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IDENTIFICATION OF CANDIDATE GENES INVOLVED IN THE ETIOLOGY OF NON-SYNDROMIC CLEFT LIP WITH OR WITHOUT CLEFT PALATE AND ISOLATED CLEFT PALATE

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1. INTRODUCTION

Cleft lip with or without cleft palate and isolated cleft palate (CL/CLP/CP) is a congenital malformation that affects the upper lip, alveolar ridge, tooth eruption, and palate fusion to different degrees. Lip and palate formation is the consequence of several processes that involve cell proliferation, cell differentiation, cell adhesion, and apoptosis. Failure anywhere in these processes can lead to formation of clefts. CL/CLP/CP is one of the most common malformations among newborns (Mooney and Siegel, 2002). Cleft palate (CP) and cleft lip and cleft lip with cleft palate (CL/CLP) are considered etiologically distinct entities, which could be explained by the fact that the lip and palate develop at different embryonic stages (Murray, 2002). The estimated prevalence in the world ranges from 1/300 to 1/2 500 births for CL/CLP and around 1/500 birth for cleft palate only and it varies depending on geographical region and different ethnicities (Stanier and Moore, 2004).

The etiology of non-syndromic CL/CLP/CP is determined by multiple, interacting genetic and environmental factors. Twenty percent of the CL/CLP/CP patients in different populations have a family history of CL/CLP/CP and twin studies showed that proband concordance rate for CL/CLP/CP was 60% in monozygotic (MZ) twins and 10% in dizygotic (DZ) twins, indicating that genetic factors play an important role in the etiology of this birth defect (Murray, 2002). Many genes are considered as susceptibility loci for non-syndromic CL/CLP/CP based on linkage and association studies in different populations. Influence of environmental factors and its interaction with genes involved in embryogenesis also plays a significant role in the CL/CLP/CP development (Stanier and Moore, 2004).

In approximately 30% of the cases CL/CLP/CP is caused by known monogenic syndromes or chromosomal aberrations, and non-syndromic

CL/CLP/CP is a complex disease with many contributing genetic factors (Schutte and Murray, 1999). Recent estimates suggest that 2-14 genes could be factors in the formation of CL/CLP/CP (Scliekelman and Slatkin, 2002).

The identification of susceptibility genes for CL/CLP/CP has been the subject of extensive research. To localize candidate genes and loci of non-syndromic clefts, several genome-wide linkage screens, genome-wide association studies and fine mapping have been published. Recent studies discovered and confirmed regions such as 1p21-p31, 1q32, 2p13, 3q27–28, 4q21-q26, 8q24, 9q21, 10q25.3, 12p11, 14q21–24, 16q24 and 17q22 (Marazita et al., 2004, Riley et al., 2007a, Marazita et al., 2009, Birnbaum et al., 2009, Mangold et al., 2009, 2010). However, despite of the many candidate genes investigated, only the *IRF6* gene has shown a convincing degree of consistency across studies and was considered to be responsible for 12%-18% of non-syndromic CL/CLP/CP cases (Zucchero et al., 2004). These results were replicated in different populations, confirming the role of the *IRF6* gene in CL/CLP/CP formation in different ethnic groups (Marazita et al., 2009). Mutation screening of more than 20 non-syndromic clefts candidate genes showed that only 2%-6% of all screened individuals have mutations in genes including *FOXE1*, *GLI2*, *JAG2*, *LHX8*, *MSX1*, *MSX2*, *SATB2*, *SKI*, *SPRY2*, *TBX10* (Vieira et al., 2005; Jezewski et al., 2003). The recent data suggest that the FGF signaling pathway may contribute to about 3%-5% of non-syndromic CL/CLP/CP cases (Riley et al., 2007b). However other genes studied, such as *TGFA*, *BCL3, PVR*, and *PVRL2* showed conflicting results in genetically diverse populations (Carreno et al., 2002, Pezzetti et al., 2007, Martinelli et al., 1998, Fujita et al., 2004).

Experiments with knockout animal models were conducted to search for new candidate genes for CL/CLP/CP. Few studies with chicks and mice identified specific roles for several major signalling pathways, including Fgf signalling pathways in midfacial morphogenesis and upper lip development

(Trumpp et al., 1999). Genetic studies of mice identified two Wnt genes involved in midfacial morphogenesis and CLP development, *WNT3* and *WNT9B* (Juriloff et al., 2001, 2004, 2005, Brugmann et al., 2007).

There is an evidence of marginally increased death rate from cardiovascular disease and cancer in CL/CLP/CP patients. Individuals with non-syndromic CL/CLP/CP have increased death rate from epilepsy, prematurity, pneumonia, aspiration, asphyxia, sepsis and suicide (Christensen et al., 2004).

In nowadays surgery can repair this defect, but despite this, orofacial clefts have lifelong implications for those affected and their families. That is why there is necessity for a better understanding of the etiology and the mechanism of cleft formation.

Our results showed very strong association between *FGFR1*, *WNT3*, *SKI*, *BMP4* and *IRF6* genes and non-syndromic CL/CLP and CP and possible interaction between 19q13 locus and non-syndromic CL/CLP, which continue to support the involvement of these genes in the development of non-syndromic clefts in Caucasians.

This study is the first study regarding identification of possible candidate genes involved in development of non-syndromic cleft lip with or without cleft palate and isolated cleft palate in Latvian population. Results of this study is step further of understanding of this complex malformation and estimating the impact of genes involved in the etiology of non-syndromic cleft lip with or without cleft palate and isolated cleft palate.

1.1. Aim of the study

The main objective of the study was identification of candidate genes involved in the etiology of non-syndromic cleft lip with or without cleft palate and isolated cleft palate.

1.2. Tasks of the study

Fulfillment of the aim required the following tasks:

1. To decide on candidate gene selection in search for significant relationships with non-syndromic cleft lip with or without cleft palate and isolated cleft palate and select genetic markers for further genotyping within the study.
2. To perform case-control association analysis for selected genes to find if genetic variations are assocated with non-syndromic CL/CLP and CP.
3. To perform case-control haplotype analysis for selected genes to find haplotypes with risk or protective effect in the development of non-syndromic CL/CLP and CP, compared to controls.
4. To carry out family-based association analysis for *BCL3*, *PVRL2*, *PVR*, *CLPTM1*, *IRF6* and *BMP4* genes in order to identify transmission distortions.
5. To perform genetic analysis for *BCL3* gene five markers (rs7257231, rs10401176, rs8103315, rs1979377 and rs2927456) for Brazilian non-syndromic cleft with or without cleft palate and isolated cleft palate cases and controls.

1.3. Hypothesis of the study

1. Diverse genes and genetic markers are involved in the etiology of non-syndromic cleft lip with or without cleft palate and isolated cleft palate in Latvian population compared to another European origin population.

1.4. Scientific novelty of the study

This study is the first study regarding identification of possible candidate genes involved in development of non-syndromic cleft lip with or without cleft palate and isolated cleft palate in Latvian population. Novel finding was *SKI*, *WNT3*, *BMP4*, *IRF6* and *FGFR1* genes role in the development of non-syndromic CL/CLP/CP in Latvian population and obtained results can be used for further studies to identify interaction between genes and environmental factors.

2. SUBJECTS AND METHODS

2.1. Subjects

In the study were included patients with non-syndromic cleft lip (CL), patients with non-syndromic cleft lip with cleft palate (CLP), patients with non-syndromic cleft palate (CP), patients with no age or sex limit and from Caucasian origin. Patients with syndromic clefts or any recognized inherited pathology and adopted patients were excluded from the study. Control group consisted from unrelated, randomly selected unaffected individuals with no family history of clefts, with no age or sex limit and from Caucasian origin. Individuals with syndromic clefts or any recognized inherited pathology and adopted individuals were excluded from the study.

The data collection was performed in accordance with the regulations issued by the Central Medical Ethics Committee of Latvia. Prior to any research procedure, all participated individuals signed an informed consent form. In the case of patients who were under 18 years of age, consent was obtained from their parents.

Patients and their parents were recruited in the Riga Cleft Lip and Palate Centre, Institute of Stomatology, Rīga Stradiņš University.

The control group consisted of 190 individuals collected at the Latvian Biomedical Research and Study Center within the framework of the national

project Genome Database of Latvian Population and 293 individuals from internal data collection of Scientific Laboratory of Molecular Genetics, Rīga Stradiņš University.

For case-control study 661 individual from Latvia were analyzed: 178 non-syndromic cleft lip with or without cleft palate and isolated cleft palate (CL/CLP/CP) patients and 483 unaffected individuals as controls. Out of all 178 non-syndromic CL/CLP/CP cases, 135 had CL/CLP (36 patients with CL, 99 patients with CLP) and 43 patients had CP.

