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SHORT COMMUNICATION

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Axis inhibition protein 2 (*AXIN2*) polymorphisms may be a risk factor for selective tooth agenesis

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Abstract Selective tooth agenesis is the most common developmental abnormality of the human dentition. To date, this abnormality has been associated only with mutations in *MSX1* and *PAX9* mutations, however it has recently been suggested that mutations of axis inhibition protein 2 (*AXIN2*) may also contribute to this complex anomaly. The protein product of this gene is a negative regulator of the Wnt-signaling pathway. We searched for *AXIN2* variants in a group of patients with tooth agenesis who did not have mutations of *MSX1* and *PAX9*. Using multi-temperature single-stranded conformational polymorphism and sequencing analysis, we identified three novel *AXIN2* gene variants: c.956 + 16A > G, c.1060-17C > T and c.2062C > T. We also observed that individuals carrying the c.956 + 16G and c.2062T alleles exhibited an increased risk of tooth agenesis. The calculated odds ratio was 2.94 (95% CI 1.104–7.816; $p = 0.026$; $p_{corr} = 0.234$) and 4.01 (95% CI 1.563–10.301; $p = 0.002$; $p_{corr} = 0.018$), respectively. Moreover, we found that the c.2062C > T transition may change exon splice enhancer-specific binding sites of the protein splicing regulators SC35 and SF2/ASF. This alternation may negatively affect the splicing process and cellular concentration of AXIN2 protein. Our findings suggest that *AXIN2* polymorphic variants may be associated with both hypodontia and oligodontia.

Keywords Hypodontia · Oligodontia · *AXIN2* · Polymorphisms

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Introduction

Hypodontia, defined as the congenital lack of one or more permanent teeth, is the most common dental abnormality found in humans and affects approximately 20% of the population worldwide (Vastardis 2000). The etiology of this anomaly is very complex and is associated with both genetic and environmental factors (Mostowska et al. 2003). Molecular studies of mice odontogenesis have shown that more than 200 genes are involved in tooth development (Thesleff and Nieminen 1996; http://www.bite-it.helsinki.fi). However, to date only a limited number of mutations of *MSX1* and *PAX9* have been shown to be associated with severe hypodontia (oligodontia) in humans (OMIM *142983 and *167416).

Based on results from familial studies, Lammi et al. (2004) have recently suggested that oligodontia may also be caused by mutations of the axis inhibition protein 2 (*AXIN2*) gene, which is localized on chromosome 17q21-q25. Mutations of this gene have been found in colorectal carcinomas and liver tumors (Salahshor and Woodgett 2005). The protein product of *AXIN2* is a negative regulator of the canonical Wnt pathway that suppresses signal transduction by promoting the degradation of β-catenin (Jho et al. 2002). AXIN2 functions as a scaffold protein, which is an essential component of a protein complex required for the phosphorylation of β-catenin (Lustig and Behrens 2003). An *AXIN2* mutation (c.1966C > T) that occurred in a Finnish family has been associated with both tooth agenesis and colon neoplasia, an observation which suggests that the lack of permanent teeth may be an indicator of colon cancer susceptibility (Lammi et al. 2004).

Analysis of *Axin2* expression patterns during mice odontogenesis revealed that this gene was expressed in the mesenchyme underlying the oral epithelium of upper and lower jaws as well as in the enamel knot, the dental papilla mesenchyme and in the layer of mesenchymal odontoblasts and preodontoblasts (Lammi et al. 2004).

The involvement of the protein product of *AXIN2* and other Wnt-signaling genes in carcinogenesis is well established, however little is known about their importance in the etiology of both hypodontia and oligodontia in humans. This gap in our knowledge led us to analyze the association of *AXIN2* gene variants with human tooth agenesis in a selected group of patients that did not have mutations in either of the *MSX1* and *PAX9* genes.

