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Carbonic Anhydrase VI Exon 2 Genetic Polymorphism in Turkish Subjects with Low Caries Experience (Preliminary Study)

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Abstract. *Aim: The aim of this study was to investigate carbonic anhydrase (CA) VI exon 2 genetic polymorphism and its possible association with low caries experience in healthy young adults. Patients and Methods: Healthy young adults with caries or who where caries-free were recruited and unstimulated whole saliva and blood samples were taken. The number of decayed, missing and filled teeth (DMFT) and oral hygiene parameters were examined. Single nucleotide polymorphism (SNP) of CA VI gene exon 2 was determined by PCR and DNA sequencing. Salivary CA activity, buffering capacity and pH were also determined. Results: Two SNPs (dbSNP: 142460367 and 142460368), which are responsible for amino acid changes, were found. The frequencies of these SNPs were not significantly different between the caries-free group and the group with caries. There was no correlation between these SNPs and the salivary parameters. Conclusion: Two SNPs found in young Turkish adults have no correlation with low caries prevalance.*

The carbonic anhydrases (CAs; EC 4.2.1.1, zinc metalloenzyme) participate in a variety of physiological processes that involve pH regulation, CO_2 and HCO_3^- transport, ion transport, and water and electrolyte balance (1, 2). They are widespread enzymes, present in mammals as different isoenzymes: some of these isozymes are cytosolic (CA I-III, VII, XIII), while others are membrane bound (CA IV, IX, XII, XIV and XV); CA VA and VB are mitochondrial (1-4). Two subtypes of CA VI are known. The A-type is thus far the only known secreted member of the α-CA family and the B-type a stress-induced cytoplasmic form (5). Three acatalytic forms are also known as carbonic anhydrase related proteins VIII, X and XI (CARP VIII, X and XI) (1-5). The CA isozymes have been purified from representatives of all types of organisms (6).

CA VI is expressed in the serous acinar cells of the parotid and submandibular glands, from where it is secreted into the saliva (7). Recent research suggests that CA VI neutralizes acidity in dental biofilm and thereby protects teeth from caries (8, 9). Low salivary concentrations of CA VI appear to be associated with an increased prevalence of caries and acid peptic diseases (9). Since CA VI is present in the enamel pellicle and gastric mucus, it may function locally in the microenvironment of dental and epithelial surfaces and accelerate the neutralization of excess acid (8). Its reported molecular weight is 39-46 kDa (10). The human CA VI (HCA VI) gene is located on chromosome 1. The gene encoding human CA VI has 8 exons and 7 introns (11). The HCA VI protein has a sequence identity of 35% to HCA II and 72 % to ovine CA, while residues involved at the active site of the enzyme are conserved. It is composed of 308 amino acids in total with a leader sequence of 12 amino acids. The isoelectric point is 6.4 (9).

The identification of genetic risk factors, which has the potential to lead to the development of diagnostic tools, is necessary for caries. Polymorphism has not been described at the CA VI locus in healthy young adults until today. The aim of this study was to investigate CA VI exon 2 genetic polymorphism and its possible association with low caries experience in healthy young adults.

Patients and Methods

Subjects. Forty-four healthy subjects with healthy teeth (no caries, n=20; 8 females, 12 males) or with low caries experience (minimal caries, n=24; 12 females, 12 males) participated in this study. Their mean ages were 21.95±2.2 (range:19-26) and 20.20±1.2 (range: 18-23) respectively. The subjects gave informed consent to participate in this study. Subjects were excluded from the study if they did not meet the inclusion criteria or did not want to participate in the study.

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None had any systemic disease. Oral health status was determined according to WHO criteria. Dental caries prevalence and the number of decayed, missing, and filled teeth (DMFT) was evaluated (12). A simplified oral hygiene index, OHI-S (Greene-Vermillion), and gingival index, GI (Leo-Silness) (13), were also calculated.

