

ORIGINAL ARTICLES/PRACE ORYGINALNE

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POLYMORPHIC VARIANTS IN *VAX1* GENE (RS7078160) AND *BMP4* GENE (RS762642) AND THE RISK OF NON-SYNDROMIC OROFACIAL CLEFTS IN THE POLISH POPULATION*

POLIMORFIZMY GENÓW: *VAX1* (RS7078160) I *BMP4* (RS762642), A RYZYKO WYSTĘPOWANIA IZOLOWANYCH WAD ROZSZCZEPOWYCH TWARZOWEJ CZĘŚCI CZASZKI W POPULACJI POLSKIEJ

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Abstract

Aim of study: *The aim of this study was to investigate the contribution of reported candidate genes: VAX1 (rs7078160) and BMP4 (rs762642) to the risk of cleft lip with or without cleft palate in the Polish population.*

Materials and methods: *Salivary DNA was obtained from 209 individuals with nonsyndromic cleft lip with or without cleft palate and 418 healthy matched control group. We performed an analysis of polymorphisms of VAX1 (rs7078160) and BMP4 (rs762642) genes. These genes are involved in facial development during pregnancy and may contribute to orofacial clefting risk. Single nucleotide polymorphisms (SNPs) were investigated by real-time PCR- based TaqMan genotyping (Light Cycler 480 II; Roche Diagnostics). To assess the clefting risk for each genotype the odds ratio (OR) was calculated.*

Results: *Conducted logistic regression did not confirm modificatory influence of rs7078160 in VAX1 gene on cleft lip with or without cleft palate risk. For AA genotype OR=1.81 (p=0.211), and for AG genotype OR=0.8 (p=0.313). Also a modificatory influence of rs762642 in BMP4 gene on orofacial clefting risk was not significant. OR=0.82 for GG genotype (p=0.471), while for GT genotype OR=1.17 (p=0.487).*

Conclusions: *No correlation between polymorphisms: rs7078160, rs762642 and nonsyndromic cleft lip with or without cleft palate risk in Polish population was observed.*

Key words: cleft lip, cleft palate, genetic variation, polymorphism

Streszczenie

Cel pracy: *Określenie ryzyka występowania rozszczepów twarzowej części czaszki przy obecności polimorfizmów: rs7078160 w genie VAX1 i rs762642 w genie BMP4 w populacji polskiej.*

Materiały i metody: *Badania przeprowadzono w grupie 209 pacjentów z rozszczepem wargi z lub bez rozszczepu podniebienia oraz 418 z grupy kontrolnej, od których wyizolowano DNA. Analizie poddano*

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wyselekcjonowane polimorfizmy dwóch genów: VAX1 (rs7078160) i BMP4 (rs762642). Geny te związane są z rozwojem twarzy podczas embriogenezy i mogą mieć wpływ na ryzyko występowania wad rozszczepowych twarzowej części czaszki. Analizę polimorfizmów pojedynczego nukleotydu (SNPs) wykonano w czasie rzeczywistym - badanie PCR (TaqMan) w aparacie LightCycler 480 II (Roche Diagnostics). Do oceny wielkości ryzyka powstania wad rozszczepowych twarzy obliczono iloraz szans (OR).

Wyniki: *Przeprowadzona analiza regresji logistycznej nie potwierdziła modyfikującego wpływu polimorfizmu pojedynczego nukleotydu genu VAX1 rs7078160 na ryzyko wystąpienia rozszczepu wargi z lub bez rozszczepu podniebienia. Dla genotypu AA iloraz szans wyniósł 1,81 (p=0,211), a dla genotypu 0,8 (p=0,313). Analiza statystyczna nie potwierdziła również modyfikującego znaczenia polimorfizmu genu BMP4 rs762642 na ryzyko wystąpienia wad rozszczepowych w obrębie twarzowej części czaszki. Iloraz szans dla genotypu GG wyniósł 0,82 (p=0,471), podczas gdy dla genotypu GT 1,17 (p=0,487).*

