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ORIGINAL ARTICLE

Complex analysis of multiple single nucleotide polymorphisms as putative risk factors of tooth agenesis in the Hungarian population

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Abstract

Objectives. The role was studied of multiple single nucleotide polymorphisms in tooth agenesis in the Hungarian population using a complex approach. ***Methods.*** Eight SNPs, *PAX9* -912 C/T, *PAX9* -1031 A/G, *MSX1* 3755 A/G, *FGFR1* T/C rs881301, *IRF6* T/C rs764093, *AXIN2*-8150 A/G, *AXIN2*-8434 A/G and *AXIN2*-30224 C/T, were studied in 192 hypodontia and 17 oligodontia cases and in 260 healthy volunteers. Case-control analysis was performed to test both allelic and genotypic associations as well as associations at the level of haplotypes. Multivariate exploratory Bayesian network-based multi-level analysis of relevance (BN-BMLA) as well as logistic regression analysis were performed. ***Results.*** Conventional statistics showed that *PAX9* SNP -912 C/T and the *MSX1* SNP changed the incidence of hypodontia, although after Bonferroni correction for multiple hypothesis testing, the effects were only borderline tendencies. Using a statistical analysis better suited for handling multiple hypotheses, the BN-BMLA, *PAX9* SNPs clearly showed a synergistic effect. This was confirmed by other multivariate analyses and it remained significant after corrections for multiple hypothesis testing ($p < 0.0025$). The *PAX9*-1031-A-*PAX9*-912-T haplotype was the most relevant combination causing hypodontia. Interaction was weaker between *PAX9* and *MSX1,* while other SNPs had no joint effect on hypodontia. ***Conclusion.*** This complex analysis shows the important role of *PAX9* and *MSX1* SNPs and of their interactions in tooth agenesis, while *IRF6, FGFR1* and *AXIN2* SNPs had no detectable role in the Hungarian population. These results also reveal that risk factors in hypodontia need to be identified in various populations, since there is considerable variability among them.

Key Words: *Gene polymorphism, SNP, hypodontia, oligodontia, PAX9, MSX1, FGFR1, IRF6, AXIN2, risk factors*

Introduction

Among congenital dental disorders, hypodontia is the most frequent one: ~ 20% of the human population is affected worldwide [1]. The etiology of tooth agenesis is not fully understood, but is presumably related to both genetic and environmental factors [2,3]. The incidence of hypodontia is highly variable. Excluding cases involving wisdom teeth, 2–54% of people are affected, depending on the population studied [4–6].

Congenital lack of six or more teeth, called oligodontia, is observed in less than 1% of the population and found to be highly heritable [7–12].

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Gene mutation studies in experimental animals have revealed the importance of a high number of genetic factors in tooth development [13]. The exact weight of the individual components is not known today, but the complexity of tooth formation is underlined by the fact that the differential expression of at least 300 genes has been identified during the study of murine tooth development [14]. However, tooth development in mice is quite different from that in humans; therefore, extrapolation of animal data requires great precaution.

In humans several mutations of the transcription factors *PAX9* and *MSX1* may lead to tooth agenesis. Mutation in the coding region of *PAX9* [13,15–19] or the deletion of it [18] primarily induce conditions with missing molars. Similar data are also available for *MSX1* [20,21].

Mutations of fibroblast growth factor receptor 1 (*FGFR1*) are also associated with tooth agenesis, as well as with cleft lip and palate [22]. Likewise, interferon regulatory factor 6 (*IRF6*) gene deletions and point mutations also were shown to be risk factors for cleft lip and palate and to be associated with premolar agenesis [3,23]. It has recently been suggested that mutations of axis inhibition protein 2 (*AXIN2*) may also contribute to these complex anomalies [24,25]. However, nucleotide changes in the genes *PAX9*, *MSX1*, *FGFR1*, *IRF6* and *AXIN2* present not only as relatively rare mutations resulting in strong phenotypic changes as described above, but also as single nucleotide polymorphisms which may represent a low-to-moderate risk for tooth agenesis [3,26–29]. Nevertheless, SNP studies on hypodontia yielded variable results depending on the ethnic groups investigated [3,5,19,25–27,30,31] and on environmental factors. Therefore, it is important to determine which polymorphisms act as actual risk factors in a given ethnic group such as, in our case, the Hungarian population.

In spite of the well established multigenic origin of tooth agenesis and the relatively large number of putative genes and polymorphisms involved, complex data analysis is missing up to now. For such a complex approach a Bayesian multivariate exploratory data analysis of associations and strong relevance represent a better choice than conventional pairwise association analysis with overstringent corrections for multiple hypothesis testing, where factors are treated independently. In a complex dependency model, approximation for a joint relevance based on independently treated posteriors of targets will yield inaccurate and overly cautious results [32–35]. Probabilistic graphical models in the Bayesian statistical framework can overcome this problem, especially in the case of complex phenotypes. Bayesian networks offer a detailed representation of relevance types, dealing with causal, acausal, and multi-target aspects. Thus, they offer a solution for the multiple hypothesis testing problem [32,36,37].

In the present work we also used complex statistical analysis, including the Bayesian Network-based Multilevel Analysis of relevance (BN-BMLA) [34] to gene polymorphisms formerly identified as associated to tooth agenesis. Altogether eight polymorphisms of the *PAX9*, *MSX1*, *FGFR1*, *IRF6* and *AXIN2* genes were examined in control and tooth agenesis groups and subjected to both conventional statistics and Bayesian Multilevel Analysis (BN-BMLA). We expected to identify multiple SNPs as risk factors in the Hungarian population and also to get an insight into their hierarchical arrangement.

Materials and methods

Tissue samples

Oral mucosal scrapings were collected from 469 patients at the Departments of Pedodontics and Orthodontics at Semmelweis University, University of Pécs and University of Szeged. Samples were frozen at −20°C until use for DNA extraction. The study was performed in conformity with the national law (23/2002 V.9 EüM) on human experimentation and with the permission of the National Ethical Committee (#84-258/2008-1018EKU). Before sample collection, fully informed written consent was received from each individual participating in the study. A detailed medical and dental examination included general health, oral hygiene, and family history, and also a panoramic X-ray examination. The primary criteria for hypodontia (Hyp) were at least one missing adult tooth germ, lack of craniofacial malformation and of any systemic disease. Criteria were the same for Oligodontia (Oli), but at least six missing teeth. The control group (Con) involved healthy volunteers with regular deciduous and adult denture without any craniofacial abnormalities. There were 260 persons in the control, 192 persons in the hypodontia, and 17 persons in the oligodontia groups. The clinical and demographic data of participants are displayed in Table I and the distribution of missing teeth in the sample population is presented in Figure 1.

DNA isolation

DNA was isolated from oral mucosal scrapings using the NucleoSpin Tissue kit (Macherey-Nagel, Düren, Germany), according to the manufacturer's instructions. Extracted DNA was stored at −20°C. Integrity of the DNA was checked by electrophoresis on 1% agarose gel and concentrations were measured using a Qubit fluorometer (Invitrogen-Life Technologies, Carlsbad, CA).

Table I. The demographic characteristics of participants.

