

# PAX9 polymorphism and susceptibility to sporadic non-syndromic severe anodontia: a case-control study in southwest China

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## ABSTRACT

Our research aimed to look into the clinical traits and genetic mutations in sporadic non-syndromic anodontia and to gain insight into the role of mutations of *PAX9*, *MSX1*, *AXIN2* and *EDA* in anodontia phenotypes, especially for the *PAX9*. Material and Methods: The female proband and her family members from the ethnic Han families underwent complete oral examinations and received a retrospective review. Venous blood samples were obtained to screen variants in the *PAX9*, *MSX1*, *AXIN2*, and *EDA* genes. A case-control study was performed on 50 subjects with sporadic tooth agenesis (cases) and 100 healthy controls, which genotyped a *PAX9* gene polymorphism (rs4904210). Results: Intra-oral and panoramic radiographs revealed that the female proband had anodontia denoted by the complete absence of teeth in both the primary and secondary dentitions, while all her family members maintained normal dentitions. Detected in the female proband were variants of the *PAX9* and *AXIN2* including *A240P* (rs4904210) of the *PAX9*, c.148C>T (rs2240308), c.1365A>G (rs9915936) and c.1386C>T (rs1133683) of the *AXIN2*. The same variants were present in her unaffected younger brother. The *PAX9* variations were in a different state in her parents. Mutations in the *MSX1* and *EDA* genes were not identified. No significant differences were found in the allele and genotype frequencies of the *PAX9* polymorphism between the controls and the subjects with sporadic tooth agenesis. Conclusions: These results suggest that the association of *A240P* with sporadic tooth agenesis still remains obscure, especially for different populations. The genotype/phenotype correlation in congenital anodontia should be verified.

**Key words:** *PAX9* transcription factor. Polymorphism. Non-syndromic Anodontia. Phenotype. Genotype.

## INTRODUCTION

Tooth agenesis is a common development anomaly in humans, which includes two types, syndromic or non-syndromic. The non-syndromic tooth agenesis with significant phenotypic variability has been classified as either sporadic or familial,

which can be inherited in an autosomal-dominant, autosomal-recessive, or X-linked mode<sup>7,32</sup>. Anodontia (OMIM 206780) is the term conventionally used in cases where teeth are completely absent<sup>19</sup>. Sporadic non-syndromic anodontia not involving associated abnormalities is extremely rare. Some case reports have suggested that anodontia of permanent teeth

is an expression of the homozygote state of the gene resulting in the pegged or missing maxillary incisor<sup>9,31</sup>. Genetic and environmental factors may be of etiologic importance to this anomaly<sup>3,15</sup>. Brook<sup>1</sup> (2009) considered that complex interactions between genetic, epigenetic and environmental factors caused dental anomalies during the long process of dental development, which is multi-factorial, multilevel, multidimensional and progressive over time. In other words, the phenotype often reveals variations between affected individuals in the same family as a result of the multi-directional role of genetic, epigenetic and environmental factors in space and time. A previous study<sup>30</sup> showed that mutations or polymorphisms of the *PAX9* (OMIM 167416), *MSX1* (OMIM 142983), *AXIN2* (OMIM 604425), and *EDA* (OMIM 300451)<sup>25</sup> genes were responsible for isolated/non-syndromic oligodontia. The reasons are still unknown for the majority of the congenital sporadic forms of missing teeth in humans. Gene-gene interactions may underlie a significant number of these cases. Numbers of studies have shown that mutations in the *PAX9* or *MSX1* cause non-syndromic oligodontia. Both the *PAX9* and *MSX1* are transcription factors expressed in the dental mesenchyme during the initiation of tooth development. As a member of the paired box domain gene family, *PAX9* regulates the cellular pluripotency and differentiation during embryonic patterning and organogenesis<sup>13,28</sup>. Its defects are associated with agenesis of the permanent molars, mostly including the maxillary first molars, maxillary second molars, and mandibular second molars<sup>32</sup>. *MSX1* belongs to the muscle segment homeobox family and is essential for the switch in odontogenic potential from the epithelium to the mesenchyme<sup>17,18</sup>. Mutations in the *MSX1* have been identified to be responsible for the missing second premolars and third molars. *AXIN2* (17q23-q24), which is known as a negative feedback regulator of the Wnt-signaling pathway, is expressed by the ectomesenchyme and regulates early organ differentiation and development. Mutations in the *AXIN2* lead to tooth agenesis affecting mostly the permanent teeth, containing the permanent molars, the lower incisors, and the upper lateral incisors in humans. The *EDA* gene (MIM #300451), encoding the protein ectodysplasin-A (*EDA*), is a type II transmembrane protein with a C-terminal TNF homology domain consisting of 10 predicted anti-parallel  $\beta$ -sheets linked by variable loops. It is identified associated with syndromic tooth agenesis that underlies X-linked hypodontia ectodermal dysplasia (XLHED; MIM #305100) and the mutation analysis of the *EDA* gene in people with isolated oligodontia were recommended in some studies<sup>4,8,26,33</sup>.

