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THE ANALYSIS OF GENE EXPRESSION ASSOCIATED
WITH
ALVEOLAR BONE RESORPTION

Miss Tawanchai Wangsai

จุฬาลงกรณ์มหาวิทยาลัย
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A Thesis Submitted in Partial Fulfillment of the Requirements
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การละลายตัวของกระดูกหลังถอนฟันเป็นส่วนหนึ่งของปัญหาซึ่งก่อให้เกิดอุปสรรคทางการรักษาทางทันตกรรมประดิษฐ์ โดยเฉพาะในขากรรไกรล่าง ความไม่สมดุลระหว่างการแสดงออกของยีนมีบทบาทสำคัญในกระบวนการสร้างและการละลายของกระดูก ซึ่งก่อให้เกิดการละลายของกระดูกขากรรไกรหลังถอนฟัน เป็นที่ทราบกันดีว่ายีน RANKL OPG MMP9 CtsK TRAP และ RUNX2 มีความเกี่ยวข้องกับกระบวนการสร้างและการละลายของกระดูก นำมาซึ่งวัตถุประสงค์ในการศึกษาครั้งนี้คือ เพื่อตรวจสอบการแสดงออกของยีน RANKL OPG MMP9 CtsK TRAP และ RUNX2 และเพื่อเปรียบเทียบความแตกต่างของการแสดงออกของยีนดังกล่าวในกลุ่มสันกระดูกว่างที่มีระดับการละลายตัวที่แตกต่างกัน และกลุ่มกระดูกรอบรากฟันคุด การศึกษานี้ได้ทำการเก็บตัวอย่างกระดูกจากสันกระดูกว่างบริเวณฟันหลังของขากรรไกรล่างในขั้นตอนการผ่าตัดฝังรากเทียม จำนวน 18 ตัวอย่าง และจากกระดูกรอบรากฟันกรามซี่ที่สามล่าง ในขั้นตอนการผ่าฟันคุด จำนวน 7 ตัวอย่าง ชิ้นกระดูกตัวอย่างถูกนำมาปั่นบด จากนั้นทำการคัดแยก mRNA และเปลี่ยนกลับเป็น cDNA เพื่อใช้เป็นแม่พิมพ์สำหรับไพรเมอร์ของยีนที่เฉพาะเจาะจงในกระบวนการปฏิกิริยาลูกโซ่พอลิเมอเรสชนิดย้อนกลับแบบเรียลไทม์ ทำการวัดรูปร่างของกระดูกบริเวณสันกระดูกว่างจำนวน 18 ตัวอย่าง ที่ตำแหน่งฝังรากเทียมโดยใช้ข้อมูลจากภาพรังสีคอมพิวเตอร์ ทำการวิเคราะห์ข้อมูลโดยจัดกลุ่มการละลายของกระดูกบริเวณสันกระดูกว่างตามอัตราส่วนระหว่างความสูงทั้งหมดของกระดูกขากรรไกรต่อความสูงกระดูกขากรรไกรส่วนล่างใต้ต่อคลองเส้นประสาทขากรรไกรล่าง โดยค่าอัตราส่วนที่น้อยกว่า 3.0 จัดเป็นกลุ่มกระดูกละลายมากและ ค่ามากกว่า 3.0 จัดเป็นกลุ่มกระดูกละลายน้อย ผลการวิเคราะห์พบว่า การแสดงออกของยีน RANKL OPG MMP9 CtsK TRAP และ RUNX2 มีความแตกต่างกันระหว่างกลุ่มสันกระดูกว่างและกระดูกรอบรากฟันคุด โดยกลุ่มกระดูกรอบรากฟันคุดมีค่าการแสดงออกของยีน RANKL OPG MMP9 CtsK TRAP และ RUNX2 สูงกว่ากลุ่มสันกระดูกละลายน้อย และกลุ่มสันกระดูกละลายมากตามลำดับ ค่าอัตราส่วนระหว่างการแสดงออกของยีน RANKL ต่อ OPG มีค่าสูงสุดในกลุ่มกระดูกรอบรากฟันคุด โดยสูงกว่ากลุ่มกระดูกละลายน้อยอย่างมีนัยสำคัญ และกลุ่มกระดูกละลายมากมีอัตราส่วนระหว่างการแสดงออกของยีน RANKL ต่อ OPG มากกว่ากลุ่มกระดูกละลายน้อยอย่างมีนัยสำคัญ นอกจากนั้นยังพบความสำพันธ์เชิงบวกระหว่างความกว้างของสันกระดูกส่วนบนกับอัตราส่วนระหว่างการแสดงออกของยีน RANKL ต่อ OPG อย่างมีนัยสำคัญ การศึกษาในครั้งนี้ได้แสดงข้อมูลที่อาจช่วยเป็นแนวทางสำหรับการคัดแยกผู้ป่วยที่มีความเสี่ยงต่อสภาวะการละลายตัวของกระดูกขากรรไกรล่างอย่างรุนแรง เพื่อให้ได้รับการรักษาที่เหมาะสมโดยเฉพาะการใช้รากเทียมช่วยในงานใส่ฟันเทียม