The transmission disequilibrium test (TDT) was carried out in Latvian 122 trios (patient with both parents, total 366 persons), from which 89 patients and their parents (total 267 persons) were divided into CL/CLP group and 33 patients with both parents (total 99 persons) - into CP group.

For additional study 606 DNA samples from Brazilian population (338 non-syndromic CL/CLP/CP cases and 268 individuals as controls) were studied in present study. In the study were included patients with non-syndromic cleft lip (CL), patients with non-syndromic cleft lip with cleft palate (CLP), patients with non-syndromic cleft palate (CP), patients with no age or sex limit and from Caucasian origin. Patients with syndromic clefts or any recognized inherited pathology were excluded from the study. Control group consisted from unrelated, randomly selected unaffected individuals with no family history of clefts, with no age or sex limit and from European origin (Portugese descent). Individuals with syndromic clefts or any recognized inherited pathology were excluded from the study. Out of all 338 cleft cases, 294 cases had non-syndromic CL/CLP and 44 cases had non-syndromic CP. Brazilian population samples were obtained at the Dental Clinics of the Hospital of Rehabilitation and Craniofacial Anomalies and Bauru Dental School, both of the University of São Paulo. Bauru, SP, Brazil. The study had local approval and was conducted with the consent of the participants and their parents or legal guardians.

2.2. DNA extraction

The genomic DNA of non-syndromic CL/CLP/CP patients and population samples was obtained from venous blood or saliva and extracted according to the established protocol of the phenol-chloroform method with slight modifications.

2.3. Genotyping using APEX-2 method

To captur all of the SNPs with minor allele frequencies (MAF) > 0.05 and $r^2 = 0.8$ in the regions of interest, 651 tagSNPs were selected based on the HapMap Phase II data, using HapMap CEU as a reference population. Multiple SNPs were selected for each gene, including 10 kb of both upstream and downstream genomic sequences.

A list of selected SNPs per gene are shown in Table 2.3.1.

Table 2.3.1.

Candidate genes and loci included in the study

Gene/Locus	Chromosomal localization	Number of genotyped SNPs^
MTHFR	1p36.3	11
LHX8	1p31.1	9
COL11A1	1p21	48
SKI	1q22-q24	20
IRF6	1q32.3-q41	11
TGFA	2p13	41
FN1	2q34	30
MSX1	4p16.3-p16.1	15
FGF2	4q26-q27*	20
FGF1	5q31	35
MSX2	5q34-q35	6
EDN1	6p24.1	15
COL11A2	6p21.3	22
FGFR1	8p11.2-p11.1	12
FOXE1	9q22	4
TBX10	11q13.2	10
MMP3	11q22.3	8
MMP13	11q22.3	20
PVRL1	11q23.3	19
COL2A1	12q13.11	33
SPRY2	13q31.1	3
BMP4	14q22-q23	4
TGFB3	14q24	8

Continuation of Table 2.3.1.

Gene/Locus	Chromosomal localization	Number of genotyped SNPs
JAG2	14q32	11
MMP25	16p13.3	7
MMP2	16q13-q21	21
CDH1	16q22.1	14
RARA	17q21	5
WNT3	17q21	17
WNT9B	17q21	12
TIMP2	17q25	26
'OFC11'	18q21**	27
BCL3	19q13.1-q13.2	4
PVRL2	19q13.2	13
CLPTM1	19q13.2-q13.3***	8
BMP2	20p12	25
MMP9	20q11.2-q13.1	6
TIMP3	22q12.3****	38
TBX22	Xq21.1	7
TIMP1	Xp11.3-p11.23	6

^ SNP - single nucleotide polymorphism;
* includes *NUDT6* gene;
** includes *SMAD2* and *SMAD4* genes;
*** includes *APOC2* gene;
**** includes *SYN3* gene

Genotyping of 651 genetic markers in forty five genes were done according to published protocol (Krjutskov et al., 2008).

2.4. Genotyping using TaqMan technology

Three markers in *BMP4*, six markers in 19q13 locus and seven markers in *IRF6* gene were selected based on recent publications regarding confirmed linkage studies and associations with non-syndromic CL/CLP/CP. Detailed information about selected markers is shown in Table 2.4.1.

Table 2.4.1.
Selected SNPs for genotyping with TaqMan probes used in the study

SNP^	Chromosomal localization	Gene	SNP localization in gene	Allele*	MAF**
rs1957860	14: 53499105	*BMP4*	~6 kb downstream of gene	C/T	0.383
rs17563	14: 53487272	*BMP4*	Exon 5	A/G	0.373
rs2071047	14: 53488161	*BMP4*	Intron 4	G/A	0.406
rs35385129	19: 49854029	*PVR*	Exon 6	C/A	0.164
rs10421283	19: 49881333	*PVR/ BCL3*	~20 kb downstream of *PVR* gene and ~62 kb upstream of *BCL3* gene	G/A	0.476

Continuation of Table 2.4.1.

SNP	Chromosomal localization	Gene	SNP localization in gene	Allele	MAF
rs2927438	19: 49933947	*BCL3*	~10 kb upstream of gene	G/A	0.190
rs419010	19: 50060160	*PVRL2*	Intron 1	T/C	0.484
rs2075620	19: 50171877	*CLPTM1*	Intron 6	A/G	0.362
rs875255	19: 50185475	*CLPTM1*	Intron 11	G/C	0.428
rs4844880	1: 207937539	*IRF6*	~88 kb upstream of gene	T/A	0.355
rs2013162	1: 208035307	*IRF6*	Exon 4	C/A	0.403
rs861019	1: 208042009	*IRF6*	Intron 1	A/G	0.399
rs2073487	1: 208043269	*IRF6*	Intron 1	T/C	0.402
rs642961	1: 208055893	*IRF6*	~10 kb downstream of gene	G/A	0.175
rs658860	1: 208057172	*IRF6*	~11 kb downstream of gene	C/T	0.181
rs2235371	1: 208030703	*IRF6*	Exon 6	C/T	0.138

^ SNP - single nucleotide polymorphism;
* Major allele is listed first; ** Minor allele frequency from: http://www.ncbi.nlm.nih.gov

Genotyping was performed using TaqMan assays (*Applied Biosystems*, USA) on automatic sequence-detection instruments 7500 Real-Time PCR System and ViiA™ 7 Real-Time PCR System (*Applied Biosystems*, USA). Reactions were carried out with the use of standard conditions as suggested by the manufacturer.

2.5. Genotyping using MALDI-TOF technology

Eight SNPs (Figure 2.5.1.) were selected to cover the entire *BCL3* gene with 1 kb distance, taking into consideration published allele frequencies (http://www.ncbi.nlm.nih.gov/sites/entrez).

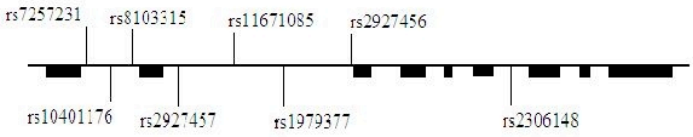

Figure 2.5.1. *BCL3* gene structure and approximate location of the SNPs selected in this study. Vertical lines indicate the approximate localization of selected SNPs within the *BCL3* gene. Black boxes indicate exons. Lines connecting boxes indicate introns.

Genotyping was performed with the use of MALDI-TOF technology using Bruker Daltonics genostrep 96 kit 10x96 (*Bruker Daltonics*, Germany) with slight modifications.

Details of selected SNPs are presented in Table 2.5.1.

Table 2.5.1.
Selected SNPs in *BCL3* gene

SNP^	Chromosomal localization	SNP localization in gene	Allele*	MAF**
rs7257231	19: 49944279	Intron 1	T/A	0.276
rs10401176	19: 49945331	Intron1	G/A	0.160
rs8103315	19: 49946008	Intron1	G/T	0.054
rs2927457	19: 49948787	Intron 2	T/G	0.051
rs11671085	19: 49949647	Intron 2	C/T	Not available
rs1979377	19: 49950842	Intron 2	G/T	0.187
rs2927456	19: 49952054	Intron 3	C/T	0.130
rs2306148	19: 49953271	Intron 6	C/T	Not available

^ SNP - single nucleotide polymorphism;
* Major allele is listed first; ** Minor allele frequency from: http://www.ncbi.nlm.nih.gov

2.6. Statictical analysis

All analyzed markers were tested for Hardy-Weinberg equilibrium in controls affecting individuals using a Pearson's chi-square test with one degree of freedom. Allele frequency differences between cleft patients and control subjects were compared for each SNP using a standard chi-test with one degree of freedom. Allelic odds ratios (ORs) and 95% confidence intervals (CIs) were estimated using the standard χ^2 test, assuming a multiplicative model. The level of statistical significance was set at α=0.05 for nominal association. Haplotype–phenotype association tests were performed with the standard chi-square test using sliding windows approach and LD block analysis. The PLINK software (Purcell et al., 2007) was used to perform case-control comparisons and to test for transmission distortions in the triad families. Bonferroni correction was applied for multiple testing correction considering the number of tests and variables (0.05/number of independent tests).

