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Lack of association of polymorphic variants of genes encoding zinc transporters with the risk of orofacial cleft-affected pregnancies

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Abstract: Maternal zinc deficiency seems to be a risk factor for orofacial clefts in offspring. This study was undertaken to investigate the involvement of polymorphic variants of genes for zinc transporters in the susceptibility of clefting. PCR-RFLP analysis was used to analyze single nucleotide polymorphisms of *SLC30A1* (rs7526700, rs2278651, rs611386), *SLC30A4* (rs2453531, rs8029246), *SLC30A5* (rs351444, rs164393, rs6886492), *SLC39A1* (rs10127484, rs11264736), and *SLC39A3* (rs759071, rs4806874, rs10415622) in mothers of children with non-syndromic cleft lip with or without cleft palate (CL/P) and control mothers. The allele, genotype, and haplotype distribution was found to be similar among case and control mothers. Also, the gene-by-gene interaction analysis conducted using the Multifactor Dimensionality Reduction approach revealed no significant interactive genetic effect on having a child with a cleft. In conclusion, our results demonstrated that the analyzed polymorphic variants of genes for zinc transporters are not implicated in abnormal palatogenesis in the investigated group of women from the Polish population.

Key words: CL/P, Zn, zinc transporters, SLC30A, SLC39A, polymorphism

Introduction

Zinc (Zn) is an essential dietary micronutrient that plays fundamental integrative roles in human physiology. The functions of Zn can be divided into three categories: structural, catalytic, and regulatory. It is estimated that 3-10% of human genes code for proteins with Zn binding domains [1]. Since Zn cannot passively diffuse across cell membranes its transport is carried out by Zn transporters. Two major unrelated families of mammalian Zn transporters have been identified: solute-linked carriers 30A (SLC30A, also called ZnTs) and SLC39A (ZIPs) [2]. Zinc uptake is mediated by the SLC39A protein family, while members of the SLC30A family transport zinc from the cytoplasm to the lumen of organelles or the extracellular space [3]. There is no storage form of Zn in the body that can be readily mobilized when dietary intake is inadequate. However, it has been shown that Zn transporter expression can be regulated by both transcriptional and post-transcriptional mechanisms in response to Zn availability to ensure an appropriate plasma concentration of this nutrient [3]. Zn transporters have been strongly implicated in embryonic, fetal, and neonatal development [2-5]. SLC30A1 and SLC30A5 play a key role in regulating delivery of maternal Zn to the developing embryo [2,5]. Cadmium exposure, which is known to be risk factor for congenital anomalies, down-regulates *Slc30a1* expression, indicating that maternal cadmium exposure may alter Zn homeostasis in the conceptus [5]. Mutations in *ZnTs* and *ZIPs* reveal their physiological functions [3]. Mutations in *SLC30A4* have been associated with a loss or reduction of Zn secretory activity of some glandural epithelial cells, however the regulation of Zn transporter proteins in histiotrophic nutrition in early pregnancy has yet to be elucidated [3,6,7]. Mutations in human *SLC39A4* cause the rare disease acrodermatitis enteropathica [8]. Interestingly, it was been found that in the mouse, maternal

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Table 1. Conditions for the identification of investigated SNPs