Until now, we do not have enough knowledge about the importance of genes in the etiology of

oligodontia in the Chinese population, although there have been some mutations identified, such as c.662C>A and c.347C>G of *MSX1* for non-syndromic autosomal-dominant oligodontia<sup>32</sup>, heterozygous C139T transition in *PAX9*<sup>34</sup>, and so on. Recently, two polymorphisms (rs2073244, rs2073246), which were located in the promoter region of the *PAX9* gene, were suggested to influence the risk of sporadic tooth agenesis in a Caucasian population<sup>24</sup>. However, it was reported that p.Ala240Pro in the *PAX9* might not be associated with tooth agenesis in a case-control study<sup>22</sup>. Therefore, whether the variants of *PAX9* have an effect on the tooth agenesis in different populations is still puzzling.

In the current study, we investigated the mutations in the *PAX9*, *MSX1*, *AXIN2* and *EDA* genes in a Chinese family and evaluated the association of gene variation and epigenetic factors with sporadic non-syndromic anodontia. Additionally, we performed genotyping analyses of a SNP (rs4904210) of the *PAX9* in subjects with sporadic tooth agenesis (cases) and 100 healthy controls in a Chinese population to test the hypothesis whether the variant A240P (rs4904210) of the *PAX9* gene is associated with susceptibility to tooth agenesis in the Chinese population.

## MATERIAL AND METHODS

All these subjects were fully informed of the study and consent was obtained from each of them. The research was approved by the Ethics Committee of West China Hospital of Stomatology, Sichuan University (2009022). All participants in the study were ethnic Han Chinese from Chengdu City or the surrounding regions in southwest China.

### Study individuals

The female proband from ethnic Han families was referred to the Department of Orthodontics, West China Hospital of Stomatology, Sichuan University (Chengdu, China). The pedigree of the family was constructed by extended interviews. Retrospective data were reviewed and panoramic radiographs were obtained to confirm the diagnosis of tooth agenesis for the proband. Panoramic radiographs were also performed for other family members to assess the dental development. These 4 subjects (including the proband) underwent complete oral examinations with a thorough clinical investigation of other ectodermal originated tissues, including the skin, hair, nails, sweat glands, ears and eyes.

### Case-control study

A total of 50 subjects with sporadic tooth agenesis (excluding the third molar) and 100 healthy controls were consecutively recruited from the West China Hospital of Stomatology, Sichuan

University (Chengdu, China) from October 2009 to November 2011. Retrospective data were reviewed and panoramic radiographs were obtained to confirm the diagnosis of tooth agenesis for the patients. Every participant underwent complete oral examinations with a thorough clinical investigation of other ectodermal originated tissues, including the skin, hair, nails, sweat glands, ears and eyes by an experienced doctor. The characteristics of the subjects were listed in Figure 1. The exclusion criteria were as follows: 1. with positive family histories; 2. with non-dental abnormalities; 3. with tooth agenesis resulted from acquired reasons, such as extraction, trauma, or others.