Collection of unstimulated whole saliva. All the subjects were instructed to refrain from smoking, eating, drinking and tooth brushing for 12 h prior to the saliva collection. Fasting unstimulated whole saliva samples were collected at the same time of day (between 08:30 and 11:00 h) by the same researcher in all cases. Before saliva collection, the mouth was rinsed with distilled water. Saliva was allowed to accumulate on the floor of the mouth and the subjects were instructed to spit into test tubes. Then saliva was allowed to accumulate on the floor of the mouth for ten minutes and the salivary flow rate was calculated (ml/min). The saliva samples were stored at –24˚C until used.

Salivary analysis. Salivary total protein, CA activity and salivary buffering capacity were determined by the methods of Lowry (14) Verpoorte (15), and Ericsson (16), respectively. Furthermore, salivary pH was measured with pH paper (pH-Indikatorpapier, Neutralit-5.5-9.0; Merck KGaA, Darmstadt, Germany).

Extraction of genomic DNA. DNA was extracted from 200 μl whole blood using a High Pure PCR Template Preparation Kit (Cat No.1 796 828; Roche, Mannheim, Germany) according to the manufacturer's guide. Blood cells were lysed by incubation with a special lysis buffer and proteinase K. Nucleic acids were bound to the glass fibers pre-packed in the High Pure filter tube. Bound nucleic acids were washed with special inhibitor removal buffer to remove of PCR inhibitory contaminants. After washing of bound nucleic acids, purification from salts, proteins and other cellular impurities, purified nucleic acids were removed using the elution buffer. At least 3-6 μg DNA in a 200 μl, was isolated by this method. The concentration of purified DNA was measured by agarose electrophoresis.

Genotype. Gene polymorphism of CA VI exon 2 was determined by PCR and DNA sequencing. The CA VI gene exon 2 region was amplified by using specific primers (Iontek, İstanbul, Turkey) (5'-TGTCTTAGAAGGGGCACT-3' sense and 5'-TACCTGTGTGG CCATTGT-3' antisense). The reactions were carried out in a volume of 50 μl containing 5 μl genomic DNA, 5 μl 10×Taq buffer (without $MgCl_2$; Fermentas, Vilnius, Lithuania), 3 μl 25 mM $MgCl_2$, 1 μl 0.2 mM dNTP, 2 μl 1 μM of the primers and 0.5 μl 2.5 units of Taq polymerase (Fermentas). The PCR cycle conditions were an initial denaturation at 95˚C for 3 min, followed by 35 cycles of denaturation at 94˚C for 1 min, annealing at 54˚C for 1 min and extension at 72˚C for 1 min, with a final extention at 76˚C for 6 min. The amplified fragment (190 bp) was used for DNA sequencing. The amplicons were sequenced with a ABI Prism 3100 Genetic Analyser using a DYNamic ET Terminator Cycle Sequencing Kit (GE Healthcare, Uppsala, Sweden).

Statisical analysis. Statistical analysis was performed by SPSS for Windows version 10.0 (SPSS inc., Chicago, IL, USA). The significance in the statistical analyses was assessed using Student's *t*-test and chi-square test between groups and Pearson correlation analysis. Relationships yielding *p*-values less than 0.05 were considered to be significant.

Results

The OHI-S and GI, salivary pH, flow rate, buffering capacity, protein levels and CA activity were not significantly different ($p>0.1$) between the subjects with healthy teeth (DMFT=0) and those with low caries experience (DMFT<6) (Table I). Non-significant negative correlation was present between DMFT and CA activity ($r=-0.246$; $p>0.1$). Two single nucleotide polymorphisms (SNPs) (dbSNP: 142460367 and 142460368) were detected which are responsible for a threonine to methionine change in codon 55 (T55M), and a methionine to leucine change in codon 68 (M68L) (Figure 1). These alterations were found in 58% and 79% and in 75 % and 75 % in the group with low caries incidence and the healthy teeth group, respectively. The frequencies were not significantly different between the groups ($p>0.1$). There was no correlation between these SNPs and the salivary parameters.

Discussion

In an earlier report, low salivary CA enzyme concentrations correlated negatively with the DMFT index, especially in individuals with poor oral hygiene (3). In the present study, the negative correlation was non-significant between the DMFT index and salivary CA activity. The main reason for the present lack of significant correlation in the subjects with low caries and with healthy teeth, may have been the good oral hygiene and gingival health of the subjects.