Wnioski: *Obecność polimorfizmów pojedynczego nukleotydu genów: VAX1 (rs7078160) i BMP4 (rs762642) nie modyfikuje w sposób istotny ryzyka wystąpienia izolowanego rozszczepu wargi z lub bez rozszczepu podniebienia w populacji polskiej.*

Słowa kluczowe: rozszczep wargi, rozszczep podniebienia, wariant genetyczny, polimorfizm

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INTRODUCTION

Head and face development constitutes one of the most complex and controlled process of embryogenesis. Abnormalities, which occurred during the crucial period between 5th and 12th week of pregnancy, can lead to lip and/ or palate clefts (1).

Isolated cleft lip with or without cleft palate (NSCL/P) is one of the most common birth defects in the head and neck region. Its prevalence rates varies from 1 in 500 to 1 in 1200 alive born children (2, 3). Immediately after birth, individuals with cleft lip and/or palate have facial deformation, feeding problems, and frequent middle ear infection. Therefore the treatment requires intervention from multiple disciplines. At the age of speech acquisition, speech therapy is often needed to correct problems resulting from muscular defects of the cleft. Compound clinical problems constitute defects in tooth development and malocclusion (4).

On orofacial clefting risk infuence genetical factors (for example: chromosome aberrations, single gene mutations, multiple genetic factors), environmental factors, or equally both (2). Because of the fact, that genetics plays considerable large part in the etiology of 90% of congenital malformations, patients with clefts ought to see genetic counselor, especially if any syndromic birth defects are likely to be present (5). Despite of the major development of molecular genetics, genetical counseling is usually based on pedigree and clinical data only. It is well known that, syndromic disorders with cleft palate are caused by genetic factors, while isolated disorders are caused in different proportions by both - genetics and environment. It is also proved, that most of the nonsyndromic inborn malformations are characterized by multifactorial hereditary pattern (6, 7).

The presence of nonsyndromic cleft lip with or without cleft palate in child is carring threefold increased risk for siblings, than average risk in the population. Cleft risk for monozygotic twins is estimated at 25-45%, while for heterozygotic 3-6% only. Risk of orofacial malformations for first-degree relatives is estimated at 4%, for second-degree relatives 0.67%, and for third-degree relatives 0.3% (8). Lack of total coincidence of orofacial clefts for monozygotic twins indicates the important role of exogenous and endogenous factors in aetiology of this type of birth defects (9).

To conduct a research polymorphisms in *VAX1* and *BMP4* genes were selected due to theirs significant and well confirmed in multiple researches (10, 11, 12, 13, 14, 15) role in orofacial development. The characteristics of chosen single nucleotide polymorphisms is presented in Table I.

AIM OF STUDY

The aim of this study was to investigate the contribution of reported candidate genes: *VAX1* (rs7078160) and *BMP4* (rs762642) to the risk of cleft lip with or without cleft palate in the Polish population.

MATERIALS AND METHODS

Patients

In research participated 627 individuals – 209 patients with cleft lip with or without cleft palate and 418 healthy matched control group. Relatives in the ascending line up to second generation represented Polish population. The study group was under orthodontic treatment in Departament of Orthodontics at Pomeranian Medical University in Szczecin (129 patients) and in Department of Dentofacial Orthopedics and Orthodontics at Wrocław Medical University (90 patients). All individuals were from 4 to 30 years (average 17.4). In research participated 91 women (43.5%) and 118 men (56.5%). Among the study group 113 individuals had unilateral cleft of the lip

Table I. Characteristics of rs7078160 and rs762642 polymorphisms.
Tabela I. Charakterystyka polimorfizmów rs7078160 i rs762642.

Polymorphism *Polimorfizm*	Gene *Gen*	Localization *Lokalizacja*	Chromosome *Chromosom*	Allele *Allel*	MAF
rs7078160	*VAX1*	Intron *Intron*	10q25.3	A/G	A=0.2658/578
rs762642	*BMP4*	Promotor *Promotor*	14q22.2	G/T	C=0.3526/767

and the hard palate, 45 individuals had bilateral cleft of the lip and the hard palate, 32 were identified with cleft palate only and 19 patients had isolated cleft lip.

