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ORIGINAL ARTICLE

Polymorphism in the CAVI gene, salivary properties and dental caries

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ABSTRACT

Objectives: Carbonic anhydrase (CA) VI is supposed to take part in pH or buffering capacity regulation, which can influence the caries risk of an individual. Its expression in the saliva can be modified by single nucleotide polymorphism (SNP). The aim was to investigate SNP in the CA VI gene in relation to active dental caries and physiochemical properties of saliva.

Materials and methods: One hundred and thirty participants aged 11–16 years were involved. Clinical examinations were carried out using standardized WHO criteria, DMFT/DMFS and white spot lesions score was evaluated. Saliva samples were examined for salivary properties and CA VI concentration. DNA evaluated in the investigation was extracted from the buccal smear. Three SNP within CAVI gene (rs2274327; rs2274328; rs2274333) were selected and genotyping was performed.

Results: In the active caries group, the mean CAVI concentration was significantly lower than in caries free group ($p = .014$). No association between increased or decreased risk of caries and analysed SNPs was found. There were some significant relations concerning SNPs and salivary buffer capacity and flow rate in rs2274327 and rs2274328.

Conclusions: Polymorphism in the CAVI gene can affect salivary properties but there is no direct connection with dental caries.

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Caries susceptibility; risk factors; buffer capacity; gustin; saliva

Introduction

Carbonic anhydrase (CA) isoenzymes constitute a group of zinc metalloenzymes, representant of which (CA II) was first discovered in 1940 by Keilin and Mann. To date, there are seven anhydrases identified, which are responsible for catalysation of the reversible hydration of carbon dioxide in the reaction: $CO_2 + H_2O <=> HCO^{3-} + H^+$ [1,2]. CAs are involved in the regulation of pH, ion, fluid and acid–base balance in several tissues [3,4]. The only secreted form of anhydrases is carbonic anhydrase VI (CAVI), which, in humans, is produced by serous acinar cell of parotid and submandibular glands and secreted into saliva [2]. The secretion of salivary CA VI changes circadian, the concentration in saliva is low at night and quickly rises after awakening [5]. The meaning of salivary flow and components for caries development can be observed on the example of patients with salivary gland dysfunctions or diseases, as well as after cancer radiotherapy in the head and neck region [6]. Although CAs are evolutionally old enzymes, their exact physiological significance in saliva is still not clear. They may take part in pH or buffering capacity regulation, which can influence the caries risk of an individual, nevertheless a study by Kivelä et al. failed to confirm this hypothesis [7]. CA VI expression in the saliva (e.g. its concentration or activity) can be modified by various factors, especially genetic, such as single nucleotide polymorphism (SNP) in the gene coding sequences [4]. SNPs constitute the most common form of human genome variability and can affect gene function [8,9]. There are some studies assessing the relationship between CA VI polymorphism and dental caries but they mainly do not focus on caries activity [10–12]. In the present paper, we aim to investigate SNP in the CA VI gene in relation to active dental caries (the first genetic report) and physiochemical properties of saliva which influence caries prevalence (the replication report).

Materials and methods

The study conformed to the STROBE guidelines (STrengthening the Reporting of OBservational studies in Epidemiology) extended with STREGA guidelines (The STrengthening the REporting of Genetic Association studies).

Study population

The study was carried out on a group of 130 participants aged 11–16 years who presented permanent dentition. The participants were recruited randomly from those attending dental treatment in the Department of Pediatric Dentistry, Medical University of Warsaw. Patients came from one ethnic group. The approval of the Bioethical Commission of Warsaw Medical University (no. KB/194/2015) was obtained and informed consent was attained from all parents/subjects who participated in the study. The inclusion criteria were lack of

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systemic diseases, no chronic medication intake, no antibiotics intake within 1 month prior to the investigation, no developmental defects of tooth hard tissues and no previous orthodontic treatment.