Family characteristic	Hypodontia group (Hyp), *n* (%)	Oligodontia group (Oli), *n* (%)	Control group (Con), *n* (%)
Total number	192	17	260
Gender distribution			
Male	84 (44%)	8 (47%)	69 (27%)
Female	108 (56%)	9 (53%)	191 (73%)
Age (mean ± SD)	18.9 ± 7.7	21 ± 6.1	26.2 ± 13.4

SNP genotyping

Allelic discrimination assays were performed in two replicates on a StepOne Real Time PCR using Taqman SNP Genotyping assays (Applied Biosystems-Life Technologies, Carlsbad, CA). SNP data are described in Table II. Genotyping reactions were performed in a final volume of 12.5 µl, with the reaction containing 3 ng genomic DNA, 6.25 µl 2× Taqman Universal PCR Master Mix and 0.625 µl 20× Taqman SNP assay. Results were analyzed using the StepOne software (Applied Biosystems-Life Technologies).

Statistical analysis

Genotype distribution and the allelic frequencies (Con, Hyp and Oli) were calculated. Deviation of the observed genotypes from the Hardy–Weinberg equilibrium were tested, while p-values < 0.05 were accepted as significant. Allelic and genotypic univariate association analyses were performed with the statistical software package of the Helmholtz Institute of Human Genetics, München, Germany (http://ihg2.helmholtz-muenchen.de/cgi-bin/hw/hwa1.pl). Pairwise linkage disequilibrium and haplotype analysis were determined using the Haploview software (http://www.broadinstitute.org/scientific-community/science/programs/medical-and-population-genetics/haploview/hap). We applied a Bayesian network-based Bayesian multilevel analysis of relevance (BN-BMLA), which enables the analysis of associations at different abstraction levels: model-based pairwise relevance (direct association), relevance of variable sets, and statistical interactions of relevant variables [34]. Results of the Bayesian multivariate statistical analysis were also confirmed with logistic regression modelling performed with SPSS using the forward variable selection method, and PIN = 0.05 as the probability threshold for variable entry, and POUT = 0.1 as the threshold of removing a variable from the model.

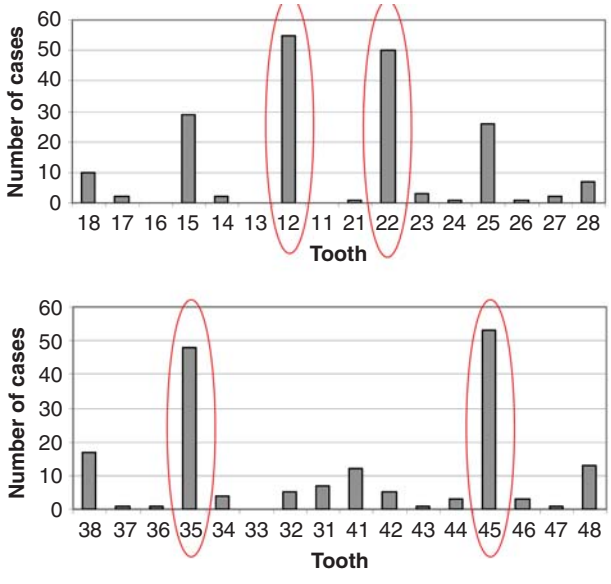

Figure 1. Distribution of tooth agenesis in upper and lower jaw of the hypodontic individuals. The most frequently missing teeth were the upper lateral incisors in the maxilla, followed by the second premolars in the mandible and the second premolars in the maxilla.

Results

In our study the number of missing teeth in the hypodontia group (192 cases) was 363. There were 73 (38%) persons with one missing tooth, 83 (43%) with two teeth, 18 (9%) with three, 15 (8%) with four and three (2%) with five. Of single missing teeth, the left lower second premolar tooth was the most frequent. Of double missing teeth, missing upper lateral incisors were the most frequent combination. The frequencies of missing tooth groups were the following, in decreasing order: 105 upper lateral incisors (28.9%), 101 lower second premolars (27.8%), 55 upper second premolars (15.2%), 30 lower third molars (8.3%) and 19 lower central incisors (5.2%) (Figure 1). One hundred and eleven (58%) of the agenesis cases were asymmetric and 81 (42%) were symmetric. The prevalence of missing teeth was higher in the posterior segment (premolars and molars 224, 62%) than in the anterior segment (incisors and canines 139, 38%), slightly higher on the right side (189, 52% vs 174, 48% on the left) and higher in the maxilla (189, 52%) than in the mandible (174, 48%). However, differences were not significant by T-test. The prevalence of oligodontia was calculated to be 8% of the agenesis cases; however, their number (17) was too low for statistical analysis.

Table II. Major data about the eight analyzed SNPs.

Gene	dbSNP, Major/minor allele (position)	rs number	TaqMan SNP Assay ID
FGFR1	T/C (26190464)	rs 881301	C__8844845_10
IRF6	T/C (1149)	rs 764093	C__2500195_10
MSX1	A/G (3755, 8755)	rs 12532	C__26933394_10
PAX9	C/T (-912, 8221)	rs 2073246	C__11921290_10
PAX9	A/G (-1031, 8102)	rs 2073244	C__11921292
AXIN2	A/G (-148, 8150)	rs 2240308	C__2577354_1_
AXIN2	A/G (-432, 8434)	rs 2240307	C__25471570_10
AXIN2	C/T (-2062, 30224)	rs 35415678	C__60544507_10

Single nucleotide polymorphisms

Control (Con), hypodontia (Hyp) and oligodontia (Oli) groups were examined for the following eight SNPs: *PAX9* -912 C/T, *PAX9* -1031 A/G, *MSX1*-8755 A/G, *FGFR1*-26190464 T/C, *IRF6*-1149 T/C, *AXIN2*-8150 A/G, *AXIN2*-8434 A/G and *AXIN2*-30224 C/T by SNP Genotyping analysis. Control populations were in Hardy Weinberg (HW) equilibrium for all studied SNPs. Of the hypodontia cases *PAX9*-912, *AXIN2*-8150 and *AXIN2*-8434 and of the oligodontia cases *FGFR1*, *AXIN2*-8434 and *AXIN2*-30224 were not in HW equilibrium.

Linkage disequilibrium (LD) analysis showed that only the *PAX9* SNPs were in LD (Figure 2A), whereas the AXIN2 8150 and 8434 SNPs were not in LD (Figure 2B). The *AXIN2* 30224 SNP was not handled by the database.

Allelic frequencies compared by Chi-square test were not significantly different between controls and Hyp or Oli cases for any SNPs. Genotype frequencies of *FGFR1*, *IRF6* and *AXIN2* SNPs did not differ in the investigated groups. On the other hand, genotype frequencies were nominally significantly different (i.e. without Bonferroni correction) under dominant and recessive models in hypodontia for *PAX9*-912, $p = 0.0128$ (OR = 1.672); *PAX9*-1031, $p = 0.0333$ (OR = 1.546); and *MSX1*, $p = 0.0143$ (OR = 2.553) SNPs compared to control (Table III). The number of Oli patients was too small for any statistical consideration. There were no significant differences in allelic frequencies or in genotypes between male and female controls or cases either. When calculating the Bonferroni correction for the eight SNPs (resulting in a decrease of acceptable significance level from $p < 0.05$ to $p < 0.00625$), all the above described tendencies can be interpreted as trends only.