The study design was approved by the Bioethics Committee of the Pomeranian Medical University in Szczecin as compatible with the GCP rules - *Good Clinical Practice* (no KB-0012/77/10). All patients, the parents or legal guardians were informed about the purpose and method of the research and had been given their informed, writing consent for a clinical trial.

Properly matched control group was consisted of individuals (avarage age of 14), whose genetic material was obtained from umbilical cord blood and storaged in Oncological Biobank in Department of Genetics and Pathology at Pomeranian Medical University in Szczecin. Oncological Biobank project was approved by Ethics Committie of the Pomeranian Medical University in Szczecin (no BN-001/174/05; 11. 10. 2005).

In the study group specific clinical and pedigree data were collected to assess the exposure to environmental factors, which are contributed to increased orofacial risk (for example: absence of folic acid supplementation in the right doses (Prevention Programme of Primal Neural Tube Defect), contagious diseases during pregnancy, chronic stress, smoking, drinking alcohol, maternal and paternal age, socioeconomic conditions as well as medications intake (16, 17, 18, 19, 20). Clinical diagnostics of existing congenital defects and differential diagnostics for monogenic syndromes, related to cleft lip with or without cleft palate was based on medical history and physical examination. Type of cleft was assessed according to World Health Organization classification - *International Statistical Classification of Diseases and Related Health Problems* – ICD 10; *Congenital malformations, deformations and chromosomal abnormalities* section Q35-Q37) (21).

Molecular analysis

Genetic material to conduct molecular analysis was obtained from salivary DNA within the framework of Oncological Biobank in Department of Genetics and Pathology at Pomeranian Medical University in Szczecin. The sample of saliva in amount of 5 ml was collected from each individual. Patients were asked not to consume any solid food 30 minutes prior to biological material collection.

Molecular analysis was performed in laboratory in the Department of Genetics and Pathology at Pomeranian Medical University in Szczecin. DNA was isolated using salting out procedure (22) or automatic Chemagen sets.

Assessment of incidence of chosen single nucleotide polymorphisms (SNPs): rs7078160 and rs762642 were investigated by real-time PCR- based TaqMan genotyping (Light Cycler 480 II; Roche Diagnostics).

Analysis of each genetic variants was conducted in the volume of 5 µl. The mixture contained: 2.5 µl LightCycler 480 Probes Master Mix (Roche Diagnostics), 0.0625 µl customed assay for specific genetic variant – TaqMan Genotyping Assay x 40 (Applied Biosystems) and 1.4375 µl deionised water (Roche Diagnostics). To perform each chemical reaction 384-well plates LightCycler 480 Multiwell Plate 384 (Roche Diagnostics) were used. Each well was filled with 4 µl of mixture and 1 µl of DNA (25 ng/µl). On each plate 4 wells contained only water to validate the results. Chemical reaction and the product analysis was followed with thermocycler LightCycler 480 Instrument and LightCycler 480 Basic Software Version 1.5 Programme (Roche Diagnostics). Fluorescence intensity of DNA samples was measured after each cycle to record the quantity of the product in a real time. Based on 58 measurements of reporter colouring FAM and VIC fluorescence, endpoint analysis was made.

Statistic analysis

To conduct statistic analysis logistic regression method was applied. To assess the risk of orofacial cleft occurrence, odds ratio (OR) was calculated with 95% confidential ratio (95% CI). As a reference groups, the most numerous subgroups were taken. The cleft risk was assessed for each genotype among the study group. Significance of particular logistic regression rates was assessed by using Wald's test. For statistic analysis we used Statistica 10.0 (StatSoft, Tulsa OK, USA) and **R 3.0.2 (**The R Foundation for Statistical Computing) Programmes. P-value of less than 0.05 was considered significant.

