of 11 variants were associated. The association test was performed using SAS version 9.1.3 (SAS Institute Inc., Cary, NC, USA).

5. Genotyping selected variants on validation set

After targeted sequencing, genotyping for the selected 11 variants was performed on an expanded sample set of 57 cases and 56 controls (the validation set). SNaPshot assay was performed using single base extension technology with the ABI PRISM SNaPShot Multiplex kit (ABI, Foster City, CA, USA) according to the manufacturer's instructions. Genotyping analysis was carried out using Genemapper software (version 4.0; Applied Biosystems, Carlsbad, CA, USA).

6. Association test

After genotyping of the 11 variants, an association test was performed on 9 variants excluding 2 variants with a minor allele frequency of zero using SAS version 9.1.3 (SAS Institute Inc., Cary, NC, USA). The frequency of each genotype, the DNA sequence at specific positions indicated by reference SNP ID numbers for 9 variants, was investigated. *P*-values, odds ratios, and 95% confidence intervals for the 9 variants were calculated. This analysis was performed on allele as well as 3 genetic models: dominant, recessive, and codominant models (Lee, et al., 2011; Lewis, 2002).

III. Results

1. Baseline characteristics of all subjects and the subjects in the validation study

Baseline characteristics of all subjects and the subjects in the validation study (i.e., genotyping) are summarized in Tables 2 and 3, respectively. The mean age and age range of all subjects were 7.2 years, 4-14 years in the case group, and 9.7 years, 5-27 years in the control group, respectively (Table 2). The male to female sex ratio among all subjects was 3.6 : 1 in the case group, and 1.2 : 1 in the control group (Table 2). Chi-squared test was used to show difference in sex ratios between cases and controls at significance level 0.05 (Table 2).

The mean age and age range of the genotyping subjects were 7.2 years, 4-14 years in the case group and 9.2 years, 5-18 years in the control group, respectively (Table 3). The male to female sex ratio in genotyping subjects was 2.4 : 1 in the case group, and 1.2 : 1 in the control group (Table 3).

Table 2. Baseline charactieristics of all subjects.

	Case group	Control group	Total	*P*-value*
Number of subjects	96	83	179	
Sex ratio (male to female)	3.6 : 1	1.2 : 1		0.001
Mean age (years)	7.2	9.7		
Age range (years)	4 – 14	5 – 27		

*Chi-squared test was used to show the difference in sex ratios (statistical significance at the $p < 0.05$ level).

Table 3. Baseline characteristics of subjects in the validation study*.

	Case group	Control group	Total
Number of subjects	57	56	113
Sex ratio (male to female)	2.4 : 1	1.2 : 1	
Mean age (years)	7.2	9.2	
Age range (years)	4 – 14	5 – 18	

*The validation study refers to genotyping.

2. Association test on the 9 variants and prediction of mRNA structure

The results of the final association test on the 9 variants are summarized in Table 4. The association test showed that *ACVR2A* (reference SNP ID number: rs1128919) was associated with differences between cases and controls (Table 4). The reference SNP ID number indicates the position of the corresponding single nucleotide polymorphism. That is, this test showed that the significant differences between cases and controls were associated with a single nucleotide polymorphism of the A or G allele in *ACVR2A* (rs1128919) (Table 4). Specifically, cases were associated with the G allele in *ACVR2A* (rs1128919) (Table 4). However, this was a synonymous variation in which the amino acid did not change whether there was an A or G allele. Thus, for better understanding of the function of *ACVR2A*, the mRNA secondary structure was predicted using the Vienna RNA v.1.8.4 software package (Figure 2).

Prediction of the mRNA secondary structure indicated that the structure and free energy of mRNA varied depending on the presence of an A or G allele at c.354, the coding sequence corresponding to *ACVR2A* (rs1128919) (Figure 2). The free energy of mRNA affects expression of the protein (Duan, et al., 2003; Hunt, et al., 2009; Lee, et al., 2011); higher mRNA free energy is associated with lower mRNA stability, which means that the rate of mRNA degradation is faster and thus the expression of its associated protein is lower. Conversely, lower mRNA free energy results in higher protein expression. The free energy of ACVR2A mRNA with the G allele at c.354 (-381.37 kcal/mol) was higher than that with

the A allele at c.354 (-384.52 kcal/mol), while stability and expression of ACVR2A mRNA with the G allele at c.354 was lower than that with the A allele at c.354 (Figure 2). Thus, cases were associated with lower stability and expression of ACVR2A mRNA with the G allele at c.354 (Table 4 and Figure 2).

Table 4. Genetic assocations in the replication cohort for 9 SNPs* in loci observed in targeted NGS*.

