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Patients with dental calculus have increased saliva and gingival crevicular fluid fetuin-A levels but no association with fetuin-A polymorphisms

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ABSTRAC: Fetuin-A is a potent inhibitor of calcium-phosphate precipitation and of the calcification process, therefore it can also be related with dental calculus. Thus, we aimed to investigate a possible relationship between fetuin-A gene polymorphism and the presence of dental calculus. A possible relationship between serum, saliva and gingival crevicular fluid (GCF) levels of fetuin-A was also investigated. Fetuin-A c.742C > T and c.766C > G polymorphisms were investigated in 103 patients with or without dental calculus. Additionally, serum, saliva and GCF fetuin-A levels of patients were compared according to dental calculus presence. A significant difference was not observed in the distribution of the fetuin-A c.742C > T and c.766C > G polymorphisms between patients with or without dental calculus. Saliva and GCF fetuin-A concentrations of patients with dental calculus were statistically higher than those without dental calculus (P=0.001, P=0.036 respectively). According to our results, fetuin-A c.742C > T and c.766C > G polymorphisms were not associated with presence of dental calculus. However, higher GCF and saliva fetuin-A levels were detected in patients with dental calculus than in patients without dental calculus, which may result from an adaptive mechanism to inhibit mineral precipitation and eventually calculus formation.

Keywords: Polymorphism, Genetic; Alpha-2-HS-Glycoprotein; Saliva; Gingival Crevicular Fluid; Dental Calculus.

Introduction

Periodontal disease is characterized by the inflammation and destruction of tooth supportive soft and hard tissues. The first response in the gingival margin occurs by the enzymatic effect of dental plaque bacteria and it increases due to the retentive effect of dental calculus formation. Beside blocking the effect of oral hygiene, dental calculus is also a reservoir for endotoxins and bacterial antibodies. It is believed that complex multifactorial factors can alter the formation process of dental calculus. The activities of some enzymes detected in dental plaque (like alkaline phosphatase, lactate dehydrogenase, and acid phosphatase) indicate that calcification of the dental plaque is not only a passive mineralization of non-viable microorganisms, but also an active and complex process supported by

enzymes.[1] Calcium and phosphate derived from saliva and the gingival crevicular fluid (GCF) are absorbed and concentrated by bacterial plaque.[2] These minerals in subgingival calculus are also derived from blood and pus entering the pocket from the surrounding soft tissues.[3] In the initial phase of dental plaque calcification, calcium phosphate supersaturation and the degradation of nucleation inhibitors are critical factors.[4] When plaque is calcified, it is named dental calculus. The development, amount and composition of dental calculus varies from case to case and also from region to region within the oral cavity.[5,6] Other factors that influence calculus formation are age, gender, eating habits, oral care, bacterial composition, host response, systemic diseases and prescribed medications.[7] Futhermore, it is thought that genetic factors can play a role on calcification. Polymorphisms of several protein genes, as fetuin-A and matrix gla protein (MGP), that affect calcium metabolism have been investigated in studies because they may potentially have a critical and key role in the calcification-related diseases.[8,9,10] Thus, the underlying mechanism of the calcification process is still unclear.

Fetuin-A is one of the inhibitor proteins of ectopic calcification that is synthesized in the liver and secreted into the circulation. Due to a high affinity of fetuin-A to hydroxyapatite, any situation that lowers fetuin-A serum concentration will increase the risk of systemic calcification.[11] It is suggested that fetuin-A inhibits formation and precipitation of the apatite precursor minerals and basic calcium phosphate (BCP).[12] Fetuin-A can inhibit unwanted calcification in circulation without inhibiting bone mineralization. The inhibitory activity of serum proteins on apatite formation was largely reduced after the specific depletion of fetuin-A from the serum. Recently, it has been reported that the inhibition is achieved by the transient formation of "calciprotein particles," soluble colloidal spheres containing fetuin-A and BCP.[12]

Fetuin-A deficiency leads to severe ectopic calcification in mice on a normal diet.[11] Low fetuin-A serum concentrations have been found to be associated with depressed cellular immunity[13] and nonspecific host defense.[14] In transgenic fetuin-A deficient mice, extraskeletal calcification, including soft tissue and peri-vertebral arterial calcification may develop.[11] It was reported that fetuin-A is a good inhibitor candidate of extraosseous calcification, particularly in patients with chronic kidney disease.[15,16] Ketteler et al.[17] reported an association between all-cause/cardiovascular mortality and low serum fetuin-A levels in hemodialysis (HD) patients. An important relationship between low serum fetuin-A levels and vascular calcification has been confirmed by several other investigators.[18,19,20]

