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Original scientific article

ANALYSIS OF *COL1A1* AND *MMP9* SINGLE NUCLEOTIDE POLYMORPHISMS IN MANDIBULAR PROGNATHISM

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Mandibular prognathism (MP) belongs to malocclusions of skeletal Class III and is characterized by overgrowth of the lower jaw with or without undergrowth of the upper jaw. MP etiology is multifactorial, including both environmental and genetic factors. It is conceivable that single nucleotide polymorphisms (SNPs) in genes controlling craniofacial development might contribute to MP. The aim of the present study was to establish a potential association between COL1A1 -1997 G>T (rs1107946) and *MMP9* -1562 C>T (rs3918242) SNPs and MP in Serbian population. This case-control study included 120 participants: 60 patients with MP and 60 controls with skeletal Class I. The two SNPs were analyzed by means of polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. The association of gene variants with MP risk was determined by calculating odds ratios (OR) and their 95% confidence intervals (CI).There was no difference in SNPs allele frequencies, and no difference could be observed in *MMP9* -1562 C>T genotypes distribution between cases and controls. However, the TT genotype of *COL1A1*-1997 G/T (rs1107946) polymorphism was associated with a two-fold increase of mandibular prognathism risk, though with a borderline statistical significance (OR 2.32, CI 0.97-5.53, p=0.055). *COL1A1*-1997 G/T (rs1107946) appears to be implicated in Class III mandibular prognathism while *MMP9* -

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1562 C/T (rs3918242) does not seem to be a risk factor for the development of this type of craniofacial anomaly.

Key words: COL1A1, mandibular prognathism**,** *MMP9,* Single Nucleotide Polymorphism

INTRODUCTION

Mandibular prognathism (MP) or skeletal Class III malocclusion with a prognathic mandible is one of the most common and severe craniofacial deformities, characterized by overgrowth of the lower jaw with or without undergrowth of the upper jaw (CHANG *et al.*, 2006). This abnormal forward projection of the mandible beyond the standard relation to the maxilla and/or cranial base leads, in the majority of patients with MP, not only to compromised facial esthetics but also to mastication and speech issues (DORACZYNSKA-KOWALIK *et al.*, 2017).

Global prevalence of MP varies between 0% and 26.7% in different populations (HARDY *et al.*, 2012); it shows the highest frequencies in East Asian and the lowest in European and American Caucasians populations (BUKHARY, 2005; XUE *et al.*, 2010). These variations in MP prevalence among different races, ethnic groups and geographic regions are due to its multifactorial aetiology. MP results from a moderate distortion of normal development, rather than from a pathological process, following complex interactions between environmental and genetic factors. Numerous known environmental contributors include atypical swallowing, mouth breathing, tongue size and pharyngeal airway alterations, hormonal disturbances, premature loss of primary teeth etc. (ZERE *et al.*, 2018).

While extremely hard to predict, mandibular growth is known to be primarily affected by heredity, although the exact patterns of transmission (autosomal recessive, autosomal dominant with complete or incomplete penetrance, polygenic) are still a subject of debate (ZERE *et al.*, 2018). Numerous studies have focused on the detection of candidate genes and their polymorphisms, including SNPs, potentially related to MP. Establishing associations between specific SNPs and MP could help to identify individuals with genetic predisposition to develop this condition. Additionally, this would aid decision making on whether to start treatment of MP earlier in childhood, predict therapy responses and even lay the foundation for gene therapy of MP in the future (LIU *et al.*, 2017).

SNPs in genes involved in craniofacial development, especially craniofacial cartilage and bone morphogenesis and metabolism, are among those that should be considered as plausible candidates for MP contributors. The *COL1A1* gene is responsible for the synthesis of the alpha 1 chain of type 1 collagen which is the most abundant protein in the bone tissue, tendons, cartilage and skin (MARINI *et al.*, 2017). *COL1A1* expression was found in the mandibular condylar cartilage, the center of mandibular growth (SHIBATA *et al.*, 2014). While some authors state that mutations in *COL1A1* affect long bones more than cranial bones (EIMAR *et al.*, 2016), SNPs in this gene have been associated with higher risk of both skeletal class II (ARDANI *et al.*, 2020) and class III (DA FONTOURA *et al.*, 2015). *MMP9* gene is responsible for the synthesis of gelatinase B, a member of matrix metalloproteinases (MMPs) family of zinc dependent enzymes with an established role in extracellular matrix (ECM) remodeling (LE *et al.*, 2007). MMP9 is one of the most abundantly expressed and functionally important MMPs in bone and cartilage cells during normal skeletal development. Its expression was found during human secondary palate formation (ŠIMIĆ BILANDŽIJA *et al.*, 2021) and the degradation of