Chulalongkorn University

ภาควิชา ทันตกรรมประดิษฐ์ ลายมือชื่อนิสิต ....

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5475812432 : MAJOR PROSTHODONTICS
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TAWANCHAI WANGSAI: THE ANALYSIS OF GENE EXPRESSION ASSOCIATED WITH ALVEOLAR BONE RESORPTION. ADVISOR: DR.JAIJAM SUWANWELA, 63 pp.

Alveolar bone resorption after tooth extraction is one of the problems that causes the difficulty in prosthetic treatment especially in mandible. Imbalance of gene expression plays an important role in bone remodeling process that leads to the atrophic mandible. RANKL, OPG, MMP9 CtsK TRAP and RUNX2 are known as the gene associated with the bone remodeling process. To examine the expression of the genes associated with the mandibular bone resorption, and to explore the differences in expression between the resorption levels and the alveolar bone of the impacted tooth, the bone samples were collected from the edentulous areas of the patients who underwent dental implant placement (n=18) and from the impacted tooth areas of the patient who had lower third molar surgical removal (n=7). The bone was homogenized then mRNA was isolated before converting to cDNA. cDNA was used with the specific primers for our interested genes in quantitative RT-PCR. The bone dimensions were measured in CT scan at the implant site of the edentulous areas (n=18). The edentulous bone resorption levels were classified by the ratio of total bone height to basal bone height: <3.0 = high resorption bone (n = 9) and >3.0 = low resorption bone (n = 9). All target gene expressions were significantly different between the edentulous bone groups and the impacted tooth alveolar bone (Kruskal-Wallis test, $p < 0.05$). The highest median of RANKL, OPG, MMP9 CtsK TRAP and RUNX2 were in the impacted tooth alveolar bone followed by the low resorption bone and the high resorption bone, respectively. The ratio of RANKL to OPG expression was highest in alveolar bone of the impacted tooth which significantly higher than the low resorption bone (Mann-Whitney U test, $p<0.05$). The high resorption bone had the ratio of RANKL to OPG expression significantly higher than the low resorption bone (Mann-Whitney U test, $p<0.05$). The relationship between the CT measurement data and the gene expressions has shown the significant positive correlation between the crestal bone width and the RANKL/OPG ratio (Pearson's correlation, $p<0.01$). Our analysis provided the data the might be useful to classify the patient who get risk of bone resorption to have the proper treatment procedures such as dental implant assisted removable denture.

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Field of Study: Prosthodontics
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Student's Signature ....
Advisor's Signature ....