Clinical examination

Clinical examinations were carried out in the dental office using standardized WHO criteria by one examiner. Children were asked to appear on an empty stomach and at least 2 h after tooth brushing and 12 h after using antibacterial mouth rinse.

Caries index, expressed as mean DMFT (plus compound – DT for decayed teeth) and DMFS and white spot lesions (WSLs) score was evaluated [13].

Sample collection for salivary analysis

Saliva samples were collected from all participants at the same time (8.00–10.00 am) in order to eliminate circadian differences in CAVI concentration [5]. For CAVI concentration, unstimulated saliva was collected using Salivette collection tube (Sarstedt AG&Co., Nümbrecht, Germany) for 5 min. After collection, the tube was stored at +4 °C and centrifuged (15 min; 1000×*g*) within 2 h. Then, unstimulated salivary flow rate was calculated and the saliva was frozen without delay at –80 °C until assay.

For evaluation of physiochemical properties of saliva (pH, buffer capacity, stimulated salivary flow rate, consistency), the commercial kit GC Saliva Check Buffer (GC Europe, Leuven, Belgium) was used, due to the manufacturer's instruction. Salivary quantity of cariogenic bacteria – *Streptococcus mutans* (SM) and *Lactobacillus spp* (LB) was evaluated with the commercial test CRT bacteria (Ivoclar Vivadent, Schaan, Liechtenstein), following the manual. The values are expressed as colony-forming units per millilitre of saliva (CFU/mL).

CAVI concentration evaluation

The saliva samples were assayed for CAVI concentration. Prior to testing, samples were brought to room temperature. Freeze/thaw cycles were avoided. Collection protocol and assay were followed according to the protocol outlined from an ELISA kit for carbonic anhydrase VI (Cloud-Clone Corp., Houston, TX) and reading were performed at 450 nm on BioTek Synergy™ Mx Microplate Reader (BioSPX B.V., Abcoude, Netherlands).

DNA sampling and extraction

DNA evaluated in the investigation was extracted from the buccal smear. The smear was collected for about 2 min using a special buccal swab (Hagmed, Rawa Mazowiecka, Poland). After the collection, the swabs were dried and stored separately in drying envelopes (Hagmed, Rawa Mazowiecka, Poland) at room temperature until DNA extraction.

DNA extraction was performed using NucleoSpin® Tissue (Macherey-Nagel GmbH & Co., Düren, Germany) commercial set due to producer's instructions. After extraction, DNA was put into TE buffer and kept in Eppendorf tubes in +4 °C until assay.

SNP selection and genotyping

Three SNPs within CAVI gene (rs2274327; rs2274328; rs2274333) were selected on the basis of known heterozygosity and minor allele frequency (MAF) >0.05 and linkage disequilibrium (LD) between pairs of selected SNPs (r^2 threshold of 0.8). Genotyping was performed with the TaqMan® SNP Genotyping Assays (Applied Biosystems, Weiterstadt, Germany) (C___1739308_1_ for rs2274327; C___1739309_1_ for rs2274328 and C___1739329_1_for rs2274333) according to the manufacturer's instructions on QuantStudio™ 12 K Flex Real Time PCR System (Thermo Fisher Scientific, Waltham, MA).

Statistical analysis

The obtained data were subjected to statistical analysis. The analyses were performed using Statistica software and R package. For the quantitative variables, descriptive statistics characterizing their variability were calculated. For binomial variables, the proportions (%) with division into groups were calculated. The comparison of the quantitative variables between two groups was carried out using a Mann–Whitney *U* and chi-square test while the comparison of three or more groups was performed using ANOVA test. In case of categorical variables (including binomial variables), chi-square test was used to compare the groups. Spearman's rank correlation coefficient was calculated. The comparison of experimental and theoretical distributions was evaluated using chi-square test (e.g. theoretical distribution defined by the Hardy–Weinberg (H–W) principle). Odd ratio was calculated to compare two groups according to probability of dental caries and particular salivary properties. In all analyses, the statistical significance level was set to .05. Missing data were excluded from the analysis.