Gene symbol	rs no.	Location[a]	SNP function[b]	Primers for PCR amplification (5' – 3')	Annealing temp. (°C)	PCR product length (bp)	Restriction enzyme	Alleles[c]
SLC30A1	rs7526700	chr1: 211739824	N/A	F: AGTACAGGATAAAGGACCAAGGT R: TTGATTTGTGGTTTTAAGGTAGG	57.4	352	*TaqI*	C/g
SLC30A1	rs2278651	chr1: 211751060	intron	F: GGTTTACACGCTCTGAATGC R: CAATACCAGCAACTCCAACG	59.7 with 10% DMSO	566	*MboI*	C/t
SLC30A1	rs611386	chr1: 211770318	N/A	F: GCCAAGGATGTTTTTCTCCA R: TGGTTTGTGGTTGTGATTGC	67	679	*MboII*	a/G
SLC30A4	rs2453531	chr15: 45779810	cds-synon Asp305Asp	F: TCAGAATGTCCCCTGACTCC R: GTCCCATGAGGCAAGTAACG	67	374	*BspPI*	A/g
SLC30A4	rs8029246	chr15: 45821158	N/A	F: CGGCCTATCCAGTGTTTATGA R: GAAAGCAACCCCCAGTAAGAT	67	360	*TaiI*	A/g
SLC30A5	rs351444	chr5: 68377251	N/A	F: GGGACATACTTCCAGCTTGC R: TTAGCTTGGCACTGAATCTCC	67	536	*HpyF3I*	C/g
SLC30A5	rs164393	chr5: 68409094	intron	F: ACCCTTGAAAAAGTCATCCTTG R: GTAAGAGAAGAGTTGGGGCTGA	67	587	*MnlI*	G/t
SLC30A5	rs6886492	chr5: 68425699	UTR-3	F: CCATGAAATACTGCAAAGATGG R: TTACAATGGAGATGCTTCCTGA	66.3	370	*TaqI*	A/g
SLC39A1	rs10127484	chr1: 153900980	N/A	F: GAAGGAGAGCAGGCGTATCA R: GCCCAGCCTAGATATTCTTATGG	67	601	*Eam1104 I*	A/g
SLC39A1	rs11264736	chr1: 153939130	intron	F: AGTGTGAAGAACATGGGCTGA R: GAAGATTTGAACGGGCTAAGG	66.3	461	*BccI*	C/t
SLC39A3	rs759071	chr19: 2728577	N/A	F: TGTGGGCTCAAGTAATCTGC R: GGGGACATCTGTGGTTGTCT	67	414	*MboI*	A/g
SLC39A3	rs4806874	chr19: 2738352	intron	F: GATGCAGAGAAGCAAGAAAGAAG R: ACAAAATTCCGCTCCTCAGTC	67	370	*NspI*	A/g
SLC39A3	rs10415622	chr19: 2739696	intron	F: ATCTCAGCTCCTCCCCTGTC R: GACTCTACCCGCACGTTAGC	66.3	532	*BseGI*	A/g

[a]Based on UCSC Human Genome Browser, February 2009 (GRCh37) assembly; [b]According to the Single Nucleotide Polymorphism database (dbSNP); [c]Uppercase denotes the more frequent allele in the control samples.

Slc39a4 haploinsufficiency results in increased sensitivity to the effects of Zn deficiency and the risk of abnormal embryonic development [8]. Recently, Kambe *et al*. [9] demonstrated that the *Slc39a1*, *2*, and *3* genes evolved to decrease sensitivity to the stress of dietary Zn deficiency in the reproducing mouse. The high degree of evolutionary conservation of *ZIP1*, *2*, and *3* suggests the possibility of these genes' involvement in protection against Zn deficiency in human pregnancies [9].

Cleft lip with or without cleft palate (CL/P) is one of the most common congenital malformations in humans. The aetiology of CL/P is highly complex and associated with both genetic and multiple environmental factors, including micronutrient deficiencies [10]. The correlation between abnormal palatogenesis and Zn dyshomeostasis has been clearly documented in laboratory animals [11]. In humans, poor maternal Zn status may be a risk factor for clefting when the micronutrient deficiency reaches a certain severity [12,13]. Observations in the rodent model indicate that the Zn transporter genes can be silent when dietary intake of Zn is normal in pregnancy, but they might be involved in buffering the micronutrient deficiency and the success of reproduction can be dramatically compromised [14,15]. Such findings have prompted the search for cleft-associated variants of Zn transporters. Therefore, the present study was designed to test single nucleotide polymorphisms (SNPs) of the *SLC30A* and *SLC39A* genes as risk factors of having a child with CL/P in the Polish population.

Materials and methods

Participants. The study was approved by the local ethics committee. Index patients with non-syndromic cleft lip with or without cleft palate were chosen by clinicians using detailed diagnostic information from medical records in the Department of Paediatrics and Department of Paediatric Surgery at the Institute of Mother and Child in Warsaw. Peripheral blood samples from 149 healthy mothers of children with orofacial clefts were obtained. In addition, 100 healthy mothers of children without congenital anomalies were used as controls (control mothers). All participating omnivorous women, aged 18-45 years, were Caucasians of Polish origin recruited from the same geographic region. Written and oral consent was obtained from all subjects.

