The Expression of Marker Genes Associated with Bone Remodeling after Tooth Extraction

การแสดงออกของยีนที่เกี่ยวข้องกับการสร้างและการละลายตัวของกระดูกหลังถอนฟัน

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ABSTRACT

The gene expression associated with the edentulous bone remodeling including gene expression of OPG, RUNX2, RANKL, CtsK, MMP9 and TRAP were examined. The expression levels were compared between the groups of different bone heights and the alveolar bone from impacted tooth. The samples were collected from the alveolar bone of 18 lower edentulous areas and 7 lower third molar impactions. Real time RT-PCR was used to examine the target gene expressions. The bone profiles of the edentulous areas were measured from the CT scan. The analysis showed that the edentulous bone had the ratio of RANKL to OPG significantly higher than impacted tooth alveolar bone (Kruskal-Wallis test, $p<0.05$), while the gene expressions were not significantly different between the impacted tooth alveolar bone and the edentulous bone. Furthermore, there was significant positive correlation (Pearson's correlation, $P<0.05$) between the crestal bone width and the ratio of RANKL to OPG.

บทคัดย่อ

การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาการแสดงออกของยีน OPG RUNX2 RANKL CtsK MMP9 และ TRAP ซึ่งเกี่ยวข้องกับการสร้างและการละลายตัวของกระดูกขากรรไกรหลังถอนฟัน และเพื่อเปรียบเทียบการแสดงออกของยีนดังกล่าวระหว่างกลุ่มสันกระดูกขากรรไกรล่างที่มีความสูงแตกต่างกันกับกลุ่มกระดูกรอบรากฟันคุด โดยทำการเก็บชิ้นกระดูกตัวอย่างจากผู้ป่วยที่มีสันกระดูกว่าง 18 คน และกระดูกรอบรากฟันกรามซี่ที่สามล่างที่เป็นฟันคุด 7 คน ตรวจสอบการแสดงออกของยีนเป้าหมายโดยวิธีการเรียลไทม์ อาร์ที-พีซีอาร์ รวมทั้งเก็บข้อมูลรูปร่างกระดูกขากรรไกรล่างจากภาพรังสีคอมพิวเตอร์ ผลการวิเคราะห์พบว่ากระดูกจากบริเวณสันกระดูกว่างมีอัตราส่วนระหว่างการแสดงออกของยีน RANKL ต่อ OPG สูงกว่ากระดูกรอบรากฟันคุดอย่างมีนัยสำคัญ ในขณะที่ไม่พบความแตกต่างของการแสดงออกของยีนแต่ละชนิดระหว่างกลุ่มสันกระดูกว่างและกระดูกรอบรากฟันคุด นอกจากนั้นยังพบความสัมพันธ์เชิงบวกระหว่างความกว้างของสันกระดูกขากรรไกรล่างส่วนบนกับอัตราส่วนระหว่างการแสดงออกของยีน RANKL ต่อ OPG อย่างมีนัยสำคัญ

Key Words: Alveolar bone resorption, Gene expression, Real time RT-PCR

คำสำคัญ: การละลายตัวของสันกระดูกขากรรไกร การแสดงออกของยีน เทคนิคเรียลไทม์ อาร์ที-พีซีอาร์

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Introduction

The number of Thai people who lost their teeth due to dental caries, periodontal disease and trauma are increasing nowadays. These patients need the prosthetic treatment to improve their mastication, speech and personality. One of the problems challenging the prosthodontist is the atrophy of the jaw bone after tooth extraction. The size of the edentulous bone is gradually reduced throughout the life, most rapidly in the first few months (Atwood, 1963; Tallgren et al., 1966; Carlsson and Persson, 1967). The resorption affects the function of prostheses especially the removable denture that relies on the quantity and architecture of the jaw bone (Jacobson and Krol, 1983). There are many factors involved in the jaw bone resorption such as anatomic factor, prosthetic factor, metabolic factor and functional factor (Atwood, 1962, 1979).