Gene (rs no.)	Genotype†	Group		*P*-value** / OR* (95% CI*)			
		Case, n (%)	Control, n (%)	Dominant‡	Recessive‡	Codominant§	Allele‖
ACVR2A (rs1128919)	AA	10 (17.54)	19 (33.93)	**0.05**	**0.02**	**0.02**	**0.01**
	AG	32 (56.14)	32 (57.14)	2.41 (1.00-5.81)	3.64 (1.22-10.85)		1.99 (1.17-3.38)
	GG	15 (26.32)	5 (8.93)				
PAX9 (rs12881240)	CC	35 (61.40)	30 (53.57)	0.40	0.74	0.74	0.40
	CT	18 (31.58)	21 (37.50)	0.73 (0.34-1.53)	0.77 (0.20-3.03)		0.77 (0.42-1.41)
	TT	4 (7.02)	5 (8.93)				
PTCH1 (rs1805155)	TT	44 (77.19)	44 (78.57)	0.86	1	1	0.71
	TC	12 (21.05)	12 (21.43)	1.08 (0.45-2.64)			1.17 (0.51-2.65)
	CC	1 (1.75)	0 (0.0)				
ACVR1 (rs2227861)	TT	47 (82.46)	47 (83.93)	0.83	1	0.83	0.84
	TC	10 (17.54)	9 (16.07)	1.11 (0.41-2.98)			1.10 (0.43-2.82)
	CC	0 (0)	0 (0)				

TBX1	CC	43 (75.44)	37 (66.07)	0.27	1	0.25	0.42
	CT	13 (22.81)	19 (33.93)				
(rs2301558)				0.63 (0.28-1.44)			0.74 (0.36-1.55)
	TT	1 (1.75)	0 (0)				
PDGFRB	AA	45 (78.95)	44 (78.57)	0.96	1	0.96	0.96
	GA	12 (21.05)	12 (21.43)				
(rs246395)				0.98 (0.40-2.41)			0.98 (0.42-2.29)
	GG	0 (0)	0 (0)				
MET	AA	53 (92.98)	53 (94.64)	0.71	1	1	0.72
	AG	4 (7.02)	3 (5.36)				
(rs33917957)				1.33 (0.29-6.25)			1.32 (0.29-6.04)
	GG	0 (0)	0 (0)				
MET	CC	53 (92.98)	53 (94.64)	0.71	1	1	0.72
	CT	4 (7.02)	3 (5.36)				
(rs35775721)				1.33 (0.29-6.25)			1.32 (0.29-6.04)
	TT	0 (0)	0 (0)				
EPHB3	GG	39 (68.42)	40 (71.43)	0.73	0.99	0.92	0.76
	GA	17 (29.82)	15 (26.79)				
(rs9881589)				1.15 (0.52-2.58)	0.98 (0.06-16.10)		1.12 (0.55-2.28)
	AA	1 (1.75)	1 (1.78)				

*SNPS=single nucleotide polymorphisms; NGS=next-generation sequencing; OR=odds ratio; CI=confidence interval.

**_P_-values were obtained using the chi-square test or Fisher's exact test (expected cell value < 5). Bold and underlined values denote statistical significance at the $p < 0.05$ level.

†Genotype is the DNA sequence at specific positions indicated by reference ID numbers for 9 SNPs.

‡Dominant and recessive models of genetic associations were based on minor alleles. The minor allele sequence is underlined in each position.

§_P_-values were obtained through 2 by 3 analysis in a codominant model.

||In addition to 3 genetic models, allele analysis was further performed based on minor alleles.

