Original Article

Salivary Gene Expression of RANK, RANKL, and OPG in Type 1 Diabetes Mellitus and Periodontal Disease Patients

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ABSTRACT

Objectives: The relationship between type 1 diabetes mellitus (T1DM) and periodontal disease may exhibit by the alteration of bone metabolism. However, evidence for this relationship is scarce and inconclusive. Thus, the aims of the present study were to investigate salivary receptor activator of nuclear factor kappa-β (RANK), receptor activator of nuclear factor kappa-β ligand (RANKL), osteoprotegerin (OPG) gene expression and the RANKL:OPG ratio in T1DM and non-T1DM. Secondary objective was to determine the relationships of RANK, RANKL and OPG gene expression to clinical parameters of T1DM and periodontal disease. **Materials and Methods:** Twenty patients with T1DM and twenty age-matched non-T1DM were recruited. Clinical periodontal parameters were measured. Total RNA was isolated from non-stimulated saliva, and the relative gene expressions of RANK, RANKL, OPG and RANKL:OPG ratio were determined by quantitative real-time polymerase chain reaction. **Results:** The T1DM group had significantly higher mean periodontal parameters than the non-T1DM group, while the mean plaque scores of both groups were not significantly different. There was a trend of higher relative gene expression of RANK, RANKL, and the RANKL:OPG ratio and lower expression of OPG in T1DM group but no statistic significant different when compared to non-T1DM. In the T1DM group, RANKL:OPG correlated with the percentage of bleeding sites, whereas RANK, RANKL, and HbA1c levels correlated with pocket depth. **Conclusions:** Bone metabolisms demonstrating by decreased OPG gene expression and upregulated of RANK, RANKL, RANKL:OPG with higher pocket depth and bleeding in T1DM may play an important role in periodontal destruction in T1DM.

Keywords: *Diabetes mellitus type 1, OPG, Periodontal diseases, RANK, RANKL*

Introduction

Type 1 diabetes mellitus (T1DM) is the most common type of diabetes mellitus (DM) in children and adolescents with increasing in the prevalence. It is an autoimmune disease characterizing a destruction of beta-cells of the pancreatic islets of Langerhans that produce the hormone insulin, leading to inadequate insulin production and chronic hyperglycemia. It has been established that poorly controlled T1DM is associated with a variety of complications, i.e., atherosclerosis, diabetic retinopathy, kidney disease, and degenerative neurological disease.[1,2] The impairment

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of the immune system in T1DM patients affects the degradation of collagen synthesis in connective tissues and alters the bone metabolism and bone density of whole-body mineral acquisition in T1DM adolescent patients with poor glycemic control.[3,4]

Periodontal disease in T1DM patients has been related to the higher incidence and severity of periodontal disease than those present in non-T1DM patient. Related mechanisms have been proposed to induce the hyperresponsive conditions of inflammation and the immune response, such as retardation of polymorphonuclear neutrophil (PMN) functions and increased levels of interleukin-6 (IL-6) and activated C-reactive protein (CRP), which eventually induce alveolar bone resorption and tooth loss.[5,6] While the relationship between periodontal disease and type 2 DM has been widely discussed, evidence of periodontal disease and the relationship between T1DM and periodontal disease is very scarce.

Factors that cause children and adolescents with T1DM to be more at risk of periodontal disease are glycemic control and the duration of disease.[7-11] Previous studies found that the blood levels of glycated hemoglobin (HbA1c) had a strong significant correlation with periodontitis in children demonstrating deeper pocket depth (PD) and more bleeding on probing (BOP) sites have been found in periodontitis with uncontrolled HbA1c levels.[7] Moreover, a study found a positive correlation between the duration of T1DM and CAL but not with PD, plaque index (PI), or gingival index (GI) in the diabetic group.[11] In contrast, there was a study reporting that PD and clinical attachment loss (CAL), of T1DM patients aged 9–17 years were not different from those of non-diabetic children and adolescents.[12] Thus, the relationship between T1DM and periodontal disease is still inconclusive.