### Mutation screening and DNA sequencing

Peripheral blood samples were obtained. DNA was extracted from blood spots by the Chelex-100 (Bio-Rad Laboratories, Hercules, California, USA)

Group	Number
Total of patients	150
Non-syndromic agenesis patients	male 13 female 31
Dental agenesis class	
Hypodontia ( $\leq 6$ teeth missing)	44
Oligodontia ( $\geq 6$ teeth missing)	6
Dental agenesis categories	
Mandibular incisor agenesis	44
Other	6

Figure 1- Number of individuals with dental agenesis

on filter papers (BFC 180, Whatman International, Maidstone, UK). The exons 1-4 of the *PAX9*, exons 1-2 of the *MSX1*, exons 2-11 of the *AXIN2* and exons 1-8 of the *EDA* were sequenced except for the intronic and promoter sequences of these genes. Primers for the *PAX9*, *MSX1*, *AXIN2* and *EDA* genes designed through the Oligo Primer Analysis Software were made by Dingan Biotech (Shanghai, China)<sup>10</sup> (Figure 2). The 50  $\mu$ L PCR reaction was carried out with the use of PCR beads, 0.3  $\mu$ M (each) primer, 5 ng/ $\mu$ l template DNA, 2.5 U Taq DNA Polymerase (Takara Biotechnology, Dalian, Liaolin, China), 0.3  $\mu$ M of dNTP final concentration, 2 mmol/L of  $MgCl_2$  and sterile distilled water. The amplification procedure contained 5 min 94°C denaturation, followed by 35 cycles of 30 sec 94°C, 30 sec 56°C, and 1 min 72°C, and 7 min 72°C extension using the ABI 9700 thermal cycles (Applied Biosystems, Foster City, California, USA). The PCR products were subjected to electrophoresis in a 2% agarose gel and purified by the BigDye Terminator Kit for analysis. Purified double-stranded PCR products were analyzed on the 3730 DNA Analyzer (Applied Biosystems, Foster City, California, USA). The sequencing results of the *PAX9*, *MSX1*, *AXIN2* and *EDA* genes were compared with the previously published human genome in the NCBI GeneBank by Using the Auto Assemble software. (Gene bank accession: *PAX9*, NC\_000014.837126773-37147012; *MSX1*, NG\_008121.1; *AXIN2*, NG\_012142.1; *EDA*, NC\_000086.697170945-97596099.)

### Statistical analysis

To estimate the associations between the

Primer name (5'→3')	Primer sequence	Size (bp)	Annealing temperature °C	PCR
Exon1 F1	CCACTGTCGCGCAGGAAC	631	67.3	Nested PCR
Exon1 R1	TTGGCTCGAGGTTGCTGA	631	64.8	Nested PCR
Exon2 F1	GGGCTCAGGCTTTAGACAC	502	61.7	Nested PCR
Exon2 R1	TTTCAAGCGATTCTCCTGC	502	63.3	Nested PCR
Exon3 F1	GACCCTTGGCTGTGAGACTC	237	63.6	Nested PCR
Exon3 R1	ACAAAAATCGCACTCTTGAT	237	59.5	Nested PCR
Exon4 F1	AATCCCAGTTACTCCAGAGGC	477	64.8	Nested PCR
Exon4 R1	ACTGGGCTAGGAGATCTGCAT	477	65.2	Nested PCR
Exon5 F1	GGCCCACTGAAGATGAAG	446	59.8	Nested PCR
Exon5 R1	GGCAAGACACCCTTTCTT	446	61.4	Nested PCR
Exon6 F1	CAGTAACATCCCAAGACAGG	347	59.6	Nested PCR
Exon6 R1	CAGTAGAGGGCATGATGGAG	347	62.3	Nested PCR
Exon7 F1	TGGCAGCTGCTTTACAAAC	435	61.8	Nested PCR
Exon7 R1	ACCCAAAGCAGGAAGTTAG	435	59.1	Nested PCR
Exon8 F1	GCCAGCTAGCACGCCTTC	566	66.2	Nested PCR
Exon8 R1	GGCCTTGTCACCCTGGAG	674	65	Nested PCR