Results

One hundred and thirty participants (56.15% of boys) took part in the investigation. The mean age of the patients (SD) was 13.25 (1.72) years. Caries (individuals with DMFT > 0) was present in 121 (93%) of individuals. Active caries (individuals with DT + WSL > 0) was present in 90 (69.23%) patients. The mean DMFT (SD) and DMFS (SD) index in the whole study group was 5.84 (4.43) and 7.94 (7.53), respectively. There were no statistically significant differences between age of patients with active dental caries and without active dental caries ($p = .174$). The sample characteristics are presented in Table 1.

The statistical analysis indicated no significant differences between salivary CAVI concentration and caries indices values, except the active caries group (DT + WSL > 0) in which

the mean CAVI concentration was significantly lower than in caries free group (p=.014). Statistically significant correlations were found between active caries and physiochemical properties of saliva and cariogenic bacteria count (Table 2).

Association between CAVI SNPs and caries prevalence

Three different polymorphisms were examined in the CAVI gene. Genotyping was successful in all individuals. Genotypes of SNPs rs2274327, rs2274328, rs2274333 were in the H–W equilibrium (p>.05). Genotype distributions are shown in Table 3. Multivariate logistic regression analysis was conducted to evaluate the effect of SNP on caries risk. No association between increased or decreased risk of caries and analysed SNPs was found. However, some significant positive correlations were found between rs2274333 A/G genotype and the presence of active WSL (active WSL > 0) (r = 0.173; p<.05).

Association between CAVI SNPs and salivary properties

In analysis of pH, buffer capacity, saliva viscosity, unstimulated saliva flow rate and stimulated saliva flow rate associations, as well as SM and LB salivary quantity, genotypes of SNPs rs2274327, rs2274328 and rs2274333 were in the H–W equilibrium (Table 4).

In multivariate logistic regression analysis, some significant results were obtained regarding rs2274327 and rs2274328, and salivary buffer capacity and salivary flow rate. The details of the analysis are provided in Table 5.

Table 1. Characteristics of the study group.

	Mean (SD)		
Parameter	DT + WSL = 0	DT + WSL > 0	p
Age (years)	12.95 (1.90)	13.40 (1.65)	.174
DMFT	3.3 (2.88)	6.98 (4.43)	<.001
DT	0 (0)	2.54 (2.70)	<.001
MT	0.05 (0.32)	0.04 (0.21)	.833
FT	3.23 (2.97)	4.39 (3.33)	.061
DMFS	4.0 (4.57)	9.69 (7.94)	<.001
Salivary pH	7.0 (0.53)	6.65 (0.58)	.001
Buffer capacity	8.3 (1.87)	6.66 (2.19)	<.001
Unstimulated salivary flow rate (mL/5 min)	1.21 (0.49)	1.40 (0.39)	.020
Stimulated salivary flow rate (mL/5 min)	5.19 (2.21)	5.13 (2.66)	.901
	n/%		
Females	18/31.6	39/68.4	.860
Males	22/30.1	51/69.9	.860
SM > 10^5 CFU/mL	11/16.2	57/83.8	<.001
LB > 10^5 CFU/mL	18/19.8	73/80.2	<.001

In Spearman's correlation rank, positive correlation of usfr and rs2274333 G/G genotype was obtained (r = 0.81; p<.05), while there was a negative correlation of usfr with rs2274333 allele A presence (r=–0.181; p<.05).

Association between CAVI SNPs and CAVI salivary concentrations

The analysis was performed in 103 individuals (in 27 remaining patients we failed to obtain the CA VI concentration). The mean salivary CAVI concentration (SD) obtained in the study was 1268 pg/mL (1493.55). In this analysis, genotypes in rs2274327, rs2274328 and rs2274333 were in the H–W equilibrium (p=.723, .920, .074, respectively).