Bayesian statistics

Because of the overly stringent handling of multiple hypothesis testing by conventional tools, particularly in a multivariate context, an additional statistical analysis was performed by Bayesian statistical methods, to identify interactions among the investigated SNPs. According to BN-BMLA analysis, the two SNPs of *PAX9* gene (*PAX9* -912 C/T: rs2073246, Pr = 0.994977 and *PAX9* -1031 A/G: rs2073244, Pr = 0.995578) showed relevant association with

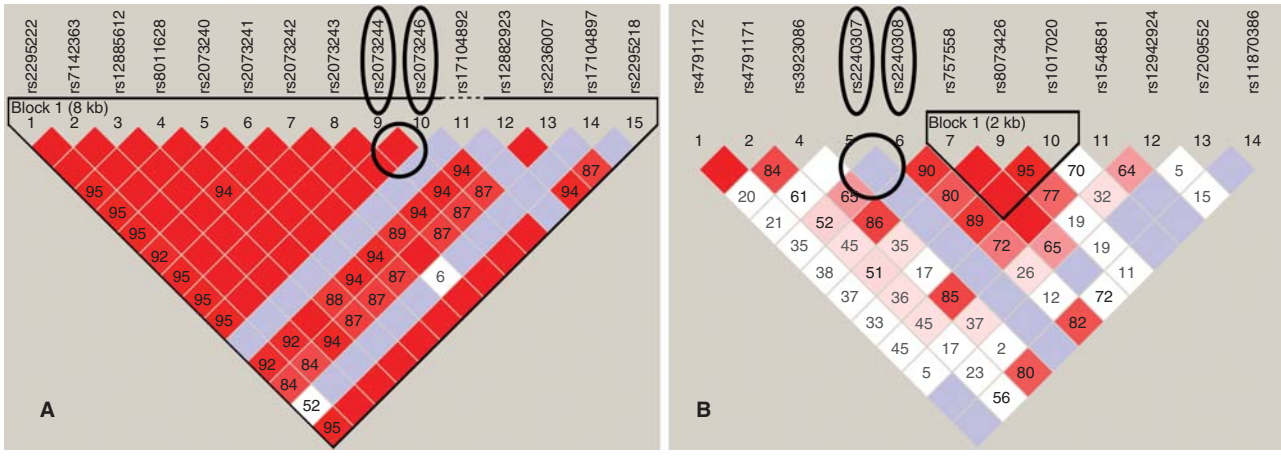

Figure 2. Pairwise linkage disequilibrium (LD) analysis of *PAX9* and *AXIN2* SNPs. (A) Pairwise linkage disequilibrium (LD) among *PAX9* SNPs (rs2073246 and rs2073244). (B) No pairwise linkage disequilibrium (LD) among *AXIN2* SNPs (rs2240308 and rs2240307). The numbers in the break-even points are the D' values

Table III. Genotype distribution and allele frequencies in hypodontia (Hyp) and control (Con) group.

				Statistical analysis (Hypodontia)		
Polymorphism	Control (n = 260) n, %	Hypodontia (n = 192) n, %	Oligodontia (n = 17) n, %	Genotypes	Genotypes	Alleles
PAX9-912 C/T						
C/C	98 (38%)	51 (27%)	7 (41%)	C/C vs C/T&T/T	C/C&C/T vs T/T	C vs T
C/T	121 (46%)	111 (58%)	9 (53%)	χ^2 = 6.19	χ^2 = 0.0	χ^2 = 2.75
T/T	41 (16%)	30 (15%)	1 (6%)	p = 0.0128*	p = 0.9668	p = 0.0974
				OR = 1.672 (1.114-2.512)	OR = 1.011 (0.605–1.688)	OR = 1.254 (0.959–1.638)
C	317 (61%)	213 (55%)	23 (68%)			
T	203 (39%)	171 (45%)	11 (32%)			
PAX9-1031 A/G						
A/A	98 (38%)	54 (28%)	7 (41%)	A/A vs A/G&G/G	A/A&A/G vs G/G	A vs G
A/G	121 (46%)	106 (55%)	9 (53%)	χ^2 = 4.53	χ^2 = 0.07	χ^2 = 2.5
G/G	41 (16%)	32 (17%)	1 (6%)	p = 0.0333*	p = 0.7977	p = 0.1142
				OR = 1.546 (1.034–2.312)	OR = 0.936 (0.565–1.551)	OR = 1.241 (0.949–1.621)
A	317 (61%)	214 (56%)	23 (68%)			
G	203 (39%)	170 (44%)	11 (32%)			
MSX1 8755 A/G						
A/A	120 (46%)	98 (51%)	7 (41%)	A/A vs A/G&G/G	A/A&A/G vs G/G	A vs G
A/G	111 (43%)	85 (44%)	8 (47%)	χ^2 = 0.9793	χ^2 = 6.00	χ^2 = 3.38
G/G	29 (11%)	9 (5%)	2 (12%)	p = 0.3224	p = 0.0143*	p = 0.0658
				OR = 1.208 (0.831–1.755)	OR = 2.553 (1.179–5.528)	OR = 0.761 (0.569–1.018)
A	351 (67%)	281 (73%)	22 (65%)			
G	169 (33%)	103 (27%)	12 (35%)			
FGFR1 T/C						
T/T	95 (37%)	75 (40%)	7 (44%)	C/C vs C/T&T/T	C/C&C/T vs T/T	C vs T
C/T	117 (45%)	85 (45%)	6 (37%)	χ^2 = 0.61	χ^2 = 0.42	χ^2 = 0.77
C/C	47 (18%)	29 (15%)	3 (19%)	p = 0.4350	p = 0.5177	p = 0.3800
				OR = 1.223 (0.737–2.029)	OR = 0.88 (0.599–1.295)	OR = 1.129 (0.861–1.482)
T	307 (59%)	235 (62%)	20 (63%)			
C	211 (41%)	143 (38%)	12 (37%)			
IRF6 1149 T/C						
T/T	189 (73%)	141 (74%)	14 (82%)	T/T vs T/C&C/C	T/T&T/C vs C/C	T vs C
T/C	61 (24%)	45 (24%)	3 (18%)	χ^2 = 0.04	χ^2 = 0.27	χ^2 = 0.13
C/C	9 (3%)	5 (2%)	0 (0%)	p = 0.8404	p = 0.6047	p = 0.7223
				OR = 0.957 (0.627–1.462)	OR = 1.339 (0.442–4.062)	OR = 0.935 (0.644–1.357)
T	439 (85%)	327 (86%)	31 (91%)			
C	79 (15%)	55 (14%)	3 (9%)			
AXIN2-8150 A/G						
A/A	65 (25%)	45 (23%)	7 (41%)	A/A vs A/G&G/G	A/A&A/G vs G/G	A vs G
A/G	142 (55%)	115 (60%)	7 (41%)	χ^2 = 0.15	χ^2 = 1.00	χ^2 = 0.1
G/G	53 (20%)	32 (17%)	3 (18%)	p = 0.7019	p = 0.3173	p = 0.7483
				OR = 1.089 (0.704–1.684)	OR = 1.28 (0.788–2.079)	OR = 0.958 (0.735–1.247)

Table III. (*Continued*).

