Comprehensive Analysis of Gene Expression Associated with Caries Susceptibility in Chromosome 2-substituted Mice

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Abstract

In this study, in order to clarify the host genetic factors that influence dental caries susceptibility, we examined the role of host genetic factors by comparing whole genome expression and salivary protein levels using C57BL/6 (B6) mice, which showed high dental caries susceptibility, and C3H and B6-Chr2^{C3H} mice, which show low dental caries susceptibility.

We analyzed gene expression in the submandibular gland by genechip microarray using submandibular glands from B6, C3H and B6-Chr2^{C3H} mice. Two genes that showed the top expression in each category among the 3 strains were analyzed by reverse transcription polymerase chain reaction (RT-PCR) and quantitative Real-Time PCR analyses. We also analyzed saliva protein concentration by the Bradford method.

On microarray analysis, the *Tmem87A* and *Capn3* genes showed the greatest differences in gene expression among the 3 strains. These genes were confirmed by RT-PCR and quantitative Real-Time PCR, and protein concentrations in C3H, B6-Chr2^{C3H} and B6 were 0.210 µg/ml, 0.177 µg/ml and 0.217 µg/ml, respectively. Protein concentrations in B6-Chr2^{C3H} were significantly lower than in B6 and C3H. Thus, *Tmem87A* and *Capn3* may be related to differences in caries susceptibility observed with the Substituted of chromosome 2 in mice. Moreover, protein levels in saliva do not affect caries susceptibility in 3 strains. This suggests that differences in caries sensitivity of B6, C3H and B6-Chr2^{C3H} are related to differences in water permeability in the salivary glands.

Keywords :

dental caries susceptibility, consomic mice, genechip microarray, chromosome2

Introduction

Dental caries is one of the most prevalent chronic diseases in pediatric dental patients and affects their growth and well-being. Dental caries is a multifactorial disease widely acknowledged to be caused by a combination of environmental and behavioral factors, and genetic predispositions. The environmental risk factors for dental caries have been studied for decades, and include dietary behaviors, bacterial flora, transmission of bacteria among hosts, hygiene, socioeconomic status and access to oral health care. However, little is known about the relationship between host genetic background and the progress of dental caries

(1-3). It is important to identify host genetic factors controlling caries susceptibility, and to clarify the host defense mechanisms influencing caries development.

In our previous study, we reported that various inbred mouse strains developed dental caries after infection with *Streptococcus mutans* (*S.mutans*), and high sucrose consumption, and it was noted that the mice could be divided into two groups, corresponding to high and low caries susceptibility; the C57BL/6 strain (B6) showed high susceptibility and the C3H strain (C3H) showed low susceptibility (4-7). As a result of quantitative trait locus (QTL) analysis, four QTLs were detected on chromosomes 1, 2, 7 and 8. In particular, the QTL on chromosome 2 showed a strong relationship with dental caries susceptibility (8, 9). For the elucidation of the causative genes for

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dental caries susceptibility, we established chromosome-substitution mice, known as consomic mice (B6-Chr2^{C3H}), in which all chromosomes except chromosome 2 were derived from B6 (10). On caries induction in B6-Chr2^{C3H} infected with *S. mutans* JC-2 (serotype c), the caries score for B6-Chr2^{C3H} was significantly lower than that in B6, and did not differ significantly from that in C3H. Salivary flow volume in B6-Chr2^{C3H} was also greater than in B6 and was significantly less than in C3H (11).

B6-Chr2^{C3H} strain showed low dental caries susceptibility and the 3 mouse strains showed differences in salivary flow volume. However, no studies have been performed to investigate the host genetic factors that influence dental caries susceptibility using B6-Chr2^{C3H}.

In this study, we investigated gene expression in the submandibular glands of 3 mouse strains that show differences in caries susceptibility. To clarify the host genetic factors that most strongly influence dental caries susceptibility, we comprehensively compared the whole genome expression in the submandibular gland. In addition, as salivary protein influences caries susceptibility, we also examined salivary protein levels in the 3 mouse strains.