Regarding T1DM and periodontal disease, the alteration of bone metabolism may affect several molecules conducive to the proliferation, differentiation and activation of both osteoblasts and osteoclasts. The receptor activator of nuclear factor kappa-β ligand (RANKL), a member of the tumor necrosis factor (TNF) ligand superfamily, is identified as a cell membrane-bound factor responsible for the stimulation of osteoclast differentiation and bone resorption. Activating its receptor activator of nuclear factor kappa-β (RANK) receptor on the surface of pre-osteoclasts triggers fusion and differentiation into mature osteoclasts and activates bone resorption.[13-15] The action of RANKL can be blocked by its soluble decoy receptor osteoprotegerin (OPG), which is a member of the TNF receptor superfamily, with structural homology to RANK and subsequently interact all the downstream molecular events that lead to osteoclast differentiation and bone resorption.[16] RANKL and OPG can be detected in gingival tissue and biological fluids, including gingival crevicular fluid, saliva, and serum. Moreover, the RANKL-OPG system controls osteoclast formation and bone resorption in normal and diseased conditions.[17] Increased RANKL concentrations and RANKL:OPG ratios and decreased salivary OPG levels have been reported to be associated with increases in PD, CAL, and the extent and severity of periodontitis.[18,19] A study of gingival tissues showed that T1DM patients with chronic periodontitis had higher RANKL mRNA levels but lower OPG mRNA levels, an increased RANKL:OPG ratio than systematically and periodontally non-T1DM individuals.[20]

As reviewed earlier, the linkage mechanisms behind these findings of T1DM and periodontal disease are not yet understood. The RANKL-OPG system may play an important role in T1DM patients with periodontal disease. Moreover, studying the RANKL-OPG system in the saliva of children or adolescents may be suitable due to the non-invasiveness of the saliva collection method, that fact that it is painless, and the convenience. There has been no study of salivary RANK, RANKL and OPG expression and the relationship between the expression of these genes and periodontal conditions in children and adolescents with T1DM. Thus, the aims of the present study were to investigate salivary RANK, RANKL, OPG gene expression and the RANKL:OPG ratio in T1DM and non-T1DM. Additionally, the secondary objective was to determine the relationships of salivary gene expressions of RANK, RANKL and OPG, clinical parameters of T1DM and periodontal disease.

Materials and Methods

The study protocol was approved by the Faculty of Dentistry/Faculty of Pharmacy, Mahidol University Institutional Review Board (MU-DT/PY IRB 2017/030.1605) and was conducted in accordance with the Helsinki Declaration of 1964, as revised in 2013. All participants provided written informed consent. The sample size in each group was determined using data from Duarte's study.[20] It was decided to have at least 14 participants in each group to have a significance level of 0.01 and power of 0.9.

Study population

Twenty patients with T1DM, aged 15 years and over, attending the Division of Pediatric Endocrinology and Metabolism, Department of Pediatrics, Siriraj

Hospital, were recruited for the present study. Another group of twenty age-matched non-diabetic individuals attending the Faculty of Dentistry, Mahidol University, was recruited as a non-diabetic control group. Non-diabetic individuals had neither systemic disease nor a history of diabetes in the family. The fasting blood sugar (FBS) test and HbA1c levels were used to confirm that the control individuals had no diabetes. Control individuals were included in the study when their FBS test results were less than 126 mg/dL (7.0 mmol/L) and HbA1c levels were less than 5.7%. T1DM patients were classified into subgroups according to their diabetes control: well controlled (HbA1c <8%) and poorly controlled (HbA1c ≥ 8%). The exclusion criteria were as follows: smoking, pregnancy, undergoing orthodontic treatment, history of taking antibiotics within 2 months, and history of scaling and/or root planing within 6 months of study entry.

Data collection

Age, sex, weight, height, and body mass index (BMI) were collected from all participants. In patients with T1DM, information related to diabetic status, such as age at diagnosis, duration, treatment regimens, and HbA1c value within 1 year of study, was collected from medical records.

Periodontal examination

All participants received periodontal examination on fully erupted permanent teeth except third molars by one examiner (R.C.). The following clinical periodontal parameters were recorded at six sites per tooth: PD, CAL, and BOP. PD was measured (to the nearest mm) from the gingival margin to the bottom of the sulcus using a mm-marked periodontal probe (PCPUNC 15, Hu-Friedy, Chicago, IL, USA). CAL was defined as the distance from the cementoenamel junction (CEJ) to the most apical penetration of the probe. BOP was evaluated within 10 seconds after probing as BOP-positive or BOP-negative. PI was recorded on six index teeth (maxillary right first molar, maxillary right lateral incisor, maxillary left first bicuspid, mandibular left first molar, mandibular left lateral incisor, and mandibular right first bicuspid). Each of the four surfaces of the index teeth (buccal, lingual, mesial, and distal) was given a score from 0–3 as previously described.[21]