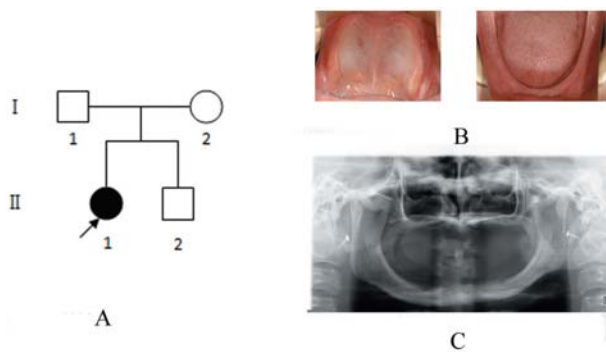
Figure 2- The sequence of EDA Primers

PAX9 polymorphism (A240P) and the risk of tooth agenesis, the difference in the allele and genotype of the PAX9 polymorphism was evaluated by the chi-square and Fisher exact tests to compare the test subjects and controls. All statistical tests were analyzed using the SPSS software (SPSS 17.0, IBM, New York, NY, USA). Differences in the frequencies of each polymorphism between the test subjects and controls were considered statistically significant if  $P \leq 0.05$ .

## RESULTS

### Clinical diagnosis/phenotype

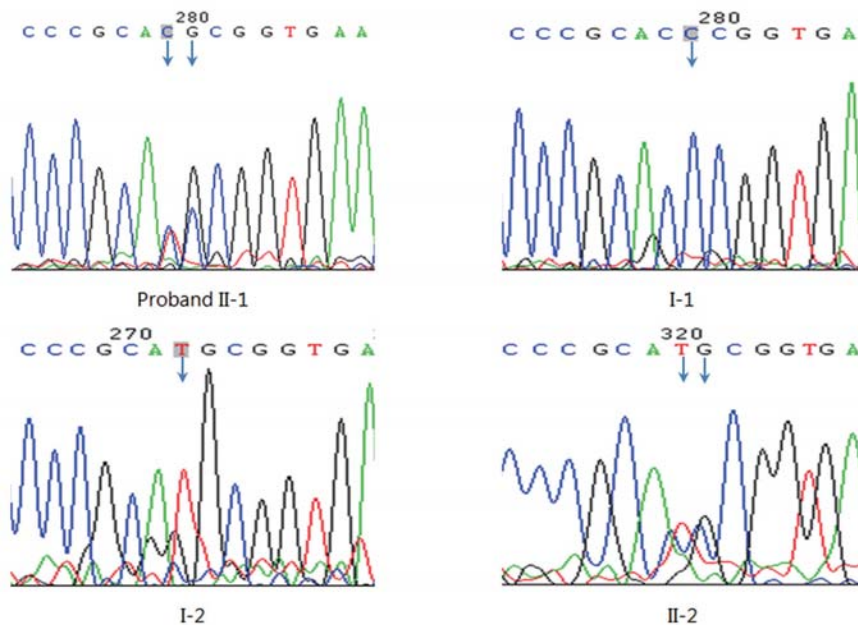
There was no history of tooth agenesis/anodontia in this family. Intra-oral and panoramic radiographs revealed that the female proband had anodontia denoted by the complete absence of teeth in both the primary and secondary dentitions (Figure 3), while all her family members maintained normal dentitions. No abnormalities were revealed in



**Figure 3-** A: Pedigree of the female proband's family. B: Tooth phenotype of the female proband with severe non-syndromic anodontia. C: Panoramic radiograph showed that the female proband (II - 1) at 21 years lacked all teeth



**Figure 4-** Maxillofacial appearance of the proband with severe dental agenesis. There is no other maxillofacial appearance defect or other unnormal