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LIST OF ABBREVIATIONS

CtsK	Cathepsin K
M-CSF	Macrophage colony-stimulating factor
MMP9	Matrix metalloprotease 9
OPG	Osteoprotegerin
PTH	Parathyroid hormone
RANK	Receptor activator of nuclear factor kappa B
RANKL	Receptor activator of nuclear factor kappa B ligand
RT-PCR	Reverse transcription polymerase chain reaction
RUNX2	Runt-related transcription factor 2
TRAP	Tartrate resistant acid phosphatase

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CHAPTER I INTRODUCTION

Demographically, the national trends of tooth loss is different, but the prevalence of people who lost their teeth due to dental caries, periodontal disease and trauma are similarly increasing with age (Sanchez-Garcia et al., 2014; Wu, Liang, Landerman, & Plassman, 2013). From the report in 2013 of the Bureau of Dental Health, Ministry of Public Health, Thailand has shown that the percentage of the people who had at least 20 teeth was decreased by 40% when the age was increased from 35-44 year-old to 60-74 year-old, and there was 7.2 % of the 60 – 74 year-old people who had fully edentulous arch.

When the teeth loss, the physiologic masticatory forces transferred via tooth root to the jaw bone are no longer persisted (Devlin & Ferguson, 1991), and the residual ridge undergoes continuing remodeling changes. The residual ridge resorption is a chronic, progressive and irreversible process (Pietrokovski, 2013). 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, the most rapid reduction is in the first few months to six months in some reports (Douglas Allen Atwood, 1963; Carlsson & Persson, 1967; Tallgren, 1966). The resorption of alveolar ridge affects the retention, stability and support of prostheses especially the removable denture that relies on the quantity and the architecture of jaw bone (Jacobson & Krol, 1983a, 1983b, 1983c). The patients who have resorbed alveolar ridge are often dissatisfied especially from the masticatory function, thus the progression of bone resorption becomes the major awareness (Huumonen et al., 2012).

Dental implant has been utilized to assist the denture in severely resorbed ridge which can greatly improve the efficiency of prosthesis. Implant supported or retained overdenture provides significant improvement in masticatory performance

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compared to the conventional completed denture (Allen & McMillan, 2003; Fueki, Kimoto, Ogawa, & Garrett, 2007). The remaining bone quality including morphology, density and quantity of the bone is important for the success of the implant (Mecall & Rosenfeld, 1991, 1992; Pietrokovski, Starinsky, Arensburg, & Kaffe, 2007).

The abnormal bone resorptions which occurred in many bone diseases such as osteoporosis, Paget's disease or hormonal imbalance have been frequently reported. The information of genes involved in the diseases has been reported but still insufficient. In jaw bone resorption, after tooth extraction, the size of the residual ridge is gradually reduced, resulting in decreasing a large amount of the jaw structure. This phenomenon has been called residual ridge reduction (Jahangiri, Devlin, Ting, & Nishimura, 1998; Suwanwela et al., 2011).

There are many factors involved in the jaw bone resorption such as anatomic factor, prosthetic factor, metabolic factor and functional factor (Douglas Allen Atwood, 1962; D. A. Atwood, 1979). Genetics is one of the impact factors influences the bone resorption (Jahangiri et al., 1998; Suwanwela et al., 2011). In the last few decades, genetic studies have been expanded extensively as a result of the completion of whole genome sequencing and the development in techniques and instruments. This provides the opportunities to discover novel information that is useful to predict and help in the abnormal conditions or diseases treatment plan.

This study investigated the genetic data, bone gene expression data of the genes associated with alveolar bone resorption and impacted tooth combining with the phenotypic data, bone morphology data from cone beam computed tomography (CBCT). The results are novel and very specific. This give us more insight to understand the process of bone remodeling after tooth extraction.

CHAPTER II REVIEW OF LITERATURE

Bone remodeling

Bone is a connective tissue which consists of two phase of mineralized extracellular matrix. The organic phase are type I collagen and non-collagenous proteins (Hill, 1998), and the inorganic phase consists of calcium and phosphorus in the form of hydroxyapatite crystals (Spector, 1994).

Bone tissue is formed through either intramembranous or endochondral ossification (Karsenty, 1999). In intramembranous ossification, which is the formation of skull, jaw and flat bones, osteoblasts differentiate directly from mesenchymal cells to form new bone without formation of a cartilaginous template. The endochondral ossification, which is the formation of long bone, mesenchymal cells differentiate to chondrocytes first and then proliferate and differentiate to form cartilaginous template before changing into mineralized bone (Chung, Lanske, Lee, Li, & Kronenberg, 1998).