In Spearman's correlation rank, positive correlation between CAVI concentration and rs2274333 allele A was found (r = 0.208; p<.05). There was a negative correlation between CAVI concentration and rs2274333 G/G genotype (r = –0.208; p<.05).

Analysis of variance (ANOVA) revealed no statistically significant differences in CAVI concentration between different genotypes of rs2274327, rs2274328 and rs2274333 (Table 6).

Discussion

The results obtained in the presented study revealed the positive correlation of salivary viscosity and cariogenic bacteria (*Streptococcus mutans*, *Lactobacillus spp.*) quantity with the caries presence, whereas there was a negative correlation with salivary pH and buffering capacity. This results confirm what is well-established knowledge in cariology, as well as are consistent with previous observations on the same subject in literature [7,11,14,15]. However, we found also a positive correlation in case of salivary flow rate, which stands in opposition to results from Frasseto et al. [16] and Kivelä

Table 3. Genotypes distribution of SNPs rs2274327, rs2274328 and rs2274333.

SNP			DT > 0	DT = 0	DT + WSL > 0	DT + WSL = 0
rs2274327	*n*/%	CC	26/38.8	24/38	33/36.6	17/42.5
		CT	29/43.3	30/47.6	41/45.5	18/45
		TT	12/17.9	9/14.4	16/17.9	5/12.5
p for H–W equilibrium			.438	.939	.601	.945
rs2274328	*n*/%	CC	17/25.4	15/23.8	23/25.5	9/22.5
		AC	31/46.3	29/46	42/46.6	18/45
		AA	19/28.3	19/30.2	25/27.9	13/32.5
p for H–W equilibrium			.546	.548	.530	.565
rs2274333	*n*/%	AA	33/49.3	28/44.4	41/45.5	20/50
		AG	28/41.8	31/49.2	43/47.7	16/40
		GG	6/8.9	4/6.4	6/6.8	4/10
p for H–W equilibrium			.986	.230	.233	.763

Table 2. The values of Spearman's correlation rank coefficient between caries indices and salivary parameters.

	Active WSL > 0	DMFT or active WSL > 0	DT	DMFT	DMFS
SM > 10^5 CFU/mL	0.381*	0.435*	0.540*	0.455*	0.428*
LB > 10^5 CFU/mL	0.448*	0.566*	0.499*	0.393*	0.380*
Salivary pH	–0.493*	–0.384*	–0.438*	–0.462*	–0.458*
Buffer capacity	–0.477*	–0.367*	–0.332*	–0.437*	–0.444*
Viscosity	–0.317*	–0.290*	–0.361*	–0.304*	–0.314*
Ss flow rate	–0.329*	–0.072	–0.085	–0.226*	–0.205

ss: stimulated saliva.
*p < .05.

et al. [7] as they indicate no significant differences between active caries and caries free group.

The previous studies analyse the relation of caries and CAVI concentration only on the basis of DMFT index, not taking caries activity into consideration. Kivelä et al. [17] reported the negative correlation of CAVI concentration and DMFT index ($r = -0.22$; $p = .001$) which was in line to outcomes by Makawi et al. [15] who discovered lower CAVI concentration in high caries risk group. In our research, we failed to confirm significant relations between salivary CAVI concentration and caries indices, except the active caries group (DT + WSL > 0), in which the concentrations were significantly lower. This findings are novel and interesting, introducing a new viewpoint on this issue. It comes to mind that, in some cases, it is a good idea not only to assess the overall DMFT index, but also the components. The components M (missing) and F (filled) reflect what was in the past and the component D is more adequate as it stands for the present caries in the particular moment (together with the present WSL count). If the enzyme concentrations is being measured at this moment, it should be compared with the parameter at the same time, as the researcher has no information about enzyme concentration in the past. Nevertheless, the results confirm that CAVI plays a specific role in the natural defence systems against dental caries.