Polymorphism	Control (n = 260) n, %	Hypodontia (n = 192) n, %	Oligodontia (n = 17) n, %	Statistical analysis (Hypodontia) Genotypes	Genotypes	Alleles
A	272 (52%)	205 (53%)	21 (62%)			
G	248 (48%)	179 (47%)	13 (38%)			
AXIN2-8434 A/G						
A/A	252 (97%)	189 (98.5%)	17 (100%)	A/A vs A/G&G/G	A/A&A/G vs G/G	A vs G
A/G	8 (3%)	2 (1%)	0 (0%)	χ^2 = 1.07	χ^2 = 1.36	χ^2 = 0.42
G/G	0 (0%)	1 (0.5%)	0 (0%)	p = 0.3016	p = 0.2440	p = 0.5188
				OR = 0.500 (0.131–1.91)	OR = 0.245 (0.01–6.048)	OR = 0.674 (0.201–2.254)
A	512 (98.5%)	380 (99%)	34 (100%)			
G	8 (1.5%)	4 (1%)	0 (0%)			
AXIN2-30224 C/T						
C/C	239 (92%)	179 (94%)	16 (100%)	T/T vs C/T&C/C	T/T&C/T vs C/C	T vs C
C/T	20 (7.5%)	12 (6%)	0 (0%)	χ^2 = 0.74	χ^2 = 0.52	χ^2 = 0.72
T/T	1 (0.5%)	0 (0%)	0 (0%)	p = 0.3908	p = 0.4697	P = 0.3960
				OR = 2.214 (0.09–54.641)	OR = 0.763 (0.366–1.592)	OR = 1.362 (0.666–2.788)
C	498 (96%)	370 (97%)	32 (100%)			
T	22 (4%)	12 (3%)	0 (0%)			

p-value was calculated by Chi-square test at 95% confidence interval.
OR, Odds Ratio (Confidence Interval).

hypodontia. This probability value (Pr) reflects the Markov blanket membership probability, which is a pairwise model-based association measure of a given variable with respect to the target variable (i.e. hypodontia in this case) (for theoretical details see Ungvari et al. [32] and Antal et al. [34]. The higher this probability value is (i.e. closer to 1) the more relevant the variable is with respect to the target variable. Therefore, both of these SNPs are considered as highly relevant. Similarly to previous studies, gender seemed to be a relevant factor with respect to hypodontia, as the corresponding odds were remarkably different in case of males and females (1.23 and 0.57, respectively). Consequently, gender was included as a covariate in all models.

Furthermore, the BN-BMLA analysis also shows that *PAX9* SNPs form a statistical interaction having a joint effect on tooth agenesis. This was validated with logistic regression. The BN-BMLA analysis was also performed with merged genotypes (heterozygote + homozygote 1 and heterozygote+homozygote 2). As a result, in addition to *PAX9* SNPs, the *MSX1* A/G SNP has also been found to show association with hypodontia (Pr = 0.815,46). Figure 3 shows a possible dependency model of Pax9 and Msx1 SNPs with respect to hypodontia. The model indicates a stronger connection between the Pax9 SNPs and hypodontia than between the Msx1 SNP and hypodontia. The 912 Pax9 SNP has a direct statistical effect on hypodontia, whereas the 1031 Pax9 SNP only modifies the effect of 912 Pax9. The Msx1 SNP has a separate main effect, although its association with hypodontia is weaker.

Table IV. Logistic regression analysis of merged genotypes.

	p-value	Exp(B)	95% CI for Exp(B) Lower	Upper
PAX9 -912 C/T (dom)	0.01017	15.402	1.914	123.963
PAX9 -1031 A/G (dom)	0.00624	17.390	2.246	134.630
MSX1 A/G (rec)	0.01498	0.354	0.153	0.817
PAX9 -1031 A/G by PAX9 -912 C/T (dom)	0.00002	0.002	0.000	0.034

CI, confidence interval; (rec), recessive model (heterozygote+homozygote1); (dom), a dominant model (heterozygote+homozygote 2).

Table V. Haploview analysis of *PAX9 -912 - PAX9 -1031* and *PAX9 -912-MSX1* haplotypes.

	Haplotype associations					
Blocks	Frequency	Case ratio	Control ratio	χ^2	*p*-value	Permutation *p*-value
PAX9 -1031(A/G) + PAX9 -912(C/T)						
AC	0.289	85.7:302.3	176.3:343.7	15.104	0.0001**	0.0045**
AT	0.041	33.3:354.7	3.7:516.3	35.192	2.99E-09**	0.00E+00**
GC	0.033	26.3:361.7	3.7:516.3	25.576	4.25E-07**	0.0001**
GT	0.638	242.7:145.3	336.3:183.7	0.432	0.5112	0.9671
MSX1 8755 (A/G) + PAX9-912(C/T)						
AC	0.094	32.3:355.7	53.0:467.0	0.902	0.3423	0.7345
AT	0.231	84.7:303.3	125.0:395.0	0.615	0.4329	0.8315
GC	0.228	79.7:308.3	127.0:393.0	1.913	0.1666	0.4623
GT	0.448	191.3:196.7	215.0:305.0	5.704	0.0169*	0.065

*$p < 0.05$, **$p < 0.01$.

Logistic regression analysis (Table IV) confirmed the strong significance of the interaction of *PAX9* -912 C/T and *PAX9* -1031 A/G (exp(B) = 0.002, 95% CI = 0.00009–0.034, *p*-value < 0.00002), and also the moderate significance of *MSX1* A/G (exp (B) = 0.354, 95% CI = 0.153–0.817, *p*-value < 0.014,98).

Haploview analysis also showed interaction between *PAX*9 -1031 and *PAX*9 -912 haplotypes. The presence of AC, AT, GC haplotypes were significantly higher ($p < 0.01$) in hypodontia than in controls, the permutation *p*-value was the highest at the AT haplotype. *MSX1-PAX9* GT haplotype frequency was only borderline significant (Table V).

Discussion

The developmental absence of one or more teeth is not an uncommon phenomenon. However, the prevalence of developmental missing teeth in the permanent dentition shows great variations between populations due to multiple factors, such as genomic and environmental elements. A recent report concerning the Caucasian population recorded prevalence rates of hypodontia between 3–11% [38]. The most often missing teeth worldwide are the lower second premolars followed by the upper second incisors [39–46]. This order is reversed in the Hungarian population as reported previously [47] and also confirmed in the present study: the order of prevalence of agenesis is upper second incisor > lower second premolar > upper second premolar > lower third molar > lower central incisor. All of these data are in line with the terminal reduction theory [48], which suggests that the reduction of the distal element of a tooth group occurs more frequently then that of a mesially placed teeth. Therefore, the second premolars, the upper second incisors, and the third molars are the most often absent [40,47,49]. These findings are also in accordance with the general rule: if only one or a few teeth are missing, the missing germ ordinarily will be the most distal tooth of any given type [50–52]. From another point of view, the data correlate with Butler's field theory which states that the most mesially situated tooth is the most stable in each morphological class. In each developmental field in a tooth group, there is a genetically stable 'key tooth', while, at the end of the field, the teeth show less stability [53,54]. It is interesting to note that the Chinese population is an outlier compared to others with their highest number of missing mandibular incisors [55].

