

Mutation analysis of the MSX1 gene exons and intron in patients with nonsyndromic cleft lip and palate

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SUMMARY

Cleft lip with or without cleft palate and cleft palate (CL/CLP/CP) is one of the most common malformations among newborns. The estimated prevalence in Latvia is 1/700. Nonsyndromic CL/CLP/CP is a complex trait determined by multiple, interacting genetic and environmental factors. MSX1 gene is one of the most important candidate-genes, which had been analyzed in relation with nonsyndromic CL/CLP/CP.

The objective of our study was to examine the etiologic role of MSX1 gene mutations in the development of nonsyndromic CL/CLP/CP in Latvian population.

Materials and methods. DNA was extracted from venous blood of 53 patients with cleft lip with or without palate. Polymerase chain reaction (PCR) was performed of selected segments of MSX1 gene. These were sequenced and analysed by comparison with reference sequence, accession Nr. AF426432 (NCBI).

Results. 16 DNA sequence variations were identified in 53 patient samples; 6 of them have not been previously described. Identified sequence variations localized in coding regions do not cause amino acid substitutions, therefore they are not considered as mutations with an etiological role in CL/CLP/CP development.

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Key words: MSX1 gene, cleft lip with or without palate

INTRODUCTION

Cleft lip with or without cleft palate (CL/CLP/CP) is one of the most common malformations among newborns [1]. The estimated prevalence in the world ranges from 1/300 to 1/2500 births for CL/CLP/CP and around 1/500 births for cleft palate only (CP) [2]. The approximate prevalence of CL/CLP/CP in Latvia is 1/700 [3]. Surgical corrections of the defect with following speech and orthodontic therapies require more than 5-10 years for the satisfactory result in the CL/CLP/CP treatment. Thus, these congenital anomalies represent major public health burden in terms of medical costs and emotional burden to patients and their families.

Most researchers suggest that about 70% of cases of CL/CLP/CP and 50% of CP are nonsyndromic, others

being accompanied by additional anomalies (syndromic [4]). CL/CLP/CP is described as a symptom in more than 300 syndromes, some of them with a known genetic background, like Van der Woude syndrome and del 22q syndrome, while in most of the cases the genetic background of nonsyndromic CL/CLP/CP is unknown [5].

Nonsyndromic CL/CLP/CP is a complex trait determined by multiple, interacting genetic and environmental factors. 20 % of the CL/CLP/CP patients in different populations have a family history of CL/CLP/CP, indicating that genetic factors play an important role in etiology of this birth defect [5,6]. Influence of environmental factors and their interaction with genes involved in embryogenesis also plays a significant role in the CL/CLP/CP development [7,8,9]. Recent studies suggest that 3 to 14 interacting genes provide a good model for genetic effects in CL/CLP/CP [10].

MSX1 gene (also known as HOX7, OFC5 and HYD1) is one of the most important candidate-genes, which had been analyzed in relation with nonsyndromic CL/CLP/CP [5,11,12,13,14]. MSX1 (muscle segment homeobox 1) gene is a member of MSX family, which plays a crucial role in the program of craniofacial morphogenesis in the development of teeth and craniofacial skeleton and has been proposed to direct terminal cell differentiation [15]. The MSX1 family of vertebrate HOX genes was originally isolated on a base of homology with *Drosophila msx* (muscle segment homeobox) gene. Human MSX1 gene maps to 4p16.1 locus and spans 4.05 kb. It contains two exons

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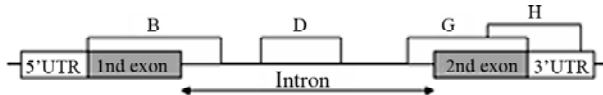


Figure. Segments of the gene MSX1

and an intron. MSX1 gene expression is associated with cyclin D1 upregulation, thus inhibiting cellular differentiation by regulating cell cycle [16].

In several past years MSX1 gene has been intensively analyzed and mutations with phenotypical manifestation have been reported. Thus mutation S202X causes Witkop syndrome, known as tooth-and-nail syndrome [17], therefore considering essential role of the gene MSX1 in a teeth and nail development. Deleted MSX1 gene was found in Wolf-Hirschhorn syndrome (WHS) patients with oligodontia, though there were also WHS patients with oligodontia, but normal MSX1 gene [18].