Associations between fetuin-A gene polymorphism and calcifications in various tissues such as kidney[8], coronary artery[21] and bone[22] have been investigated. In the literature, there is no study investigating the relationship between fetuin-A gene polymorphism and dental calculus. Thus, in the present study, we evaluated the relationship between fetuin-A gene polymorphism and the presence of dental calculus, and also the possible relationship between the polymorphisms with serum, saliva and GCF levels of fetuin-A.

Methodology

Study population

The study population comprised a total of 103 individuals (48 males, 55 females; age range 20–55 years). Forty-nine patients had dental calculus (age range 20–49 years) and 54 patients did not have dental calculus (age range 20–55 years). The sample size was calculated as 88 considering 90% power and a significance level of 0.05 (effect size = 0.30). The participants of this study were chosen from patients referred to the Periodontology Department. Individuals who brushed their teeth at least once a day were included in the study. Patients with uncontrolled diabetes mellitus or any systemic diseases that may influence periodontal tissues and calculus formation, pregnant women, smokers, and people who had periodontal treatment and used antinflammatory drugs in the previous 6 months were excluded from the study.

Participants were included according to their gingival index (GI),[23] probing pocket depth (PPD) and radiographic bone loss scores after periodontal and radiographic examination. The clinical measurements of GI and PPD were assessed in six areas per tooth:

1) mesio-buccal; 2) disto-buccal; 3) mid-buccal; 4) mesio-lingual; 5) disto-lingual; and 6) mid-lingual. All clinical assessments were performed by a calibrated examiner (G.E.D.) who used a manual periodontal probe during the patients' first visit for clinical examinations. PPD was measured in millimeters as the distance from the gingival margin to the base of the pocket. After periodontal examination, patients who had bleeding on probing, GI between 1 and 2, and PPD ≤ 3 mm remained in the scores. On the other hand, for standardization, gingivitis patients were included, but periodontitis patients who had PPD ≥ 3 mm and radiographic bone loss were excluded from the study.

The approval of the Local Ethics Committee of Ataturk University Faculty of Dentistry was obtained (AU-DF-EC: 2011.8.17-011). All patients were briefly informed and written informed consent was obtained before participation.

Assessment of dental calculus and plaque index

Lingual surfaces of the mandibular anterior regions were assessed bilaterally for dental calculus formation. The occurrence of deposits in each region was dichotomously judged by inspection and probing as present (score 1) and included in the group with dental calculus or absent (score 0) and included in the group without dental calculus.

The thickness of microbial dental plaque on the tooth surface near the marginal gingiva was assessed using PI of Silness and Löe. [24] After the teeth were dried, the microbial dental plaque was scraped with a periodontal probe and evaluated by unaided eye.

Serum sampling

Serum samples were collected one day after the clinical examinations for standardization. Blood samples were drawn into vacutainer tubes. After centrifugation the obtained serum samples were stored at −80°C until biochemical measurements.

Saliva sampling

Saliva samples were collected one day after the clinical examinations to prevent contamination from gingival bleeding. They were collected according to the unstimulated saliva collection procedure. It was suggested to each subject not to drink, eat, perform oral hygiene or chew 120 min before and during the procedure. Saliva samples were collected into a tube to avoid interaction between any substance in the toothpaste and other molecules. Collected samples were stored at −80°C until the biochemical analysis.

GCF sampling

GCF was collected to evaluate its fetuin-A levels. To prevent the contamination from gingival bleeding, samples were collected one day after clinical examinations using filter paper strips (Oraflow Periopaper 8000®, 593520, NY, USA) from the pocket sites of mandibular anterior teeth with dental calculus in the group who had dental calculus and from the labial surfaces of mandibular anterior region in the group without dental calculus. The area was isolated to prevent samples from being contaminated by saliva. The sample site was gently air-dried and all supragingival plaque was removed. The paper strips were inserted into the crevice until mild resistance was felt and left in place for 30 seconds. Strips contaminated by bleeding were discarded and viable strips were placed into coded Eppendorf tubes and stored at −80°C until analysis.