In the present study, we investigated also the SNP in the CAVI gene and its relation to CAVI salivary concentration, caries incidence and physiochemical properties of saliva. Our examination revealed some significant correlations of SNP in rs2274333 (positive correlation of allele A and negative correlation of allele G) but there were no significant differences in the distribution on genotypes. The issue of SNP relation with CAVI concentration is rarely investigated; however, the recent study of Aidar et al. [18] confirmed our observations in rs2274333. What is more, a connection with rs2274327 T/T genotype was discovered (significantly lower CAVI concentrations comparing to C/T or C/C genotypes).

The outcomes in research evaluating the association between SNP in the CAVI gene and caries prevalence on the basis of caries indices (DMFT and the compounds, WSL) are still ambiguous [10,19–21]. The present results are in line to some previous observations as we found no definitive evidence in multivariate logistic regression analysis; however, some significant correlations of rs2274333 and active WSL presence were found. Sengul et al. [22] reported no statistically significant interaction between carious and non-carious

Table 6. Salivary CAVI concentration in different genotypes of rs2274327, rs2274328 and rs2274333.

SNP	*n*	Mean salivary CAVI concentration (SD) (pg/mL)	*p*
rs2274327 C/C	40	1231 (1739)	.209
rs2274327 C/T	47	1485 (1394)	
rs2274327 T/T	16	722 (938)	
rs2274328 A/A	25	1082 (1308)	.545
rs2274328 A/C	52	1443 (1698)	
rs2274328 C/C	26	1166 (1304)	
rs2274333 A/A	48	1253 (1378)	.344
rs2274333 A/G	50	1373 (1650)	
rs2274333 G/G	5	347 (104)	

Table 4. *p* Values of the Hardy–Weinberg equilibrium in salivary properties assessment.

	pH	Buffer capacity	Viscosity	Unstimulated saliva flow rate	Stimulated saliva flow rate	SM rate	LB rate
rs2274327	0.650	0.160	0.749	0.642	0.853	0.850	0.579
rs2274328	0.608	0.180	0.695	0.077	0.695	0.714	0.518
rs2274333	0.523	0.398	0.165	0.179	0.323	0.216	0.618

Table 5. OR (95%CI) for association between CA VI polymorphisms and salivary properties.

		Dominant model		Co-dominant model		Recessive model	
Salivary property	SNP (rs)	OR (95%CI)	*p*	OR (95%CI)	*p*	OR (95%CI)	*p*
pH	2274327	1.08 (0.53;2.20)	.445	0.96 (0.59;1.56)	.439	0.74 (0.29;1.89)	.636
	2274328	1.13 (0.51;2.52)	.678	1.00 (0.63;1.60)	.606	0.90 (0.42;1.92)	.661
	2274333	0.78 (0.39;1.56)	.254	0.74 (0.42;1.28)	.444	0.41 (0.10;1.68)	.717
Buffer capacity	2274327	0.96 (0.41;2.25)	.035*	1.34 (0.75;2.42)	.061	2.74 (1.00;7.48)	.365
	2274328	0.40 (0.16;0.98)	.444	0.69 (0.39;1.24)	.090	0.96 (0.38;2.42)	.035*
	2274333	1.11 (0.49;2.56)	.248	0.93 (0.48;1.81)	.245	0.37 (0.04;3.01)	.594
Viscosity	2274327	0.94 (0.46;1.92)	.582	1.03 (0.63; 1.69)	.574	1.25 (0.49;3.18)	.726
	2274328	1.14 (0.51;2.57)	.892	1.09 (0.68;1.75)	.939	1.10 (0.51;2.36)	.802
	2274333	1.17 (0.59;2.36)	.075	0.92 (0.53;1.61)	.070	0.31 (0.06;1.50)	.368
usfr	2274327	1.23 (0.59;2.57)	.550	1.05 (0.63;1.7)	.430	0.83 (0.31;2.22)	.473
	2274328	1.39 (0.60;3.26)	.041*	0.87 (0.54;1.42)	.035*	0.51 (0.22;1.17)	.149
	2274333	0.94 (0.46;1.92)	.042*	0.76 (0.43;1.36)	.069	0.17 (0.02;1.41)	.739
ssfr	2274327	0.82 (0.34;1.96)	.968	0.89 (0.48;1.65)	.802	0.93 (0.28;3.04)	.664
	2274328	1.11 (0.40;3.06)	.900	1.07 (0.60;1.93)	.986	1.10 (0.43;2.79)	.886
	2274333	1.37 (0.58;3.24)	.248	1.07 (0.55;2.10)	.178	0.40 (0.05;3.32)	.325
SM rate	2274327	0.90 (0.44;1.82)	.904	0.95 (0.58;1.55)	.799	1.00 (0.39;2.56)	.743
	2274328	1.33 (0.60;2.97)	.546	1.25 (0.78;2.00)	.933	1.38 (0.64;2.95)	.661
	2274333	0.77 (0.39;1.54)	.152	0.97 (0.56;1.69)	.108	2.26 (0.56;9.14)	.274
LB rate	2274327	0.86 (0.39;1.86)	.189	0.76 (0.45;1.30)	.348	0.51 (0.19;1.32)	.918
	2274328	1.58 (0.68;3.66)	.806	1.21 (0.72;2.02)	.427	1.07 (0.47;2.46)	.286
	2274333	1.11 (0.52;2.35)	.485	1.17 (0.64;2.15)	.592	1.78 (0.36;8.81)	.980