Materials and methods

Study population

Peripheral blood samples from 55 unrelated Caucasian patients with selective tooth agenesis were obtained from the Department of Orthodontics at the University of Medical Sciences in Poznan. The selection criteria were agenesis of at least one permanent tooth, excluding third molars, and the lack of other craniofacial malformations or systemic diseases. Analysis of dental diagrams and panoramic radiographs revealed that the most commonly missing teeth were the second premolars, followed by the lateral and central incisors. Of the 55 patients, 29 were missing from one to six permanent teeth (52.73%; hypodontia), whereas the lack of more than six permanent teeth was observed in 26 individuals (47.27%; oligodontia). In the group of patients with hypodontia, those missing the upper lateral incisors were the most numerous (65.52%). The control groups consisted of 102 healthy individuals that had normal primary and permanent dentition and did not show any craniofacial abnormalities. The experiments were approved by the local Ethics Committee, and written and oral consent was obtained from all participants.

DNA analysis

DNA was isolated from peripheral blood lymphocytes by salt extraction. Ten exons of *AXIN2* were PCR amplified with the use of specific primers (primer sequences available for request). Amplified regions were screened for DNA sequence variants by single-stranded conformational polymorphism analysis performed in a temperature gradient (MSSCP) utilizing of the DNA Pointer Mutation Detection System (Kucharczyk TE, Warsaw, Poland). All DNA products that exhibited changes in mobility in a polyacrylamide gel were purified by the GelOut system (A&A Biotechnology, Warsaw, Poland) and sequenced using the ABI Prism BigDye Terminator v3.1 Cycle Sequencing kit and ABI 310 genetic analyzer (Applied Biosystems, Foster City, Calif.). Genotyping of all identified polymorphisms was carried out by PCR followed by the appropriate restriction enzyme digestion and was performed according to the manufacturer's instructions (Fermentas, Vilnius, Lithuania). DNA fragments were separated

Table 1 Conditions for the identification of *AXIN2* polymorphisms

Polymorphism[a]	Primer		Fragment length (bp)	Annealing temperature (°C)	Restriction enzyme
	Forward	Reverse[b]			
c.148C > T	CCACGCCGATTGCTGAGAGG	TTCCGCCTGGTGTTGGAAGAGACAT	242	63.2	*Mph*1103I
c.432T > C	CCTGGAGAGGGAGAAATGC	CATCACCGACTGGATCTCG	253	50.9	*Esp*3I
c.956 + 16A > G	GGGTTTCTGGTGAGGGTCAGT	CAAGCAAGCCCCACGGAAGG	209	65.6	*Mls*I
c.1060-17C > T	CCCACCTAGCCTGCTGAAC	TAGGCCGCATTACCTCTCG	206	61.4	–[c]
c.1365A > G	ACGTCTTCCCTTTCAGGATG	GGTACTGCGAATGGTGGTG	248	55.6	*Msp*I
c.1386C > T	TGCGTAGGGAGCCGAATGTTG	GTGGTCCGGGGAGCGGGATC	294	63.2	*Taq*I
c.1712 + 19G > T	CACCACCACTACATCCACCAC	GCTCCCACCTCACACCTG	249	61.2	*Cpo*I
c.2062C > T	TCCTTCTGTTTTCTCTCTGCTCATTCC	AGCGTGTTGGGTGGGGTCG	209	65.6	*Taq*I
c.2141 + 73G > A	GTGTCGAAGCCCCCAAAG	CTTGATCCTCCATCTCACAGC	370	63.2	*Bse*RI

[a]Single nucleotide polymorphisms (SNPs) numbered in relation to cDNA positions (NCBI, NT_010783 and NM_004655)
[b]The underlined letters denote modified bases necessary for the creation of specific restriction sites
[c]Analysis using single-stranded conformational polymorphism analysis performed in a temperature gradient (MSSCP)

Table 2 Allele frequencies of *AXIN2* polymorphisms in patients with tooth agenesis and in the controls