Biochemical analysis

On the day of the analysis, serum, saliva and GCF samples were centrifuged for 10 minutes at 5000×g in a microcentrifuge before assessment. Serum, saliva and GCF fetuin-A levels were measured using a commercially available ELISA kit (BioVendor, GmbH, Heidelberg, Germany).

Analysis of Fetuin-A polymorphisms

Genomic DNA was extracted from 200 µL samples of EDTA-anticoagulated peripheral blood samples using a commercial kit (QIAamp DNA Blood Mini Kit, Qiagen, City, Country). Analyses of the fetuin-A c.742C > T and c.766C > G polymorphisms were performed with the polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) technique according to previously published protocols.[8,25] PCR mixes of 25 µL were set up, containing 1XTaq buffer with KCl, 1.5 mM $MgCl_2$, 0.2 Mm dNTPs, 0.25 mM of

each primer (Operon), 1 U of Taq DNA polymerase (Fermentas, St. Leon-Rot, Germany), and 100 ng DNA. The PCR conditions were as follows: initial denaturation for 5 minutes at 95°C; 35 cycles of denaturation for 1 minute at 94°C, annealing for 1 minute at 59°C, and extension for 1 minute at 72°C; and a final extension for 15 minutes at 72°C. The 366-bp PCR product was digested with 3.75 U of *Hin1*II (Fermentas) restriction endonuclease overnight at 37°C, and the digested products were separated by electrophoresis on a 3% (w/v) agarose gel and visualized using ethidium bromide. The c.742T allele contains a unique *Hin1*II restriction site, which yields 165- and 201-bp products, whereas the c.742C allele remained undigested. Analysis of the fetuin-A c.742C > T polymorphism was performed with the primers F5′-CCTCCCACAAGCAGAAAC-3′ and R5′-TGATGATTCCGCATACCC-3′. The 366-bp PCR products were digested with 3.75 U of *Hin1*II (Thermo Scientific) restriction endonuclease, and the digested products were separated by electrophoresis on a 3% (w/v) agarose gel and visualized using ethidium bromide. The c.742T allele contains a unique *Hin1*II restriction site, which yields 165- and 201-bp products. Analysis of the fetuin-A c.766C > G polymorphism were performed with the primers F5′-GTCACCCCTCCTTGTAAC-3′ and R5′-CCCCAATGAGACCACA-3′. The PCR products (405 bp) were digested at 37°C with 5 U of SacI (Thermo Scientific), and the digested products were separated on 3% agarose gel. Allele C does not contain the SacI site, whereas allele G yields 193- and 212-bp fragments.

Statistical analysis

Results of serum, saliva and GCF fetuin-A levels were reported as mean and standard deviation, and compared between groups using Mann–Whitney U test. Comparison of the distribution of fetuin-A genotypes according to dental calculus presence was done by the *chi*-square test. The mean ages of cases with and without dental calculus were compared with one-way ANOVA test. P<0.05 was considered statistically significant.

Results

Our study group consisted of 110 patients (58 males, 52 females) who were referred to the Periodontology Department of Ataturk University. Patients with extreme fetuin-A serum values because of unknown systemic inflammatory conditions were excluded from the study (n = 7). A total of 103 subjects were analyzed for fetuin-A genotype and serum, saliva and GCF concentrations of fetuin-A in patients with/without dental calculus. The mean ages of cases with and without dental calculus were 26.41±7.44 and 28.04±7.48 years, respectively. The age range of patients were not significantly different between groups (p = 0.270). In terms of plaque index (PI) there were no differences between the patients with (1.00 ± 0.79) and without dental calculus (0.77 ± 0.79) (p = 0.105). Distribution of fetuin-A c.742C > T and c.766C > G polymorphisms according to dental calculus situation are shown in Table 1. The number of patients with TT genotype were only 4, thus these cases were included in the CT genotype group. Similarly, the number of GG genotypes were only 3 and these cases were included in the CG genotype group. When the genotype distribution of the fetuin-A c.742C > T and c.766C > G polymorphisms were compared, no significant difference was found between patients with or without dental calculus (p = 0.285, p = 0.115, respectively) (Table 1). Saliva and GCF fetuin-A concentrations of patients with dental calculus were statistically higher than those without dental calculus (p = 0.036, p = 0.001, respectively) (Table 2). Serum fetuin-A concentrations in patients with dental calculus were not statistically different than in patients without dental calculus (p = 0.223). Median, minimum and maximum values of serum, saliva and GCF fetuin-A according to groups can be seen in Table 2. Serum, saliva and GCF fetuin-A concentrations were not different between subjects carrying the fetuin-A c.742C > T and c.766C > G polymorphism and those

Table 1. Fetuin-A genotype distribution of cases according to groups (presence or absence of dental calculus).