Meckel's cartilage, a supporting tissue of the embryonic mandible in mammals (TSUZURAHARA *et al.*, 2011). Although, to our knowledge, there are no studies regarding MMP9 contribution to MP, SNPs in the *MMP9* gene have previously been associated with anterior open bite malocclusion (KÜCHLER *et al.*, 2017) and temporomandibular disorders (MILOSEVIC *et al.*, 2015).

The aim of this study was to assess the possible association between *COL1A1* -1997 G>T (rs1107946) and *MMP9* -1562 C>T (rs3918242) SNPs and mandibular prognathism in Serbian population.

MATERIALS AND METHODS

Patients

The present case-control study was approved by the Ethical Committee of the School of Dental Medicine, University of Belgrade and carried out in full agreement with the Declaration of Helsinki. All participants signed an informed consent. The study involved 120 unrelated participants of Serbian ethnicity, divided into two groups: 60 Class III individuals diagnosed with mandibular prognathism, 32 males and 28 females, and 60 Class I individuals, 25 males and 35 females, as controls. Extra- and intra-oral clinical examinations were performed to evaluate occlusal relationship, later confirmed by study cast analyses and lateral cephalogram analyses. The inclusion criteria for the study group were: finished growth and development, a mandibular base/cranial base length ratio ≥5 mm (measured on lateral cephalograms according to Schwartz), cephalometric value of SNB angle ≥ 82°, and the presence of at least two parameters from the following: ANB angle of the centric jaw (relative sagittal position of the maxilla and mandible) ≤ 0° 24, Wits appraisal ≤ - 2 mm, negative overjet, Class III molar relationship on both sides of the arch, according to Angle's classification, concave or flat profile. The inclusion criteria for the control group were: completed growth and development, normally developed and orthognathic mandible, ANB angle of 1–5°; and the Wits appraisal of 0–2 mm, overjet of 2 mm, Class I molar occlusion on both sides of the arch, according to Angle's classification, and good profile.

The patients were excluded from the study if exhibiting the following: severe facial trauma, systemic diseases (cardiovascular and endocrinological), congenital anomalies such as cleft lip and/or palate, disorders of endocranial development, teeth number and/or morphology abnormalities, previous orthodontic treatment and retrognathic or underdeveloped maxilla, i.e. individuals with SNA angle less than < 79°

COL1A1 -1997 G>T and MMP9 -1562 C>T single nucleotide polymorphisms (SNPs) genotyping

Genomic DNA was isolated using standardized desalting method from buccal swabs obtained during orthodontic control examinations, and was stored at -20°C awaiting further analysis. Polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) method was used for the analysis of the two SNPs. The PCR reactions were carried out in a total volume of 25 μL, consisting of a half of volume PCR Master Mix (2X) (Thermo Fisher Scientific, Vilnius, Lithuania), 200 nM of each primer (*Col1A1* Fwd: CACCCTGCCCTAGACCAC; *Col1A1* Rev: GAAAATATAGAGTTTCCAGAG; *MMP9* Fwd: GCCTGGCACATAGTAGGCCC; *MMP9* Rev: CTTCCTAGCCAGCCGGCATC), and 0.2 μg of genomic DNA. After amplification, products were digested using FastDigest Restriction

Enzymes (Thermo Fisher Scientific, Vilnius, Lithuania), Eco31I for *Col1A1* and Sph1 for *MMP9* and the digestion products were separated on 8% PAA gels, stained with ethidium bromide and visualized under UV light.

After the digestion of the *Col1A1* product, the presence of two bands – 212bp and 81bp corresponded to the GG genotype, while the presence of a single band – 293bp corresponded to the TT genotype. The heterozygous genotype GT displayed all three bands (Figure 1).