A number of previous investigations found symmetrical hypodontia to be more frequent than asymmetrical [40,42–44,56]. However, in the Hungarian population we found an asymmetrical prevalence, with right maxillary lateral incisor agenesis and more missing posterior teeth. Furthermore, our results are in line with most previous findings about similar frequencies at the right and left sides and in different genders [5,40–42,44,46,57], but not with other papers which report significant differences in this respect [58,59].

The main purpose of our study was to examine in the Hungarian population the role of single nucleotide polymorphisms already identified in other populations as genetic/genomic risk factors in tooth agenesis. The aim of the complex analysis was to investigate epistatic effects and interactions between several associated SNPs, since previous studies only analyzed the contribution of genes to hypodontia separately. In the context of multigenic diseases like tooth agenesis, it is reasonable to examine hypothetical network models of genes allowing the detection of potential interaction of factors leading to germ loss.

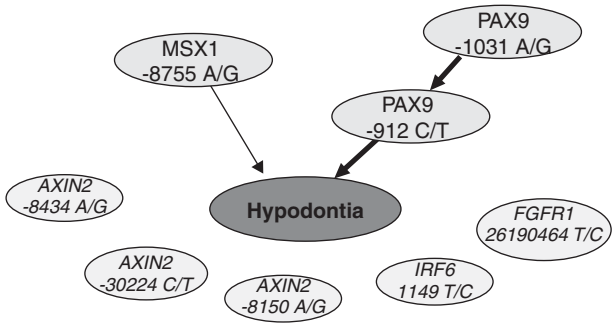

Figure 3. A potential dependency model in hypodontia. Three SNPs in two genes were found relevant in connection with hypodontia phenotype. The thickness of the visible arrows demonstrate the strength of their effect on the development of tooth agenesis. The two Pax9 SNPs together (the major TT genotype for Pax9-912 and the major GG genotype for Pax9-1031) have stronger relevance than e.g. Msx1-8755 or Pax9-912 alone. The Pax9-1031-SNP has no individual effect, but through the PAX9-912 together they are intensifying each others influence. At our sample number the remaining five SNPs were not associated (neither calculating the conventional, nor the BN-BMLA statistical approaches) to hypodontia in the Hungarian population.

We investigated eight different potential polymorphic positions in the *AXIN2*, *FGFR1*, *IRF6*, *MSX1* and *PAX9* genes. This particular panel of genetic polymorphisms was selected because they had either been associated previously with hypodontia [3,26] or these genes have already been shown to code important molecules in the process of tooth agenesis [14,20]. Indeed, in all cases we could demonstrate the existence of wild type and rare alleles as suggested before [15,26].

We found that the differences in the genotype frequencies of *PAX9* -912 C/T (rs 2073246) and -1031 A/G SNPs (rs 2073244) were nominally significant individually between controls and agenesis cases only when compared by traditional statistics without Bonferroni correction. However, they are jointly important factors for hypodontia, as indicated by the BN-BMLA method used for the analysis of strong relevance. In our population, the major TT genotype for *PAX9*-912 and the major GG genotype for *PAX9*-1031 increased the incidence of tooth agenesis. The effect of the -912 SNP was more direct on hypodontia while the -1031 SNP acted through the -912 SNP, while *MSX1* seemed to be a weaker contributor.

The haplotype level analysis performed by Haploview indicated that the two *PAX9* SNPs are linked (Table V) and also provided evidence that *PAX9* SNPs haplotypes have a strong effect on hypodontia prevalence, whereas single SNPs have a limited one.

The performed Bayesian statistical analysis also showed that the *PAX9 -912* and *-1031* formed a triade with patients' state, revealing that Pax9-1031 SNP had a marked joint effect together with the -912 SNP, while its individual effect was only weak (Figure 3). This interaction was confirmed by traditional statistics. In addition, the analysis showed that the *MSX1* SNP is similarly strongly relevant with potential synergistic effect with *PAX9* SNPs (Figure 3). *IRF6*, *FGFR1* and *AXIN2* SNPs were not associated to hypodontia, neither alone, nor together with other SNPs, although the power of the sample size for these SNPs was relatively low (< 0.2).

The biological basis of the joint effect of -912 and -1031 Pax9 SNPs is not fully understood. We performed two *in silico* analyses for DataBase of CpG islands and Analytical Tool (DBCAT) analysis (http://dbcat.cgm.ntu.edu.tw [60]) and the *cis*-Regulatory Elements in the Mammalian Genome (Cremag) analysis (www.cremag.org, [61],) to examine putative binding sites for transcription factors and methylation sites in this region. Although these SNPs are not found in either the core promoter region or in CPG islands, they are in a well conserved region of Pax9 upstream region in primates [62], so they could participate in the regulation in an as yet not defined manner. For example, if the SNPs are located in a promoter attachment site, a two nucleotide change in the sequence may decrease this attachment. For Pax9, appropriate gene expression level is necessary for complete function. Interestingly, Santagati et al. [62] have found that Pax9 regulatory elements interspersed in a wider genomic interval, even in the region of neighboring genes. Pax9 is a member of a synteny group, formed by the *Mbip—Nkx2-1—Nkx2-9—Pax9—Slc25a21—Mipol1—Foxa1* genes, and genomic region around Pax9 maintained the physical association, possibly reflecting a fixed multigenic transcriptional domain whose members cannot be separated without compromising their normal function [62].

Our Bayesian multivariate analysis of the interaction of Pax9 and Msx1 could be detected in the form of a joint strong relevance of *PAX9* and *MSX1* (Pr = 0.8150). As Figure 3 shows, *PAX9* SNPs were in linkage and they intensified each other's effect and the −1031 SNP had an effect through the other SNP. The two Pax9 SNPs together (the major TT genotype for Pax9-912 and the major GG genotype for Pax9-1031) have stronger relevance than, e.g., Msx1-8755 or Pax9-912 alone. As it is found in an unknown but highly conserved regulatory sequence, probably the 1031 SNP alone causes minor loss of function, but potentiated with further nucleotide changes the regulatory site can be even less accessible.