Saliva sample collection

All participants refrained from eating, drinking, and brushing 1 hour (h) before saliva collection. Then, 5–10 ml of non-stimulated whole saliva was collected in a sterile test tube and stored on ice.[22] Two hundred microliters of saliva were transferred to a new eppendorf tube, and then 1 mL of saliva protection (RNeasy Protect Saliva reagent, Qiagen, Hilden, Germany) was added to the saliva and briefly vortexed to protect RNA. The saliva samples were stored at -80°C until further analysis of RANK, RANKL and OPG gene expression.

RNA extraction and cDNA synthesis

Total RNA was isolated from saliva (RNeasy Protect Saliva Mini kit, Qiagen, Hilden, Germany) according to the manufacturer's recommendation. An aliquot of the saliva mixture was sequentially centrifuged, and the supernatant was removed. Then, the pellet was washed with a series of buffers. Elute RNA was retrieved, and the amount of total RNA was further calculated. The concentration of RNA was determined by the 260/280 nm absorbance (Nanodrop 1000, USA). Then, 1 µg of RNA was used to synthesize cDNA (High-Capacity cDNA Synthesis Kit, Applied Biosystems, Carlsbad, CA). Mixtures of random primers, reverse transcriptase enzymes, dNTPs, and RNA were incubated at 37°C for 2 h and at 85°C for 5 minutes (min).

Quantitative real-time polymerase chain reaction (real-time PCR) for RANK, RANKL, and OPG.

Relative gene expression of RANK, RANKL, and OPG was performed by real-time PCR using a PCR kit (Quantinova™ SYBR Green PCR Kit, Qiagen, Hilden, Germany). β-Actin was used as an internal control. Real-time PCR primers were originally designed from GenBank using Primer3Plus software and through the BLAST network service of the National Center for Biotechnology Information (Bethesda MD, USA), including OPG with GenBank No. NM_002546.4 (product size 185 base pairs (bp); forward primer 5'-GGCAACACAGCTCACAAGAA -3' and reverse primer 5'-CGGTAAGCTTTCCATCAAGC-3') and β-actin with GenBank No. NM_001101.3 (product size 184 bp; forward primer 5'-AGAGCTACGAGCTGCCTGAC-3' and reverse primer 5'-AGCACTGTGTTGGCGTACAG-3')withanannealing temperature (AT) of 60°C. For the RANK gene, the primer sequences used were according to Dorotheou *et al.*[23] with GenBank No. NM_003839.4 (product size 101 bp; forward primer 5'- GCTTTCCCAGTGTGTGTTCA-3' and reverse primer 5'-AAAGTCCCACCACATGTTCC-3') with an AT of 54°C. For RANKL, the primer sequences used were according to Wada *et al.*[24] with GenBank No. NM_003701.4 (product size 196 bp; forward primer 5'-ACCAGCATCAAAATCCCAAG -3' and reverse primer 5'-CCCCAAAGTATGTTGCATCC-3') with an AT of 59°C. Briefly, each cDNA sample was mixed with 5 µM forward and reverse primers and 25 µl of SYBR Green mixture in a 96-well plate. The conditions were pre-incubation at 95°C for 10 min, followed by 50 cycles

of denaturation at 95°C for 15s, primer annealing at 54–60°C for 1min and an extension step at 95°C for 15s. Quantitative data of relative gene expression was analyzed by software (CFX Manager™ software, Bio-Rad, Swiss). The RANKL:OPG ratio was calculated from the average relative gene expression of RANKL and OPG in each group.

Statistical analysis

Data were analyzed using a software program (SPSS for Windows version 18, SPSS Inc., Chicago, IL, USA). Descriptive statistics were used to summarize patient characteristics. The Shapiro-Wilks test was used to test for normality. The independent t-test or Mann-Whitney U test was used for comparisons between two groups according to normality tests. The Pearson correlation coefficient or Spearman rank correlation were used to assess the relationship between gene expression and clinical periodontal parameters in both groups. In the T1DM, the relationships between diabetic status (the levels of HbA1c and diabetes duration) and clinical periodontal parameters and gene expression were analyzed with Pearson correlation or Spearman rank correlation analysis. Independent t-tests or Mann-Whitney U tests were used for comparisons between subgroups of the T1DM group. Gene expression experiments were performed in triplicate from all subjects. Significant differences or correlates were established at $P < 0.05$.