Materials and Methods

Mice

C57BL/6CrSlc (B6) and C3H/HeSlc (C3H) mice, and consomic mice (B6-Chr2^{C3H}) were used in this study. Twenty-day-old B6 and C3H mice were purchased from Sankyo Lab Service Co. (Tokyo, Japan), and B6-Chr2^{C3H} mice were maintained in our laboratory. All mice were kept in clean racks at room temperature (25 ± 1°C), with a relative humidity of 55 ± 5%, and a 12-hour light/dark cycle. Mice were fed *ad libitum* on a commercial diet (MR Breeder; Nihon Nohsan Co., Kagawa, Japan), and were provided pure bottled water. The animal-use protocols of this study were reviewed and approved by the Nihon University Institutional Review Board (ECA 08-0012).

RNA isolation and microarray analysis

For gene expression analysis, submandibular glands were dissected from the 3 mouse strains at 49 days of age. Total RNA was individually extracted from submandibular glands using FastRNA Pro Green Kit (MP Biomedicals, Santa Ana, CA), according to the manufacturer's instructions. The concentration and purity of the RNA were determined spectrophotometrically. RNA samples were analyzed on 1 %

agarose gels by electrophoresis, followed by staining with ethidium bromide in order to verify the integrity of 18S and 28S ribosomal RNA. First-strand cDNA was synthesized from 500 ng of total RNA using a Gene Chip 3'IVT Express Kit (Affymetrix Inc. Santa Clara, CA). Second-strand cDNA was synthesized from 10 µL of first-strand cDNA, and biotin-labeled amplified RNA (aRNA) was transcribed from cDNA. aRNA (15 µg) was fragmented to obtain optimal array sensitivity and minimize the required amount of magnesium. Fragmented aRNA was hybridized to a Mouse Genome 430 2.0 Array (Affymetrix Inc.) for 16 hours at 45°C. The array was washed and stained with streptavidin-phycoerythrin using a Fluidics Station (Affymetrix Inc.). Digital images were obtained using a Gene Array Scanner (Affymetrix Inc.).

Statistical analysis of microarray data

The resulting data from the 3 mouse strains were analyzed using Affymetrix Expression Console Software (version 1.1, Affymetrix) and Ingenuity Pathways Analysis (IPA) (12). Affymetrix Microarray suite 5.0 (Mas5) was used to obtain the generated signal values and detection calls (Present (P), Absent (A) or Marginal (M)), and the data were exported for further analysis. On analysis of gene expression data, we divided data into two categories. Category 1 included present calls in C3H and B6-Chr2^{C3H} and absent calls in B6, while signal intensity of C3H and B6-Chr2^{C3H} was more than 300 and existed on chromosome 2. Category 2 included present calls in the 3 mouse strains and gene expression of B6 was 2-fold lower (upregulation) than the gene expression of C3H and B6-Chr2^{C3H} strains based on signal value, and existed on chromosome 2. IPA was used to cluster the genes for possible biological pathways and to explore the inter-relationships between network genes with particular patterns.

RT-PCR and Quantitative Real-Time PCR analysis

In order to verify the microarray data, two genes from different categories and the housekeeping gene (β -actin) (positive control) were analyzed by reverse transcription polymerase chain reaction (RT-PCR) and quantitative Real-Time PCR analyses.

For PCR analysis, the submandibular glands were dissected from 3 mice from each strain at 49 days of age. Total RNA was individually extracted from the submandibular glands using an RNeasy Mini kit (QIAGEN, Valencia,

Table 1 Primer used by RT-PCR and Real-Time PCR

Gene	Sequence	exon position	size
RT-PCR			
Calpain3	Foword : 5'-AGGTGGGAACACCACAGAAGCCA-3' Reverse: 5'-CGGTCCTTGCGCCGGTTCTT-3'	exon5 exon10	675
Transmenbrane87a	Foword : 5'-GCACTTCCGGCCTTGCTGGA-3' Reverse: 5'-ACATGTAGCTTGCAAAAACAGCCGT-3'	exon19 exon20	335
Real-Time PCR			
Calpain3	Foword : 5'-ACACAGACCAGGAAAGTGGAG-3' Reverse: 5'-CTTGTGTTTGTTCACCACTG-3'	exon14 exon15	127
Transmembrane87a	Foword : 5'-GCACTCGTGTATGTGCTGT-3' Reverse: 5'-CCACTTCATGCAAGAATACC-3'	exon16 exon18	122

CA), in accordance with the manufacturer's instructions. cDNA was synthesized from this total RNA using PrimeScript II 1st strand cDNA Synthesis kit (Takara, Tokyo, Japan).