For performing data statistical analysis, non-syndromic CL/CLP/CP patients were divided into 2 groups - non-syndromic cleft palate (CP) patient

group and non-syndromic cleft lip and cleft lip with cleft palate patient group (CL/CLP), because it has been considered that cleft lip and cleft lip with cleft palate develop at similar embryonic stages.

3. RESULTS

In this chapter results are shown according to the methods used for genotyping. All together three different genotyping methods were used.

3.1. Genotyping using APEX-2 method

Six hundred and fifty one markers in 44 genes were analyzed for 106 cleft lip and cleft lip with cleft palate (CL/CLP) patients, 29 cleft palate (CP) patients and 182 healthy and unrelated individuals as controls.

In the Table 3.1.1. all markers which remained associated after correction for multiple testing in the CL/CLP and CP sample set are presented.

The strongest association with CL/CLP was found for SNP rs16824948, which is located in *SKI* gene, where the allele T was associated with increased risk (p-value = 0.0013×10^{-14}; OR = 6.376; 95% CI = 4.039-10.07). Obtained association remained statistically significant after Bonferroni correction.

SNP rs11655598 in *WNT3* gene showed very strong association (p-value = 0.0053×10^{-11}; OR = 5.925; 95% CI = 3.593-9.772) with CL/CLP, which remained significant after correction for multiple testing. Allele G was associated with increased risk for CL/CLP.

Also allele C for SNP rs7829058 in *FGFR1* gene showed increased risk (p-value = 0.0024×10^{-5}; OR = 7.991; 95% CI = 3.435-18.59) for non-syndromic CL/CLP.

The strongest association with CP was found for SNP rs11655598, located in *WNT3* gene, where the allele G was associated with increased risk (p-value = 0.0039×10^{-11}; OR = 9.495; 95% CI = 4.879-18.34).

SNP rs16824948 in *SKI* gene showed very strong association (p-value = 0.0011×10^{-7}; OR = 6.777; 95% CI = 3.577-12.84) with CP, which remained significant after correction for multiple testing. Allele T was associated with increased risk for CP.

Rs7829058 in *FGFR1* gene showed increased risk (p-value = $0.0002x10^{-6}$; OR = 13.16; 95% CI = 4.93-35.1) for non-syndromic cleft palate, whereas the allele C was associated with increased risk for cleft palate phenotype.

Table 3.1.1.

Most significant results of single-marker association analysis associated with non-syndromic CL/CLP and CP

Genes	SNP[^]	Alle-les[#]	MAF[**]		p-value	OR[^^]	95% CI[##]
			Cases	Contr.			
CL/CLP							
SKI	rs16824948	C/T	0.382	0.088	$0.0013x10^{-14}$	6.376	4.039-10.07
FGFR1	rs7829058	G/C	0.137	0.019	$0.0024x10^{-5}$	7.991	3.435-18.59
WNT3	rs11655598	C/G	0.307	0.069	$0.0053x10^{-11}$	5.925	3.593-9.772
CP							
SKI	rs16824948	C/T	0.397	0.088	$0.0011x10^{-7}$	6.777	3.577-12.84
FGFR1	rs7829058	G/C	0.207	0.019	$0.0002x10^{-6}$	13.16	4.93-35.1
WNT3	rs11655598	C/G	0.414	0.069	$0.0039x10^{-11}$	9.459	4.879-18.34

[^] SNP - single nucleotide polymorphism; [#] Major allele is listed first; [**] MAF - minor allele frequency; [^^] OR - odds ratio; [##] 95% CI - 95% confidence interval

Haplotype-association analysis was performed using two different approaches. Haplotype analysis was applied using two to five SNP slinding window approach for the genes *SKI*, *WNT3* and *FGFR1* which were strongly associated with CL/CLP and CP phenotype in single-marker association analysis. Second approach was haplotype-based association analysis within LD blocks for all genes.

In the Table 3.1.2. and Table 3.1.3. best results (p value ≤0.0001) of haplotype analysis using sliding window in *SKI* gene are presented.

The strongest association with CL/CLP in *SKI* gene was found for nine haplotypes, which were associated with higher risk of disease.

Table 3.1.2.

The best results of haplotype analysis in *SKI* gene associated with non-syndromic CL/CLP

**SNP 1	SNP 2	SNP 3	SNP 4	SNP 5	Frequency		p-value
					Cases	Contr.	
rs262683	rs2460000	rs263533	rs16824948	rs903910	*	*	*
T	G	T	T	C	0.112	0.001	$0.0172x10^{-8}$
rs2460000	rs263533	rs16824948	rs903910	rs4648625	*	*	*
G	T	T	C	T	0.109	0.003	$0.0106x10^{-7}$
rs263533	rs16824948	rs903910	rs4648625	rs6673129	*	*	*
C	T	C	T	*	0.13	0.003	$0.0132x10^{-8}$
C	T	C	*	*	0.137	0.003	$0.0034x10^{-8}$
C	T	*	*	*	0.291	0.079	$0.0176x10^{-8}$
rs16824948	rs903910	rs4648625	rs6673129	rs12045693	*	*	*
T	C	T	C	A	0.116	0.003	$0.0282x10^{-8}$
T	C	T	C	*	0.134	0.007	$0.0092x10^{-7}$
T	C	T	*	*	0.228	0.013	$0.0062x10^{-14}$
T	C	*	*	*	0.233	0.014	$0.0392x10^{-15}$

*Empty cell; **SNP - single nucleotide polymorphism

Very strong association with CP was found for seven haplotypes, which all were associated with higher risk for CP.

Table 3.1.3.

The best results of haplotype analysis in *SKI* gene associated with non-syndromic CP

**SNP 1	SNP 2	SNP 3	SNP 4	SNP 5	Frequency		p-value
					Cases	Contr.	
rs2460000	rs263533	rs16824948	rs260507	rs903910	*	*	*
G	T	T	C	C	0.261	0.004	$0.0029x10^{-16}$
G	T	T	C	*	0.203	0.007	$0.0136x10^{-11}$
rs263533	rs16824948	rs260507	rs903910	rs4648625	*	*	*
T	T	C	C	T	0.218	0.008	$0.0034x10^{-11}$
T	T	C	C	*	0.219	0.008	$0.0061x10^{-11}$
rs16824948	rs260507	rs903910	rs4648625	rs6673129	*	*	*
T	C	C	T	C	0.219	0.006	$0.0045x10^{-12}$
T	C	C	T	*	0.218	0.006	$0.0075x10^{-12}$
T	C	C	*	*	0.22	0.007	$0.0126x10^{-12}$

*Empty cell; **SNP - single nucleotide polymorphism

Table 3.1.4. presents the best haplotype-based association results (p value ≤0.0001) in *FGFR1* gene.

We found strongest association between two *FGFR1* haplotypes and CL/CLP. Both haplotypes were associated with higher risk for CL/CLP. Strongest association was found with CP for three *FGFR1* haplotypes, which were associated with higher risk too.

Table 3.1.4.

The best results of haplotype analysis in *FGFR1* gene associated with non-syndromic CL/CLP and CP

**SNP 1	SNP 2	SNP 3	SNP 4	SNP 5	Frequency		p-value
					Cases	Contr.	
CL/CLP							
rs6996321	rs7829058	rs13279569	*	*	*	*	*
G	C	G	*	*	0.138	0.019	$0.0185x10^{-5}$
rs7829058	rs13279569	rs328300	*	*	*	*	*
C	G	*	*	*	0.147	0.019	$0.0045x10^{-5}$
CP							
*	rs6996321	rs7829058	rs13279569	rs328300	*	*	*
G	C	*	T	*	0.106	0.003	$0.0026x10^{-4}$
G	C	*	*	*	0.165	0.018	$0.0021x10^{-4}$
*	rs7829058	rs13279569	rs328300	*	*	*	*
C	G	*	*	*	0.185	0.019	$0.0331x10^{-7}$

*Empty cell; **SNP - single nucleotide polymorphism

In the Table 3.1.5. best results of haplotype analysis of *WNT3* gene are presented.

Strongest association between CL/CLP and *WNT3* gene haplotypes were found for four haplotypes, which were associated with increased risk for this cleft phenotype. Strongest association with CP and *WNT3* gene was found for five haplotypes. All haplotypes were associated with higher risk for CP.

Table 3.1.5.