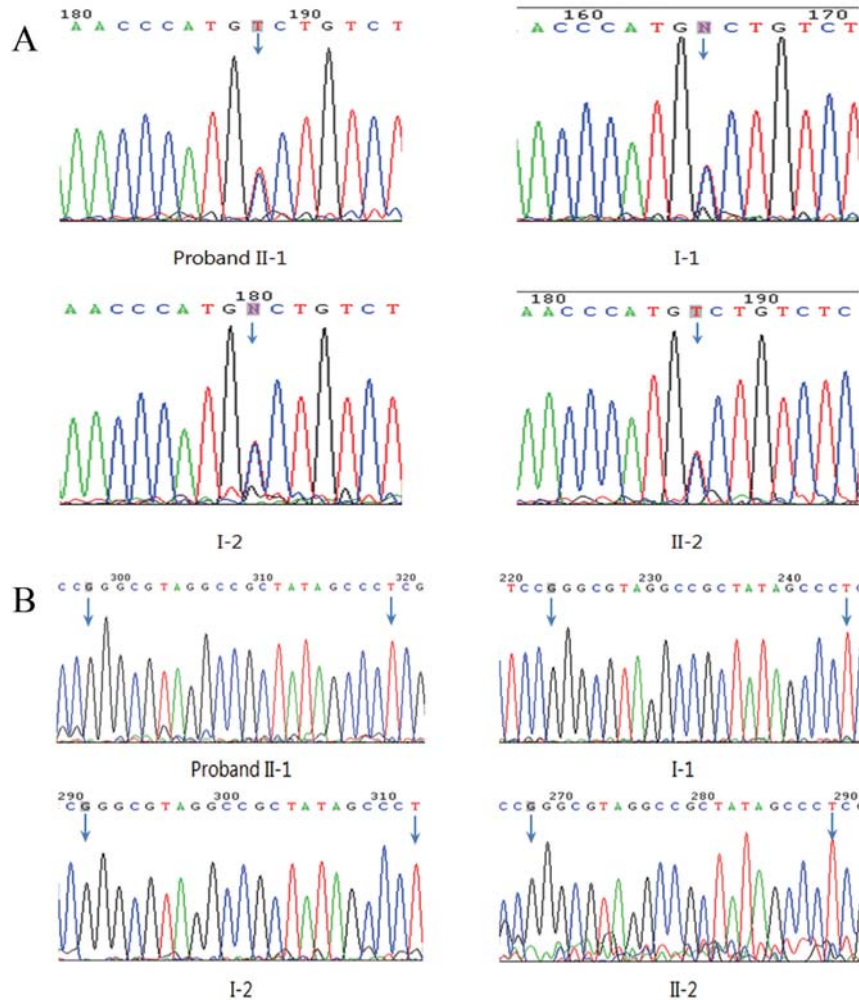


**Figure 5-** DNA-sequencing chromatograms of exon3 of PAX9 in the female proband (II-1) and her family members. A variant of PAX9 gene {G718C (rs4904210)} was detected in the heterozygous state in the female proband (II-1). The same variant in the homozygous state was present in her father (I-1) and heterozygous state in her young brother (II-2), but not in her unaffected mother (I-1)

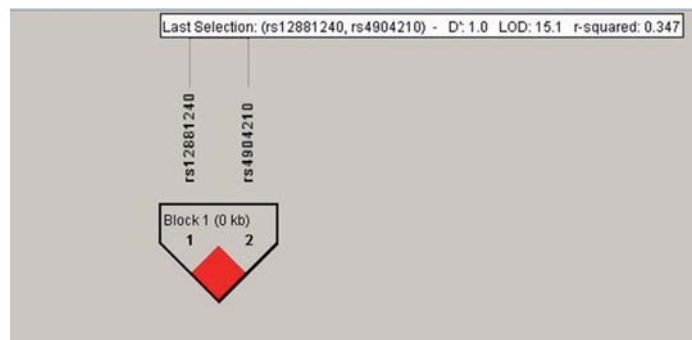


the sweat glands, hair, and nails through clinical examination of the proband (Figure 4) and her family members. Additionally, the distance between the orbits or eyebrows, and the development of the

eyebrow bone were genetically normal. No other clinical features indicating a syndrome or systemic disorder were found.