Genotyping. DNA was isolated from peripheral blood lymphocytes by salt extraction. All 13 analyzed polymorphic variants of *SLC30A1,*

Table 2. Genotype frequencies of the investigated polymorphisms in mothers of children with CL/P and control mothers

Gene	rs no.	Genotype	CL/P mothers (frequency)	Control mothers (frequency)	Odds ratio (95% CI)	p[a]
SLC30A1	rs7526700	CC	90 (0.63)	56 (0.57)	Referent	-
		CG	52 (0.36)	40 (0.40)	0.809 (0.476 - 1.375)	0.4328
		GG	2 (0.01)	3 (0.03)	0.415 (0.067 - 2.561)	0.3794[b]
		CG + GG	54 (0.37)	43 (0.43)	0.781 (0.464 - 1.316)	0.3533
		MAF	0.19	0.23	-	-
SLC30A1	rs2278651	CC	33 (0.24)	24 (0.25)	Referent	-
		CT	76 (0.54)	49 (0.50)	1.128 (0.597 - 2.133)	0.7101
		TT	31 (0.22)	24 (0.25)	0.939 (0.444 - 1.986)	0.8700
		CT + TT	107 (0.76)	73 (0.75)	1.066 (0.582 - 1.951)	0.8357
		MAF	0.49	0.50	-	-
SLC30A1	rs611386	GG	86 (0.58)	56 (0.57)	Referent	-
		AG	59 (0.40)	39 (0.39)	0.985 (0.582 - 1.667)	0.9554
		AA	3 (0.02)	4 (0.04)	0.488 (0.105 - 2.266)	0.4400[b]
		AG + AA	62 (0.42)	43 (0.43)	0.939 (0.561 - 1.571)	0.8101
		MAF	0.21	0.24	-	-
SLC30A4	rs2453531	AA	71 (0.48)	51 (0.53)	Referent	-
		AG	68 (0.46)	42 (0.44)	1.163 (0.687 – 1.970)	0.5741
		GG	9 (0.06)	3 (0.03)	2.155 (0.555 – 8.359)	0.3598[b]
		AG + GG	77 (0.52)	45 (0.47)	1.229 (0.735 – 2.056)	0.4317
		MAF	0.29	0.25	-	-
SLC30A4	rs8029246	AA	72 (0.50)	55 (0.58)	Referent	-
		AG	58 (0.41)	35 (0.37)	1.266 (0.732 - 2.188)	0.3979
		GG	13 (0.09)	5 (0.05)	1.986 (0.668 - 5.906)	0.2106
		AG + GG	71 (0.50)	40 (0.42)	1.356 (0.805 - 2.287)	0.2532
		MAF	0.29	0.24	-	-
SLC30A5	rs351444	CC	55 (0.37)	30 (0.32)	Referent	
		CG	73 (0.49)	44 (0.46)	0.905 (0.506 - 1.619)	0.7363
		GG	21 (0.14)	21 (0.22)	0.545 (0.257 - 1.156)	0.1117
		CG + GG	94 (0.63)	65 (0.68)	0.789 (0.457 - 1.362)	0.3938
		MAF	0.39	0.45	-	-
SLC30A5	rs164393	GG	63 (0.43)	45 (0.47)	Referent	-
		GT	73 (0.50)	41 (0.43)	1.272 (0.740 - 2.185)	0.3834
		TT	10 (0.07)	10 (0.10)	0.714 (0.274 - 1.859)	0.4892
		GT + TT	83 (0.57)	51 (0.53)	1.162 (0.693 - 1.951)	0.5686
		MAF	0.32	0.32	-	-
SLC30A5	rs6886492	AA	89 (0.64)	58 (0.60)	Referent	-
		AG	45 (0.32)	32 (0.33)	0.916 (0.523 - 1.606)	0.7605
		GG	6 (0.04)	7 (0.07)	0.559 (0.179 - 1.746)	0.3112
		AG + GG	51 (0.36)	39 (0.40)	0.852 (0.500 - 1.451)	0.5557
		MAF	0.20	0.24	-	-
SLC39A1	rs10127484	AA	37 (0.25)	21 (0.21)	Referent	-
		AG	74 (0.50)	58 (0.58)	0.724 (0.383 - 1.368)	0.3193
		GG	37 (0.25)	21 (0.21)	1.000 (0.469 - 2.133)	1.000
		AG + GG	111 (0.75)	79 (0.79)	0.797 (0.434 - 1.465)	0.4654
		MAF	0.50	0.50	-	-
SLC39A1	rs11264736	CC	42 (0.30)	22 (0.23)	Referent	-
		CT	69 (0.48)	56 (0.58)	0.645 (0.345 - 1.206)	0.1683
		TT	31 (0.22)	19 (0.19)	0.855 (0.396 - 1.845)	0.6890
		CT + TT	100 (0.70)	75 (0.77)	0.698 (0.385 - 1.268)	0.2370
		MAF	0.46	0.48	-	-
SLC39A3	rs759071	AA	81 (0.56)	51 (0.53)	Referent	-
		AG	47 (0.33)	38 (0.39)	0.779(0.448 - 1.354)	0.3749
		GG	16 (0.11)	8 (0.08)	1.259 (0.503 - 3.155)	0.6222
		AG + GG	63 (0.44)	46 (0.47)	0.862 (0.514 - 1.446)	0.5743
		MAF	0.27	0.28	-	-
SLC39A3	rs4806874	AA	68 (0.46)	44 (0.44)	Referent	-
		AG	63 (0.42)	48 (0.48)	0.849 (0.498 – 1.448)	0.5484
		GG	18 (0.12)	8 (0.08)	1.456 (0.583 – 3.636)	0.4195
		AG +GG	81 (0.54)	56 (0.56)	0.936 (0.562 – 1.558)	0.7990
		MAF	0.33	0.32	-	-
SLC39A3	rs10415622	AA	64 (0.45)	38 (0.39)	Referent	-
		AG	61 (0.43)	50 (0.51)	0.724 (0.418 - 1.254)	0.2487
		GG	16 (0.12)	10 (0.10)	0.950 (0.391 - 2.305)	0.9097
		AG + GG	77 (0.55)	60 (0.61)	0.762 (0.451 - 1.288)	0.3092
		MAF	0.33	0.36	-	-