Bone remodeling process consist of two major phases: the formation phase and the resorption phases. Under normal conditions, during the first three decades of life, the bone remodeling occurs without significantly change, and the bone mass remains relatively constant (Eriksen, 1986; Ortman, 1962). As time passes, there are many factors adversely affect to the bone structure, and significant bone loss occurred by the imbalance in the bone formation and resortpion (Y, Valds-Flores, Orozco, & Velzquez-Cruz, 2013) leading to bone loss in many diseases such as osteoporosis, some metastatic carcinoma and multiple myeloma (Fohr, Dunstan, & Seibel, 2003).

In the bone formation phase, osteoblast cells which derived from mesenchymal stem cells deposit the bone organic phase, collagenous bone matrix to enhance bone mineralization (Aubin, 1998). Mature osteoblasts undergo apoptosis

and differentiate into osteocytes or become inactive bone lining cells. Osteocytes which differentiated from osteoblasts are in the bone matrix, inside the lacuna spaces. They have mechanosensors which respond to the mechanical signals and further generate the signals to other cells involved in bone formation and resorption (Cowin, Moss-Salentijn, & Moss, 1991; Lanyon, 1993).

In the resorption phase, the clastic cells are responsible for the resorption of mineralized matrix of bone, dental hard tissue and calcified cartilage. All clastic cells derived from hematopoietic stem cells that will proliferate and differentiate into the mature clastic cells. The osteoclasts, one of the clastic cells, are responsible in general bone resorption processes, and the tooth eruption also requires osteoclast to form an eruption pathway (Wise, Frazier-Bowers, & D'Souza, 2002). In different bone sites, osteoclasts have the same function, but different in some site-specific characters. 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). In long bone, osteoclasts are formed faster than the jaw bone because of the difference in the cellular composition of bone marrow. On the other hand, the jaw osteoclasts are larger in sizes and have more tartrate resistant acid phosphatase (TRAP) activity (de Souza Faloni et al., 2011).

TRAP is mainly expressed in multinucleated cells, macrophages, and also in some epithelial cells and neurons (Lang, Schultzberg, & Andersson, 2001). The enzyme has been extensively used as a marker for osteoclast, and it has been suggested that TRAP could be used as a marker for pathological bone resorptive condition (Halleen et al., 2000). TRAP promotes the destruction of collagen by generating destructive reactive oxygen species into the resorption lacunae (Bonucci & Nanci, 2001; Halleen et al., 1999). Figure 1 shows the origin of the bone cells: osteoblast, osteoclast and osteocyte.

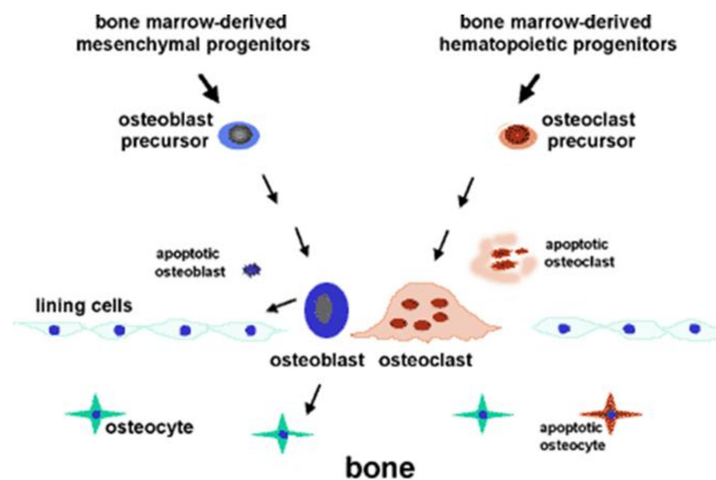

Figure 1 The origin of bone cells (Oursler & Bellido, 2003)