**Figure 6-** DNA-sequencing chromatograms of exon2 of *AXIN2* in the female proband (II-1) and her family members  
 A. c.148C>T (rs2240308) in *AXIN2* gene was detected in the heterozygous state in the female proband (II-1), and the same variant was present in her unaffected younger brother (II-2) and her parents.  
 B. c.1365A>G (rs9915936) and c.1386C>T (rs1133683) of *AXIN2* gene were found in the homozygous state in the female proband (II-1), and the same variant was present in her unaffected family members in the homozygous state



**Figure 7-** Pairwise linkage disequilibrium (LD) between the two *PAX9* single nucleotide polymorphisms (SNPs), determined using HAPLOVIEW software. The strength of LD between the two SNPs was measured with the  $D'$  statistic. The numbers in the boxes are the  $D'$  value

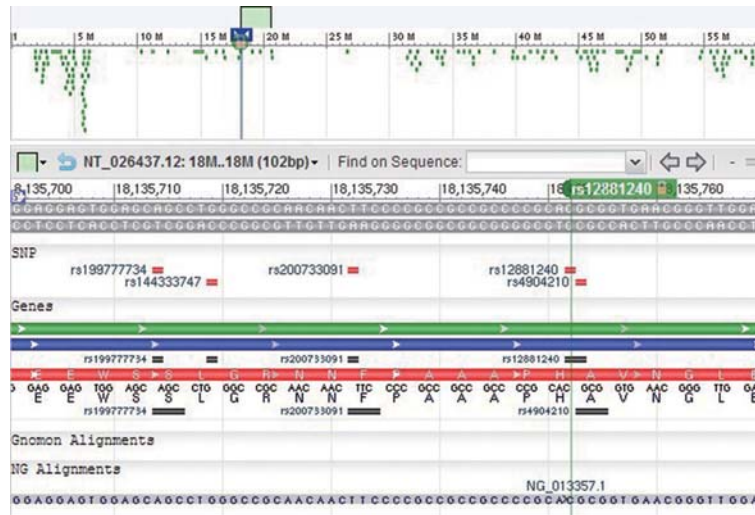
**Mutation analysis**

Variants of the *PAX9* and *AXIN2* were detected in the female proband, including G718C (p.Ala240Pro) in the exon3 of the *PAX9*, c.148C>T (p.Pro50Ser) in the exon2, c.1365A>G (p.Pro455Pro) in the exon6 and c.1386C>T (p.Pro462Pro) in the exon6 of the *AXIN2*. The same variants were present in her unaffected younger brother (Figures 5, 6). The

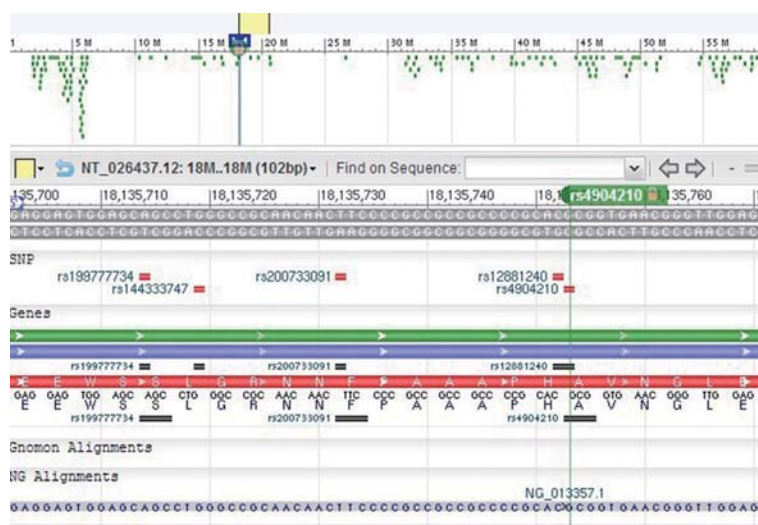
*PAX9* variations were in a different state in her parents (Figures 7). Mutations in the *MSX1* and *EDA* genes were not identified in either the proband or her family members.