RT-PCR was carried out using a Gene Amp PCR System 9700 (Applied Biosystems, Foster City CA). Primer sets are listed in Table 1. PCR products were electrophoresed on 4% agarose gels.

Real-Time PCR was carried out using DNA Engine Opticon (BioRad, Hercules, CA) with a DYNAMO SYBR Green qPCR Kit (Finnzymes, Espoo, Finland). The PCR protocol entailed an initial incubation at 95°C for 5min, followed by 40 cycles of 94°C for 15 s, 56°C for 30s, and 72°C for 30s. Primer sets are listed in Table1. Gene expression levels in Real-Time PCR were calculated using the $\Delta\Delta C_t$ method with normalization against-actin(13)

Measurement of total protein in saliva

Stimulated saliva was collected from three mice of each strain at 49 days of age. Mice were weighed and anesthetized with an intraperitoneal injection of 50mg/kg sodium pentobarbital. These mice were then injected intraperitoneally with pilocarpin (10mg/kg) to harvest stimulated saliva. Five minutes after injection of pilocarpin, secreted saliva was collected for 30min using a micropipette. Protein concentration in saliva was measured using a Bio-rad Protein Assay Kit (BioRad Japan, Tokyo, Japan) based on the method of Bradford (14). Total salivary protein was determined using a standard curve prepared using albumin as a reference.

Statistical analysis of Real-Time PCR and salivary protein data

Statistical analysis of variations in Real-Time PCR and

salivary protein were performed by Tukey's test as a post-hoc comparison of each group after ANOVA for multiple comparisons.

Results

Gene expression in submandibular glands

We initially compared the gene expression patterns in the 3 mouse strains. In a total of 24,896 genes on the genechip array, 592 genes were classified as present in C3H, B6-Chr2^{C3H} and absent in B6. Of these 592 genes, there were 517 with a signal intensity of more than 100 and 127 genes with signal intensity of more than 300, and 12 of these were located on chromosome 2 (Table2). On the other hand, 15,894 genes were classified as present calls in the 3 mouse strains. Of these, 72 showed that gene expression that was 2-fold lower (upregulation) in B6 than in C3H and B6-Chr2^{C3H}, and 14 of these were located on chromosome 2 (Table3).

Ingenuity Pathways Analysis

In order to analyze the role of differences in gene expression using Expression Console Software, two categories were analyzed by IPA. The 12 genes probe sets derived from category 1 were related to Cell Cycle, Reproductive System Development and Function, Connective Tissue Development and Function in associated network functions. The 14 genes probe sets derived from category 2 were related to Tissue Morphology, Cell Death and Survival, Connective Tissue Development and Function in associated network functions (Table4).

RT-PCR and Quantitative Real-Time PCR

RT-PCR and quantitative Real-Time PCR analysis were used to confirm the microarray data, and we analyzed data

Table2 Genes of category 1

Gene Symbol	C3H signal value	B6-Chr2 ^{C3H} signal value	Public ID	Probe Set ID
<i>Capn3</i>	983	1386	AF127766	1426043_a_at
<i>Galnt3</i>	800	825	BC003765	1427749_at
<i>Tnks1bp1</i>	689	644	BQ031120	1427051_at
<i>Pak6</i>	684	603	BB818370	1455200_at
<i>AW555355</i>	612	408	AW555355	1444273_at
<i>Pigu</i>	570	701	AW538039	1427928_s_at
<i>Pofut1</i>	511	380	BE946126	1443881_at
<i>Pbx3</i>	490	493	NM_016768	1421193_a_at
<i>Serinc3</i>	471	616	NM_012032	1417816_s_at
<i>Pmpca</i>	391	677	AK004549	1424661_at
<i>Usp8</i>	365	545	AB045709	1422883_at
<i>Harbi1</i>	351	447	BM231455	1423794_at