[a]Chi-square analysis; [b]Fisher exact test; MAF, minor allele frequency.

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Table 3. Haplotypes and their association with maternal risk of having CL/P progeny

Haplotype	Frequency	Case, control ratios (freq.)	Chi-square	p value
SLC30A1 (rs7526700, rs2278651, rs611386)				
CTG	0.296	0.298, 0.293	0.013	0.9106
CCG	0.279	0.301, 0.246	1.843	0.1746
GCG	0.198	0.182, 0.224	1.335	0.2479
CTA	0.192	0.184, 0.204	0.308	0.5789
CCA	0.024	0.024, 0.025	0.005	0.9420
GTA	0.010	0.011, 0.008	0.093	0.7607
SLC30A4 (rs2453531, rs8029246)				
AA	0.697	0.675, 0.729	1.646	0.1995
GG	0.267	0.292, 0.229	2.370	0.1237
GA	0.034	0.032, 0.036	0.044	0.8341
SLC30A5 (rs351444, rs164393, rs6886492)				
CGA	0.437	0.447, 0.421	0.307	0.5796
GTA	0.221	0.207, 0.244	0.914	0.3389
GGG	0.104	0.095, 0.119	0.754	0.3853
CGG	0.086	0.086, 0.086	0.000	0.9973
CTA	0.064	0.081, 0.037	3.821	0.0506
GGA	0.060	0.064, 0.055	0.155	0.6935
GTG	0.028	0.021, 0.038	1.280	0.2580
SLC39A1 (rs10127484, rs11264736)				
AC	0.499	0.495, 0.505	0.047	0.8288
GT	0.466	0.464, 0.469	0.014	0.9057
GC	0.029	0.034, 0.020	0.781	0.3768
SLC39A3 (rs759071, rs4806874, rs10415622)				
AAA	0.646	0.653, 0.636	0.147	0.7010
GGG	0.264	0.264, 0.264	0.000	0.9898
AGG	0.042	0.043, 0.040	0.014	0.9051
AGA	0.017	0.017, 0.017	0.002	0.9629
GAG	0.017	0.014, 0.021	0.296	0.5867
AAG	0.014	0.009, 0.022	1.357	0.2440