For determining the rate of resorption, genetic is one of the impact factors influences the bone resorption (Jahangiri et al., 1998). Many studies explored some genes playing a role in development, differentiation and function of bone cell. RUNX2 is the key regulator of osteoblast cell function depending on up- or down- regulation of the other genes. RUNX2 is significantly associated with bone mineral density (BMD) and peak bone mass (PBM) in human (Zanatta et al., 2012). The communications among osteoblast and stromal cells influence the osteoclast cell differentiation through their receptors.

From the basis of bone formation, the sites of bone are different in their molecular mechanisms. Maxilla and mandible including alveolar bone are formed by neural crest cell, while the axial and appendicular bones are formed by mesoderm (Akintoye et al., 2006). The gene expressions in the different sites of bone are also different. The RANKL/OPG ratio is higher in long bone than in the jaw bone. However, the jaw osteoclast is larger in size and it has the higher level of TRAP activity (Ana Paula, 2011). The osteoclasts are responsible in bone resorption process, and the tooth eruption also requires osteoclast to form an eruption pathway (Wise et al., 2002). The integrin $\alpha_v\beta_3$ receptors are highly expressed on the osteoclast cell surfaces and bound to arginyl-glycyl-aspartyl (RGD)-peptide in mineralized bone matrix then form the ruffled border at the sealing zone (Fisher et al., 1993). Osteoclast synthesizes hydrochloric acid through the ruffled border to dissolve hydroxyapatite, then proteases such as cathepsin and matrix metalloprotease (MMPs) could reach and degrade bone organic matrix (Troen et al., 2006; Kalervo, 2000). Previous studies have discovered that cathepsin K (Ctsk) and matrix metalloproteinase 9 (MMP-9) are the main proteases in the resorption process (Reponen et al., 1994; Sundaram et al., 2007). The higher expression levels of CtsK and MMP-9 in osteoarthritis patient have found to be associated with the higher bone resorption. Furthermore, there are other genes such as receptor activator of nuclear factor kappa-B ligand (RANKL), osteoprotegerin (OPG), Tartrate-Resistant Acid Phosphatase (TRAP) which their expressions and differences in balance between them affect the resorption and the formation of bone (Logar et al., 2007).

RANKL is on the surface of osteoblast cell. It has been reported to be one of the regulators in promoting the differentiation, maturation, function and survival of osteoclast. RANKL increase osteoclast function by inducing actin ring formation

and cytoskeletal rearrangement (Fuller et al., 1998; Yasuda et al., 1998a; Burgess et al., 1999), while OPG which expressed from many tissue sites can also bind to RANKL, and the binding results in inhibition of osteoblast and osteoclast cell-to-cell signaling (Yasuda et al., 1998b). OPG is known as the bone protector by limiting osteoclast formation and activity (Simonet et al., 1997).

TRAP is mainly expressed in macrophages and osteoclasts (Lang et al., 2001). The enzyme has been extensively used as a marker for osteoclast, and it could be used as a marker for pathological bone resorption (Halleen et al., 2000). It generates destructive reactive oxygen species at resorption lacunae (Halleen et al., 1999; Bonucci and Nanci., 2001).

There are several methods that have been used to study the gene profile. Real time RT-PCR is one of the well-known techniques that has become the standard technology for the quantification of nucleic acids (Carter et al., 2012; Fleige et al., 2006). In this study, some specific genes: RANKL, OPG, TRAP, MMP-9, CtsK and RUNX2 were observed by the real time RT-PCR technique. The gene expression profiles of the edentulous bone samples and the alveolar bone samples of impacted teeth were compared. Furthermore, since the computed tomography (CT scan) is the most consistent with direct measurements (Peker et al., 2008), the edentulous gene expressions were also correlated to their bone profiles from CT scans.

Objectives of the study

The objectives of this study were to examine the gene expressions of OPG, RUNX2, RANKL, CtsK, MMP9 and TRAP. The expression levels were compared between the groups of different edentulous bone height and the alveolar bone from impacted tooth.