usfr: unstimulated saliva flow rate; ssfr: stimulated saliva flow rate.
*$p < .05$.

group while evaluating CAVI SNP in rs2274327 in children. Similar results were obtained previously by Yarat et al. [20] who claim that there are no differences in the frequency of genotypes in rs2274327 and rs2274328. On the other hand, Li et al. [10] discovered that patients with rs170342907 T/T genotype were more susceptible to caries than individuals with C/C genotype but in other rs' no associations were found.

In the analysis of the salivary physiochemical properties and cariogenic bacteria count, some significant results were obtained in case of salivary buffer capacity, which are in accordance with the reports from Aidar et al. [18], as well as Peres et al. [11]. This findings regarded rs2274327. A positive association between buffer capacity and CA VI polymorphism was discovered also by Yildiz et al. [21] when dividing the patients into three groups, regarding the salivary pH (high; intermediate; low). What is novel in our study, we also found a significant differences in rs2274328, what was not previously reported. Individuals with C/C genotype were more likely to have lower salivary buffer capacity than in A/A genotype. What is more, the previous studies failed to prove the relation between other salivary parameters and SNP in the CAVI gene [11,19,20]. Differently to them, the multivariate analysis in our study showed significant differences in genotypes distribution in rs2274328 (genotype C/C is more likely to have higher unstimulated saliva flow rate than in genotypes A/C or A/A) which is a kind of a new discovery. Also, a significant correlations in rs2274333 were found when analysing unstimulating saliva flow rate what was not formerly reported.

In conclusion, the obtained results indicate that SNP in the CAVI gene can affect gene expression, and this can be one of the possible mechanisms which can contribute to better understanding of the disparity in some salivary facilities such as buffer capacity and salivary flow in humans. The practitioners, nevertheless, should remember that this is a complex issue which can be influenced by many secondary factors, not only the genetics. The mentioned salivary parameters are factors associated with susceptibility to dental caries; however, SNP in the CA VI gene was not associated with the risk of caries directly. One of the limitations of the present study could be the group size although we tried to specify the minimal group size on the basis of MAF in the population. We tried to avoid other potential risk of bias by performing any procedure (clinical examination; laboratory procedures; data analysis) by one examiner under control of other examiners and following directly the study assumptions and inclusion criteria. Though, further studies are necessary for evaluation and confirmation of our conclusions. This studies should involve larger numbers of participants from various ethnic groups because of the different alleles and genotypes distributions across populations.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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