The best results of haplotype analysis in *WNT3* gene associated with non-syndromic CL/CLP and CP

**SNP 1	SNP 2	SNP 3	SNP 4	SNP 5	Frequency		p-value
					Cases	Contr.	
CL/CLP							
rs199496	rs11658976	rs11655598	rs12452064	rs199494	*	*	*
G	A	G	G	A	0.277	0.063	$0.0248x10^{-10}$
rs11658976	rs11655598	rs12452064	rs199494	rs7218567	*	*	*
A	G	G	A	C	0.274	0.064	$0.0084x10^{-9}$
rs11655598	rs12452064	rs199494	rs7218567	rs111769	*	*	*
G	G	A	C	T	0.29	0.07	$0.0034x10^{-9}$
G	G	*	*	*	0.289	0.068	$0.0098x10^{-9}$
CP							
rs11658976	rs11655598	rs12452064	rs199494	rs7218567	*	*	*
A	G	*	*	*	0.383	0.064	$0.0078x10^{-9}$
rs11655598	rs12452064	rs199494	rs7218567	rs111769	*	*	*
G	G	A	C	T	0.411	0.07	$0.0006x10^{-9}$
G	G	A	C	*	0.404	0.069	$0.0366x10^{-10}$
G	G	A	*	*	0.402	0.068	$0.0041x10^{-9}$

Continuation of Table 3.1.5.

SNP 1	SNP 2	SNP 3	SNP 4	SNP 5	Frequency		p-value
					Cases	Contr.	
G	G	*	*	*	0.416	0.069	$0.0049x10^{-10}$

*Empty cell; **SNP - single nucleotide polymorphism

After performing haplotype-based association analysis within LD blocks for all genes, all together 114 different haploblocks were generated compared CL/CLP patients and controls and 111 haploblocks - compared CP patients and controls. Only one haplotype in *FGF1* gene showed strong association (p value ≤0.0001) with CL/CLP, but not with CP.

Haplotype rs34002-rs250092-rs34010-rs250103-rs34013 (TGGAT), which was associated with higher risk of this cleft phenotype, is shown in the Table 3.1.6.

Table 3.1.6.

Results of haplotype analysis within LD blocks in *FGF1* gene associated with non-syndromic CL/CLP

**SNP 1	SNP 2	SNP 3	SNP 4	SNP 5	Frequency		p-value
					Cases	Contr.	
rs34002	rs250092	rs34010	rs250103	rs34013	*	*	*
T	G	G	A	T	0.105	0.001	$0.0082x10^{-7}$
T	G	T	A	T	0.222	0.359	0.0007

*Empty cell; **SNP - single nucleotide polymorphism

3.2. Genotyping with MALDI-TOF technology

3.2.1. *BCL3* gene

Eight markers were selected for genotyping with MALDI-TOF technology and three SNPs (rs2927457, rs11671085, and rs2306148) were not informative, therefore were not considered for further analysis.

To perform case-control association study five SNPs in *BCL3* gene were analyzed in 129 non-syndromic cleft lip and cleft lip with or without cleft palate (CL/CLP) patients, 39 non-syndromic cleft palate (CP) patients and 335 unrelated unaffected individuals as control group in Latvian population. In order to analyze these markers in another population from European origin, 606 DNA samples from a Brazilian population (294 non-syndromic CL/CLP cases,

44 CP cases and 268 unrelated and unaffected individuals with no family history of CL/CLP/CP) were studied. Transmission disequilibrium test (TDT) was performed for 109 trios (affected sib with both parents) from Latvian population, out of all 85 sibs and their parents were divided in CL/CLP group and 24 trios - in CP group.

In the Table 3.2.1. results for case-control comparisons with CL/CLP and CP in Latvian population are showed, while in Table 3.2.2. results of single marker association analysis with CL/CLP and CP in Brazilian population are showed.

We did not find any significant association of analyzed markers in *BCL3* gene between CL/CLP or CP cases and controls in population from Latvia or Brazil. The only suggestive evidence for association was for SNP rs10401176 with CL/CLP in the Latvian cohort (p-value = 0.042; OR = 0.609; 95% CI = 0.377-0.986) where allele A was associated with decreased risk for non-syndromic CL/CLP, but it was not significant after Bonferroni correction.

Table 3.2.1.

BCL3 gene results of case-control analysis associated with non-syndromicCL/CLP and CP in Latvian population

Gene	SNP^	Alleles#	MAF**		p-value	OR^^	95% CI##
			Cases	Contr.			
CL/CLP							
BCL3	rs7257231	A/T	0.165	0.181	0.5976	0.897	0.6-1.341
BCL3	rs10401176	G/A	0.1	0.154	0.042	0.609	0.377-0.986
BCL3	rs8103315	G/T	0.1	0.075	0.23	1.375	0.816-2.317
BCL3	rs1979377	T/G	0.096	0.108	0.6148	0.878	0.53-1.456
BCL3	rs2927456	C/T	0.057	0.073	0.391	0.758	0.403-1.43
CP							
BCL3	rs7257231	A/T	0.129	0.181	0.3079	0.672	0.311-1.45
BCL3	rs10401176	G/A	0.097	0.154	0.2254	0.588	0.247-1.401
BCL3	rs8103315	G/T	0.032	0.075	0.2134	0.413	0.098-1.74
BCL3	rs1979377	T/G	0.048	0.108	0.1426	0.422	0.129-1.383
BCL3	rs2927456	C/T	0.016	0.073	0.0886	0.208	0.028-1.531

^SNP - single nucleotide polymorphism; # Major allele is listed first

** MAF - minor allele frequency; ^^ OR - odds ratio; ## 95% CI - 95% confidence interval

Table 3.2.2.

BCL3 gene results of case-control analysis associated with non-syndromic CL/CLP and CP in Brazilian population

Gene	SNP[^]	Aleles[#]	MAF[**]		p-value	OR[^^]	95% CI[##]
			Cases	Contr.			
CL/CLP							
BCL3	rs7257231	A/T	0.277	0.302	0.4013	0.884	0.663-1.179
BCL3	rs10401176	G/A	0.125	0.117	0.72	1.076	0.72-1.608
BCL3	rs8103315	G/T	0.109	0.094	0.4713	1.174	0.759-1.817
BCL3	rs2927456	C/T	0.111	0.078	0.0929	1.478	0.935-2.337
CP							
BCL3	rs7257231	A/T	0.274	0.302	0.6075	0.871	0.514-1.475
BCL3	rs10401176	G/A	0.191	0.117	0.0707	1.773	0.947-3.319
BCL3	rs8103315	G/T	0.155	0.094	0.1016	1.76	0.888-3.486
BCL3	rs2927456	C/T	0.095	0.078	0.603	1.242	0.548-2.815

^SNP - single nucleotide polymorphism; [#] Major allele is listed first

[**] MAF - minor allele frequency; [^^] OR - odds ratio; [##] 95% CI - 95% confidence interval

Haplotype based association analysis was performed to find any possible association with CL/CLP and CP in Latvian and Brazilian populations.

The strongest associations with CL/CLP in Latvian population were found for four *BCL3* gene haplotypes, which all were associated with an increased risk of CL/CLP (Table 3.2.3.).

Table 3.2.3.

The best results of haplotypes analysis in _BCL3_ gene associated with non-syndromic CL/CLP in Latvian population

SNP^ 1	SNP 2	SNP 3	SNP 4	SNP 5	Frequency		p-value
					Cases	Contr.	
rs7257231	rs10401176	rs8103315	rs1979377	rs2927456	*	*	*
A	G	T	T	T	0.101	0.034	0.0007
rs7257231	rs10401176	rs8103315	rs1979377	*	*	*	*
A	G	T	T	*	0.099	0.039	0.0005
rs7257231	rs10401176	rs8103315	*	*	*	*	*
A	G	T	*	*	0.098	0.038	0.0006
rs10401176	rs8103315	rs1979377	*	*	*	*	*
G	T	T	*	*	0.096	0.039	0.0009

*Empty cell; ^SNP - single nucleotide polymorphism

Haplotype rs7257231-rs10401176 (TA) showed weak association with CP (p-value = 0.0345) in Latvian population and it was associated with lower risk of this cleft phenotype (data not shown).

Haplotype analysis in Brazilian population showed that haplotypes rs10401176-rs810315 (GG) (p-value = 0.0078), rs7257231-rs10401176-rs810315 (TAG) (p-value = 0.0321) and rs10401176-rs810315-rs2927456 (GGC) (p-value = 0.0357) revealed borderline association with CP but haplotypes did not showed significant association with CL/CLP (data not shown).

The transmission disequilibrium test was carried out in Latvian non-syndromic CL/CLP and CP individuals and their parents in order to identify transmission distortions. No association was found for any analyzed markers with CL/CLP or CP (data not shown).