SLC30A4, SLC30A5, SLC39A1, and *SLC39A3* were identified using PCR followed by appropriate restriction enzyme digestion (PCR-RFLP). DNA fragments were separated using electrophoresis on 2% agarose gel and visualized using ethidium bromide staining. Primer sequences and conditions for PCR-RFLP analyses are presented in Table 1. The selection of SNPs was conducted through the use of the genome browser of the International HapMap Consortium (www.hapmap.org). SNPs were prioritized according to their validation status, functional relevance and importance, and minor allele frequency MAF>0.1 in the Caucasian population.

Statistical analysis. The differences in allele and genotype frequencies between case mothers and controls were determined using standard χ^2 and Fisher exact tests. The odds ratio (OR) and associated 95% confidence intervals (95% CI) for mothers of children with CL/P versus control mothers were also calculated. Haplotype analysis was performed using Haploview 4.0 (http://www.broadinstitute.org/mpg/haploview). Associations between the investigated polymorphisms and the risk of having a child with an orofacial cleft were tested using the nonparametric and genetic model-free Multifactor Dimensionality Reduction (MDR) approach (MDR version 2.0 beta 5). Statistical significance was evaluated using a 1,000-fold permutation test (MDR permutation testing module 0.4.9 alpha). A p-value of <0.05 was considered statistically significant.

Results

For all analysed polymorphisms of the *SLC30A1, SLC30A4, SLC30A5, SLC39A1*, and *SLC39A3* genes there was no evidence for both allelic and genotyping association with the risk of being a case mother in the Polish population. The genotyping results are presented in Table 2. No deviation from Hardy-Weinberg equilibrium in either CL/P mothers or control mothers at the 0.05 level was observed. The minor allele frequency for all SNPs was at least 0.19. Haplotype analysis did not detect differences in haplotype distribution between case and control mothers (Table 3). Moreover, the exhaustive MDR analysis revealed no significant interactive genetic effect on having a child with CL/P for all analysed SNPs (Table 4). All possible two-, three- and four-way SNP interactions were tested using 10-fold cross validation in an exhaustive search (considering all possible SNP combinations). All of the "best models" (showing highest testing balanced accuracy and cross validation consistency >5 out of 10) did not reach the statistical significance evaluated using a 1,000-fold permutation test.

Discussion

Genetic studies in humans have shown associations between expression profiles of genes for Zn transporters and chronic diseases (diabetes, asthma) as well as carcinogenesis [16,17]. Since craniofacial malformations arise from misregulation of normally coordinated tissue patterns during early embryogenesis [10], another interesting aspect of Zn transporter function is the possibility of these proteins' involvement in early embryonic nutrition. It has been demonstrated that the expression of the *Slc30a1* gene is highly active in the yolk sac of the developing mouse embryo, and this expression is partially dependent on maternal Zn intake [18]. Due to the key role of Zn in human physiology, the aim of this study was to verify possible contributions of Zn transporter gene SNPs to orofacial clefting. To our knowledge, the present report is the second association study examining the potential role of genes encoding Zn transporters in the aetiology of non-syndromic structural malformations in affected progeny. In a sample of Dutch triads of patients and their parents, no association was observed between the 1069C>T (rs2272662) variant of *SLC39A4* and spina bifida [19]. In our study the investigated SNPs were not found to be associated with the women's risk of having children with CL/P, analyzed either separately or in combination. We analyzed three polymorphisms of the gene for the SLC30A1 protein, which plays an essential function in transporting maternal Zn into the embryo, and two polymorphic variants of the gene for SLC39A1 protein, which has been detected in a wide variety of tissues and cell types [3].