Materials and Methods

Bone samples

The subjects were more than 25-year-old consenting patients, 7 samples from the alveolar bone of lower third molar impacted teeth, and 18 samples from the alveolar bone of lower posterior edentulous areas. The samples were collected from the impacted tooth removal surgeries and the dental implant surgeries, respectively. The patients who have relevant medical history or have worn any removable dental prosthesis were excluded. Bone samples were frozen in liquid nitrogen until the isolation process.

RNA isolation

The bone was homogenized within lysis reagent (Qiazol®, Qiagen, Inc., USA) by the bead-based homogenizer (PowerLyzer™, Mo Bio Laboratories, Inc., USA), which the bead tubes were pre-chilled in liquid nitrogen (Carter et al., 2012). The mRNA was extracted by the spin-column based method (PureLink® RNA Mini Kit, Life Technologies, Inc., USA). The quantification of nucleic acid was performed using a spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific, Inc., USA), and the quality of the RNA was verified using an electrophoresis method (Bioanalyzer, Agilent Technologies, USA).

Real time RT-PCR

We used CFX96™ real time RT-PCR system (Bio-Rad laboratories, Inc., USA) to observe the gene expressions. We designed primers by using primer designing tool software (Primer3 and BLAST, http://www.ncbi.nlm.nih.gov/tools/primer-blast/), (Table 1).

Table 1 The primers used in real time RT-PCR

Gene		Primer sequences
OPG	forward	TCAGGTTTGCTGTTCCTACA
	reverse	GTTCTTGTGAGCTGTGTTGC
RUNX2	forward	CAGCCCAGAACTGAGAAACT
	reverse	ACAGATGGTCCCTAATGGTG
RANKL	forward	GCCAGTGGGAGATGTTAGAC
	reverse	ATAGCCCACATGCAGTTTCT
CtsK	forward	ATGACCAGTGAAGAGGTGGT
	reverse	AGAGTCTGGGGCTCTACCTT
MMP9	forward	CCTTCTACGGCCACTACTGT
	reverse	CCAGTACTTCCCATCCTTGA
TRAP	forward	GCTATCTGCGCTTCCACTAT
	reverse	GAGGCCTCGATGTAAGTGAC

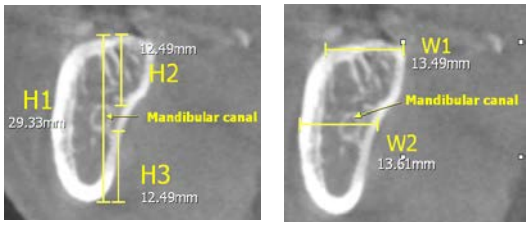
GAPDH was used as a reference gene for normalization and amplified in the separated tube. Isolated RNA was reverse transcribed to cDNA and used as the template in real time PCR by using the reverse transcription kit (Sensiscript®,Qiagen, Inc., USA) and the real time PCR Master Mix (SYBR® FAST Universal kit, Kapa Biosystems, Inc., USA) respectively.

Gene expression analysis

The gene expressions of all samples (n=25) were normalized with the reference gene, GAPDH. The relative quantification was performed according to the delta Ct method (Livak and Schnittgen, 2001), by using the gene expression analysis software (CFX Manager™, Bio-Rad, USA).

Computed tomography (CT)

The CT images of edentulous areas (n=18) were examined. The examinations were performed with cone beam computed tomography (CB MercuRay, Hitachi medico Technology Corp., Japan). The data were manipulated by CB work version 2.12 software. At the implant site, the cross-sectional image of the mandible was examined as described in Fig. 1. To test the precision, the measurements were repeated 3 times with no reference to the original data.