3.3. Genotyping using TaqMan technology

3.3.1. 19q13 locus

In present study were analyzed seven markers in 19q13 locus, which contains *PVR*, *BCL3*, *PVRL2* and *CMPTM1* genes. We performed case control comparisons for all seven markers between 113 non-syndromic CL/CLP/CP patients (86 patients with CL/CLP and 27 patients with CP only) and 148 unrelated and unaffected individuals as controls in Latvian population. Transmission disequilibrium test (TDT) was performed for 66 trios (affected sib with both parents), out of all 52 sibs and their parents were divided in CL/CLP group and 14 trios - in CP group.

We did not find any significant association of analyzed markers in 19q13 locus between CL/CLP or CP cases and controls (data not shown).

In present study haplotype based association analysis in 19q13 locus was performed. We did not find any association of analyzed haplotypes with CL/CLP in Latvian population. Haplotypes rs419010-rs2075620 (CG) (p-value = 0.0156), rs419010-rs2075620-rs875255 (CGC) (p-value = 0.0161), rs2927438-rs419010-rs2075620-rs875255 (GCGC) (p-value = 0.0279) and rs2927438-rs419010-rs2075620 (GCG) (p-value = 0.0305) showed very weak

association with CP and these haplotypes were associated with an increased risk of CP.

The transmission disequilibrium test was carried out in Latvian non-syndromic cleft lip with or without cleft palate and isolated cleft palate individuals and their parents in order to identify transmission distortions. Borderline association was found only for one marker in *BCL3* gene (rs10421283) (p-value = 0.0477) with CL/CLP but not with CP, which did not remain significant after Bonferroni correction.

3.3.2. *BMP4* gene

To perform case-control comparisons in *BMP4* gene three SNPs were analyzed for 127 cleft lip with or without cleft palate (CL/CLP) patients, 37 cleft palate (CP) patients and 190 unrelated and healthy individuals with no family history of non-syndromic CL/CLP/CP as controls in Latvian population. Transmission disequilibrium test was performed for 65 trios (affected sib with both parents), out of all 38 sibs and their parents were divided in CL/CLP group and 27 trios - in CP group.

The strongest association with CL/CLP was found for SNP rs2071047, where the allele A was associated with decreased risk (p-value = 0.0087; OR = 0.63; 95% CI = 0.446-0.891) for CL/CLP. Obtained association remained statistically significant after Bonferroni correction. SNP rs17563 showed borderline association (p-value = 0.0178; OR = 0.666; 95% CI = 0.476-0.933) with CL/CLP, which did not remain significant after correction for multiple testing. We did not find any association of analyzed SNPs in *BMP4* gene with isolated CP.

Haplotype based association analysis was performed to find any additional possible association in *BMP4* gene with CL/CLP and CP in Latvian population.

The strongest association with CL/CLP was found for haplotype rs17563-rs2071047 (AA) (p-value = 0.0087) which was associated with decreased risk for the disease. Two additional haplotypes rs2071047-rs1957860 (AC) (p-value = 0.0165) and rs17563-rs2071047-rs1957860) (AAC) (p-value = 0.0184) also were associated with CL/CLP, where both haplotypes showed protective effect for disease.

Haplotype analysis did not show any association with CP (data not shown).

The transmission disequilibrium test was carried out in Latvian non-syndromic cleft lip with or without cleft palate and isolated cleft palate individuals and their parents to identify transmission distortions. We found borderline association between SNP rs1957860 (p value = 0.0455; OR = 3.0; 95% CI = 0.968-9.302) and CP. No association was found for any analyzed markers with CL/CLP (data not shown).

3.3.3. *IRF6* gene

We performed case-control analysis in *IRF6* gene seven SNPs for 85 cleft lip and cleft lip with or without cleft palate (CL/CLP) patients, 27 cleft palate (CP) patients and 148 unrelated unaffected individuals as controls in Latvian population. Transmission disequilibrium test was performed for 63 trios (affected sib with both parents), out of all 49 sibs and their parents were divided in CL/CLP group and 14 trios - in CP group.

The strongest association with CL/CLP was found for SNP rs658860, where the allele T was associated with decreased risk (p-value= 0.0244×10^{-3}; OR = 0.412; 95% CI = 0.272-0.625) for CL/CLP. Obtained association remained statistically significant after Bonferroni correction. SNP rs642961 showed strong association (p-value = 0.0019; OR = 2.141; 95% CI = 1.315-3.488) with CL/CLP, which also remain significant after correction for multiple testing. Allele G was associated with decreased risk for CLP/CP. Similar results

were obtained for rs658860 with CP (p-value = $0.0378x10^{-5}$; OR = 0.412; 95% CI = 0.036-0.289), where allele T was also associated with decreased risk for disease and the association reamained significant after Bonferroni correction.

Haplotype based association analysis was performed to find any additional possible association with CL/CLP and CP in Latvian population.

The strongest associations with CL/CLP were found for nine *IRF6* haplotypes, from which seven haplotypes were associated with an increased risk for CL/CLP, but two haplotypes were associated with lower risk of CL/CLP.

Table 3.3.3.1. shows best results of haplotype analysis (p-value ≤0.001) in *IRF6* gene between CL/CLP patients and controls.

We found very strong association for two *IRF6* haplotypes, where one haplotype rs642961-rs658860 (GT) (p-value = $0.0378x10^{-5}$) was associated with higher risk of this CP and other haplotype rs642961-rs658860 (GC) (p-value = $0.0195x10^{-4}$) was associated with lower risk for CP.

In the Table 3.3.3.2. are presented best results of haplotype analysis (p-value ≤0.001) in *IRF6* gene between CP patients and controls.

Table 3.3.3.1.

Best results of haplotype analysis in *IRF6* gene associated with non-syndromic CL/CLP in Latvian population

**SNP 1	SNP 2	SNP 3	SNP 4	SNP 5	Frequency		p-value
					Cases	Controls	
rs2013162	rs861019	rs2073487	rs642961	rs658860	*	*	*
C	G	T	G	C	0.006	0.184	$0.0155x10^{-6}$
A	A	C	G	C	0	0.13	$0.0114x10^{-4}$
C	A	*	*	*	0.265	0.14	0.0009
rs861019	rs2073487	rs642961	rs658860	*	*	*	*
G	T	G	C	*	0.006	0.185	$0.0131x10^{-6}$
A	C	G	C	*	0	0.13	$0.0113x10^{-4}$
rs2073487	rs642961	rs658860	*	*	*	*	*
T	G	C	*	*	0.006	0.187	$0.0078x10^{-6}$
C	G	C	*	*	0	0.131	$0.0085x10^{-4}$
rs642961	rs658860	*	*	*	*	*	*
G	C	*	*	*	0.006	0.318	$0.0093x10^{-13}$
G	T	*	*	*	0.747	0.549	$0.0244x10^{-3}$

*Empty cell; **SNP - single nucleotide polymorphism

Table 3.3.3.2.

The best results of haplotype analysis in *IRF6* gene associated with non-syndromic CP in Latvian population

SNP 1	SNP 2	SNP 3	SNP 4	SNP 5	Frekvence		p-value
					Cases	Controls	
rs2013162	rs861019	rs2073487	rs642961	rs658860	*	*	*
C	G	T	G	C	0	0.20	0.0004
C	A	T	G	T	0.058	0.004	0.001
A	A	C	G	T	0.481	0.26	0.0013
rs861019	rs2073487	rs642961	rs658860	*	*	*	*
G	T	G	C	*	0	0.199	0.0004
A	C	G	T	*	0.481	0.259	0.0013
rs2073487	rs642961	rs658860	*	*	*	*	*
T	G	C	*	*	0	0.196	0.0005
C	G	T	*	*	0.481	0.255	0.001
rs642961	rs658860	*	*	*	*	*	*
G	T	*	*	*	0.923	0.549	0.0378×10^{-5}
G	C	*	*	*	0	0.318	0.0195×10^{-4}

*Empty cell; **SNP - single nucleotide polymorphism

We performed transmission disequilibrium test to identify transmission distortions between Latvian non-syndromic CL/CLP/CP individuals and their parents. We found strong association with CP for rs642961 (p-value = 0.0039; OR = 0.091; 95% CI = 0.012-0.704), which remain significant after correction for multiple testing, but borderline association was found between rs658860 (p-value = 0.0067; OR = 0.1; 95% CI = 0.013-0.781) and CP. Both markers were associated with lower risk for CP. The same markers showed significant association with CL/CLP, but they were associated with increased risk for CL/CLP phenotype (rs642961 (p-value = 0.0035; OR = 3.143; 95% CI = 1.343-7.357), rs658860 (p-value = 0.0054; OR = 3.0; 95% CI = 1.275-7.057)) even after Bonferroni correction.