Jejewski et al. [11] performed full MSX1 gene sequencing in 917 persons with nonsyndromic CL/CLP/CP and reported 16 (2%) potentially etiologic mutations. The results of the direct sequencing of MSX1 gene performed by two more groups of researchers [19,20] also suggest that point mutations in this gene underlie approximately 2% of CL/CLP/CP cases [11,19,20].

There is a necessity for further gene MSX1 studies due to the small number of publications related to MSX1 role in CL/CLP/CP development and possible differences between populations.

The objective of our study was to examine the etiologic role of MSX1 gene mutations in the development of nonsyndromic CL/CLP/CP in Latvian population.

MATERIAL AND METHODS

In the study 53 patients with non-syndromic CL/CLP/CP (10 with cleft lip only (CL), 4 with cleft palate only (CP) and 39 with cleft lip and palate (CLP)) were recruited. 53 venous blood samples were collected in collaboration

with Riga Stradins University, Institute of Stomatology, Riga Cleft Lip and Palate Centre. The data collection was performed in accordance with the regulations issued by the Central Medical Ethics Committee of Latvia. All the participating families signed an informed consent form.

DNA was extracted from venous blood using standard phenol/chloroform extraction protocol. PCR was performed for 4 segments of the gene MSX1 (Figure) named B, D, G and H. For every segment forward (F) and reverse (R) primers were generated. A PCR product of B segment covers the 1st exon coding region and partially intron; the PCR product of G segment covers the 2nd exon coding region and partially intron; the PCR product of H segment covers 2nd exon and 3' non-coding region; the PCR product of D segment covers the middle part of the intron.

The primer set contained a) previously reported primers (Electronic appendix 1: <http://genetics.uiowa.edu/publications/peterj/PeterMSX190902.html>) [11]) and b) modified primers for B fragment: F 5'-GGCTGCTGACATGAC-TTCTT-3' ($T_m = 57.3^\circ\text{C}$) and R 5'-AGGTCTGGAACCT-TCTTCCTG-3' ($T_m = 59.8^\circ\text{C}$).

PCR was performed in 20 μl volume containing 10-20 ng DNA/ μl ; 25 $\mu\text{mol/l}$ of dNTP mix; 2,5 mmol/l MgCl_2 ; Taq buffer + $(\text{NH}_4)_2\text{SO}_4$ pH 8.0; 0.5 $\mu\text{mol/l}$ of each primer; and 0.01 unit Taq polymerase/ μl .

PCR products were visualized on the 2% agarose gel, and then extracted from agarose (DNA extraction kit "Fermentas"). DNA sequencing was performed in a 10 μl reaction using 1 μl of ABI Big Dye Terminator, 0.16 μl of 0.2 $\mu\text{mol/l}$ sequencing primer, 3 μl of 5x Seq buffer, 3 μl ddH₂O and 2.5 ng/100 bp of DNA template. Following a denaturation stage at 96°C for 2 minutes, the samples were prepared for sequencing at 96°C for 30 seconds 50°C for 15 seconds and 60°C for 4 minutes for 25 cycles. DNA was precipitated by cold 96% ethanol and 3M ammonium acetate. Analyses were performed on ABI 3100. The results were compared to the published sequence Nr. AF426432 in NCBI database. MSX1 gene for the B segment was analyzed in 41 patient (82 alleles), D segment in

Table 1. Results of MSX1 gene analysis

Nr.	Segment	Sequence variations	Locus	Sequence variations/all alleles	Allelic frequency
1.	B	C68A, Ala23Glu*	1 st exon, non-coding	3/82	0.037
2.	B	C101G, Ala34Gly*	1 st exon, non-coding	10/82	0.122
3.	B	G246A, Ala82Ala	1 st exon, coding	5/82	0.061
4.	B	C330T, Gly119Gly*	1 st exon, coding	4/82	0.049
5.	B	del.496-507(GCCGGGTGGGG) homozygous*	Intron	16/82	0.195
6.	B	del.496-507(GCCGGGTGGGG) heterozygous*	Intron	19/82	0.232
7.	D	G1633C	Intron	1/102	0.010
8.	D	A1831G	Intron	1/102	0.010
9.	G	delT2763*	Intron	6/46	0.130
10.	G	A2626G*	Intron	4/46	0.087
11.	G	C2651A*	Intron	5/46	0.109
12.	G	A2731G*	Intron	8/46	0.174
13.	G	A2801G	Intron	1/46	0.022
14.	G	C3226T*	2 nd exon, non-coding	6/46	0.131
15.	G	Homozygous delG3283	2 nd exon, non-coding	46/46	1
16.	G	delG3288C	2 nd exon, non-coding	1/46	0.022
17.	H	A3227G*	2 nd exon, non-coding	22/80	0.275
		Heterozygous – 12 patients			
		Homozygous – 5 patients			

*Described previously by Jejewski P.A [11]

51 patients (102 alleles), G segment in 23 patients (46 alleles), H segment in 40 patients (80 alleles).