Table3 Genes of category 2

Gene Symbol	C3H/B6	B6-Chr2 ^{C3H} /B6	Public ID	Probe Set ID
<i>Tmem87a</i>	10	9	BC027354	1424454_at
<i>Rbm45</i>	5	6	BB821609	1437904_at
<i>Elf5</i>	4	4	BC012424	1419556_at
<i>Gchfr</i>	4	3	BB610454	1435750_at
<i>Ubr1</i>	3	2	BG075178	1458033_at
<i>Slc25a12</i>	3	5	BB526231	1444489_at
<i>I700007K13Rik</i>	3	2	AK005731	1428705_at
<i>Sord</i>	3	3	AV253518	1438183_x_at
<i>Dhtkd1</i>	3	3	BB667395	1457027_at
<i>Fahd2a</i>	2	2	BI872590	1431189_a_at
---	2	2	AI746468	1439960_at
<i>Zfp106</i>	2	3	BC025424	1425098_at
---	2	3	AW495875	1435129_at
<i>Dtwd1</i>	2	2	BC020149	1424697_at

Table4 Identification in molecular networks generated in category 1 and 2 by IPA

Top Functions ^a	Focus Molecules ^b	Molecules in Network ^c
Category 1		
Cell Cycle, Reproductive System Development and Function, Connective Tissue Development and Function	11	HAB1, CAPN3, GALNT3, PAK6, PBX3, PIGU, PMPCA, POFUT1, SERINC3, TNKS1BP1, USP8
Category 2		
Tissue Morphology, Cell Death and Survival, Connective Tissue Development and Function	12	DHTKD1, DTWD1, ELF5,FAHD2A, GCHFR, RBM45, RPUSD2, SLC25A12, SORD, TMEM87A, UBR1,ZFP106

^a Transcripts with significantly different expression (focus genes) were analyzed by IPA for generation networks and assessment of function.

^b Number of focus genes in the network.

^c Assignment of function to a network is based on literature stored in the IPA Knowledge Base.

Figure 1-A. RT-PCR of *Calpain3*.

The PCR product of 3 strains were confirmed by template from cDNA as positive band around 675bp. No band was detected by template from genome 3 strains and DW as negative control around same position.

Figure 1-B. RT-PCR of *Tmem87a*.

The PCR product of 3 strains were confirmed by template from cDNA as positive band around 335bp. No band was detected by template from genome 3 strains and DW as negative control around same position.

from 2 genes, *Capn3* and *Tmem87A*, which showed the largest differences in expression among B6, and C3H and B6-Chr2^{C3H}. RT-PCR assays showed high density for the cDNA products of C3H and B6-Chr2^{C3H} in *Capn3* and *Tmem87A* (Figs.1-A and 1-B). These results were correlated with the results of the microarray assays for two samples, and quantitative Real-Time PCR data confirmed the differences in microarray data with regard to the expression of *Capn3* and *Tmem87A*. The relative abundance in B6 after normalization against β -actin was significantly lower than in B6-Chr2^{C3H} and C3H (Figs.2-A and 2-B).

Salivary protein

Table 5 summarizes the relative differences of total saliva volume, protein concentration and total protein volume in the 3 strains. The largest saliva volume was seen in C3H at 220 μ l, followed by B6-Chr2^{C3H} at 213 μ l and B6 at 81.6 μ l. Total saliva volume in B6-Chr2^{C3H} and C3H was signifi-

Figure 2-A. Real-Time PCR of *Calpain3*.

Figure 2-B. Real-Time PCR of *Tmem87a*.

Each bar corresponds to the mean ($\pm$ SD) for each strain of mouse.

cantly higher than in B6. Protein concentrations in C3H, B6-Chr2^{C3H} and B6 were 0.210 μ g/ml, 0.177 μ g/ml and 0.217 μ g/ml, respectively. Protein concentration in B6-Chr2^{C3H} was significantly lower than in B6 and C3H. Total protein volume in C3H, B6-Chr2^{C3H} and B6 was 0.0460ng, 0.0377ng and 0.0177ng, respectively, and total protein volume in B6-Chr2^{C3H} and C3H was significantly lower than in B6.