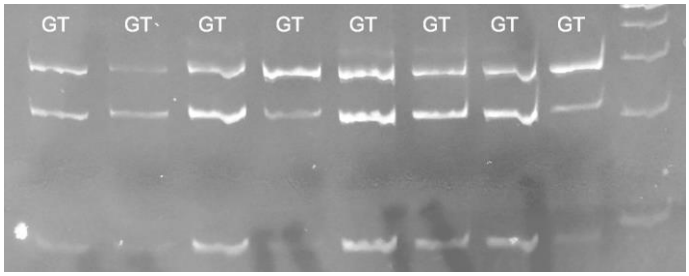

Figure 1. Genotypes of *COL1A1* SNP after RFLP analysis; the last lane contains DNA ladder.

After the digestion of the *MMP9* product, the presence of a single band – 435bp corresponded to the CC genotype, while the presence of two bands – 247 and 188bp corresponded to the TT genotype. Heterozygotes showed the presence of all three bands (Figure 2).

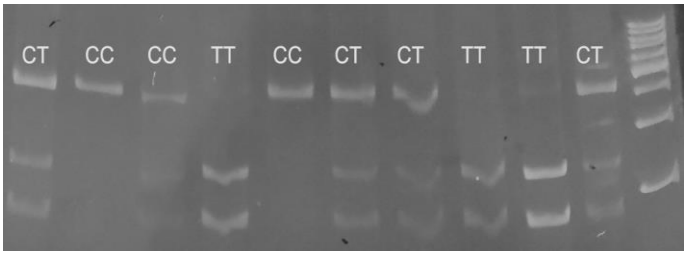

Figure 2. Genotypes of *MMP9* SNP after RFLP analysis; the last lane contains DNA ladder.

Statistical analysis

The expected frequency of variants in the control group was analyzed by the Hardy-Weinberg equilibrium test. Possible differences in genotype and allele frequency distributions were determined using Pearson's chi-square test and Fisher exact test. The association of gene variants with risk of disease was investigated using unconditional logistic regression analysis,

odds ratios (ORs) and their 95% confidence intervals (CIs). Statistical calculations were performed using SPSS Statistics for Windows Software, version 22.0 (IBM Corp, Armonk, NY, USA). *P* values of < 0.05 and was considered statistically significant.

RESULTS

Genotype distributions for *COL1A1*-1997 G/T (rs1107946) in Class III patients with mandibular prognathism and Class I controls deviated from Hardy-Weinberg equilibrium (HWE) (P<0.05), whilst the genotype frequencies of *MMP9* -1562 C/T in both study groups were in HWE (P>0.05). No statistically significant difference was observed between Class III and Class I individuals, regarding the allelic distribution for neither *COL1A1*-1997 G/T (rs1107946) nor *MMP9* -1562 C/T (rs3918242) single nucleotide polymorphisms (P=0.12 and P=0.13, respectively).

Homozygous carriers of the variant allele T for the *COL1A1*-1997 G/T (rs1107946), had an approximately two-fold increase of risk for mandibular prognathism development (Odds Ratio 2.32, 95% Confidence Interval 0.97-5.53) in the recessive model (GG+GT vs. TT), although with borderline statistical significance (P=0.055).

No significant difference was observed for the genotype frequencies of *MMP9* -1562 C/T (rs3918242) single nucleotide polymorphisms between Class III individuals with mandibular prognathism and Class I controls (Table 1).

Table 1. Genotype frequencies of COL1A1-1997 G/T (rs1107946) and MMP9 -1562 C/T (rs3918242) single nucleotide polymorphisms in Class III patients with mandibular prognathism and Class I controls

SNP	Study group	Control group	P*	OR (95% CI)
COL1A1 -1997 G/T (rs1107946)	N = 60 (%)	N = 60 (%)		
GG	2 (3.3)	5 (8.3)	Ref	
GT	39 (65)	45 (75)	0.451	2.17 (0.40-11.80)
TT	19 (31.7)	10 (16.7)	0.103	4.75 (0.78-29.02)
GG vs. GT+TT	2 (3.3) vs 58 (96.7)	5 (8.3) vs 55 (91.7)	0.439	2.64 (0.49-14.16)
GG+GT vs. TT	41 (68.3) vs 19 (31.7)	50 (83.3) vs 10 (16.7)	**0.055**	2.32 (0.97-5.53)
MMP9 -1562 C/T (rs3918242)	N = 60 (%)	N = 60 (%)		
CC	39 (65)	43 (71.7)	Ref	
CT	16 (26.7)	17 (28.3)	0.920	1.04 (0.46-2.33)
TT	5 (8.3)	0 (0)	ND	
CC vs. CT+TT	39 (65) vs 21 (35)	43 (71.7) vs 17 (28.3)	0.431	1.36 (0.63-2.95)
CC+CT vs. TT	55 (91.7) vs 5 (8.3)	60 (100) vs 0 (0)	ND	

MMP9 – matrix metalloproteinase 9; COL1A1 - Collagen, type I, alpha 1; SNP – Single Nucleotide Polymorphism; OR - Odds Ratio; CI - Confidence Interval; ND – not determined.