Polymorphism	dbSNP ID	Localization	Protein effect	Allele	Number (frequency)		X^2	p	p_{corr}
					Tooth agenesis	Controls			
c.148C > T	rs2240308	Exon 1	Pro50Ser	C	57 (0.52)	98 (0.49)	0.225	0.635	–
				T	53 (0.48)	102 (0.51)			
c.432T > C	rs2240307	Exon 1	Ile144Ile	T	109 (0.99)	198 (0.99)	0.0061	0.938	–
				C	1 (0.01)	2 (0.01)			
c.956 + 16A > G	–	Intron 2	–	A	98 (0.89)	196 (0.96)	5.851	**0.016**	0.144
				G	12 (0.11)	8 (0.04)			
c.1060-17C > T	–	Intron 3	–	C	100 (0.91)	180 (0.88)	0.529	0.467	–
				T	10 (0.09)	24 (0.12)			
c.1365A > G	rs9915936	Exon 5	Pro455Pro	A	11 (0.10)	23 (0.115)	0.164	0.686	–
				G	99 (0.90)	177 (0.885)			
c.1386C > T	rs1133683	Exon 5	Pro462Pro	C	44 (0.40)	75 (0.375)	0.311	0.577	–
				T	64 (0.60)	125 (0.625)			
c.1712 + 19G > T	rs7219582	Intron 5	–	G	100 (0.93)	177 (0.885)	1.298	0.255	–
				T	8 (0.07)	23 (0.115)			
c.2062C > T	–	Exon 7	Leu688Leu	C	96 (0.87)	196 (0.96)	8.505	**0.0035**	**0.0315**
				T	14 (0.13)	8 (0.04)			
c.2141 + 73G > A	rs4072245	Intron 7	–	G	89 (0.89)	187 (0.93)	1.086	0.297	–
				A	11 (0.11)	15 (0.07)			

either in 1.5% agarose or 10% polyacrylamide gels. Conditions for PCR-restriction fragment length polymorphism (RFLP) analyses are shown in Table 1. The only one exception was the 1060-17C > T polymorphism, for which genotyping was done by means of MSSCP analysis.

Statistical analysis

The differences in allele and genotype frequencies between patients and controls were determined using standard Chi-square and Fisher exact tests. The odds ratio (OR) and associated 95% confidence interval (95% CI) for subjects with tooth agenesis versus controls were also calculated. The Bonferroni correction for multiple comparisons was applied. Both the p values, before the correction (p) and after the correction (p_{corr}), were determined. The linkage disequilibrium (LD) coefficient (D') and correlation coefficient (r^2) were used to determine the extent of LD using thresholds $D' > 0.33$ and $r^2 > 0.1$ (Long et al. 2004). A log of the likelihood odds ratio (LOD) > 5.4 was considered to be highly significant, whereas LOD > 3.6 was considered to be significant (Lander and Kruglyak 1995). All of these values were calculated between pairs of nine single nucleotide polymorphisms (SNPs) using the Haploview software (Barrett et al. 2005).

Results and discussion

The sequence analysis of exons and exon-intron boundaries of *AXIN2* performed in our patients with selective tooth agenesis revealed six known polymorphisms of this gene: c.148C > T, c.432T > C, c.1365A > G, c.1386C > T, c.1712 + 19G > T and c.2141 + 73G > A. Moreover, we found three novel gene variants, of which two were localized in intronic sequences (c.956 + 16A > G and c.1060-17C > T) and one, a synonymous polymorphism, was localized in exon 7 (c.2062C > T, Leu688Leu; Table 2). Our results indicated that individuals carrying the c.956 + 16G and c.2062T alleles had an increased risk of selective tooth agenesis (Table 3). The calculated OD for c.956 + 16A > G transition was 2.94 (95% CI: 1.104–7.816;

Table 3 Association between *AXIN2* genotypes and tooth agenesis

Polymorphism	Genotype	Controls n (%)	Tooth agenesis n (%)	Odds ratio (95% CI)	p^a	p_{corr}
c.956 + 16A > G	Homozygous AA	94 (92.16)	44 (80.00)	Referent		
	Combined AG + GG	8 (7.84)	11 (20.00)	2.94 (1.104–7.816)	0.026	0.234
	Mutant allele frequency[b]	0.04	0.11[c]	–	–	–
c.2062C > T	Homozygous CC	94 (92.16)	41 (74.55)	Referent		
	Combined CT + TT	8 (7.84)	14 (25.45)	4.01 (1.563–10.301)	**0.002**	**0.018**
	Mutant allele frequency[b]	0.04	0.13[d]	–	–	–