RESULTS

Conducted logistic regression did not confirmed the correlation between modificatory influence of single nucleotide polymorphism rs7078160 (*VAX1* gene) and cleft lip with or without cleft palate risk (tab. II). Values of odds ratio for AA and AG genotypes were respectively: 1.81 (0.71-4.58), p=0.211 and 0.8 (0.52-1.23), p=0.313.

The analysis of specific types of clefts risk and rs7078160 polymorphism *VAX1* gene (tab. III), showed that, AG genotype increases over threefold risk of isolated cleft lip occurrence (OR=3.26, p=0.05) and slightly increases the risk of unilateral cleft of the lip and the hard palate occurrence (OR=1.13, p=0.001) in relation to other types of orofacial clefts.

Logistic regression did not confirm modificatory influence of rs762642 polymorphism BMP4 gene on orofacial clefting risk (tab. IV). Odd ratio for GG genotype OR=0.82 (0.49-1.4) p=0.471, while for GT genotype OR=1.17 (0.76-1.79) p=0.487.

The analysis of specific types of clefts risk and rs762642 polymorphism *BMP4* gene (tab. V) showed that, only the risk of unilateral cleft of the lip and the hard palate occurrence is significant decreased with GG genotype (OR=0.72, p=0.001), and GT genotype (OR=0.97, p=0.001) in relation to other phenotypes of orofacial clefts.

DISCUSSION

Intensification of research on the aetiology of isolated cleft lip with or without cleft palate confirmed the assumption of multifactorial background of the congenital

Table II. Cleft lip with or without cleft palate risk and rs7078160 polymorphism *VAX1* gene.

Tabela II. Ryzyko wystąpienia izolowanego rozszczepu wargi z lub bez rozszczepu podniebienia dla polimorfizmu genu VAX1 rs7078160.

Nucleotide polymorphism ***Polimorfizm nukleotydu***	**OR**	**95% CI**	**p (Wald's test)**
Ref.=GG			
AA	1.81	0.71-4.58	0.211
AG	0.80	0.52-1.23	0.313

Table III. Specific types of clefts risk and rs7078160 polymorphism VAX1 gene.

Tabela III. Ryzyko wystąpienia poszczególnych typów wad rozszczepowych dla polimorfizmu genu VAX1 rs7078160.

ICD-10	**Cleft type** ***Typ rozszczepu***	**OR**	**95% CI**	**p**
Ref.: Q35	Cleft palate only *Rozszczep podniebienia*			
AA nucleotide polymorphism ***Polimorfizm nukleotydu AA***				
Q36	Rozszczep wargi *Cleft lip only*	0.13	–	1.00
Q37.0	Bilateral cleft of the lip and hard palate *Rozszczep podniebienia twardego z obustronnym rozszczepem wargi*	0.00	–	1.00
Q37.1	Unilateral cleft of the lip and the hard palate *Rozszczep podniebienia twardego z jednostronnym rozszczepem wargi*	0.00	–	1.00
AG nucleotide polymorphism ***Polimorfizm nukleotydu AG***				
Q36	Cleft lip only *Rozszczep wargi*	3.26	0.75-14.17	0.05
Q37.0	Bilateral cleft of the lip and hard palate *Rozszczep podniebienia twardego z obustronnym rozszczepem wargi*	1.56	0.44-5.45	1.00
Q37.1	Unilateral cleft of the lip and the hard palate *Rozszczep podniebienia twardego z jednostronnym rozszczepem wargi*	1.13	0.37-3.43	0.001

Table IV. Cleft lip with or without cleft palate risk and rs762642 polymorphism BMP4 gene.

Tabela IV. Ryzyko wystąpienia izolowanego rozszczepu wargi z lub bez rozszczepu podniebienia dla polimorfizmu genu BMP4 rs762642.

Nucleotide polymorphism *Polimorfizm nukleotydu*	**OR**	**95% CI**	**p (Wald's test)**
Ref.=TT			
GG	0.82	0.49-1.40	0.471
GT	1.17	0.76-1.79	0.487

Table V. Specific types of cleft risk and rs762642 polymorphism BMP4 gene.