Obtained results were compared with previous publications in detail with Jezewski P.A. For this reason mutation nomenclature used was the same as used in article of Jezewski P.A.

Statistical analysis of results was not performed due to the small number of the patient samples.

RESULTS

16 sequence variations were identified in 53 patient samples; 6 of them have not been previously described [11]. Results are represented in the Table 1.

Fragment B analysis revealed sequence variations C68A and C101G localised in the 1st exon non-coding region, G246A (Ala82Ala) and C330T (Gly119Gly) in coding region, and homozygous deletion del.496-507 (GCCGGGTGGGG) in non-coding region.

Fragment D analysis revealed 2 sequence variations G1633C and A1831G in non-coding region.

Fragment G analysis in intron revealed sequence variations delT2763, A2626G, C2651A, A2731G and A2801G, C3226T and delG3288C in 2nd exon non-coding region. Homozygous delG3283 was identified in all analyzed samples; presumably this is indicating an error in a reference sequence.

Fragment H analysis revealed sequence variation A3227G in the 2nd exon, homozygous or heterozygous state which is localized in non-coding region.

DISCUSSION

In our study of Latvian CL/CLP/CP patients we iden-

tified 16 sequence variations in the gene MSX1, localised in both coding and non-coding regions. Identified sequence variations localized in coding regions do not cause an amino acid substitution, therefore they are not considered as mutations with an etiological role in CL/CLP/CP development. We are going to continue studies to ascertain their role in the development of CL/CLP/CP by comparison their frequency in unaffected (without cleft lip and or palate) Latvian population.

Due to sequence variation delG3283 in homozygous state in all analyzed samples, we had identified error in the reference sequence Nr. AF426432.

Sequence variants represented in more than 1% of population we identify as SNPs. 13 SNPs were found, 9 of them were previously described, therefore their frequency compared with previous publication. (Table 2). [11].

Several SNPs frequencies showed differences between populations. SNP C68A frequency in our study was 3.7% versus 0 in previous publication for Caucasians. SNP C330T frequency in our study is 4.9%, versus previous publication for Asians had 19% and Caucasians 16%. Considerable difference between populations attributes to SNP A2731G, which in Caucasian populations had frequencies 24% and 17% (our study) versus Asian population, where SNP A2731G frequency was 0.6%.

CONCLUSIONS

16 DNA sequence variations were identified in 53 patient samples; 6 of them have not been previously described. Identified sequence variations localized in coding regions do not cause amino acid substitutions, there-

Table 2. MSX1 gene SNP allelic frequencies in different populations.

Nr.	SNP	Population	Allelic frequency for CL/CP patients	Allelic frequency in control population
1.	del.496-507 (GCCGGGTG GGG)	South American	0.275	ND
		Caucasian	0.410	0.463
		Caucasian (present study)	0.267	ND
2.	A3227G	Caucasian	0.625	ND
		Caucasian (present study)	0.275	ND
3.	C68A	S-American	0.005	0.038
		Caucasian	0	0
		Caucasian (present study)	0.037	ND
4.	C101G	S-American	0.064	0.049
		Caucasian	0.424	0.227
		Caucasian (present study)	0.122	ND
5.	C330T	S-American	0.009	0.070
		Asian	0.190	0.081
		Caucasian	0.158	0.087
		Caucasian (present study)	0.049	ND
6.	delT2763	Caucasian	0.383	0.571
		Asian	0.040	ND
		Caucasian (present study)	0.130	ND
7.	A2626G	Caucasian	0.072	ND
		Asian	0.012	ND
		Caucasian (present study)	0.087	ND
8.	C2651A	Caucasian	0.187	0.148
		Asian	0.030	ND
		Caucasian (present study)	0.109	ND
9.	A2731G	Caucasian	0.240	ND
		Asian	0.006	ND
		Caucasian (present study)	0.174	ND

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fore they are not considered as mutations with an etiological role in CL/CLP/CP development.

The size and the profile of the sample do not allow making critical statements about etiological role of identified MSX1 gene sequence variations; nevertheless we had identified several SNPs, which show considerable differences between both patient populations and control samples.

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