[a]Chi-square analysis
[b]All populations are in Hardy-Weinberg equilibrium, $p > 0.1$
[c]Significantly different from controls: $p = 0.016$
[d]Significantly different from controls: $p = 0.0035$

$p = 0.026$). However, after Bonferroni correction the p value was no longer statistically significant. The calculated OR for the c.2062C > T transition was 4.01 (95% CI: 1.563–10.301; $p = 0.002$), and the p value remained statistically significant after Bonferroni correction as well ($p_{corr} = 0.018$).

In addition, in patients with tooth agenesis very strong LD was detected only between the c.956 + 16A > G and c.2062C > T polymorphisms ($D' = 0.903$, $r^2 = 0.745$, LOD = 8.53): among 14 individuals with the c.2062C > T polymorphism, 11 were also carriers of the c.956 + 16A > G intronic variant (78.50%). In contrast, among the control group the LD ($D' = 0.468$, $r^2 = 0.219$, LOD = 2.60) was less significant compared with patients: the c.2026C > T and c.956 + 16A > G polymorphisms occurred simultaneously only in 4 of 12 healthy individuals (33.33%).

It is worth noting that the synonymous transition c.2062C > T may constitute a risk factor for selective tooth agenesis since it may disrupt exonic splicing enhancer (ESE) sequences involved in the splicing process. These motifs are *cis*-elements playing a crucial role in correct splice-site identification. They can act either by enhancing or by repressing splicing and serve as binding sites for specific proteins that are needed to form the spliceosome (Cartegni et al. 2002). Point mutations disrupting the ESE sequences might be associated with genetic diseases, including spinal muscular atrophy or Marfan syndrome (Caputi et al. 2002; Cartegni and Krainer 2002). Using ESE motif prediction tools (http://www.rulai.cshl.edu/tools/ESE/index.html), we found that polymorphism c.2062C > T might exert an effect on splice site prediction. This gene variant may eliminate the binding site (motif CTCCCCTG) for the SC35 protein and create one additional binding site (motif CTCCCTT) for the protein complex SF2/ASF.

SC35 and SF2/ASF proteins have been found in the pre-spliceosomal E complex and have also been implicated in many aspects of constitutive and regulated splicing (Blencowe 2000). The SC35 motif can promote the splicing process, whereas the SF2/ASF protein can repress it (Wang et al. 1998; Liu et al. 2000). Thus, we suggest that replacement of the SC35 binding site by SF2/ASF – following c.2062C > T transition – may negatively affect splicing efficiency and the cellular concentration of AXIN2 and lead to abnormalities in tooth development (Fig. 1).

The results of the pairwise LD analysis may indicate that the risk of selective tooth agenesis is connected with the simultaneous occurrence of both novel *AXIN2* polymorphisms c.956 + 16A > G and c.2062C > T. Our analysis of splice sites predictions subsequent to the c.956 + 16A > G transition using the NETGENE2 program (http://www.cbs.dtu.dk/services/NetGene2/) revealed