Results

Characteristics of the participants in non-T1DM and T1DM group

Twenty patients with T1DM and twenty non-T1DM of age-matched individuals were included in the present study. [Table 1] shows the characteristics of the participants in both groups. The T1DM and non-T1DM groups did not differ with respect to age ($P = 0.953$). However, patients with T1DM had a significantly higher mean BMI than the non-T1DM group ($P \leq 0.001$). The median HbA1c levels of the T1DM and non-T1DM groups were 8.11 (range, 6.82–11.76) and 4.75 (range, 4.2–5.2), respectively. Differences between these two groups were statistically significant ($P \leq 0.001$). All diabetic patients were treated with insulin. In addition, four diabetic patients had dyslipidemia and received simvastatin; two persons received calcium carbonate, and nine persons received vitamin D2. Most of the patients with T1DM (60%) had dental visits only when they had symptoms; similarly, 70% of the non-T1DM group went to see dentists only when they had problems. Five percent of the T1DM group and 25% of the non-T1DM group had never seen a dentist.

Table 1: Characteristics of the participants in both groups

	T1DM (n=20)	Non-T1DM (n=20)	*P*
Age, years	18.39 ± 1.99	18.35 ± 2.01	0.953
Gender, n (%), male	11 (55%)	4 (20%)	
female	9 (45%)	16 (80%)	
BMI, kg/m2	24.25 ± 3.77	19.72 ± 1.64	<0.001*
Reported frequency of dental visit, n (%)	6 (30%)	1(5%)	
2 visit/year	1 (5%)	0	
1 visit/year	12 (60%)	14 (70%)	
When have symptoms	1 (5%)	5 (25%)	
Never			
HbA1c	8.11	4.75	<0.001**

* Independent t-test, statistically significant at $P < 0.05$.

** Mann-Whitney U test, statistically significant at $P < 0.05$

T1DM, type 1 diabetes mellitus; BMI, body mass index; HbA1c, glycated hemoglobin

Clinical periodontal parameters

Patients with T1DM had more gingival inflammation and a higher percentage of full mouth gingival bleeding than the non-T1DM group, while PI was not significantly different ($P = 0.456$). The T1DM group had significantly deeper PD, greater CAL, and a higher percentage of BOP sites than the non-T1DM group ($P \leq 0.001$, $P = 0.002$, and $P \leq 0.001$, respectively, [Table 2]).

RANK, RANKL and OPG gene expression between non-T1DM and T1DM group

The relative mean mRNA levels of the RANK and RANKL genes, and the RANKL:OPG ratio demonstrated higher in the T1DM group than in the non-T1DM group, whereas the mean mRNA levels of the OPG gene in the T1DM group were lower than those in the non-T1DM group. However, statistical analysis showed that there was no significant difference in the mean relative mRNA levels of the RANK, RANKL, and OPG genes and the RANKL:OPG ratios between the T1DM and non-T1DM groups ($P = 0.676$,

Table 2: Clinical periodontal parameters of the study population

	T1DM (n=20)	Non-T1DM (n=20)	*P*
Plaque index	0.90	1.10	0.456
Probing depth, mm	2.67 ± 1.51	2.37 ± 0.19	<0.001*
Clinical attachment level, mm	1.81	1.16	0.002**
Bleeding on probing sites, %	54.17	21.72	<0.001*