The cells involved in bone remodeling

Osteoclast

In low physical activity and fractured bone condition, calcium serum levels are decreased leading to stimulate the parathyroid hormone (PTH) from the parathyroid glands (Bronner, Farach-Carson, & Farach-Carson, 2003). PTH promotes bone resorption by increasing osteoclastogenesis and the resorptive activity of mature osteoclasts. The osteoclastogenesis requires growth factors which mainly are macrophage-colony stimulating factor (M-CSF) and receptor activator of nuclear factor kappa B ligand (RANKL). M-CSF is secreted by many kind of cells including osteoblasts, fibroblasts, monocytes and endothelial cells in response to resorptive stimuli such as PTH (Hofstetter, Balaga, Jost-Al Brecht , Leunig, & Felix, 2003). (Weir et al., 1993). M-CSF induces the expression of receptor activator of nuclear factor kappa B (RANK), the receptor for RANKL (Yasuda, Shima, Nakagawa, Yamaguchi, et al., 1998), and activates other genes which are required for osteoclast differentiation (Kawaguchi and Noda, 2000). RANKL is on the surface of osteoblast cell. It induces actin ring formation and

cytoskeletal rearrangement resulting in increasing osteoclast cell function (Burgess et al., 1999; Fuller, Wong, Fox, Choi, & Chambers, 1998; Yasuda, Shima, Nakagawa, Yamaguchi, et al., 1998). The RANKL/RANK interaction is influenced by another factor called osteoprotegerin (OPG) that expressed from many tissue sites. OPG can also bind to RANKL, and the binding results in inhibition of osteoblast and osteoclast cell-to-cell signaling (Coetzee & Kruger, 2004; Yasuda, Shima, Nakagawa, Mochizuki, et al., 1998) (Figure 2). OPG is known as the bone protector by limiting osteoclast formation and activity (Simonet et al., 1997).

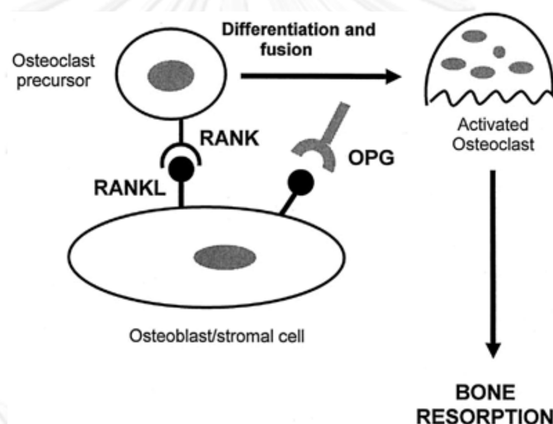

Figure 2 The relationship among RANK/RANKL/OPG system

RANKL generates a signal through RANK expressed on osteoclast progenitor cells. OPG counteracts this communication by competitive binding and neutralizing RANKL (Coetzee & Kruger, 2004).

The balance of expressions between RANKL and OPG plays an important role in the differentiation of osteoclast (Faccio et al., 2005; Geusens, 2012). Disruption of this balance can lead to inappropriate levels of osteoclast formation and excessive bone loss. The binding of RANKL to RANK also promotes other genes that regulate the expression of osteoclastic genes (B. Boyce & Xing, 2007).

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Osteoclast in Bone resorption

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 which is rich in actin and cell adhesion molecules (Fisher et al., 1993; Schlesinger, Blair, Teitelbaum, & Edwards, 1997). In the ruffle membrane, osteoclast synthesizes hydrochloric acid from vacuolar H^+-ATPases and chloride channels (Edwards, Cohen, Xu, & Schlesinger, 2006) which secrete H^+ and Cl^- ions into the sealing zone to form HCl to dissolve hydroxyapatite (Vaananen & Laitala-Leinonen, 2008), then proteases such as cathepsin and matrix metalloprotease (MMPs) could reach and degrade bone organic matrix (Troen, 2006).