Genotype	Presence of dental calculus	Absence of dental calculus	p
742			
CC	37	37	$X^2 = 0.285$, $df = 1$
CT-TT	12	17	$p = 0.513$
766			
CC	34	44	$X^2 = 0.115$, $df = 1$
CG-GG	15	10	$p = 0.173$

carrying fetuin-A CC genotype [p = 0.97, p = 0.832, p = 0.391, respectively for fetuin-A 742 (Table 3) and p = 0.541, p = 0.596, p = 0.735 respectively for fetuin-A 766 (Table 4)].

Discussion

In the present study, analysis of fetuin-A c.742C > T and c.766C > G polymorphisms was carried out, and serum, saliva and GCF fetuin-A levels were investigated in patients with or without dental calculus. Fetuin-A c.742C > T and c.766C> G polymorphisms were found not to be associated with dental calculus. On the other hand, GCF and saliva fetuin-A concentrations were significantly higher in patients with dental calculus than in patients without dental calculus. To our knowledge, this is the first study investigating the association between fetuin-A gene polymorphisms and dental calculus.

Dental calculus occurs when bacterial plaque has undergone mineralization due to the precipitation of mineral salts. However, not all the plaque becomes calcified[6] and some patients with deficient oral hygiene may not present dental calculus; the reason for this situation is still unknown. Genetic factors are known to play an important role in determining an individual's susceptibility to stone disease and can act in the calcification process. Fetuin-A is a powerful inhibitor of calcium-phosphate precipitation. It has the capacity to form a complex with calcium and phosphate, thereby transporting and clearing the insoluble calcium-phosphate salt, and preventing its extra-skeletal deposition.[26] Thus, fetuin-A gene polymorphism and levels can be related with dental calculus. The relationship of fetuin-A gene polymorphism with some diseases have been investigated. Roos et al.[27] evaluated the association between fetuin A c.766C > G polymorphism and arterial function in subjects with normal renal function and they concluded that the patients with this polymorphism demonstrated high aortic stiffness and increased

Table 2. Median, minimum and maximum levels of fetuin-A in serum, saliva and gingival crevicular fluid (GCF) according to dental calculus situation (presence or absence).

Dental calculus	Serum fetuin-A (μg/mL)	Saliva fetuin-A (μg/mL)	GCF fetuin-A (ng/sample)
	Median (min-max)	Median (min-max)	Median (min-max)
Present n = 49	55.65 (12.01–96.25)	0.068 (0.00–6.71)	29.49 (1.39-937.92)
Absent n = 54	60.92 (12.51–99.83)	0.041 (0.00–3.95)	18.12 (1.09-883.86)
p (Mann-Whitney U test)	0.223	0.001	0.036

Table 3. Median, minimum and maximum levels of fetuin-A in serum, saliva and gingival crevicular fluid (GCF) of patients according to *fetuin-A* 742 genotypes.

Fetuin-A 742	Serum fetuin-A (μg/mL)	Saliva fetuin-A (μg/mL)	GCF fetuin-A (ng/sample)
	Median (min-max)	Median (min-max)	Median (min-max)
CC n = 74	59.35 (12.01–99.83)	0.054 (0.00–6.71)	21.48 (1.09–937.92)
CT-TT n = 29	52.93 (12.30–96.43)	0.057 (0.01–3.95)	27.53 (2.32–327.14)
p (Mann-Whitney U test)	0.097	0.832	0.391

Table 4. Median, minimum and maximum levels of Fetuin-A in serum, saliva and gingival crevicular fluid (GCF) of patients according to *fetuin-A* 766 genotypes.