**Results of association with the *PAX9* polymorphism with tooth agenesis**

No significant association with tooth agenesis



**Figure 8-** The gene chromatograms of c.717C>T (rs12881240) single nucleotide polymorphisms (SNPs) in *PAX9*



**Figure 9-** The gene chromatograms of c.718G>C (rs4904210) single nucleotide polymorphisms (SNPs) in *PAX9*

**Table 1-** Difference analysis of genotype (The differences of two constituent ratio analysis, conducting chi-square statistics) Sample size: 150

genotype		Patient		Control		$\chi^2$ value	P value
		N	proportion(%)	N	proportion(%)		
C717T (rs12881240)	C	70	70	148	74	0.537	0.464
	T	30	30	52	26		
G718C (rs4904210)	G	52	52	104	52	0	1
	C	48	48	96	48		

**Table 2-** Difference analysis of gene frequency (the differences of two constituent ratio analysis, conducting chi-square statistics) Sample size: 150

genotype	Patient		Control		$\chi^2$ value	P value
	N	Proportion (%)	N	Proportion (%)		
C717T (rs12881240)					3.194	0.200*
C/C	25	50	51	51		
C/T	20	40	46	46		
T/T	5	10	3	3		
G718C(rs4904210)					0.503	0.778
C/C	10	20	17	17		
G/C	28	56	62	62		
G/G	12	24	21	21		

for alleles of the polymorphism (A240P) (Figures 8, 9) was observed between the test subjects and controls ( $P=1$ ) (Table 1 and Figure 7). In addition, the genotype frequencies seemed to distribute no differences ( $P=0.778$ ) (Table 2).

## DISCUSSION

The female proband had anodontia denoted by the complete absence of teeth in both the primary and secondary dentitions, while all her family members maintained normal dentitions. A known mutation (G718C transversion) in the exon 3 of the *PAX9* resulted in the corresponding mutant A240P in the female proband and her family members (I - 1, II - 2), whereas this mutation was not detected in her mother (I - 2). In the current research, there were no mutations in the *MSX1* and *EDA* genes in the proband and her family members. Mostowska, et al.<sup>14</sup> (2006) suggested that the variants c.148C>T, c.1365A>G and c.1386C>T of *AXIN2* had no significant effect on the risk of tooth agenesis in a case-control study performed on 55 subjects with tooth agenesis and 102 healthy controls. There was yet no family history of these indications in the families. Human *PAX9*, highly conserved, consists of coding and non-coding regions. A240P is located in the coding region and probably contributes to structural and functional changes of the protein. This mutation in sporadic oligodontia was reported as polymorphic in many African, American, and European subpopulations. Paixão-Côrtés, et al.<sup>21</sup> (2011) detected a complex involving the *PAX9* related to some dental morphological differences between Sub-Saharan Africans and non-Africans, which is probably associated with the different patterns of evolution in Africa and in other continents. Pawlowska<sup>23</sup> (2010) found an A240P mutation in sporadic oligodontia with all the molars lacking. Another study<sup>10</sup> reported a Chinese proband missing most of the anterior teeth had an A240P in the *PAX9*. However, it did not establish

the presence or absence of an association of this mutation with the *PAX9* associated phenotype. In a case-control study performed on 102 subjects with tooth agenesis and 116 healthy controls<sup>22</sup>, Pan, et al.<sup>22</sup> (2008) suggested that the A240P mutation with *PAX9* had no significant effect on the risk of tooth agenesis. In the present study, we investigated the associations between the A240P *PAX9* gene and sporadic tooth agenesis in a southwestern Han Chinese population, which yet found no evidence for A240P on the risk of sporadic tooth agenesis in our population. It seemed that A240P might have no susceptibility with sporadic tooth agenesis for Chinese people. Otherwise, a pedigree study of tooth agenesis indicated that the mutations in the *PAX9* gene showed the third molars missing<sup>12</sup>. The rejection of the sample missing the third molar is also the reason why we got the negative results. What we can see from this is that the association of A240P with sporadic tooth agenesis still remains obscure, especially for different populations.