* Pearson's Chi-square or Fisher exact test, significant at $P \leq 0.05$.

DISCUSSION

Although it is well established that Class III mandibular prognathism can be associated with a wide range of environmental factors, the phenomenon of familial aggregation is also evident, suggestive of genetic factors playing a role in its etiology. Various genetic models of inheritance have been proposed, including autosomal dominant, autosomal recessive, but also polygenic threshold model (LITTON *et al.*, 1970; WOLFF *et al.*, 1993; IWAGAKI, 1938). Numerous linkage analyses on Korean, Japanese, Chinese and Estonian pedigrees reported on several genetic loci related to Class III, including 1p22.1, 1q32.2, 1p36, 4p16.1, 6q25, 12pter-p12.3, 12q22-q23, 14q24.3-31.2, and 19p13.2 (YAMAGUCHI *et al.*, 2005; JANG *et al.*, 2010; LI *et al.*, 2010; LI *et al.*, 2011; NIKOPENSIUS *et al.*, 2013; CHEN *et al.*, 2015). Autosomal dominant model of inheritance with variable expressivity and incomplete penetrance was described as the most acceptable genetic model in MP cases with Mendelian inheritance. Although the extent of the involvement of genetic constitution in the pathogenesis of MP remains unclear, some individuals are evidently more prone to mandibular prognathism than others.

Comprehension of the mechanisms behind genetic etiology may contribute to a better management of MP and improvement of treatment outcomes. It might aid the clinicians to screen for individuals with MP susceptibility during childhood and plan an early management prior to the MP phenotype development. In patients with more pronounced mandibular development, where early childhood orthodontic treatment might not suffice and orthognathic surgery is foreseeable, unveiling the predisposition may help reduce the adverse effects of over-treatment. Literature data have demonstrated that genetic components related to MP development are involved not only in the craniofacial bones' ontogenesis, but also related to the masticatory muscles and the condyle, implicating them as novel localized treatment targets, in addition to mandible bones. Finally, linking the genetic polymorphisms and post-treatment phenotype of MP patients might help predict individuals with adverse/favorable surgery responses and thus offer a more precise decision regarding clinical protocol. Linkage analyses have, thus far, implicated multiple loci as candidates behind the mandibular prognathism phenotype, and within them certain candidate genes, including *COL2A1* and *COL1A1* (DORACZYNSKA-KOWALIK *et al.*, 2017).

COL1A1 encodes the major component of type I collagen, the fibrillar collagen found in most connective tissues, including cartilage. During human fetal development, COL1A1 is expressed in the Meckel's cartilage perichondrium, the mandibular bone periosteum, the mandibular condylar cartilage (HINTON, 2014) and the cranial base cartilage (SHIBATA *et al.*, 2014). *COL1A1* is closely related to temporomandibular joint growth and development and is, as such, an important determinant of mandibular length and height. Mutations in the *COL1A1* and *COL1A2* genes are associated with clinical manifestations of osteogenesis imperfecta and Ehlers-Danlos syndrome, including abnormal craniofacial growth, dental malocclusion, and dentinogenesis imperfecta. Previous studies have found an association between a SNP (rs2249492) in the *COL1A1* gene and Class II (ARDANI *et al.*, 2020), as well as Class III malocclusion (DA FONTOURA *et al.*, 2015). More recently, two SNPs located in the promoter of *COL1A1* at positions –1997 G/T (rs1107946) and –1663 indel T (rs2412298) have been associated with bone mineral density (BMD) and have in some populations been reported to interact with an intronic polymorphism (rs1800012) regulating jointly the *COL1A1* expression