4. DISCUSSION

Many genes are involved and regulate the development of the craniofacial region. Different growth factors (e.g., FGFs, TGFs, PDGFs, EGFs, BMPs and respective receptors), signaling molecules (e.g., WNT family, SHH and respective receptors), cell adhesion molecules (PVRL1) and transcription factors (e.g., MSX, DLX, LHX, PRRX and BARX family and respective receptors) are encoded by genes which might be involved in the development of non-syndromic cleft lip with or without cleft palate and isolated cleft palate.

The human fibroblast growth factors (FGFs) and their cell surface receptors (FGFRs) are a complex family of signaling molecules, that play important roles in a variety of processes of embryogenesis and tissue homeostasis (Itoh and Ornitz, 2004; Chen and Deng, 2005; Dailey et al., 2005; Eswarakumar et al., 2005; Krivicka-Uzkurele et al., 2008). Riley et al. (2007a) performed genome-wide linkage scan in 220 multiplex extended Filipino kindreds for cleft lip with or without cleft palate and identified a novel region at 8p11-23 that is likely to be involved in non-syndromic cleft lip with or without cleft palate. Genes within this region, including *FGFR1* gene, which is localized at 8p12, are considered as possible candidate genes for non-syndromic CL/CLP or CP. Genetic variations in *FGFR1* gene in interaction with non-syndromic CL/CLP or CP have been analyzed in many populations, but observed results are controversial between different populations and races (Riley, 2007a, 2007b; Menezes et al., 2008; Mostowska, 2010; Butali, 2011; Wang, 2011). In our study, after performing the single marker association analysis, one marker in *FGFR1* gene (rs7829058) showed the strongest evidence of association with both non-syndromic cleft lip and cleft lip with cleft palate (p-value = 0.0024×10^{-5}; OR = 7.991; 95% CI = 3.435-18.59) and isolated cleft palate (p-value = 0.0002×10^{-6}; OR = 13.16; 95% CI = 4.93-35.1), which remained significant after Bonferroni correction. We performed haplotype analysis of *FGFR1* gene and observed the association with non-

syndromic isolated cleft palate. In another study to enlarge the study population, we pooled together our samples with samples from Estonia and Lithuania. Results of this study showed that SNP rs7829058 is associated with non-syndromic CL/CLP, but the association did not remain significant after Bonferroni correction (p-value = 0.0137; OR = 1.457; 95% CI = 1.079-1.968) (Nikopensius et al., 2011). Single marker association analysis in *FGF1* gene showed that SNP rs34010 (p-value = 0.0002; OR = 0.485; 95% CI = 0.331-0.71) is associated with protective effect for non-syndromic CL/CLP, but this association did not reach significant results after Bonferroni correction. When we compared isolated cleft palate samples with control samples, we found that SNP rs34016 in *FGF1* gene is associated with increased risk for CP (p-value = 0.006, OR = 2.934; 95% CI = 1.322-6.512), however this association also did not remain significant after correction for multiple comparisons. Haplotype analysis in *FGF1* gene showed positive association with non-syndromic CL/CLP, but not with CP phenotype. Riley et al. (2007a) performed study, where they found that both linkage and association results were positive (recessive multipont HLOD = 1.07) for markers in *FGFR1* gene. The same author in other study (2007b) sequenced the coding regions and performed association testing on 12 genes (*FGFR1*, *FGFR2*, *FGFR3*, *FGF2*, *FGF3*, *FGF4*, *FGF7*, *FGF8*, *FGF9*, *FGF10*, *FGF18*, and *NUDT6*) in population from Iowa and Philippine and used protein structure analyses to predict the function of amino acid variants. They identified few likely disease-causing mutations, including one nonsense mutation (R609X) in *FGFR1* and other missense variants in *FGFR1*, *FGFR2* and *FGFR3* genes. Structural analysis of *FGFR1* variants suggested that identified mutations would impair the function of the proteins through different mechanisms. They also performed SNPs genotyping and found an association between non-syndromic CLP and SNP rs13317 in *FGFR1* gene (p-value = 0.03). The case-control study results in Brazilian population performed by Menezes et al. (2008) partially corroborate the

association data, presented by Riley et al. (2007b), in which several genes including *FGFR1* gene, demonstrated a trend for association with non-syndromic CLP with or without dental anomalies. Differencies in the frequencies of the alleles of each polymorphism between cases and controls by each cleft subphenotype were assessed by using OR and 95% CI and they found modest association between SNP rs13317 and right unilateral CLP with tooth agenesis. Mostowska et al. at 2010 published the study, where authors analyzed genes encoding transcription factors such as *FGF10* and *FGFR1* in Polish population. They analyzed two SNPs (rs6987534 and rs328300) in *FGFR1* gene for allelic association, but none of both SNPs were associated with non-syndromic CL/CLP or CP. These markers were analyzed in our study, but we did not find any association between both markers and non-syndromic CL/CLP or CP in our population. Only evidence that these markers could be involved in the etiology for non-syndromic CL/CLP or CP in Latvian population, were results of haplotype analysis, where haplotypes of *FGFR1* gene including both SNPs, showed increased risk for non-syndromic CL/CLP and CP phenotype. Very similar results were found by Wang et al. at 2011 after testing SNPs in 10 genes coding for fibroblast growth factors and their receptors (including *FGF2* and *FGFR1* gene) in Asian and Maryland case-parent trios ascertained through a child with non-syndromic CL/CLP. They found that *FGFR1* yielded evidence of linkage and association in the TDT, confirming previous evidence. Haplotypes consisting of three SNPs (rs6987534, rs6474354 and rs10958700) in *FGFR1* gene were nominally significant among Asian trios similarly to haplotype based association results found in our study. Negative association results were found by Butali et al. at 2011 after performing genotype association studies and direct sequencing on the *FGFR1* and *FGFR2* genes in Nigerian population. These results can be explained by cleft etiology between different races. The prevalence of

CL/CLP/CP in Africa has been reported as relatively lower compared to other populations.

SKI is a proto-oncogene that is required for development of the central nervous system and skeletal muscle, and is involved in specifying selected cranial neural-crest-derived craniofacial structures (Berk et al., 1997). Relatively small amount of studies exist on *SKI* gene and its possible role in the development of CL/CLP or CP. Vieira et al. (2005) reported direct sequencing approach to study 20 candidate genes in Philippines for CL/CLP/CP and the sequencing results suggested that rare point mutations in *FOXE1*, *GLI2*, *JAG2*, *LHX8*, *MSX1*, *MSX2*, *SATB2*, *SPRY2*, *TBX10* and *SKI* gene may be causes of non-syndromic CL/CLP and the linkage disequilibrium data supported a larger, not yet specified, role for variants in or near *MSX2*, *SKI* or *JAG2* genes. To identify genetic variants within the *SKI* gene and investigate the potential association between *SKI* polymorphisms and risk for orofacial clefts, Lu with colleagues (2005) re-sequenced the gene. They identified one novel polymorphism (257C>G) in exon 1, which was associated with the decreased risk (OR = 0.6; 95% CI = 0.3-1.0) for CL/CLP in Californian population. This SNP is located very close to the promoter region so it is possible that this may be in linkage disequilibrium with sequence variants in upstream regulatory regions. In our study we analyzed twenty SNPs in *SKI* gene, which is located at 1q22-q24, for allelic association with non-syndromic CL/CLP or CP. SNP rs16824948 was significantly associated with non-syndromic CL/CLP (p-value = 0.0013×10^{-14}; OR = 6.37; 95% CI = 4.039-1.07) and CP (p-value = 0.0011×10^{-7}; OR = 6.777; 95% CI = 3.577-12.84), where the allele T was associated with increased risk for CL/CLP and CP and this association remained significant after correction for multiple testing. We performed haplotype analysis and the results of this analysis showed similar results. Unfortunately after pooling data together with Estonians and Lithuanians, we did not observe any significant (p-value ≤ 0.05) association with analyzed

markers in *SKI* gene and CL/CLP, but SNP rs12562937 showed borderline association with CP (p-value = 0.0143; OR = 0.534; 95% CI = 0.321-0.889), which did not remain significant after Bonferroni correction (Nikopensius et al., 2010; Nikopensius et al., 2011). Our results support previous positive findings for *SKI* gene role in the etiology of non-syndromic CL/CLP/CP, but additional studies are necessary to replicate obtained results in other populations.