MSX1 and PAX9 proteins are key transcription factors in the early dental development having a crucial role in the formation of odontogenic mesenchyme [63]. Their molecular interaction is at the level of the transcription [21,64] described by a number of studies. Msx1 and Pax9 proteins are key mesenchymal transcription factors which establish cross-talk between dental tissues in the early phases of odontogenesis [14,65–69]. They both play an important role

in the maintenance of mesenchymal Bmp4 expression, which ultimately drives dental morphogenesis. Either Pax9 or Msx1 nullizygosity in mice results in an arrest of tooth formation at the bud stage [70,71]. Knock out mouse studies also revealed that Msx1 was reduced in Pax9 null mutants, but Pax9 expression was not influenced in Msx1 null mice, implying that Pax9 must be positioned upstream to Msx1 [70,71]. Ogawa et al. [72] confirmed the interaction of Pax9 and Msx1 first at the protein level, by coimmunoprecipitation and GST interaction, actually showing that the protein complex formed by Msx1 and Pax9 inhibits DNA binding by the paired domain of Pax proteins and the homeodomain of Msx1. Both *in vitro* and *in vivo* they demonstrated that there is a physical association between the two proteins. In a subsequent study [21], the interaction of Msx1 and Pax9 was also revealed in transciptional regulation. It demonstrated that Pax9 is able to directly regulate Msx1 expression and interact with Msx1 at the protein level to enhance its ability to transactivate Msx1 and Bmp4 expression during tooth development. Findings demonstrate the partnership of Pax9 and Msx1 in a signaling pathway that involves Bmp4. Furthermore, the regulation of Bmp4 expression by the interaction of Pax9 with Msx1 was shown to be at the level of transcription and through formation of a protein complex it determines the fate of the transition from bud to cap stage during tooth development [21]. Furthermore, a recent study indicated that the zinc finger transcription factor Osr2 forms stable protein complexes with the Msx1 protein and also interacts weakly with the Pax9 protein providing another link for the interactions of Msx1 and Pax9 during early tooth development [73].

Our results are in concordance with Peres et al. [26], who found genotype frequencies of both *PAX9* -912 and -1031 SNPs significantly different between controls and tooth agenesis patients, but they did not investigate complex effects. In their study the -912 SNP variation yielded more significant differences than in our case, but their tooth agenesis group included mostly third molar hypodontia. The third molar hypodontia is thought to be linked to the 8981 SNP (rs4904210) [74], so the -912 SNP is probably not a primary causative factor for third molar agenesis, but it may still represent a certain level of risk. On the contrary, an investigation in a Chinese population found no significant effect of -1031 rs2073244 on tooth agenesis, although, when grouped together with three other *PAX9* SNPs (rs 2073245, 2073247, 4904210), an AGGC haplotype was found to decrease the risk of tooth agenesis [55]. Studies with other *PAX9* SNPs yielded variable results [74–77]. In humans severe phenotype changes result from the mutation of the coding region of *PAX9* gene [13,15–19] or from the deletion of *PAX9* [18].

Our study shows that in the Hungarian population the minor GG genotype for *MSX1*-3755 SNP significantly increased the incidence of tooth agenesis. *MSX1* is normally expressed in dental mesenchyme, but it is not present in the dental epithelium during the development of bud cap and the bell stages of tooth development [29,78,79]. Most probably, mutations in the coding region of *MSX1* primarily lead to missing premolars [20]. The only population level study in this respect was carried out in Rio de Janeiro, Brazil. Those data suggest that there is a relationship between missing teeth and the genes *MSX1*, *PAX9* and *TGFA* (which is actually regulated by *MSX1* and *PAX9*), but further studies are needed to understand the exact relationship between these factors [20].

We found no significant difference in *FGFR1* (rs 881301), *IRF6* (rs 764093) and *AXIN2* SNPs (rs 2240308, rs 2240307, rs 35415678). These SNPs were all previously found to be associated to tooth agenesis [3,24]. The *IRF6* gene locus appears to contribute to rare syndromes, non-syndromic oral clefts, and tooth agenesis, preferentially incisors and premolars [3,80,81]. The *FGFR1* signaling pathway regulates facial development evidenced by its mutation, which causes the autosomal dominant Kallmann syndrome [22]. *IRF6* mutations appear to be associated with cleft lip and palate and with premolar agenesis [3]. Vieira et al. [3] found a significant association between *IRF6* (rs17015215, rs7802), *FGFR1* (rs881301) gene polymorphisms and tooth agenesis in an admixed population from Brazil which is predominantly Caucasian with Portuguese ancestry. However, they could not replicate these findings in a population from Ohio, which is also of Caucasian origin, but predominantly involving descendents of Northern Europeans [3]. Similar to the Ohio population, we did not find a significant association between *IRF6* (rs764093) and *FGFR1* (rs881301) SNPs and tooth agenesis in Hungarians, suggesting genomic heterogenity among different Caucasian populations. Our *FGFR*1 rare allele frequency (38%) was similar to that of the Ohio population (39%), in which no association with hypodontia was observed either [3].

AXIN2 is a scaffold protein, which phosphorylates β-catenin, a member of the Wnt pathway of embryonic development. First it was found to be associated with a Finnish oligodontia family [24]. A later finding suggested that *AXIN2* polymorphic variants may be associated with both hypodontia and oligodontia [24]. Three novel *AXIN2* gene variants were identified (c.956+16A>G, c.1060-17C>T and c.2062C>T). Individuals carrying the c.956+16G and c.2062T alleles exhibited an increased risk of tooth agenesis. The rare allele frequency for *AXIN2* 30224 C/T (rs 35415678) was much lower in the Hungarian population than in the Polish one (3% and 13%, respectively). Due to these small allele frequencies a larger

sample size is required to detect a potential association. The frequencies of the other two *AXIN2* SNPs were similar.

In conclusion, among the investigated single nucleotide polymorphisms *PAX9* and *MSX1* but not *IRF6*, *FGFR1* and *AXIN2*, SNPs seem to have an important role in tooth agenesis in the Hungarian population. Our data also reveal the importance of population level genomic studies and provide evidence for the necessity of risk factor evaluation in different populations. It also confirmed that Bayesian multivariate analysis provides a normative solution for the correction of multiple hypothesis testing and Bayesian network based methods, particularly in the Bayesian statistical framework, allow the analysis of strong relevance even in the case of multiple target variables and small sample size.

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References

[1] Vastardis H. The genetics of human tooth agenesis: new discoveries for understanding dental anomalies. Am J Orthod Dentofacial Orthop 2000;117:650–6.

[2] Mostowska A, Kobielak A, Trzeciak WH. Molecular basis of non-syndromic tooth agenesis: mutations of MSX1 and PAX9 reflect their role in patterning human dentition. Eur J Oral Sci 2003;111:365–70.

[3] Vieira AR, Modesto A, Meira R, Barbosa AR, Lidral AC, Murray JC. Interferon regulatory factor 6 (IRF6) and fibroblast growth factor receptor 1 (FGFR1) contribute to human tooth agenesis. Am J Med Genet A 2007;143:538–45.

[4] Hunstadbraten K. Hypodontia in the permanent dentition. ASDC J Dent Child 1973;40:115–17.

[5] Endo T, Ozoe R, Kubota M, Akiyama M, Shimooka S. A survey of hypodontia in Japanese orthodontic patients. Am J Orthod Dentofacial Orthop 2006;129:29–35.

[6] Lidral AC, Reising BC. The role of MSX1 in human tooth agenesis. J Dent Res 2002;81:274–8.

[7] Gabris K, Tarjan I, Csiki P, Konrad F, Szadeczky B, Rozsa N. [Prevalence of congenital hypodontia in the permanent dentition and its treatment]. Fogorv Sz 2001;94:137–40.

[8] Polder BJ, Van't Hof MA, Van der Linden FP, Kuijpers-Jagtman AM. A meta-analysis of the prevalence of dental agenesis of permanent teeth. Community Dent Oral Epidemiol 2004;32:217–26.