(a) The bone heights (b) The bone widths

Fig 1 (a) The measurements at the implant site: total bone height (H1) was the distance from the alveolar crest to the lower border of the mandible parallel to the mid-sagittal plane. Alveolar bone height (H2) was the distance from the alveolar crest to the superior border of mandibular canal. Basal bone height (H3) was the distance from the inferior border of mandibular canal to the lower border of the mandible. (b) Crestal bone width (W1) and the total bone width (W2) were the bucco-lingual bone width at superior portion of trabeculae bone and at mandibular canal level, respectively.

Data analysis

The edentulous bone heights were grouped into 2 groups: H1 $\leq$ 30 mm (n = 9) and H1 > 30 mm (n = 9). The Kruskal-Wallis Test was used for the statistical analysis among the median of the edentulous bone samples and the impacted tooth bone samples (n = 7), p value of <0.05 was considered significant. Correlations between the gene expressions and the radiographic measurements of edentulous bone samples were analyzed by Pearson's correlation analysis.

Results

From the data analysis of real time RT-PCR, the relative quantity gene expressions between the edentulous bone samples and the impacted tooth bone samples were different (Fig 2). Mean values of bone measurement data were described in table 2.

Table 2 Descriptive statistics of the radiographic measurements of the edentulous bones

	Min	Max	Mean	SD
H1 (mm)	17.01	53.06	32.6189	8.62331
H2 (mm)	9.45	28.00	17.8515	4.74086
H3 (mm)	5.38	21.17	11.1594	4.22050
H1/H3	2.03	4.48	3.0866	0.64198
W1 (mm)	2.76	16.19	6.4419	2.89423
W2 (mm)	5.62	27.53	13.9585	5.10329

The edentulous bone heights, H1, were grouped as previous described: H1 $\leq$ 30 mm (n = 9) and H1 > 30 mm (n = 9). The impacted tooth alveolar bone (n = 7) was compared to the edentulous bone groups.

There were no significant differences in the gene expressions between the groups, but there was significant difference in the ratio of RANKL to OPG between the groups ($p<0.05$) as described in table 3.

Multiple comparison analysis revealed significant differences of RANKL/OPG ratio between impacted tooth bone and H1 $\leq$ 30 mm group ($P<0.05$), and between impacted tooth bone and H1 > 30 mm group ($p<0.01$). The others had no significant differences.

Table 3 The Kruskal-Wallis test of gene expressions between the impacted tooth alveolar bone and the edentulous bone groups: H1 $\leq$ 30 mm and H1 > 30 mm, p value of <0.05 was considered significant

Gene	Group	Median	Mean Rank	Sig.
OPG	Impacted tooth bone	0.128160	15.00	0.307
	H1 $\leq$ 30 mm	0.010680	10.00	
	H1 > 30 mm	0.113610	14.44	
RUNX2	Impacted tooth bone	0.012980	14.29	0.460
	H1 $\leq$ 30 mm	0.002540	10.56	
	H1 > 30 mm	0.142570	14.44	
RANKL	Impacted tooth bone	0.020560	12.29	0.438
	H1 $\leq$ 30 mm	0.013770	11.11	
	H1 > 30 mm	0.419850	15.44	
CtsK	Impacted tooth bone	0.013560	14.00	0.136
	H1 $\leq$ 30 mm	0.001420	9.22	
	H1 > 30 mm	0.414580	16.00	
MMP9	Impacted tooth bone	0.004890	13.14	0.421
	H1 $\leq$ 30 mm	0.002140	10.67	
	H1 > 30 mm	0.042560	15.22	
TRAP	Impacted tooth bone	0.011090	13.43	0.601
	H1 $\leq$ 30 mm	0.001550	11.11	
	H1 > 30 mm	0.031080	14.56	
RANKL/OPG	Impacted tooth bone	0.195369	6.14	0.015
	H1 $\leq$ 30 mm	1.289720	15.44	
	H1 > 30 mm	1.336008	15.89	