* Independent t-test, statistically significant at $P < 0.05$

** Mann-Whitney U test, statistically significant at $P < 0.05$

T1DM, type 1 diabetes mellitus

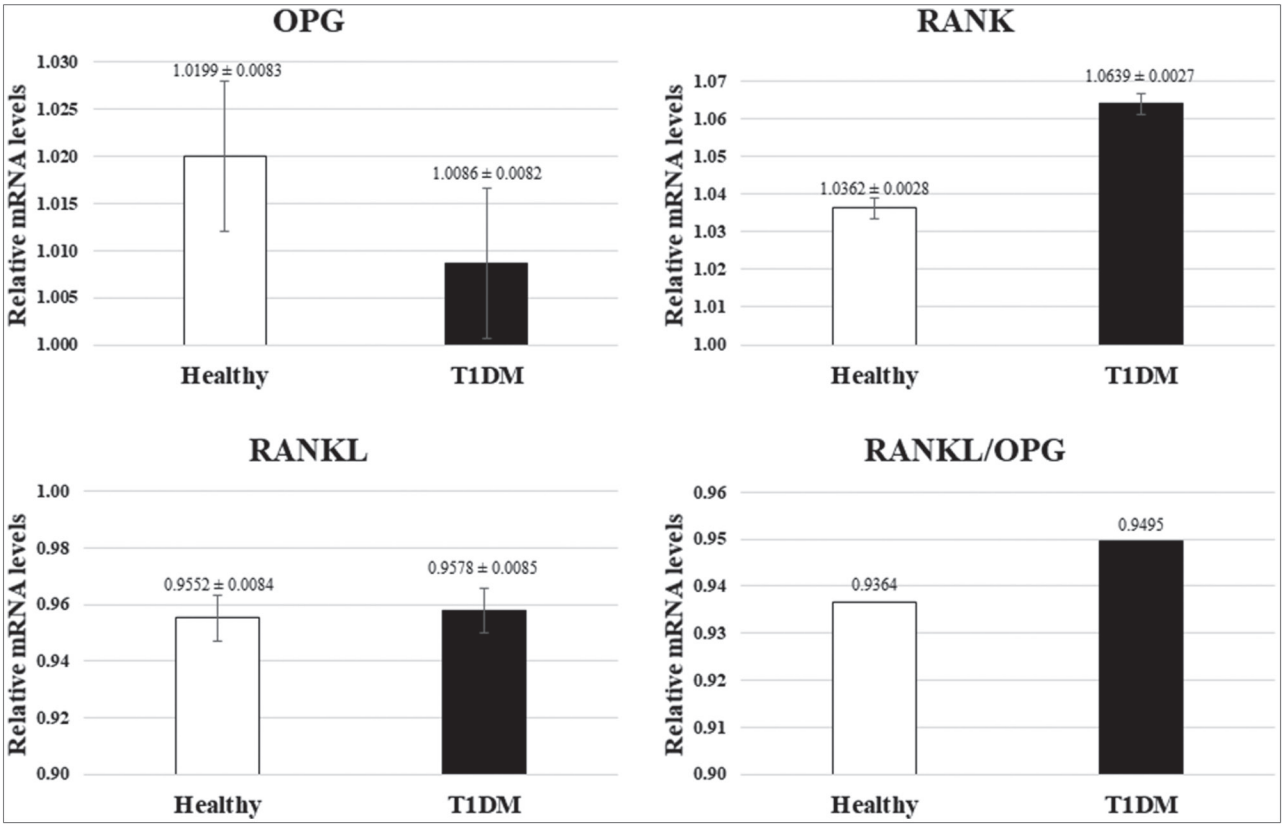

Figure 1: OPG, RANK, RANKL gene expression and RANKL:OPG ratio were demonstrated comparison between T1DM (n = 20) and non-T1DM control (n = 20) group by quantitative RT-PCR. There was no statistic significant different at $P < 0.05$ in all genes between T1DM and non-T1DM control

P = 0.814, P = 0.979, and P = 0.754, respectively, [Figure 1]).

Correlations of RANK, RANKL and OPG gene expression with clinical periodontal parameters

To evaluate the relationship between RANK, RANKL and OPG gene expression and clinical periodontal conditions, statistical analysis demonstrated that salivary RANK and RANKL gene expression had a positive correlation with probing depth in the T1DM group (r = 0.533, P = 0.015, and r = 0.544, P = 0.013, respectively, [Table 3]). The RANKL:OPG ratio had a positive correlation with percentage of BOP in the T1DM group (r = 0.495, P = 0.026, respectively, [Table 3]).

Correlations among HbA1c levels, diabetes duration and clinical periodontal parameters and the RANKL/OPG ratio

In the T1DM group, HbA1c levels had a positive correlation with the PD (r = 0.617, $P \leq 0.001$). No correlation between the duration of diabetes and periodontal parameters was found [Table 4].