Previous studies have discovered that cathepsin K (Ctsk) and matrix metalloproteinase (MMPs), especially MMP-9, are the main proteases in the resorption process (Sundaram et al., 2007). It was discovered that humans lacking CtsK exhibit pycnodysostosis, which is characterized by short skeleton and osteosclerosis (Utokpat, Panmontha, Tongkobpetch, Suphapeetiporn, & Shotelersuk, 2013; Xue et al., 2011). CtsK and MMPs are transported via lysosomes from the endoplasmic reticulum, then released into the resorption lacuna (Delaisse et al., 2003). MMPs are considered to be necessary for the migration of precursor and immature osteoclasts to the bone surface (Inui et al., 1999), while CtsK degrades type I collagen and other matrix proteins (Figure 3) (Troen, 2004). CtsK is stimulated and up-regulated by RANKL in human osteoclasts (Motyckova et al., 2001). Another study reported that the higher expression of CtsK, MMP-9, TRAP, RANKL, OPG, and osteocalcin, and the interaction between them associated with the higher bone resorption and formation in osteoarthritis patient (Logar et al., 2007).

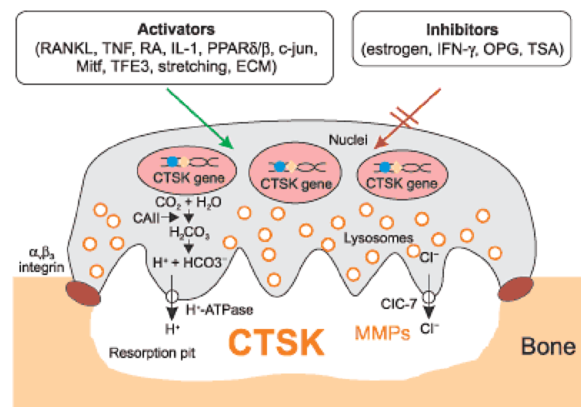

Figure 3 The role of CtsK and MMPs in bone resorption

CtsK and MMPs are transported via lysosomes from the endoplasmic reticulum, then released into the resorption lacuna (Delaisse et al., 2003; Troen, 2004).

Osteoblast

Osteoblasts response for the deposition and mineralization of bone matrix. They differentiate from mesenchymal stem cells by signaling from several transcription factors especially Runt-related transcription factor 2 (RUNX2) (Wang et al., 2014). Osteoblasts express receptors for hormones including PTH (Dempster, Cosman, Parisien, Shen, & Lindsay, 1993), estrogen (B. F. Boyce, Hughes, Wright, Xing, & Dai, 1999) and corticosteroids. Furthermore, osteoblasts release of RANKL which plays an important role in osteoclast differentiation (Yamashita, Takahashi, & Udagawa, 2012).

RUNX2 has been considered as a key transcription factor for promoting early stages of osteoblast differentiation (Karsenty, 2007). RUNX2 starts its function in pre-osteoblasts, up-regulated in immature osteoblasts and down-regulated in mature osteoblasts. It activates genes that enhance osteoblast differentiation and promote cell maturation (Pratap et al., 2003). The previous study has shown that bone mineral density (BMD) and peak bone mass (PBM) in human are associated with RUNX2 expression (Zanatta, Valenti, Donatelli, Zucal, & Dalle Carbonare, 2012). The

communication among osteoblast and stromal cells influence the osteoclast cell differentiation through their receptors.

Osteocyte

Osteocytes are derived from osteoblasts and located within the bone matrix,. They perform many roles in bone biology (Dallas & Bonewald, 2010), including detection in mechanical loading change. Osteocytes have cellular projections which are located in the canaliculi in the mineralised matrix. The cellular projections allow osteocytes to communicate with other cells and the bone surface (Aarden, Burger, & Nijweide, 1994).

Post-extraction remodeling and residual ridge resorption

After tooth extraction, the consequence of inflammatory reactions is immediately occurred. Blood clot transforms to be a granulation tissue within a few days, then epithelial cells penetrate into the granulation tissue within the first week and the soft tissue is restored.

The proliferate and differentiate osteoprogenitor cells migrate from the ruptured periodontal ligament and from woven bone (Kingsmill, 1999). The osteoid of the new bone can be detected at the bottom of the socket by the end of first week and mineralized after 3 weeks. After 6 weeks, trabecular bone formation is observed (Kubilius, Kubilius, & Gleiznys, 2012).