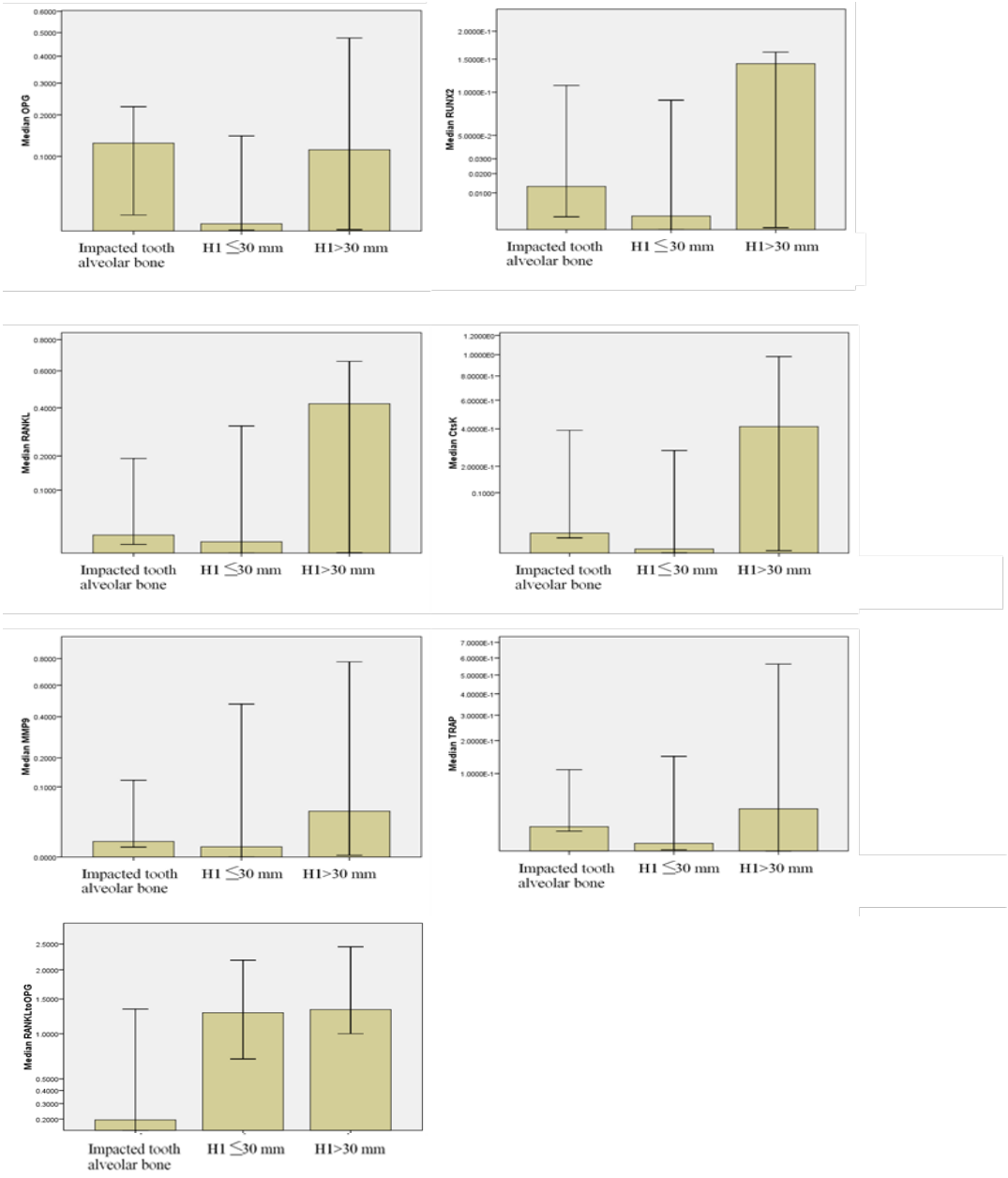


Fig 2 The medians of OPG, RUNX2, RANKL, CtsK, MMP9 and TRAP expression, and the ratio of RANKL to OPG between the impacted tooth alveolar bone and the edentulous bone groups. The edentulous bone which H1 > 30 mm expressed the highest level of RUNX2, RANKL, CtsK, MMP9 and TRAP, and had the highest RANKL/OPG ratio. The impacted tooth alveolar bone expressed the highest level of OPG.