Among the various factors of tooth agenesis (genetic factors, environmental factors, radiotherapy, and so on)<sup>28</sup>, the genetic factor is known as an important role in the development of tooth agenesis. It is shown that dental agenesis may be caused by several independent defective genes, acting alone or in collaboration with others. Kangas, et al.<sup>11</sup> (2004) reported that dental anomalies (the size and shape of teeth, tooth agenesis or supernumerary teeth) were determined by a set of genes in tooth development. *PAX9* and *AXIN2* mutations have been ruled out in a number of instances with severe oligodontia reported by Gerits, et al.<sup>6</sup> (2006). Their mutations, however, partly account for the development of tooth agenesis<sup>2,5,29</sup>. However, our research suggested other genes were not yet associated with tooth agenesis. Some critical non-coding regions (eg. intronic and promoter sequences) of the *PAX9*, *MSX1*, *AXIN2* and *EDA* or changes in protein-protein interactions between these genes implicated it may attribute

to anodontia in this family. Another possibility is a change in protein–protein interactions between the *PAX9* and *MSX1*. Developmental studies have shown that the *PAX9* and *MSX1* are co-expressed in the mesenchyme during the bud stage of tooth development. Gerits, et al.<sup>6</sup> (2006) found *in vivo* that mutated *MSX1* and *PAX9* proteins had alterations in their protein spatial structure, thereby influencing their thermo-stability and/or the three-dimensional folding, which disturbed the normal functional activities of the mutant protein and changed its DNA-binding capacity and interactions with other transcriptional factors. Their expression seems to be requisite for the expression of BMP4 that plays an important role in tooth development<sup>25</sup>. Paixão-Côrtes, et al.<sup>20</sup> (2011) suggested that the derived A240P *PAX9* is related with third molar agenesis and that it may have a recessive pattern of inheritance with variable expressivity. On the other hand, one *MSX1* gene derived allele appeared in agenesis affected individuals only. In conclusion, common variants located out of the DNA binding domain of these two transcription factor genes can also be related to tooth agenesis. Another study found direct evidence for a genetic interaction between heterozygous *PAX9* and *MSX1* loss-of-function mutations which result in oligodontia in mice<sup>16</sup>. Thus, we suppose that interaction between the *PAX9* mutation and the *MSX1* may be another reason for the anodontia of the probed.

The female probed lacked all the teeth, while her younger brother with the same gene variants of the *PAX9* and *AXIN2* did not present any developmental abnormalities in the dentition (Figures 4, 5). The etiology in these cases most probably consists of multiple factors: not only mutations found in different genes but also other factors such as epigenetic regulation and environmental factors that may result in different phenotypes. In our study, it may be considered that the epigenetic regulation is a factor that results in the different phenotypes between the female probed and her brother with the same gene mutations. The definition of epigenetic can refer to the addition or removal of methyl groups to DNA or acetylation of histones, while the broader definition is an alteration in gene expression without changes in nucleotide sequencing, including interaction between cells at a tissue level<sup>1,27</sup>. Previous studies support the view that epigenetic factors can influence the number or position of affected teeth, although there is a relatively strong genetic basis to missing or extra teeth<sup>27</sup>. It is considered that epigenetic factors play a critical role in tooth development that can create variability at all phenotypical-somatic and behavioral levels. For example, histone demethylase regulate the differentiation of dental stem cell<sup>1</sup>. Furthermore, it is supposed

that epigenetic influences the spatial arrangement of cells and the timing of the interactive signaling, contributing to differences in tooth size and shape, number and dental asymmetry in MZ co-twins<sup>1,27</sup>. Maybe there existed certain epigenetic influences which lead to the differences of phenotype between the probed and her brother. Thus, a further study of epigenetic biomarkers is needed to explain the reasons for observed differences.

Moreover, there may be other as-yet-unknown factors associated with severe anodontia and relevant to severe non-syndromic anodontia in the Chinese population. Therefore, it is still a question why the same genotype can lead to different phenotypes. In view of this multifactorial nature of dental anomalies, specific mutations of single genes and their acting background should be explored. The genotype/phenotype correlation in congenital anodontia cannot be verified, as there was only one pedigree analyzed. So, further genotype/phenotype investigations are required in the future.

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