It has been discovered that Wnt signalling pathway plays a crucial role in craniofacial development, and three previously reported studies have concluded that genetic variations in *WNT3* and *WNT9B* genes might be associated with CL/CLP or CP in humans in different populations (Chiquet, 2008; Menezes, 2010; Mostowska, 2012). Twenty-nine single nucleotide polymorphisms in *WNT3* and *WNT9B* genes, located in 17q21, were analyzed in our study for association with non-syndromic CL/CLP and CP in case-control population. One marker in *WNT3* gene (rs11655598) showed the strongest evidence of association with both non-syndromic CL/CLP (p-value = 0.0053×10^{-11}; OR = 5.925; 95% CI = 3.593-9.772) and isolated cleft palate (p-value = 0.0039×10^{-11}; OR = 9.495; 95% CI = 4.879-18.34). This association remained significant after Bonferroni correction. Haplotype based association analysis supports this finding as well. Chiquet et al. (2008) analyzed thirty-eight SNPs in seven *WNT* family genes (*WNT3*, *WNT3A*, *WNT5A*, *WNT7A*, *WNT8A*, *WNT9B* and *WNT11*) in Hispanic and European American population and SNPs in three genes (*WNT3A*, *WNT5A* and *WNT11*) were significantly associated with non-syndromic CL/CLP after correction for multiple testing. Multiple haplotypes in *WNT* family genes were associated with non-syndromic CL/CLP too. Menezes et al. (2010) performed analysis for thirteen SNPs spanning six *WNT* genes (*WNT3*, *WNT3A*, *WNT5A*, *WNT8A*, *WNT9B* and *WNT11*) based on recent publications regarding confirmed associations with non-syndromic cleft lip with or without cleft palate in humans (Chiquet et al.,

2008) or in animal models (Juriloff et al., 2005; Juriloff et al., 2006; Lan et al., 2006) to test for association with CL/CLP and CP subphenotypes in Brazilian population. They found that individuals carrying variant alleles in *WNT3* presented an increased risk for "all clefts" (CL/CLP/CP) and cleft lip and cleft lip with or without cleft palate (CL/CLP). SNP rs142167, located in the 5'UTR of *WNT3* gene, showed association with the phenotype "all clefts" (p-value = 0.0003; OR = 1.61; 95% = 1.29-2.02), cleft lip with palate (CLP) (p-value = 0.001; OR = 1.6; 95% CI = 1.26-2.02) and "unilateral CLP" (p-value = 0.002; OR = 1.65; 95% = 1.27-2.13). Under a nominal value of 0.05, SNP rs9890413 in the same gene also showed an association with "all clefts" (p-value = 0.03; OR = 1.46; 95% CI = 1.12-1.74), with CLP (p-value = 0.02; OR = 1.46; 95% CI = 1.16-1.84) and "unilateral CLP" (p-value = 0.04; OR = 1.46; 95% CI = 1.13-1.89) but SNP rs142167 in *WNT3* was associated with "unsuccessful bilateral" cleft subphenotype (p-value = 0.03; OR = 1.57; 95% CI = 1.17-2.11). The results of the haplotype analysis also supported the associations found for the single SNPs. We analyzed two SNPs (rs111769 and rs2165846) described in previously mentioned studies (Chiquet et al., 2008; Menezes et al., 2010) and SNP rs111769 was associated with non-syndromic CP. This association however did not remain significant after Bonferroni correction (p-value = 0.0195; OR = 1.931; 95% CI = 1.105-3.374). Mostowska et al. (2012) analyzed fourteen SNPs in six *WNT* genes (*WNT3*, *WNT3A*, *WNT5A*, *WNT8A*, *WNT9B* and *WNT11*) and authors found that one *WNT3* gene variant rs3809857 revealed a significant association with the risk of non-syndromic cleft lip and cleft lip with or without cleft palate (CL/CLP) whereas allele T was associated with decreased risk for clefts in Polish population (p-value = 0.015; OR = 0.492; 95% CI = 0.276-0.879). Moreover, haplotype analysis revealed that *WNT3* is significantly associated with non-syndromic CL/CLP. Three SNPs (rs12452064, rs2165846 and rs4968282), analyzed in Polish population, were also included in our study also, but none of them showed any significant

association with non-syndromic CP in both Latvian and Polish populations (p-value ≥0.05). SNP rs4968282 showed borderline association with non-syndromic CL/CLP in our population, but observed association did not remain significant after multiple testing (p-value = 0.0444; OR = 0.654; 95% CI = 0.431-0.991). This finding was not confirmed in Polish population. In the Baltic study SNP rs11653738 in *WNT3* gene showed association with CP, but it lost its significancy after correction for multiple testing (p-value = 0.0064; OR = 1.518; 95% CI = 1.123-2.053) (Nikopensius et al., 2010). Two SNPs (rs4968282 and rs1105127) in *WNT9B* gene showed association with non-syndromic CL/CLP (p-value = 0.0013; OR = 0.688; 95% CI = 0.548-0.865 and p-value = 0.0377; OR = 1.239; 95% CI = 1.012-1.518, respectively), which did not remain significant after Bonferroni correction (Nikopensius et al., 2011). Our results further support previous findings that *WNT3* gene is one of the susceptibility genes for non-syndromic CL/CLP/CP in Caucasians.

Linkage and association studies in different populations showed significant association with 19q13 locus also called OFC3 (orofacial 3) locus containing number of following genes, *PVR, PVRL2, BCL3,* and *CLPTM1*, but results were controversial (Stanier and Moore, 2004; Wyszynski et al., 1997; Martinelli et al., 1998; Beaty et al., 2001; Fujita et al., 2004; Morkuniene et al., 2007; Park et al., 2009). In this study, we tested SNPs in *BCL3, CLPTM1, PVR* and *PVRL2* genes in families and individuals from Latvia for association with CL/CLP and CP. *BCL3* polymorhisms were also tested in study involving non-syndromic CL/CLP and CP patients and controls from Brazil. We did not find any significant association after correction for multiple testing of analyzed markers in 19q13 locus and CL/CLP or CP phenotypes compared cases and controls in Latvian population, or between SNPs in *BCL3* gene and non-syndromic CL/CLP or CP in Brazilian population. Only indication of possible association was found between *BCL3* SNP rs8103315 (p-value = 0.0396; OR = 0.245; 95% CI = 0.058-1.04) and CP, and between *BCL3* SNP rs4803750 (p-

value = 0.0449; OR = 0.496; 95% CI = 0.247-0.996) and CL/CLP, but obtained association did not remain significant after Bonferroni correction. Haplotype analysis in *BCL3* gene showed strong association with CL/CLP in Latvian population and borderline association with CP in Brazilian population. Despite the possitive association between haplotypes and non-syndromic CL/CLP and CP, but no significant association between single marker and CL/CLP or CP, means that haplotypes in *BCL3* gene probably do have some functional effect, which have to be clarified. Such marginal results in Brazilian individuals were not unexpected. A previous study with Brazilian families did not observe any suggestion of transmission disequilibrium between *BCL3* and non-syndromic cleft lip with or without cleft palate (Gaspar et al., 2002). Population based association studies are less sensitive than family based association studies such as TDT and if any possitive association is found from family studies, it will provide strong evidence. We have also performed TDT in Latvian non-syndromic CL/CLP and CP individuals and their parents in order to identify transmission distortions. Only SNP rs10421283 in *BCL3* gene showed borderline association (p-value = 0.0477) with CL/CLP, but not with CP. These findings corroborate previous studies, where an excess of parental transmission of *BCL3* alleles to cleft probands were detected (Maestri et al., 1997; Park et al., 2009). Warrington et al. (2006) studied 19q13 locus and they found an association between non-syndromic cleft lip with or without cleft palate and the *PVR* gene in two independent populations (Iowa and South America) that remained significant after correction for multiple testing. We however did not find any association between markers in *PVR* and *PVRL2* genes and non-syndromic CL/CLP or CP in Latvian population, similar to Danish and Italian populations (Warrington et al., 2006; Pezzetti et al., 2007). In the Baltic study two markers in *PVRL2* gene (rs519113 and rs2075642) showed association with CL/CLP, but this association did not remained significant after Bonferroni correction (p-value = 0.0039; OR = 0.702; 95% CI = 0.552-0.894 and p-value =

0.0206; OR = 1.347; 95% CI = 1.046-1.733, respectively) (Nikopensius et al., 2011). SNP rs6859 in *PVRL2* gene and two SNPs (rs5127 and rs16979595) in *CLPTM1* gene showed association with cleft palate, but lost its association after correction for multiple testing (p-value = 0.0472; OR = 1.35; 95% CI = 1.003-1.816, p-value = 0.0146; OR = 1.494; 95% CI = 1.081-2.064, p-value = 0.0288; OR = 1.457; 95% CI = 1.038-2.046, respectively) (Nikopensius et al., 2010). If the 19q13 locus has some impact in the development of non-syndromic CL/CLP or CP then only as a low penetrance or as a modifier locus.