There were the relationships between the measurement data and the gene expression profiles. Total bone height (H1) had significant positive correlations to OPG, RUNX2, CtsK, MMP9, TRAP ($p<0.05$) and RANKL ($p<0.01$). Alveolar bone height (H2) had significant positive correlations to OPG, RUNX2 and RANKL ($p<0.05$).Basal bone height (H3) had significant positive correlations to OPG, RUNX2, CtsK, MMP9, TRAP ($p<0.05$) and RANKL ($p<0.01$). The ratio of total bone height (H1) to basal

bone height (H3) had no significant correlation with any target gene. Crestal bone width (W1) had significant positive correlation to the ratio of RANKL to OPG ($p<0.01$). Total bone width (W2) had no significant correlation with any target gene. The result of correlations was shown in the table 4.

Table 4 Correlations between the measurement data and the gene expression profiles of the edentulous bone: Total bone height (H1) and basal bone height (H3) had significant correlations to all target genes. Alveolar bone height (H2) had significant correlations to OPG, RUNX2 and RANK. The ratio of total bone height (H1) to basal bone height (H3) had no significant correlation with any target gene. Crestal bone width (W1) had significant correlation to the ratio of RANKL to OPG, and total bone width (W2) had no significant correlation with any target gene

		OPG	RUNX2	RANKL	CtsK	MMP9	TRAP	RANKL /OPG
H1	Correlation	0.573*	0.570*	0.653**	0.512*	0.541*	0.538*	0.353
	Sig.	0.013	0.014	0.003	0.030	0.020	0.021	0.150
H2	Correlation	0.547*	0.485*	0.540*	0.396	0.426	0.424	0.227
	Sig.	0.019	0.041	0.021	0.104	0.078	0.080	0.366
H3	Correlation	0.522*	0.544*	0.641**	0.510*	0.541*	0.531*	0.402
	Sig.	0.026	0.020	0.004	0.031	0.020	0.023	0.098
H1/H3	Correlation	-0.130	-0.191	-0.278	-0.240	-0.253	-0.241	-0.281
	Sig.	0.606	0.447	0.265	0.337	0.311	0.335	0.259
W1	Correlation	0.053	0.120	0.331	0.169	0.166	0.180	0.633**
	Sig.	0.833	0.635	0.179	0.503	0.509	0.475	0.005
W2	Correlation	0.168	0.193	0.146	0.180	0.192	0.194	-0.099
	Sig.	0.505	0.442	0.563	0.474	0.445	0.442	0.697

Discussion

To date, no single factor alone has been stated to contribute the resorption of the bone after tooth extraction. Our strategies were to examine the gene expression associated with the bone remodeling at the edentulous area, and to compare the gene expression profiles between the groups of different edentulous bone heights and the alveolar bone from impacted tooth.

From the results, the highest OPG level was expressed from the impacted tooth alveolar bone. Although, there was no significant difference, but it might be explained by the basis of tooth eruption. The impacted tooth is the tooth that fails to erupt in the right position at the right time. The eruption is a process that involves the interaction of cells of the tooth structure, dental follicle, and alveolar bone (Wise et al., 2002). At the specific time, the reduction

of OPG in dental follicle allows for osteoclast formation in alveolar bone (Wise et al., 2000b), then other signaling cascades regulate the bone remodeling during the eruption.

Since our data showed that the impacted tooth alveolar bone not only expressed the higher OPG level, but also expressed lower RANKL, MMP9, CtsK and TRAP than the edentulous bone, it could be suggested that the bone in the impacted tooth areas were less active in resorptive remodeling than the edentulous bone site. This was confirmed by the RANKL/OPG ratio which was significantly higher in the edentulous bone than the impacted tooth alveolar bone.