[9] Nieminen P. Genetic basis of tooth agenesis. J Exp Zool B Mol Dev Evol 2009;312B:320–42.

[10] Bergendal B, Norderyd J, Bagesund M, Holst A. Signs and symptoms from ectodermal organs in young Swedish individuals with oligodontia. Int J Paediatr Dent 2006;16:320–6.

[11] Schalk-van der Weide Y, Beemer FA, Faber JA, Bosman F. Symptomatology of patients with oligodontia. J Oral Rehabil 1994;21:247–61.

[12] van den Boogaard MJ, Creton M, Bronkhorst Y, van der Hout A, Hennekam E, Lindhout D, et al. Mutations in WNT10A are present in more than half of isolated hypodontia cases. J Med Genet 2012;49:327–31.

[13] Frazier-Bowers SA, Guo DC, Cavender A, Xue L, Evans B, King T, et al. A novel mutation in human PAX9 causes molar oligodontia. J Dent Res 2002;81:129–33.

[14] Thesleff I. Epithelial-mesenchymal signalling regulating tooth morphogenesis. J Cell Sci 2003;116:1647–8.

[15] Jumlongras D, Lin JY, Chapra A, Seidman CE, Seidman JG, Maas RL, et al. A novel missense mutation in the paired domain of PAX9 causes non-syndromic oligodontia. Hum Genet 2004;114:242–9.

[16] Stockton DW, Das P, Goldenberg M, D'Souza RN, Patel PI. Mutation of PAX9 is associated with oligodontia. Nat Genet 2000;24:18–19.

[17] Nieminen P, Arte S, Tanner D, Paulin L, Alaluusua S, Thesleff I, et al. Identification of a nonsense mutation in the PAX9 gene in molar oligodontia. Eur J Hum Genet 2001;9:743–6.

[18] Das P, Stockton DW, Bauer C, Shaffer LG, D'Souza RN, Wright T, et al. Haploinsufficiency of PAX9 is associated with autosomal dominant hypodontia. Hum Genet 2002;110:371–6.

[19] Lammi L, Halonen K, Pirinen S, Thesleff I, Arte S, Nieminen P. A missense mutation in PAX9 in a family with distinct phenotype of oligodontia. Eur J Hum Genet 2003;11:866–71.

[20] Vieira AR, Meira R, Modesto A, Murray JC. MSX1, PAX9, and TGFA contribute to tooth agenesis in humans. J Dent Res 2004;83:723–7.

[21] Ogawa T, Kapadia H, Feng JQ, Raghow R, Peters H, D'Souza RN. Functional consequences of interactions between Pax9 and Msx1 genes in normal and abnormal tooth development. J Biol Chem 2006;281:18363–9.

[22] Dode C, Levilliers J, Dupont JM, De Paepe A, Le Du N, Soussi-Yanicostas N, et al. Loss-of-function mutations in FGFR1 cause autosomal dominant Kallmann syndrome. Nat Genet 2003;33:463–5.

[23] Kondo S, Schutte BC, Richardson RJ, Bjork BC, Knight AS, Watanabe Y, et al. Mutations in IRF6 cause Van der Woude and popliteal pterygium syndromes. Nat Genet 2002;32:285–9.

[24] Mostowska A, Biedziak B, Jagodzinski PP. Axis inhibition protein 2 (AXIN2) polymorphisms may be a risk factor for selective tooth agenesis. J Hum Genet 2006;51:262–6.

[25] Lammi L, Arte S, Somer M, Jarvinen H, Lahermo P, Thesleff I, et al. Mutations in AXIN2 cause familial tooth agenesis and predispose to colorectal cancer. Am J Hum Genet 2004;74:1043–50.

[26] Peres RC, Scarel-Caminaga RM, do Espirito Santo AR, Line SR. Association between PAX-9 promoter polymorphisms and hypodontia in humans. Arch Oral Biol 2005;50:861–71.

[27] Mostowska A, Biedziak B, Trzeciak WH. A novel c.581C>T transition localized in a highly conserved homeobox sequence of MSX1: is it responsible for oligodontia? J Appl Genet 2006;47:159–64.

[28] Wang J, Jian F, Chen J, Wang H, Lin Y, Yang Z, et al. Sequence analysis of PAX9, MSX1 and AXIN2 genes in a Chinese oligodontia family. Arch Oral Biol 2011;56:1027–34.

[29] Suazo J, Santos JL, Jara L, Blanco R. Parent-of-origin effects for MSX1 in a Chilean population with nonsyndromic cleft lip/palate. Am J Med Genet Part A 2010;152A:2011–16.

[30] De Coster PJ, Marks LA, Martens LC, Huysseune A. Dental agenesis: genetic and clinical perspectives. J Oral Pathol Med 2009;38:1–17.