Fetuin-A 766	Serum fetuin A (μg/mL)	Saliva fetuin A (μg/mL)	GCF fetuin A (ng/sample)
	Median (min-max)	Median (min-max)	Median (min-max)
CC n = 78	58.33 (12.01 –99.83)	0.051 (0.00–6.71)	22.71 (1.39–937.92)
CG-GG n = 25	55.25 (13.73–96.25)	0.065 (0.00–3.22)	26.77 (1.09–474.02)
p (Mann-Whitney U test)	0.541	0.596	0.735

cardiovascular risk. Geroldi et al.[28] noted an association between fetuin-A gene polymorphism and Alzheimer disease in which some calcifications could be seen in various parts of the brain. However, in HD patients, distribution of fetuin-A c.766C > G polymorphism was not different from normal individuals.[9] In another study, fetuin-A c.742C > T and c.766C > G polymorphisms was evaluated in pseudoxanthoma elasticum disorder (characterized by calcified dystrophic elastic fibers in skin, retina, and arteries) and no meaningful difference was detected when compared to healthy subjects.[25] In the present study, fetuin-A c.742C > T and c.766C> G polymorphisms were not associated with the presence of dental calculus. Different ethnic populations, study subjects, and sample sizes may cause differences in genotype distribution among studies.

We also compared serum, saliva and GCF fetuin-A levels according to fetuin-A c.766C > G or c.742C > T polymorphisms. There are some studies indicating that the fetuin-A gene polymorphism (c.766C > G) can be associated with reduced serum fetuin-A levels.[20,25] In their cohort study, Osawa et al.[29] evaluated the importance of fetuin-A gene polymorphism in chronic renal failure patients and they showed that the patients with c.766C > G polymorphism had significantly lower serum fetuin-A levels and higher all-cause and cardiovascular mortality rates. Aksoy et al.[8] showed that patients with CG genotype had lower serum fetuin-A concentrations compared with those carrying CC genotype when fetuin-A c.766C > G polymorphism were examined. Conversely, another study indicated that reduction in serum fetuin-A levels was not associated with fetuin-A c.766C > G polymorphism.[9] Another study in diabetic patients reported that sequence variants in fetuin-A gene affect coronary artery calcification, even though no relationship between c.766C > G or c.742C > T polymorphisms and serum fetuin-A levels could be found.[21] Hendig et al.[25] documented an association between serum fetuin-A levels and genotype. In our study, we found that serum fetuin-A concentrations of patients with fetuin-A c. 766C > G or c.742C > T polymorphisms were not different from those carrying CC genotype, which agrees with Cozzolino et al.[9] and Lehtinen et al.[21] results. Additionally, no association between fetuin-A c.742C > T and c.766C > G polymorphisms and saliva and GCF fetuin-A levels of patients was found. We were the first to investigate fetuin-A levels in saliva and GCF and fetuin-A c.742C > T and c.766 C > G polymorphisms in relation to dental calculus. Therefore, we could not compare our results with the literature.

Comparing serum, saliva and GCF fetuin-A levels according to the presence of dental calculus, we found that serum fetuin-A concentration was not different, but GCF and saliva fetuin-A concentrations were higher in patients with dental calculus than in those without dental calculus. Aksoy et al.[8] found that serum fetuin-A concentrations were significantly lower in patients with renal stone disease than in healthy controls. In a recent study, urinary fetuin-A levels were found to be lower in patients with urolithiasis than in healthy subjects.[30] The results of previous studies[8, 30] were not parallel with our results, according to which it may be speculated that fetuin-A proteins leaked to saliva and GCF from serum in order to inhibit dental calculus. Also, local releasing of fetuin-A may be considered. Furthermore, dental calculus complexes may be a reason of such high concentrations of fetuin-A in GCF and saliva. Fetuin-A concentration may be increased as a compensatory mechanism to protect oral cavity from dental calculus accumulation by cleaning calcium and phosphates. Another reason for higher fetuin-A concentration in GCF and saliva in patients with dental calculus than in patients without dental calculus may be due to a higher affinity to hydroxyapatite, which is the major mineral of dental calculus. Also, it has been documented that serum calcium and phosphate levels are correlated with fetuin level.[31] Thus, according to our study it may be claimed that fetuin-A levels were increased in local circulations, such as GCF and saliva, to inhibit dental calculus formation.

Conclusion

In this study, we found that fetuin-A c.742C > T and c.766C > G polymorphisms were not associated with presence of dental calculus. Despite this, higher GCF and saliva fetuin-A levels were detected in patients with dental calculus, which may be caused by adaptive mechanisms to inhibit mineral precipitation and eventually calculus formation.

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