The secretion of CA VI into the saliva has been observed to follow a circadian periodicity, its concentration being very low during sleep and rising rapidly to the daytime level after awakening and breakfast (17, 18). In the present study, to eliminate circadian differences in CA, the saliva samples were collected at the same time in the morning.

The CA VI concentration in saliva has been shown not to be directly associated with regulating salivary pH or buffer capacity and the enzyme may have a different role or may participate in these processes together with other CAs (19). Estrogens and androgens are known to regulate CA expression in some tissues. However CA VI secretion is not affected by the hormonal alterations associated with pregnancy and is not involved in regulating the actual salivary buffering capacity (20), which was consistent with the findings of the present study in which no significant correlation was found between salivary buffering capacity and CA activity.

The association between SNP of CA VI gene and caries suspectibility in healthy young adults has not been studied previously. Genetic studies on CAs have mainly related to CA II, III, IV, VIII and X (21-23). However Peres *et al.* (24) studied the association between SNPs in exon 2 of the CA VI gene and caries suspectibility or salivary buffer capacity in children and reported the same two SNPs as we report here. They also found no relationship between the SNPs and caries experience, which

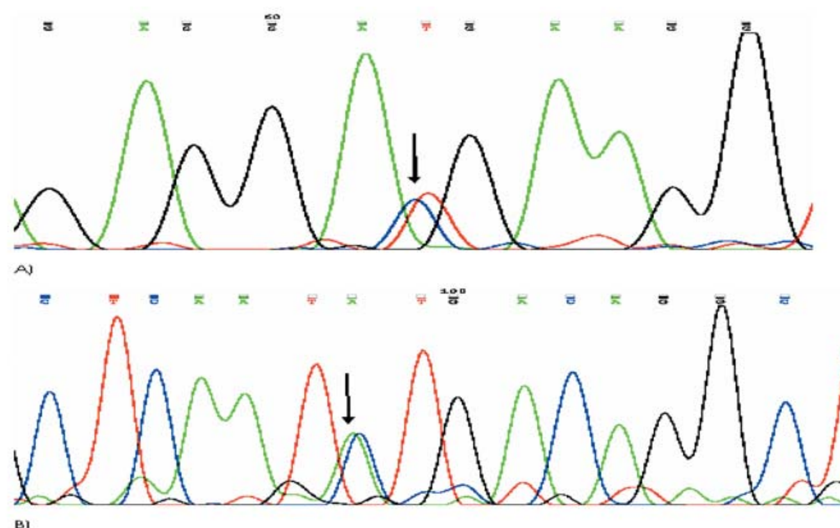

Figure 1. *Chromatogram analysis showing the polymorphic sites within exon 2 of the CA VI gene. The arrows indicate the SNP sites. A: Transition of C to T in codon 55, which leads to a threonine to methionine change. B: Transversion of A to C in codon 68 which leads to a methionine to leucine change.*

Table I. *Dental and salivary parameters.*

	DMFT=0 (n=20)	DMFT<6 (n=24)
OHI-S	0.13±0.005	0.14 ±0.010
GI	0.016±0.008	0.010±0.009
pH	7.02±0.41	7.17±0.35
Salivary flow rate (ml/min.)	0.48±0.22	0.52±0.24
Salivary buffering capacity	1.62±0.32	1.50±0.29
Salivary total protein (mg/dl)	131.48±48	132.00± 49
Salivary CA activity (Units/g protein)	26.69±6.6	27.18±10.4

Values are given as mean±standard deviation. CA: Carbonic anhydrase, DMFT: number of decayed, missing and filled teeth; OHI-S: oral hygiene index; GI: gingival index.

was consistent with the present results. While the rs2274327 (C/T) polymorphism was associated with a decrease of salivary buffering capacity in the healthy children, in the present study, there was no correlation between SNPs and salivary buffering capacity. This divergence in results may be explained by the variation in age and number of the subjects studied.

In conclusion, two SNPs (dbSNP: 142460367 and 142460368) occur in healthy young Turkish adults, but there is no correlation between caries prevalance and these SNPs.

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