[31] Matalova E, Fleischmannova J, Sharpe PT, Tucker AS. Tooth agenesis: from molecular genetics to molecular dentistry. J Dent Res 2008;87:617–23.
[32] Ungvari I, Hullam G, Antal P, Kiszel PS, Gezsi A, Hadadi E, et al. Evaluation of a partial genome screening of two asthma susceptibility regions using bayesian network based bayesian multilevel analysis of relevance. PLoS ONE 2012;7:e33573.
[33] Stephens M, Balding DJ. Bayesian statistical methods for genetic association studies. Nat Rev Genet 2009;10: 681–90.
[34] Antal P, Millinghoffer A, Hullám G, Szalai C, Falus A. editors. A Bayesian view of challenges in feature selection: feature aggregation, multiple targets, redundancy and interaction. Workshop on new challenges for feature selection in data mining and knowledge discovery. ECML PKDD Antwerpen, Belgium; 2008.
[35] Balding DJ. A tutorial on statistical methods for population association studies. Nat Rev Genet 2006;7:781–91.
[36] Baurley JW, Conti DV, Gauderman WJ, Thomas DC. Discovery of complex pathways from observational data. Stat Med 2010;29:1998–2011.
[37] Chen GK, Thomas DC. Using biological knowledge to discover higher order interactions in genetic association studies. Genet Epidemiol 2010;34:863–78.
[38] Larmour CJ, Mossey PA, Thind BS, Forgie AH, Stirrups DR. Hypodontia–a retrospective review of prevalence and etiology. Part I. Quintessence Int 2005;36:263–70.
[39] Ajami BA, Shabzendedar M, Mehrjerdian M. Prevalence of hypodontia in nine- to fourteen-year-old children who attended the Mashhad School of Dentistry. Indian J Dent Res 2010;21:549–51.
[40] Muller TP, Hill IN, Peterson AC, Blayney JR. A survey of congenitally missing permanent teeth. J Am Dent Assoc 1970; 81:101–7.
[41] Rolling S, Poulsen S. Oligodontia in Danish schoolchildren. Acta Odontol Scand 2001;59:111–12.
[42] Rolling S. Hypodontia of permanent teeth in Danish schoolchildren. Scand J Dent Res 1980;88:365–9.
[43] Salama FS, Abdel-Megid FY. Hypodontia of primary and permanent teeth in a sample of Saudi children. Egypt Dent J 1994;40:625–32.
[44] Silva Meza R. Radiographic assessment of congenitally missing teeth in orthodontic patients. Int J Paediatr Dent 2003;13: 112–16.
[45] Ng'ang'a RN, Ng'ang'a PM. Hypodontia of permanent teeth in a Kenyan population. East Afr Med J 2001;78: 200–3.
[46] Aasheim B, Ogaard B. Hypodontia in 9-year-old Norwegians related to need of orthodontic treatment. Scand J Dent Res 1993;101:257–60.
[47] Gabris K, Fabian G, Kaan M, Rozsa N, Tarjan I. Prevalence of hypodontia and hyperdontia in paedodontic and orthodontic patients in Budapest. Community Dent Health 2006;23: 80–2.
[48] de Beer GR. Embryos and ancestors. Oxford: Clarendon Press; 1951. p 58–9.
[49] Brook AH. A unifying aetiological explanation for anomalies of human tooth number and size. Arch Oral Biol 1984;29: 373–8.
[50] Schalk-van der Weide Y, Bosman F. Tooth size in relatives of individuals with oligodontia. Arch Oral Biol 1996;41: 469–72.
[51] Jorgenson RJ. Clinician's view of hypodontia. J Am Dent Assoc 1980;101:283–6.
[52] Fekonja A. Hypodontia in orthodontically treated children. Eur J Orthodont 2005;27:457–60.
[53] Dahlberg AA. The changing dentition of man. J Am Dent Assoc 1945;32:676–90.
[54] Butler PM. editor. Studies of the mammalian dentition. Differentiation of the post-canine dentition. In Proceedings of the Zoological Society of London, London 1939;109B:1–36.
[55] Pan Y, Wang L, Ma J, Zhang W, Wang M, Zhong W, et al. PAX9 polymorphisms and susceptibility to sporadic tooth agenesis: a case-control study in southeast China. Eur J Oral Sci 2008;116:98–103.
[56] Endo T, Ozoe R, Yoshino S, Shimooka S. Hypodontia patterns and variations in craniofacial morphology in Japanese orthodontic patients. Angle Orthod 2006;76:996–1003.
[57] Dolder E. Deficient dentition. Statistical survey. Dent Rec 1937;57:142–3.
[58] Nordgarden H, Jensen JL, Storhaug K. Reported prevalence of congenitally missing teeth in two Norwegian counties. Community Dent Health 2002;19:258–61.
[59] Rosenzweig KA, Garbarski D. Numerical aberrations in the permanent teeth of grade school children in Jerusalem. Am J Phys Anthropol 1965;23:277–83.
[60] Kuo HC, Lin PY, Chung TC, Chao CM, Lai LC, Tsai MH, et al. DBCAT: database of CpG islands and analytical tools for identifying comprehensive methylation profiles in cancer cells. J Comput Biol 2011;18:1013–17.
[61] Piechota M, Korostynski M, Przewlocki R. Identification of cis-regulatory elements in the mammalian genome: the cREMaG database. PLoS ONE 2010;5:e12465.
[62] Santagati F, Abe K, Schmidt V, Schmitt–John T, Suzuki M, Yamamura K, et al. Identification of Cis–regulatory elements in the mouse Pax9/Nkx2–9 genomic region: implication for evolutionary conserved synteny. Genetics 2003;165:235–42.
[63] Miletich I, Sharpe PT. Normal and abnormal dental development. Human molecular genetics. 2003;12 Spec No 1:R69–73.
[64] Nakatomi M, Wang XP, Key D, Lund JJ, Turbe–Doan A, Kist R, et al. Genetic interactions between Pax9 and Msx1 regulate lip development and several stages of tooth morphogenesis. Dev Biol 2010;340:438–49.
[65] Jernvall J, Thesleff I. Reiterative signaling and patterning during mammalian tooth morphogenesis. Mech Dev 2000; 92:19–29.
[66] Peters H, Neubuser A, Balling R. Pax genes and organogenesis: Pax9 meets tooth development. Eur J Oral Sci 1998; 106 Suppl 1:38–43.
[67] Peters H, Wilm B, Sakai N, Imai K, Maas R, Balling R. Pax1 and Pax9 synergistically regulate vertebral column development. Development 1999;126:5399–408.
[68] Tucker AS, Al Khamis A, Sharpe PT. Interactions between Bmp–4 and Msx–1 act to restrict gene expression to odontogenic mesenchyme. Dev Dyn 1998;212:533–9.
[69] Vainio S, Karavanova I, Jowett A, Thesleff I, Identification of BMP–4 as a signal mediating secondary induction between epithelial and mesenchymal tissues during early tooth development. Cell 1993;75:45–58.
[70] Peters H, Neubuser A, Kratochwil K, Balling R. Pax9–deficient mice lack pharyngeal pouch derivatives and teeth and exhibit craniofacial and limb abnormalities. Genes Dev 1998;12:2735–47.
[71] Satokata I, Maas R. Msx1 deficient mice exhibit cleft palate and abnormalities of craniofacial and tooth development. Nat Genet 1994;6:348–56.
[72] Ogawa T, Kapadia H, Wang B, D'Souza RN. Studies on Pax9–Msx1 protein interactions. Arch Oral Biol 2005;50:141–5.
[73] Zhou J, Gao Y, Zhang Z, Zhang Y, Maltby KM, Liu Z, et al. Osr2 acts downstream of Pax9 and interacts with both Msx1 and Pax9 to pattern the tooth developmental field. Dev Biol 2011;353:344–53.
[74] Pereira TV, Salzano FM, Mostowska A, Trzeciak WH, Ruiz–Linares A, Chies JA, et al. Natural selection and molecular evolution in primate PAX9 gene, a major determinant of tooth development. Proc Natl Acad Sci U S A 2006;103: 5676–81.

[75] Pawlowska E, Janik–Papis K, Wisniewska–Jarosinska M, Szczepanska J, Blasiak J. Mutations in the human homeobox MSX1 gene in the congenital lack of permanent teeth. Tohoku J Exp Med 2009;217:307–12.

[76] Paixao–Cortes VR, Braga T, Salzano FM, Mundstock K, Mundstock CA, Bortolini MC. PAX9 and MSX1 transcription factor genes in non–syndromic dental agenesis. Arch Oral Biol 2011;56:337–44.

[77] Bianch FJ, de Oliveira TF, Saito CB, Peres RC, Line SR. Association between polymorphism in the promoter region (G/C–915) of PAX9 gene and third molar agenesis. J Appl Oral Sci revista FOB 2007;15:382–6.

[78] Mackenzie IC, Rittman G, Gao Z, Leigh I, Lane EB. Patterns of cytokeratin expression in human gingival epithelia. J Periodontal Res 1991;26:468–78.

[79] Huang YQ, Ma J, Ma M, Deng Y, Li YD, Ren HW, et al. Association between MSX1 variants and oral clefts in Han Chinese in western China. DNA Cell Biol 2011;30:1057–61.

[80] Vieira AR. Unraveling human cleft lip and palate research. J Dent Res 2008;87:119–25.

[81] Vieira AR, Seymen F, Patir A, Menezes R. Evidence of linkage disequilibrium between polymorphisms at the IRF6 locus and isolate tooth agenesis, in a Turkish population. Arch Oral Biol 2008;53:780–4.