T1DM subgroups

Comparisons between subgroups revealed that the well-controlled group had a higher mean weight but a lower mean BMI than the poorly controlled group but there were no statistically significant differences (P = 0.617 and P = 0.706, respectively, [Table 5]). There were no significant differences in age or height (P = 0.260 and P = 0.112, respectively, [Table 5]). Diabetes duration ranged from 3 to 19 years, interestingly, duration was more prolonged in the well-controlled group (11.48 ± 5.32 years) than in the poorly controlled group (8.49 ± 4.39); however, the difference between the two groups was not significant (P = 0.188). Glycemic control between the well-controlled and poorly controlled groups was demonstrated by the mean HbA1c level, which showed a statistically significant difference (P = 0.001). Clinical periodontal parameters, and saliva gene expression of the RANK, RANKL and RANKL/OPG ratio were not significantly different between subgroups [Table 5].

Discussion

The present study was conducted in a group of patients with T1DM and age-matched systematically non-T1DM with a mean age of eighteen years. Therefore, severe periodontitis was not expected to be

Table 3: Relationship between RANK, RANKL and OPG gene expression and clinical periodontal conditions

Variable	RANK		RANKL		OPG		RANKL/OPG	
	T1DM	Non-T1DM	T1DM	Non-T1DM	T1DM	Non-T1DM	T1DM	Non-T1DM
%BOP								
r	0.233	-0.446	0.483	-0.184	-0.055	0.345	0.495	-0.206
P	0.323	0.49	0.031*	0.438	0.818	0.137	0.026**	0.383
PD								
r	0.533	0.048	0.544	-0.329	0.317	0.224	0.140	-0.144
P	0.015*	0.840	0.013*	0.156	0.173	0.342	0.557	0.545
CAL								
r	0.105	0.036	-0.116	-0.226	-0.052	-0.110	0.006	0.087
P	0.659	0.880	0.627	0.339	0.828	0.645	0.980	0.715

1* Spearman rank correlation, statistically significant at $P < 0.05$

** Pearson correlation coefficient, statistically significant at $P < 0.05$

BOP, bleeding on probing; PD, probing depth; CAL, clinical attachment level; T1DM, type 1 diabetes mellitus; RANK, receptor activator of nuclear factor kappa-β; RANKL, receptor activator of nuclear factor kappa-β ligand; OPG, osteoprotegerin.

Table 4: Relationship between diabetic status (HbA1c levels and diabetes duration) and clinical periodontal parameters, and RANKL/OPG ratio

Factor	% BOP	PD	CAL	RANKL/OPG
HbA1c				
r	-0.218[1]	0.617	-0.09	-0.403
P	0.356	0.00*	0.707	0.078
Duration				
r	0.171	0.073	0.083	0.406
P	0.47	0.76	0.729	0.075

[1]* Spearman rank correlation, statistically significant at $P < 0.05$

** Pearson correlation coefficient, statistically significant at $P < 0.05$

BOP, bleeding on probing site; PD, probing depth; CAL, clinical attachment level; RANKL, receptor activator of nuclear factor kappa-β ligand; OPG, osteoprotegerin; HbA1c, glycated hemoglobin.

found in the present study; however, the periodontal status of patients with T1DM showed more gingival inflammation, a higher percentage of BOP, deeper PD, and greater CAL than non-T1DM. These findings are consistent with previous reports on the periodontal status of children with T1DM.[25-28] Moreover, subgingival bacterial challenge in patients with T1DM did not differ from that in non-diabetic patients[29] and suggested that an exaggerated inflammatory response accelerated periodontal breakdown in patients with T1DM.[30,31] Recent meta-analysis confirmed that T1DM was a relevant risk factor for the development of periodontitis. A patient was considered affected by periodontitis if he/she had at least one site with any level of attachment loss or radiographic bone loss on at least two non-adjacent teeth. Almost one out of five patients with T1DM were also affected by periodontitis. The proportion of patients affected by periodontitis in subjects with T1DM was more than double in comparison with non-diabetics.[32]