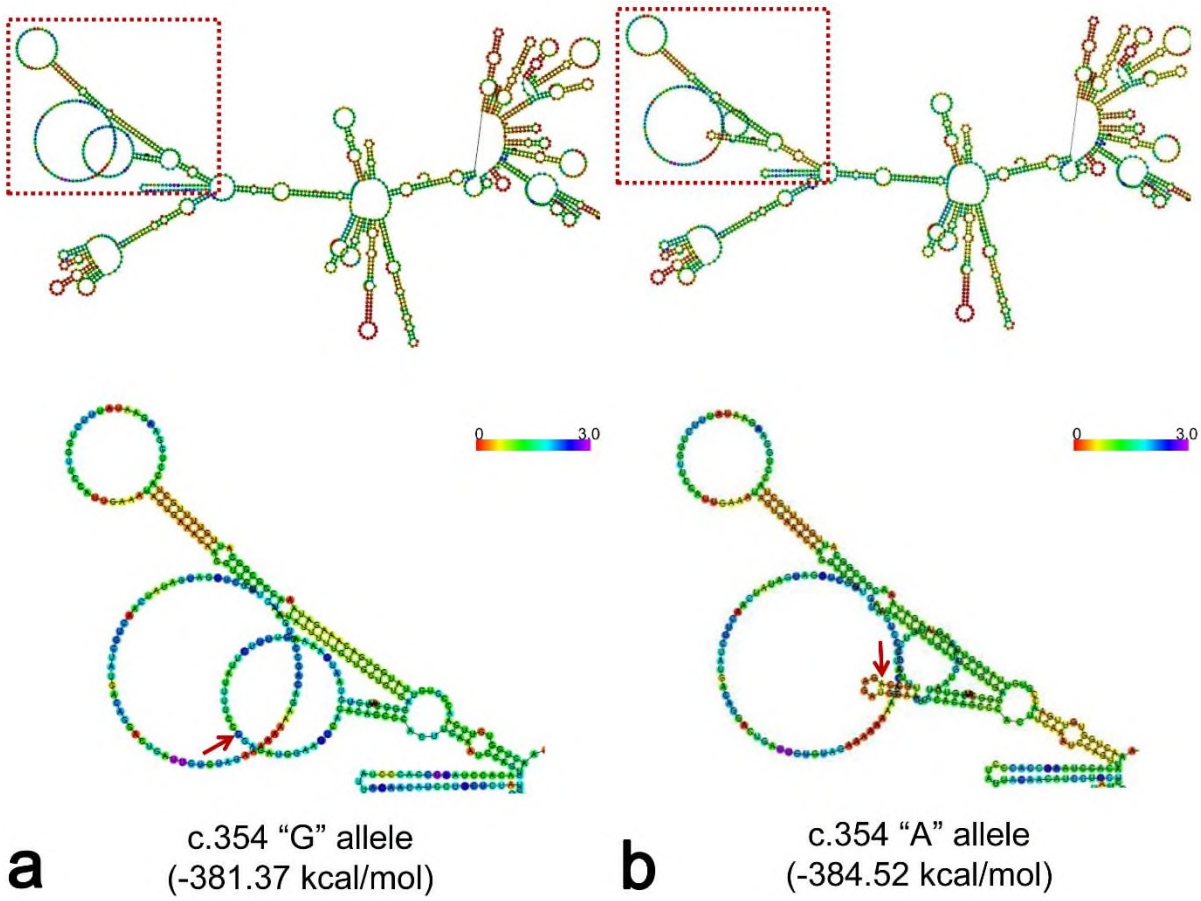

Figure 2. mRNA structure according to the rs1128919 (*ACVR2A*, c.354G>A) genotype. The figures below are enlargements of the red dotted boxes in panels (a) and (b). The energies in panels (a) and (b) are free energies in the secondary structure of ACVR2A mRNA according to the c.354 G or A allele. Red arrows indicate the location of the c.354 G and A alleles. Base pair probabilities calculated by the web server are color coded 0-3, with higher numbers corresponding to higher confidence. Note the lower stability of ACVR2A mRNA with the c.354 G allele.

IV. Discussion

The results of this study suggest that the formation of human mesiodens is associated with a synonymous variation in *ACVR2A* (rs1128919). Based on these results, the *ACVR2A* (rs1128919) variation does not alter the structure or morphology of the protein involved in this gene, but changes protein expression depending on mRNA stability (Figure 2). This implies that formation of human mesiodens can be induced by changes in ACVR2A expression.

ACVR2A encodes the activin type IIA receptor protein (**ACVR2A**) involved in odontogenesis (Ferguson, et al., 1998; Heikinheimo, et al., 1998; Kwon, et al., 2017). Related to this, bone morphogenic protein (**BMP**) is a ligand that binds TGF-β type II receptor including BMP type II receptor (**BMPR2**) and ACVR2A (Manero, 2013; Olsen, et al., 2015). BMP signaling activity induces odontogenesis via epithelial-mesenchymal tissue interaction (Tucker, et al., 1998; Vainio, et al., 1993). BMP binds to TGF-β type II receptors including BMPR2 and ACVR2A in the cell membrane, stimulates an intracellular signaling pathway called the SMAD (small mother against decapentaplegic) pathway, while activin competing with BMP works in opposition after binding with ACVR2A (Manero, 2013; Olsen, et al., 2015). That is, a decrease in ACVR2A may further activate BMP-induced odontogenesis. Therefore, this study suggests that a synonymous variation from the A to the G allele in *ACVR2A* (rs1128919) may activate BMP signaling by

decreasing expression of ACVR2A, leading to mesiodens formation.

Animal studies have confirmed that BMP and its antagonist are involved in the formation of mesiodens. Munne, et al. (2009) reported that supernumerary incisors were formed by increased WNT and BMP in mice with a Sostdc 1 deficiency as a BMP antagonist. Murashima-Suginami, et al. (2007) reported that the deficiency of USAG-1, an antagonist of BMP, inhibited the apoptosis of odontogenic-mesenchymal cells and caused the formation of supernumerary incisors. In this regard, Murashima-Suginami, et al. (2008) have demonstrated that enhanced BMP signaling in USAG-1 deficient mice induces supernumerary tooth formation.