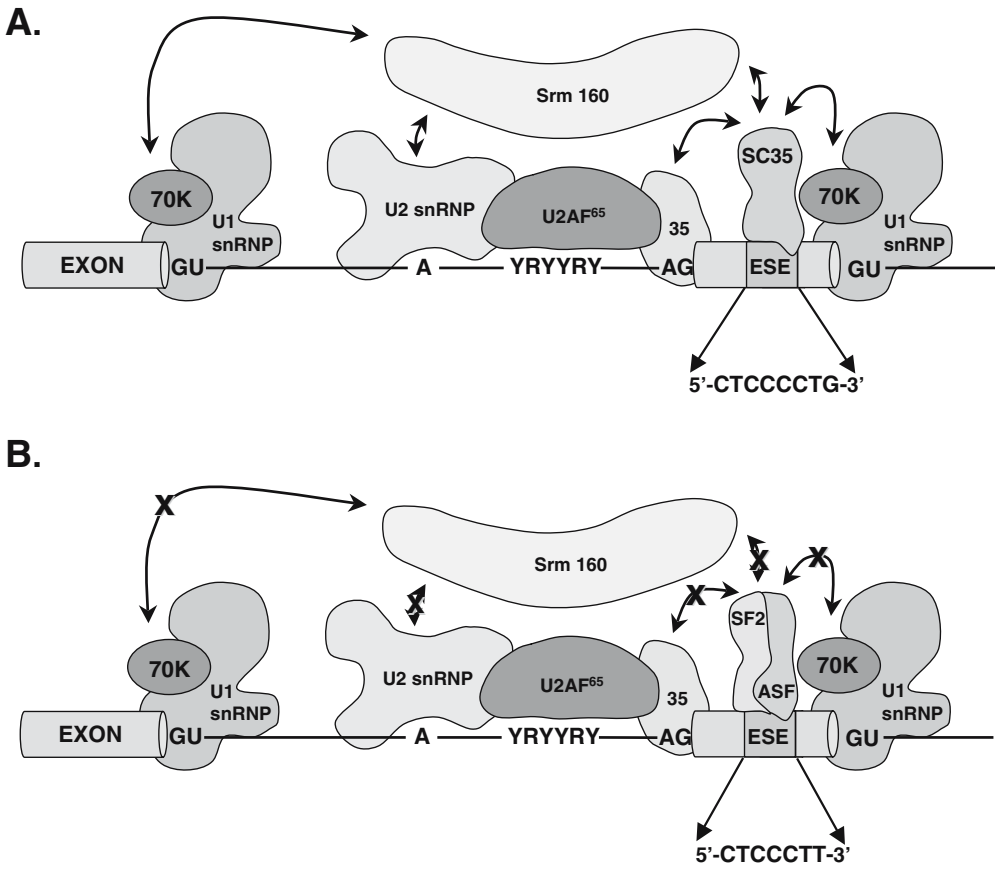

Fig. 1 Hypothetical models of the involvement of exonic splicing enhancer sequences in splicing (adapted from Cartegni et al. 2002). The c.2062C > T transition resulting in the replacement of SC35 binding site by SF2/ASF may negatively affect splicing efficiency and the cellular concentration of AXIN2 (**a**, **b**). *U2AF* Splicing factor ($U2AF^{65}$ + $U2AF^{35}$), *snRNP* small nuclear ribonucleoprotein, *Srm 160* splicing co-activator

that this polymorphism may also exert a weak effect on splicing as it creates an additional donor-splicing site within the sequence of exon 2 (5′-ACGGACAGCA^GTGTgtgagt-3′; confidence 0.24). Based on these results, we assume that the negative effect of c.2062C > T transition on splicing may be intensified by the c.956 + 16A > G variant.

Molecular studies of mice odontogenesis have demonstrated that a number of Wnt genes are expressed in the developing teeth and that changes in their expression may be one of the factors determining tooth agenesis (Zhang et al. 2005). The first evidence for a crucial role of the Wnt pathway in odontogenesis comes from the functional analysis of *Lef1*. The protein product of this gene is a transcription factor that plays a role in activating Wnt target genes and it is also a critical factor affecting the survival of the epithelial cells during tooth morphogenesis (Sasaki et al. 2005). Mice homozygous for the *Lef1* deletion died shortly after birth and showed abnormalities in epithelial-mesenchymal interactions, which led to an arrest of tooth development at the bud stage (Kratochwil et al. 1996). Defects in odontogenesis were also observed in mice overexpressing Wnt-signaling inhibitors, including Dickkopf and secreted frizzled-related protein 3 (Sarkar and Sharpe 2000; Andl et al. 2002).

In conclusion, we provide here the first experimental evidence that the risk of both hypodontia and oligodontia may be related to *AXIN2* polymorphic variants. Our results also confirm the importance of the Wnt pathway in tooth development and suggest that other factors – as yet unknown – may be associated with this common developmental anomaly in addition to the mutations of *MSX1* and *PAX9*. However, an understanding the exact function of AXIN2 in odontogenesis requires further detailed analysis of each stage of this process.

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