The results from the present study showed that there was no significant difference in the relative mRNA expression levels of RANK, RANKL, and OPG and the RANKL:OPG gene ratio between the T1DM and non-T1DM groups; however, the trend of the protective effect represented by OPG was higher in the non-T1DM group, and in contrast, RANK, RANKL, and the ratio of RANKL:OPG gene expression were higher in the T1DM group. To our knowledge, the present study is the first to study salivary RANK, RANKL and OPG gene expression in patients with T1DM in adolescence. Previously, expression of the RANKL and OPG genes with respect to T1DM and periodontitis was studied in serum and gingival tissue.[20,33] Serum concentrations of bone metabolism markers showed that T1DM had significantly lower RANKL to OPG ratios and higher OPG concentrations than non-T1DM.[33] However, when indicated in gingival tissues, RANKL and RANKL:OPG mRNA levels showed higher in T1DM with periodontitis than non-T1DM without periodontitis, whereas OPG mRNA levels demonstrated lower. Results were suggested that a diabetic state may promote an imbalance in the RANKL:OPG system in favor of osteoclastogenesis.[20] In vivo study demonstrated that high glucose and LPS treatment significantly promoted the protein expression of RANKL and RANKL/OPG ratio, while inhibited the protein expression of OPG. The maximum RANKL/OPG ratio was observed in the high glucose and LPS group, followed by the LPS group.[34] Moreover, the inconsistencies in the relationship between OPG levels and diabetes and periodontitis may reflect the diverse roles of OPG at the local and systemic levels, as has been

Table 5: Characteristics, clinical periodontal parameters, and mean relative mRNA levels of RANK, RANKL, OPG genes and RANKL:OPG in T1DM group

	Well-controlled (n=10) mean ± SD (range)	Poorly controlled (n=10) mean ± SD (range)	*P*
Age, years[1]	18.53 ± 1.44 (15.75 - 20.75)	17.58 ± 2.12 (15 – 20.67)	0.260
Height, cm.	169.55 ± 11.32 (148.5 – 180.5)	164.27 ± 7.53 (154 – 177.6)	0.112
Weight, kg	68.70 ± 12.96 (45.5 – 86.5)	65.72 ± 13.23 (49.6 – 88.3)	0.617
BMI, kg/m^2	23.71 ± 2.71 (19.5 – 28)	24.39 ± 4.89 (18.8 – 33.4)	0.706
Duration, years	11.48 ± 5.32 (3.5 – 17.92)	8.49 ± 4.39 (3 – 19.17)	0.188
HbA1c	7.48 ± 0.35 (6.82 – 7.88)	9.42 ± 1.31 (8.12 – 11.76)	0.001*
Plaque index	1.02 ± 0.35 (0.29 – 1.54)	0.93 ± 0.39 (0.29 – 1.67)	0.607
Probing depth, mm	2.64 ± 0.10 (2.45 – 2.78)	2.71 ± 0.17 (2.37 – 2.96)	0.281
Clinical attachment level, mm	1.70 ± 0.34 (0.85 – 1.93)	1.64 ± 0.47 (0.6 – 2.19)	0.850
Bleeding on probing sites, %	54.84 ± 13.56 (37.33 – 79.17)	52.94 ± 20.64 (20.24 – 88.02)	0.799
RANK	0.967 ± 0.176 (0.667 – 1.190)	1.161 ± 0.366 (0.785 – 1.852)	0.226
RANKL	0.949 ± 0.090 (0.717 – 1.003)	0.966 ± 0.072 (0.803 – 1.002)	0.545
OPG	0.989 ± 0.089 (0.801 – 1.131)	1.029 ± 0.083 (0.879 – 1.189)	0.313
RANKL/OPG	0.967 ± 0.129 (0.723 – 1.242)	0.836 ± 0.092 (0.99 – 1.136)	0.641

* Independent t-test, statistically significant at $P < 0.05$, ** Mann-Whitney U test, statistically significant at $P < 0.05$. Subgroups of type 1 diabetes group into well-controlled (HbA1c < 8) and poorly control (HbA1c ≥ 8) groups. T1DM, type 1 diabetes mellitus; BMI, body mass index; HbA1c, glycated hemoglobin; RANK, receptor activator of nuclear factor kappa-β; RANKL, receptor activator of nuclear factor kappa-β ligand; OPG, osteoprotegerin.

previously discussed.[35] Our findings may support the local role of OPG in the inhibition of bone resorption.

In accordance with previous studies, our study demonstrated high levels of RANK and RANKL and the RANKL:OPG ratio and lower OPG gene expression in T1DM; however, we found very slight alterations in the mean RANK, RANKL, and OPG expression and the RANKL:OPG ratio in patients with T1DM compared to the non-T1DM group, even though the clinical periodontal parameters showed higher inflammation and destruction. Salivary OPG gene expression had a negative correlation with the percentage of bleeding sites in the T1DM group. These results suggested that higher BOP in the T1DM group were related to decreased OPG gene expression. Low levels of the anti-resorptive factor OPG are associated with sites showing periodontal destruction.[36,37] Greater CAL found in the T1DM group could be the outcome of the relationship between OPG gene expression and pathological changes in the periodontium.