There are few studies reported in humans regarding to *BMP4* gene showing positive association with non-syndromic CL/CLP or CP. Results of meta-analysis of 13 genome scans identified six regions on five chromsomes with HLODs ≥3.2 and one of these regions was at 14q21-25 displaying evidence of linkage with non-syndromic cleft lip with or without cleft palate (Marazita et al., 2004). Based on this discovery, Lin et al. (2008) performed case-control study of *BMP4* gene polymorphisms and found association between 538T/C polymorphism (rs17563) and non-syndromic CL/CLP in Chinese population. The results showed that the 538C allele carriers were associated with a significantly increased risk of non-syndromic CL/CLP compared with the noncarriers (p-value = 0.005; OR = 1.52; 95% CI = 1.13-2.03). There is a study, where mutation analysis of *BMP4* gene have been performed, and it showed significant overrepresentation of *BMP4* mutations in cases with a range of lip and orbicularis oris muscle (OOM) defects and an absence of mutations in more than 500 control samples. These findings support a role for *BMP4* in the pathogenesis of non-syndromic cleft lip with or without cleft palate (Suzuki et al., 2009). Suazo et al. (2010) analyzed the association between *BMP4* gene three SNPs (rs762642, rs2855532 and rs1957860) and non-syndromic CL/CLP in 150 unrealated trios from Chilean population. Obtained results showed that there are no significant transmission distortions for individual SNPs as it was observed for haplotypes rs1957860-rs762642 (T-

T (p-value = 0.018) and C-T (p-value = 0.015)). Thus, despite the positive association detected between these haplotypes and non-syndromic clefts, associated haplotypes probably do not have a functional effect on *BMP4* expression or protein activity but possibly reflect non-syndromic cleft lip with or without cleft palate susceptibility changes, which are in linkage disequilibrium with these polymorphisms. These findings support a role for *BMP4* in non-syndromic cleft lip with or without cleft palate in the admixed Chilean population. In the present study after performing case-control comparisons, we found association between genetic variations in *BMP4* gene and CL/CLP but not with CP. The strongest association with CL/CLP was found for SNP rs2071047, which is located in intron 4, where the allele A was associated with decreased risk (p-value = 0.0087; OR = 0.63; 95% CI = 0.446-0.891) for CL/CLP. Obtained association remained statistically significant after Bonferroni correction. SNP rs17563, which is located in exon 5, showed only the borderline association (p-value = 0.0178; OR = 0.666; 95% CI = 0.476-0.933) with CL/CLP. Allele A was associated with decreased risk for CL/CLP. Haplotype analysis showed similar results to association analysis - no association was found between haplotypes in *BMP4* gene and isolated cleft palate phenotype, but two haplotypes showed protective effect for non-syndromic CL/CLP. Transmission disequilibrium test performed to detect any transmission distortions in Latvian trios showed controversial results compared to the single marker association. No association was found between SNPs in *BMP4* gene and CL/CLP as it was described in case-control study but SNP rs1957860, which is located ~ 6kb downstream of gene, showed borderline association with CP (p-value = 0.0455; OR = 3.0; 95% CI = 0.968-9.302). Our results support previous findings that *BMP4* gene plays significant role in the development of non-syndromic CL/CLP and CP. Obtained results showed that *BMP4* gene could be involved in the development of non-syndromic cleft palate (CP) as a contributor, but it could have protective effect in the

susceptibility for non-syndromic cleft lip and cleft lip with or without cleft palate (CL/CLP).

There are many studies regarding to *IRF6* as one of the main genes in the development for non-syndromic CL/CLP/CP. In the present study we found strong association between *IRF6* gene SNPs and non-syndromic CL/CLP and CP. The strongest association with CL/CLP and CP was found for SNPs rs658860 and rs642961, both located ~10-11 kb downstream of the *IRF6* gene. Observed association was strongly confirmed by case-control analysis, haplotype analysis and transmission disequilibrium test. Recent study in Chinese population showed association between *IRF6* gene SNP rs2235371, located in exon 6, where TDT and HHRR (haplotype-based haplotype relative risk) analysis showed association with non-syndromic cleft lip (CL) (Li et al., 2012). The same SNP was analyzed in our study, but we did not find any association with CL/CLP/CP. These results could be explained by the fact that we did not performed case-control comparisons between cleft lip (CL) individuals and controls. It is possible that SNP rs2235371 could be associated with CL in Latvian population, but sample size in the present study is too small to test it. Classically, cleft lip only and cleft lip with cleft palate are categorized together because these two phenotypes are thought to have the same genetic etiology, whereas cleft palate have different genetic background (Harville, 2005), but obtained results in recent studies suggest that cleft lip only and cleft lip with cleft palate might be separate entities with different etiology and pathogenesis (Jugessur et al., 2011). Similar study was performed in Honduran population (Larrabee et al., 2011), where SNPs rs642961 and rs2235371 were analyzed. They found the association between rs2235371 and non-syndromic CL/CLP in both case-control (p-value = 0.01) and family-based association (p-value = 0.01) studies, but no association was found for rs642961, which is proposed to have potential biological significance to *IRF6* expression and function (Pan et al., 2010). Results obtained in this study, are contrary to ours

and another study performed by Shi et al. (2011), where both SNPs were analyzed in Chinese population. Obtained results could be explained by different populations analyzed. Studies, reported previous, suggesting that different populations may be affected by different polymorphisms in *IRF6* gene. In the Baltic study marker rs17389541 showed association with CP (p-value = 0.0006; OR = 1.726; 95% CI = 1.263-2.358) supported by analysis of haplotypes including this polymorphism. This is a novel implication of *IRF6* in non-syndromic CP susceptibility and there is a necessarity to replicate obtained results in other populations.

Failure to replicate an association of SNPs for known cleft genes, such as *MSX1* gene, with non-syndromic CL/CLP or CP can be caused by allelic or locus heterogeneity in the etiology of cleft formation. Number of patients and controls analyzed in this study could be to small and number of selected single nucleotide polymorphisms in each gene can be insufficient to achieve full gene coverage. Last, we have analyzed only individual genes, but not interaction between genes and environmental factors, which can also be a very important factor in the development for CL/CLP/CP. Our plans are to start analysis of interaction between genes and environmenatl factors in very near future.

In summary, our results continue to support the involvement of *FGFR1*, *WNT3*, *SKI*, *BMP4* and *IRF6* genes in non-syndromic CL/CLP and CP in humans and shows possible association between 19q13 locus and non-syndromic CL/CLP and CP. Despite all findings we need to perform additional studies to identify potentially functional variants in these genes and replication studies in different populations not only in Caucasians for genes, which showed modest evidence for association with non-syndromic CL/CLP or CP.

One may argue that almost all investigated SNPs are localized in introns or intergenic regions and do not alter transcription factor binding sites or have any other potentially damaging effect. We should also consider that these SNPs, possibly neutral, might also be in linkage disequilibrium with an

etiologic variant, which could explain the results observed in this study. Additionally, the results of many performed GWAS showed the SNPs associated with some disease are considered to be functional.

In conclusion, the results of our study stated that the non-syndromic CL/CLP/CP is very complex malformation and that there are still many undiscovered genes involved in the etiology of this malformation and only few genes have a major role in the development of non-syndromic cleft lip with or without cleft palate and isolated cleft palate.

CONCLUSIONS

1. Six hundred and seventy five genetic markers were selected for further genotyping within present study located in selected forty five candidate genes to search for significant relationships with non-syndromic CL/CLP and CP.
2. Case-control analysis showed that genetic variants in *SKI*, *FGFR1, WNT3* and *IRF6* genes contribute susceptibility to both non-syndromic cleft lip and cleft lip with or without cleft palate (CL/CLP) and cleft palate only (CP) in Latvian population. *BMP4* gene could have protective effect in the susceptibility for non-syndromic cleft lip and cleft lip with or without cleft palate (CL/CLP).
3. Haplotype analysis showed significant association between haplotypes in *SKI*, *FGFR1*, *WNT3* and *IRF6* genes and non-syndromic CL/CLP and CP, and between haplotypes in *BMP4* and *BCL3* genes and non-syndromic CL/CLP.
4. Results of family based association analysis showed significant association between *IRF6* gene and non-syndromic CL/CLP and CP.
5. Comparative analysis for *BCL3* gene five markers showed association between *BCL3* gene haplotypes and non-syndromic isolated cleft palate in Brazilian population.

PUBLICATIONS

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Approbation

1. Pre-defence of the thesis was held in joint meeting of Department of Biology and Microbiology, Rīga Stradiņš University, Institute of Stomatology, Rīga Stradiņš University and Latvian Association of Human Genetics at April 23, 2012, Stomatology Institute, Rīga Stradiņš University, Riga, Latvia.
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