Expression of *ACVR2A* in the median region of the palate suggests the role of *ACVR2A* in the formation of mesiodens. The TGF-β2 signaling protein plays an important role in the fusion of midfacial processes during palatogenesis, and the TGF-β type II receptor, or a TGF-β2 binding receptor, is a serine/threonine kinase with a domain similar to the activin type IIA receptor (Behnan, et al., 2005). In Albertson et al.'s study of zebrafish, which have genes similar to humans, abnormal fusion of the maxillary process was observed in zebrafish deficient in acvr2a, which was found to be more severe than when acvr2b was deficient (Albertson, et al., 2005). Mutation in genes for the TGF-β receptor cause cleft palate (Iwata, et al., 2012; Shiang, et al., 1993). BMP signaling, which is related to ACVR2A, plays an important role in palatogenesis and cleft palate formation (Boyne, et al., 1998; Zhang, et al., 2002).

In this study, the prevalence of mesiodens was significantly higher in males than in females (Table 2). To verify that *ACVR2A* is a causative gene for mesiodens, the sex-based difference in the prevalence of mesiodens needs to be better understood (Rajab and Hamdan, 2002; Ramesh, et al., 2013). Welle, et al. (2008) and Braga, et al. (2012) have shown that the activin receptor is associated with sex differences. Lee, et al. (2005) reported that the rate of muscle increase in female mice is larger than that of male mice after mutations in activin type II receptors, suggesting the need for additional studies on these sex differences. Future functional studies are needed to identify differences in the expression of ACVR2A in the median region of the palate between males and females.

In this study, few samples were analyzed compared to the estimated mesiodens prevalence of 2.7-3.4% in the Asian population (Davis, 1987; Khandelwal, et al., 2011). Additional studies with larger sample sizes are needed. In addition, this study was limited to Korean subjects, and further studies on samples from diverse ethnic groups are needed to generalize these findings.

Additional functional studies of levels and the differences of ACVR2A expression between sexes in the median region of the palate and signaling pathways associated with formation of mesiodens at the molecular level may support the findings of this study.

V. Conclusion

This study suggests that the formation of human mesiodens is associated with a synonymous variation in *ACVR2A* (rs1128919). This study implies that there is a decrease in the expression of ACVR2A which is associated with formation of human mesiodens.

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국문요약

인간 정중 과잉치와 *ACVR2A* 유전자의 동의 변이와의 연관

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(지도교수 송 제 선)

과잉치는 정상 치아 수 외의 추가적인 치아를 의미한다. 정중 과잉치는 상악 정중 구개 부위의 상악 중절치 주위에 위치하는 과잉치이다. 정중 과잉치에 관한 병인은 아직 명확하게 규명되지 않았다. 지금까지 인간 과잉치의 원인 유전자에 관한 연구가 수행되었지만, 비증후군, 비가족력의 인간 정중 과잉치의 원인 유전자를 규명한 연구는 없었다. 본 연구의 목적은 비증후군, 비가족력 인간 정중 과잉치와 연관된 원인 유전자를 규명하는 것이다.

연세대학교 치과대학병원에 내원한 환자 중에서, 대조군 83명, 사례군

96명을 대상자로 선정하였다. 대상자의 타액 샘플을 자가 채취하고, DNA를 추출하였다. 표적 패널은 치아 발달과 관련된 101 개의 표적 유전자로 구성되었다. 우선, 발견 세트 (대조군 27 샘플, 사례군 39 샘플) 에서 표적 패널에 대해 표적 차세대 염기서열 분석이 수행되었고, 통계적으로 유의한 후보 변이들이 선택되었다. 이후 검증 세트 (대조군 56 샘플, 사례군 57 샘플) 에서 선택된 9 개의 변이들에 대해 유전형 분석한 후에 연관성 테스트가 수행되었다.

연관성 테스트 결과, 9 개 변이들 중, *ACVR2A* (rs1128919) 동의 변이가 통계적으로 유의한 것이 확인되었다. 또한, 인 실리코 분석을 수행하여 *ACVR2A* (rs1128919) 의 G 변이를 갖는 ACVR2A mRNA 의 불안정성을 입증했다.

본 연구는 인간 정중 과잉치가 *ACVR2A* (rs1128919) 동의 변이와 연관이 있다는 것을 보여준다. 이를 통해 본 연구는 ACVR2A 발현양의 감소가 인간 정중 과잉치 형성과 연관이 있다는 것을 제시한다.

핵심되는 말: 인간 정중 과잉치, 동의 변이, *ACVR2A* (rs1128919)