Tabela V. Ryzyko wystąpienia poszczególnych typów wad rozszczepowych dla polimorfizmu genu BMP4 rs762642.

ICD-10	**Cleft type** ***Typ rozszczepu***	**OR**	**95% CI**	**p**
Ref.: Q35	Cleft palate only *Rozszczep podniebienia*			
GG nucleotide polymorphism ***Polimorfizm nukleotydu GG***				
Q36	Cleft lip only *Rozszczep wargi*	0.67	0.08-5.31	1.00
Q37.0	Bilateral cleft of the lip and hard palate *Rozszczep podniebienia twardego z obustronnym rozszczepem wargi*	0.70	0.17-2.91	1.00
Q37.1	Unilateral cleft of the lip and the hard palate *Rozszczep podniebienia twardego z jednostronnym rozszczepem wargi*	0.72	0.22-2.38	0.001
GT nucleotide polymorphism ***Polimorfizm nukleotydu GT***				
Q36	Cleft lip only *Rozszczep wargi*	1.67	0.34-8.08	1.00
Q37.0	Bilateral cleft of the lip and hard palate *Rozszczep podniebienia twardego z obustronnym rozszczepem wargi*	1.20	0.37-3.86	1.00
Q37.1	Unilateral cleft of the lip and hard palate *Rozszczep podniebienia twardego z jednostronnym rozszczepem wargi*	0.97	0.35-2.66	0.001

anomaly and led to the identification of many genes co-responsible for its occurrence (23).

Studies carried out during animal trials confirmed the essential role of the *VAX1* gene in the morphogenesis of one's head and face (24). In the course of two independent genome wide association studies, the *VAX1* gene, primarily rs7078160 polymorphism to be exact was identified as a potential risk factor conducive to the occurrence of congenital anomalies (10, 11).

This assumption was substantiated by the results of research conducted on the Estonian population, in which case a high correlation between the presence of *VAX1* polymorphism and the presence of congenital malformations to one's face was observed (12).

Mostowska at al. (13) identified more than a fourfold increase in the risk of cleft defects of the facial skeleton part in the presence of rs7078160 polymorphism of *VAX1* gene. Nevertheless, our own research results carried out on the Polish population did not confirm the importance of the modifying influence of rs7078160 polymorphism of *VAX1* gene on the risk of causing isolated defects of the facial skeleton part.

Although the studies in question conduced by *Mostowska* at al. (13) were carried out on a study group comprising similar numbers, individuals with isolated cleft lip and isolated cleft palate were not part thereof. The fact of geographical differences may be of significance as well. The study group comprised

people living in central and southern parts of Poland (13).

The influence of the modifying effect of the presence of rs7078160 polymorphism on the risk of isolated cleft defects found no corroboration during studies conducted on the Chinese population. According to observations set out by the authors themselves, the validity of the research was largely limited by a non-scholastic selection of the study group, scarcity of study groups, and poorly chosen methods of statistical analysis (25). Not without significance is the fact that the population was clearly ethnically divergent from the ones encompassed by genome wide association studies.

Second polymorphism analyzed in the course of this study has not yet been described with reference to the native population. Literature data on the rs762642 polymorphism of *BMP4* gene point out that the genetic alteration is clearly a factor responsible for the formation of isolated cleft lip or cleft palate on an animal model (26).

It was assumed that the *BMP4* gene was directly associated with congenital facial anomalies, although previous attempts to identify specific polymorphisms were not successful (27). The results of two studies carried out on Chilean and Chinese populations (14, 15) point at a correlation between the presence of rs762642 and increased risk of cleft defects. Our own studies did not confirm this assumption in relation to the native population.

CONCLUSION

Presence of single nucleotide polymorphisms: rs7078160 (*VAX1* gene) and rs762642 (*BMP4* gene) do not significantly modify non-syndromic cleft lip with or without cleft palate risk in the Polish population.

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