The higher expressions of these genes in the edentulous bone could be explained by our bone collecting time. In this study, the samples were collected more than six months after tooth extraction. Most of the dimensional changes of the alveolar bone after tooth extraction took place during the first three months of healing (Johnson et al., 1969; Schropp et al, 2003), but gradually loss throughout the life resulting in the loss of alveolar bone, which the loss in width greater than the loss in height (Tan et al., 2009).

There was no significant difference of gene expression between the different edentulous bone heights: $\leq$ 30 mm and > 30 mm. This might be caused by all bone samples were collected from the minimally to moderate compromised edentulous jaw (Thomas et al.,2002) as a result of the limitation in collecting the bone mass from severe resorption ridge, which might show other significant data.

It has been noticed that the ratio of RANKL to OPG did not significantly correlate to the bone height, but to the crestal bone width (W1). It might be implied that at the time more than six months after tooth extraction, the more RANKL overexpressed, the more alveolar crest width revealed. It was supported by the previous studies stated that the crest of the residual ridge and the sharp edges of the alveolar processes are reduced after tooth loss (Atwood, 1963; Pietrokovski and Massler, 1967; Enlow et al., 1976), and the recent study reported that the bone width closed to the mandibular canal was greater than the bone width at the alveolar crest (Braut et al., 2012). Furthermore, there were significant positive correlations ($p<0.05$) between all target gene expressions to the total bone height (H1) and the basal bone height (H3).

Since the panoramic radiographic studies revealed that the distance from the lower edge of mental foramen to the lower border of mandible is about one third of the total height of mandible, the original and the reduction in height of bone then be estimated (Wical and Swoope, 1974). There was longitudinal study (Packota et al., 1988) relied on this one-third distance to calculate the bone loss that had occurred. In addition, the recent study reported that the alveolar bone height of the posterior mandible was relatively constant (Braut et al., 2012). From the reported studies, to eliminate the difference according to the individual jaw size, we measured the distance from the mandibular canal and estimated the resorption by calculating the ratio of the total bone height (H1) to the basal bone height (H3). However, the ratio did not significantly correlate with the gene expressions.

There was no significant correlation between the total edentulous bone width (W2) and the gene expression. It might be postulated that there was approximately constant of bone remodeling, and the

major loss in bone width occurred in the superior part to the mandibular canal.

The obstacle of this study was the RNA isolation from the mandibular bone particle. The bone particle was very hard texture and rich in mineral, but small number of cells. We found that the stabilizing reagent could not penetrate throughout the bony tissue resulting in dramatically degradation of total RNA. The best yield was received from the bead based homogenizing which the bead tube was pre-chilled in liquid nitrogen before homogenization.

We suggested that this study should be extended in the future to collect samples from the more aggressive bone resoption and the shorter period of time after tooth extraction. It might give more data to be used as the guideline for the treatment plan.

Conclusions

This study gave the descriptive data of the gene expressions from the edentulous bone and the alveolar bone of impacted tooth. To compare with the groups of edentulous bone, more than 6 months after tooth extraction, the edentulous bone had significantly higher level of RANKL/OPG ratio than the impacted tooth alveolar bone. Furthermore, there was significant positive correlation between the crestal bone width and RANKL/OPG ratio in the edentulous bone.

Acknowledgement

The author would like to acknowledge Assistant Professor Dr.Atiphan Pimkhaokham, Assistant Professor Dr.Keskanya Subbalekha, Department of Oral and Maxillofacial Surgery, Associate Professor Dr.Mansuang Arksornnukit and Assistant Professor Dr.Pravej Serichetaphongse, Department of Prosthodontics, for their kindly suggestion and helping in sample collection. Furthermore, I would like to thank Associate Professor Dr. Prasit Pavasant, Research Unit for Mineralized Tissue, Department of Anatomy, for facilitating lab session.

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