and affecting the BMD (HUSTED *et al.*, 2009; JIN *et al.*, 2009; JIN *et al.*, 2006). In the present study, association between *COL1A1*-1997 G/T (rs1107946) SNP and MP was established, but only with a borderline significance, probably due to the relatively small sample size. Interestingly, *COL1A1*-1997 G/T (rs1107946) in Class III patients with mandibular prognathism and Class I controls deviated from Hardy-Weinberg equilibrium (HWE). Deviations from HWE may be due to genotyping errors, but as repeated genotyping was consistent with the initial genotyping, this reason was rejected. Deviations may as well be due to chance or genetic factors which include a heterozygous advantage, population admixture, population substructure, inbreeding, etc. (LI and LEAL, 2009). In the present study, the most probable explanation for HWE deviation is *chance*, being the consequence of a relatively small sample size.

Matrix metalloproteinases (MMPs) represent a family of zinc and calcium-dependent proteolytic enzymes, secreted by immune cells in response to inflammatory stimuli, capable of degrading various components of the extracellular matrix, such as interstitial and basement membrane collagens, fibronectin, laminin, and proteoglycan core proteins (KONTOGIORGIS *et al.*, 2005). They also play an important role in the remodeling of bone. MMP-9 has been found to be involved in the orthodontic periodontal reshaping, i.e. in the response to forces during tooth movement (GRANT *et al.*, 2013). The changes of MMP-9 expression levels have also been demonstrated in several animal models of experimentally created malocclusion (WANG *et al.*, 2010; WANG *et al.*, 2014). Additionally, the high remodeling capability of the temporomandibular joint affects the mandibular condylar remodeling responses. Hence, it was reasonable to hypothesize that the same SNPs that were proposed as risk factors for the temporomandibular disorders in Serbian population could also be risk factors for malocclusion development (18). However, the present study did not find association between this SNP and Class III malocclusion.

CONCLUSION

This study suggests the involvement of *COL1A1*-1997 G/T (rs1107946) in the complex process of Class III mandibular prognathism development, paving the path for future research into the role of this polymorphism, on larger cohorts. On the other hand, *MMP9* -1562 C/T (rs3918242) does not appear to be a risk factor for the development of this type of maxillofacial deformities.

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ANALIZA POLIMORFIZAMA *COL1A1* I *MMP9* GENA U MANDIBULARNOM PROGNATIZMU

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Izvod

Mandibularni prognatizam (MP) ubraja se u malokluzije III skeletne klase i karakterišu ga prerazvijenost donje vilice sa nerazvijenošću gornje vilice ili bez nje. Etiologija prognatizma je kompleksna i uključuje kako faktore sredine tako i naslednu osnovu. Stoga je očekivano da polimorfizmi pojedinačnih nukleotida (SNP) u okviru gena koji kontrolišu kraniofacijalni razvoj mogu doprineti razvoju mandibularnog prognatizma. Cilj ove studije bio je utvrđivanje moguće asocijacije između *COL1A1* -1997 G>T (rs1107946) i *MMP9* -1562 C>T (rs3918242) polimorfizama i mandibularnog prognatizma u srpskoj populaciji. U ovu studiju uključeno je 120 učesnika: 60 pacijenata sa prognatizmom i 60 kontrola sa skeletnom I klasom. Genotipovi su određeni metodom lančane reakcije polimeraze i analizom dužine restrikcionih fragmenata (PCR-RFLP). Asocijacija sa rizikom od nastanka mandibularnog prognatizma određena je računanjem odnosa šansi (OR) i njihovog 95% intervala poverenja (CI). Frekvence alela za oba ispitivana polimorfizma nisu pokazale značajnu razliku između dve studijske grupe ispitanika (P=0.12 and P=0.13, redom). Takođe, nije bilo razlike ni u distribuciji genotipova za *MMP9* polimorfizam. Ipak, TT homozigoti za *COL1A1*-1997 G/T (rs1107946) polimorfizam pokazali su asocijaciju sa razvojem mandibularnog prognatizma (OR 2.32, 95% CI 0.97-5.53) mada sa graničnom statističkom značajnošću (P=0.055). Moguća je uloga *COL1A1*-1997 G/T (rs1107946) polimorfizma u razvoju mandibularnog prognatizma ali je neophodna potvrda u vidu dodatnih istraživanja na većem uzorku pacijenata. *MMP9* -1562 C/T (rs3918242) polimorfizam verovatno nije faktor rizika za pojavu ovog kraniofacijalnog deformiteta.

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