Table 4. Results of gene-gene interactions analyzed by MDR method

Genes and rs numbers	Testing balanced accuracy[a]	Cross validation consistency[b]	p value[c]
SLC39A1 rs10127484	0,5729	9 / 10	0,281
SLC30A4 rs2453531, *SLC39A1* rs10127484	0,5862	6 / 10	0,186
SLC30A1 rs7526700, *SLC30A1* rs2278651, *SLC30A4* rs8029246	0,4920	2 / 10	0,895
SLC30A1 rs611386, *SLC30A1* rs2278651, *SLC39A1* rs10127484, *SLC39A1* rs11264736	0,4855	2 / 10	0,922

[a]Testing balanced accuracy is the accuracy of classification of cases and controls in the testing dataset calculated as (Sensitivity+Specificity)/2; [b]Cross validation consistency is the number of times the model was selected as the best model after 10-fold cross-validation runs; [c]Significance of accuracy (empirical p value based on 1,000 permutations).

It has been shown that mRNA levels of both *SLC30A1* and *SLC39A1* may be potential Zn status indicators in the human lymphoblastoid cell line [20]. In women of childbearing age supplemented with Zn, the expression of *SLC39A1* in leucocytes has been reported to decrease significantly, whereas there were no changes in the mRNA levels of SLC30A1 [20]. Therefore, SLC39A1 mRNA expression is considered a potential biomarker of Zn status in humans. In contrast to these predictions, the allele and genotype distribution of *SLC30A1* and *SLC39A1* polymorphic variants was found to be similar among the investigated mothers of CL/P affected children and controls.

In the present study, the allele distribution of *SLC30A5* rs351444 showed the highest difference between case and control mothers. Moreover, the risk of having a CL/P affected child for the maternal rs351444 GG genotype compared with the wild type tended to be decreased (OR=0.55; 95%CI: 0.26-1.16; p=0.11). Interestingly, haplotype analysis of *SLC30A5* polymorphic variants (rs351444, rs164393, and rs6886492) showed borderline association between the CTA haplotype and increased risk of clefting (p=0.051).

Expression of *SLC30A4* appears to be independent of Zn status, which is contrary to *SLC30A1, SLC30A5, SLC39A1*, and *SLC39A3* [21]. It is noteworthy that there was no evidence for association of polymorphic variants of SLC30A4 with CL/P risk. The analysis of epistasis is helpful in determining the true contribution of genetic factors to congenital anomaly susceptibility [22]. However, in our study the exhaustive MDR analysis revealed no significant interactive effect of polymorphic variants of the genes for Zn transporters on abnormal palatogenesis. There are no previously published clinical association studies of the SNPs analyzed in the present report, and so we are unable to compare our results with data from other studies.

However, our negative results do not exclude the potential role of polymorphisms of Zn transporter genes in the aetiology of CL/P. The results might be influenced by the sample size, which may not be large enough to detect a modest effect of the analyzed gene variants. We cannot exclude that SNPs in genes for Zn transporters may influence palatogenesis via the embryonic, rather than the maternal, genotype. In humans, some of genes for Zn transporters may have a restricted expression to only fetal tissues [23]. Therefore, the exclusion of the investigated SNPs and other variants of genes encoding Zn transporters as risk factors of CL/P in the Polish population requires further analyses, which should be performed in larger groups of case and control mothers as well as in CL/P-affected children. The relatively high and fluctuating frequency of orofacial clefts is attributable to the sensitivity of facial development to environmental insult. Recent progress in our understanding of the molecular functions of Zn transporters and their clinical associations has revealed that additional analysis involving polymorphisms of *SLC30A8* and *SLC39A14* may throw a new light on the aetiology of CL/P. Maternal diabetes and infection with the common cold during early pregnancy are well known risk factors for abnormal palatogenesis [10,24]. SLC39a14 plays a major role in hypozincemia induced by inflammation, which is among the classical changes observed during acute infections [3]. In rodents, dietary Zn supplementation decreases the frequency of malformations caused by bacterial lipopolysaccharides [25]. The association between some polymorphisms of *SLC30A8* and the onset of diabetes mellitus is well documented in humans [3].

In summary, the presented results did not support any association between maternal polymorphisms of *SLC30A* and *SLC39A* genes and the risk of having an infant with CL/P in the investigated Polish population. However, given the high heterogeneity of the association between the maternal Zn blood level and the risk of clefting [13], we suspect that genes acting in Zn homeostasis could be involved in CL/P onset and deserve further investigation.

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