Discussion

The relationship between host genetic background and progress of dental caries is poorly understood. In this study, we evaluated the role of genetic factors on chromosome 2 in order to clarify the factors influencing dental caries susceptibility. Our previous study showed that host genetic factors on chromosome 2 influence caries susceptibility (7-9). Thus, we established B6-Chr2^{C3H}, in which chromosome 2 derived from B6 was replaced with chromosome 2 derived from C3H (10). This B6-Chr2^{C3H} strain showed

Table 5 Saliva protein concentration, amount of saliva, total saliva protein in 3 strains

Strain	Protein concentration (μ g/ ml)	Total saliva volume (μ l)	Total protein volume (μ g)
B6 (n=3)	0.217($\pm$0.0093)	81.6($\pm$14.1)	0.0177($\pm$0.0028)
C3H (n=3)	0.209($\pm$0.0064)	220($\pm$35.6)	0.0460($\pm$0.0068)
B6-Chr2^{C3H} (n=3)	0.177($\pm$0.0038)	213($\pm$26.2)	0.0377($\pm$0.0041)

Data are expressed as the mean $\pm$ SD.

significantly lower caries scores than B6, and showed no significant differences with C3H. In addition, the amount of saliva in the B6-Chr2^{C3H} strain was greater than that in B6 strain and lower than that in C3H(11).

We suspected that the differences in saliva volume may influence differences in caries susceptibility among the 3 mouse strains. Thus, we believed that differences in saliva volume affect gene expression in the submandibular gland, and that gene expression in the submandibular gland led to differences in caries susceptibility. On microarray analysis, we focused on the Calpain3 (*Capn3*) and transmembrane protein 87A genes (*Tmem87A*), which showed significantly different expression. *Capn3* is a Ca²⁺-activated cysteine protease and we believe that *Capn3* is related to caries susceptibility because salivary fluid secretion is Ca²⁺-dependent (15). However, there have been no reports on *Capn3* expression in the salivary glands. The molecular identity of *Tmem87A* is unknown. However, Young *et al.* (16) reported that transmembrane protein 16A (*Tmem16A*, which is also known as anoctamin 1 (ANO1)) is a bona fide calcium (Ca²⁺)-activated chloride (Cl⁻) channel that is activated by intracellular Ca²⁺ and Ca²⁺-mobilizing stimuli. As *Tmem87A* and *Tmem16A* are transmembrane proteins that have similar molecular structures, we believe that *Tmem87A* plays a similar role as *Tmem16A*. Thus, *Capn3* and *Tmem87A* are related to quantity of secreted saliva.

Our data suggest that *Tmem87A* and *Capn3* are genetic factors involved in the differences in dental caries sensitivity among the 3 mouse strains. Culp *et al.* show that caries score is significantly increased in aquaporin5 (*Aqp5*)-deficient mice(17), and Ma *et al.* reported that *Aqp5* affects salivary fluid secretion in the salivary gland (18, 19). However,

differences in *Aqp5* gene expression were not observed among the 3 mouse strains. We believe that there are multiple genetic factors related to caries. In addition, *Aqp5* is located on chromosome 15 in mice. Therefore, caries susceptibility in B6-Chr2^{C3H}, in which chromosome 2 is replaced, does not have a direct influence.

On IPA, differences among B6, and C3H and B6-Chr2^{C3H} were related to connective tissue development and function of the submandibular gland. Thus, the number and structure of the acinus and ducts in the submandibular glands differ among the 3 strains.

Host factors for dental caries susceptibility are influenced by salivary parameters such as buffer capacity, cariogenic bacteria counts, pH, salivary secretion, total saliva volume and total protein content (20). Orino *et al.* reported that the salivary flow volume in B6-Chr2^{C3H} was significantly lower than in C3H, and significantly higher than in B6 (10). This study showed similar results. Dental caries susceptibility may thus be related to total saliva concentration (21) and salivary components (22, 23). Therefore, because the proteins in saliva may affect caries sensitivity, we measured salivary protein concentration in the 3 mouse strains. Protein concentration of B6-Chr2^{C3H} was significantly lower than in B6 and C3H. However, the total protein volume in B6-Chr2^{C3H} and C3H was significantly lower than in B6. Furthermore, when saliva from the 3 mouse strains was placed in medium with *S. mutans*, the colony counts did not significantly differ (unpublished data). Our data suggest that salivary proteins do not participate in the differences in caries susceptibility in the 3 mouse strains.

In conclusion, our results suggest that *Tmem87A* and *Capn3* on chromosome 2 play a role in caries susceptibility. Moreover, salivary protein does not affect caries susceptibil-

ity in the 3 strains. This suggests that differences in caries sensitivity between B6, C3H and B6-Chr2^{C3H} are based on differences in water secretion and transportation in the salivary gland.