The relationship of HbA1c with PD in the present study is in accordance with previous reports on the relationship between diabetes control and the periodontal pocket. Patients with a long-term duration of poor diabetes control had greater PD and had lost more tooth attachments and proximal bone than the controlled insulin-dependent diabetes subjects.[38,39] One percentage increase in HbA1c was independently associated with an average increase in bleeding on probing of 25% and with an increase in the rate of sites with pocket depth >3 mm of 54%.[40] The results of the present study again confirm that the severity of periodontal conditions is modified by the diabetic state and the metabolic control of diabetes. Our findings that there was no difference in clinical periodontal parameters between the well-controlled and poorly controlled subgroups may be because of the small numbers of patients in each group. In addition, it has been reported that the longer the duration of diabetes is, the more periodontal destruction and inflammation, the deeper the PD, the more BOP sites and the greater clinical attachment loss observed.[10,11]

Compared to gingival crevicular fluid sampling, which is collected by utilizing paper strips inserted into the gingival crevice, saliva sampling is a painless, non-invasive method used to easily collect an adequate amount of saliva for study. Therefore, saliva sampling is more suitable for sampling in children and adolescents. Whole saliva is a pooled sample containing locally derived and systemically derived markers. Studies have been conducted to investigate the possibility of using saliva for diagnosis and monitoring of periodontal disease. Host-derived enzymes in saliva have been suggested to be the most promising indicators of periodontal disease.[41] The salivary RANKL:OPG ratio is a potential diagnostic tool with high positive predictability (95%) for the presence of periodontitis.[42] There have been limited studies regarding RANKL and OPG gene expression in saliva. RANKL and OPG gene expression detected in the present study suggested that saliva might be a useful source for studying the RANKL-OPG system in the relationship between

periodontal disease and diabetes. Further investigations are needed to evaluate the possibility of utilizing saliva for monitoring the disease activity of periodontal disease in the presence of diabetes.

The present study was a cross-sectional study. Further studies with larger sample sizes are needed to verify the results of the present study and confirm the results at the protein level. Longitudinal studies concerning the effect of glycemic control on the RANK-OPG system and the stability of the periodontal status should be investigated. However, the clinical findings indicating that there was more periodontal destruction and inflammation in the T1DM group should be recognized by dentists. Since patients with T1DM are generally young, periodontal disease might be overlooked. Once patients have been diagnosed with T1DM, optimal plaque control should be set as a goal through education and motivation of the parents and patients to prevent periodontal disease progression. Within the limitations of the present study, the results of the present study suggest that patients with T1DM had more periodontal destruction than the non-T1DM group.

Conclusion

Given this finding, there was no significant difference in the mean relative salivary mRNA levels of the RANK, RANKL, and OPG genes and the RANKL:OPG ratio between the 2 groups. However, decreased salivary OPG gene expression was observed in T1DM, as was also seen in the positive correlation between HbA1c levels and PD. Therefore, decreased salivary OPG gene expression may associate with periodontal inflammation and OPG may play an important role in periodontal destruction in T1DM.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

Authors' contributions

Rachanin Chairatnathrongporn: Literature search, Methodology, Data collection, Formal analysis, Writing-original draft. Kallapat Tansriratanawong: Literature search, Methodology, Data collection, Formal analysis, Writing-original draft. Jeerunda Santiprabhob: Methodology, Data collection. Chatkoew Boriboonhirunsarn: Methodology, Data collection. Ananya Promsudthi: Literature search, Methodology, Data collection, Formal analysis, Writing-original draft.

Ethical policy and institutional review board statement

The study protocol was approved by the Faculty of Dentistry/Faculty of Pharmacy, Mahidol University Institutional Review Board (MU-DT/PY IRB 2017/030.1605) and was conducted in accordance with the Helsinki Declaration of 1964, as revised in 2013. All participants provided written informed consent.

Patient declaration of consent

The authors certify that they have obtained all appropriate patient consent forms.

Data availability statement

The data set used in the current study is available on request from Dr. Ananya Promsudthi (E-mail: ananya.pro@mahidol.ac.th).

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