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References

- Lehner T, Lamb JR, Welsh KL, Batchelor RJ: Association between HLA-DR antigens and helper cell activity in the control of dental caries. *Nature*, 292: 770-772, 1981.
- Yoshida N, Yoshida K, Nakamura H, Iwaku M, Ozawa H: Immunohistochemical localization of HLA-DR-positive cells in unerupted and erupted normal and carious human teeth. *J Dent Res*, 75: 1585-1589, 1996.
- Borass JC, Messer LB, Till MJ: A genetic contribution to dental caries, occlusion, and morphology as demonstrated by twins reared apart. *J Dent Res*, 67: 1150-1155, 1988.
- Kurihara Y, Naito T, Obayashi K, Kurihara Y, Moriwaki K: Caries susceptibility in inbred mouse strains and inheritance patterns in F1 and backcross (N2) progeny from strains with high and low caries susceptibility. *Caries Res*, 25: 341-346, 1991.
- Maeda T, Ogiwara K, Kurihara Y: Study on the inheritance patterns of caries susceptibility in Inbred Mice. *Jpn J Ped Dent*, 30: 618-623, 1992.
- Obayashi K: Caries susceptibility of Balb/c mice to caries induction by *Streptococcus* serotype c, d, g strains. *Jpn J Ped Dent*, 22: 87-95, 1984.
- Nariyama M, Shimizu K, Uematsu T, Maeda T: Identification of chromosomes associated with dental caries susceptibility using quantitative trait locus analysis in mice. *Caries Res*, 38: 79-84, 2004.
- Suzuki N, Kurihara Y, Kurihara Y: Dental caries susceptibility in mice is closely linked to the H-2 region on chromosome 17. *Caries Res*, 32: 262-265, 1998.
- Uematsu T, Nariyama M, Shimizu K, Maeda T: Mapping of effected gene (s) to dental caries susceptibility on mouse chromosome 2. *Ped Dent J*, 13: 75-81, 2003.
- Nomi M, Shimizu K: Establishment of consomic mice to clarify host susceptibility to caries. *Ped Dent J*, 19: 74-81, 2009.
- Orino D, Shimizu K: Reduced dental caries susceptibility in chromosome 2-substituted consomic mice. *Int J Oral-Med Sci*, 9: 234-240, 2011.
- Ingenuity Systems, www.ingenuity.com, Redwood City, CA.
- Livak KJ, Schmittgen TD: Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2^{-ΔΔCt} Method. *Methods*, 25: 402-408, 2001.
- Bradford M. M: A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. *Anal Biochem*, 72: 248-254, 1976.
- Qi B, Narita T, Satoh K, Guo MY, Katsumata-Kato O, Murakami M: Characteristics of neurokinin A-induced salivary fluid secretion in perfused rat submandibular gland. *Arch Oral Biol*, 55: 737-744, 2010.
- Young YD, Hawon Cho, Jae Yeon Koo, Min Ho Tak, Yeongyo Cho, Won-Sik Shim: TMEM16A confers receptor-activated calcium-dependent chloride conductance. *Nature*, 455: 1210-1216, 2008.
- Culp DJ, Quivey RQ, Bowen WH, Fallon MA, Pearson SK, Faustoferri R: A Mouse Caries Model and Evaluation of Aqp5^{-/-} Knockout Mice. *Caries Res*, 39: 448-454, 2005.
- Ma T, Song Y, Gillespie A, Carlson EJ, Epstein CJ, Verkman AS: Defective Secretion of Saliva in Transgenic Mice Lacking Aquaporin-5 Water Channels. *J Biol Chem*, 274: 20071-20074, 1999.
- Tradtrantip L, Tajima M, Li L, Verkman AS: Aquaporin water channels in transepithelial fluid transport. *J Med Invest*, 56: 179-184, 2009.
- Leone CW, Oppenheim FG: Physical and chemical aspects of saliva as indicators of risk for dental caries in humans. *J Dent Educ*, 65: 1054-1062, 2001.
- Masakuma K, Inaba R, Iwata H: Salivary calcium and total protein in relation to dental caries. *Nihon Eiseigaku Zasshi*, 50: 886-892, 1995.
- Scannapieco FA: Saliva-bacterium interactions in oral microbial ecology. *Crit Rev Oral Biol Med*, 5: 203-248, 1994.
- Lenander-Lumikari M, Lommaranta V: Saliva and Dental Caries. *Adv Dent Res*, 14: 40-47, 2000.