After healing of the wounds, residual ridge remodeling results in loss of the alveolar bone crest, simultaneously during the first 3 months (Schropp, Wenzel, Kostopoulos, & Karring, 2003). After 6 months, it has been reported that the mean reduction of the alveolar bone height is 0.2 mm more apical to the baseline at the mesial and distal sides of the extracted tooth (Schropp et al., 2003). Atraumatic tooth

extraction can preserve alveolar bone and also has less destruction to mucoperiostium which can decrease the amount of bone loss (Fickl, Zuhr, Wachtel, Bolz, & Huerzeler, 2008).

The resorption of maxillary and mandibular bone are different. The pattern of bone loss in maxilla results in reduction in palatal width and length as well as height (D. M. Watt & Likeman, 1974). While in mandible, the majority loss occurs from the labial aspect anteriorly and from the lingual aspect posteriorly. The vertical height of mandible in dentate patient has been examined in previous study. The basal bone from the lower edge of mental foramen to the lower border of mandible is about one-third in proportion to the total mandibular bone height (Wical & Swoope, 1974). In edentulism, the vertical height loss is greatest in the anterior region. Overall, the average arch width of edentulous mandible is greater than that of edentulous maxilla (Carlsson & Persson, 1967; Parkinson, 1978; David M. Watt & MacGregor, 1986). The pattern of residual ridge resorptions were classified into six stages as described in Figure 4. (Douglas Allen Atwood, 1963; Cawood & Howell, 1988). These patterns of bone loss have found to be constant intra-individual, but there are inter-individual variations (Tallgren, 1972).

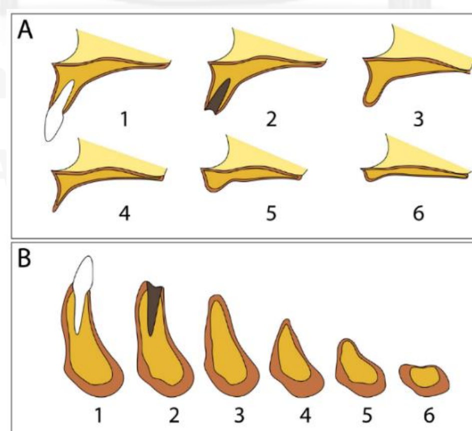

Figure 4 Bone loss following tooth extraction

Bone loss in maxilla (A) and mandible (B) were classified into 1. Pre-extraction stage, 2. Post-extraction stage, 3. High well rounded ridge stage, 4. Knife-edge shaped ridge

stage, 5. Low well-rounded ridge stage and 6. Depressed bone level stage (Cawood & Howell, 1988).

A few in vitro studies have revealed the association between genes and alveolar bone loss, for examples, the inhibition of RANKL by administration of OPG reduced osteoclastogenesis and inhibited alveolar bone destruction and caused a decrease in alveolar bone loss in several models of periodontal disease (Jin et al., 2007; Teng et al., 2000).

Quantitative RT-PCR (real-time RT-PCR)

Several methods have been used to study gene profiles. Quantitative or real-time reverse transcription PCR, qRT-PCR, is one of the well-known techniques that becomes a standard technology for quantification of nucleic acids (Carter et al., 2012; Fleige et al., 2006). The advantages of qRT-PCR are the possibility to monitor the production of amplification products during each cycle of PCR and the possibility to allow abundance target genes and samples analysis with less inter-assay variation than conventional PCR which increases the reliable results. qRT-PCR including 2 main steps: the reverse transcription step, converts mRNA to cDNA, and PCR step, the amplification and detection of target gene. Detection chemistry can be either specific, probe, or non-specific, non-probe based. The non-probe based chemistry which widely used is SYBR green dye. The binding of the dye to the single strand cDNA would be unbound during the cycle and exhibits the fluorescence signal that can be detected by the sensor. The quantification output can be calculated from the copy number of unknown target relative to an external standard curve. The external standard curve is the linear regression of the threshold cycle (C_T) of the unknown sample dilution series, while the internal standard is the comparison of the C_T of the target sample to the reference

gene, then the product can be compared between samples (Bustin, Benes, Nolan, & Pfaffl, 2005).

In this study, specific marker genes: RANKL, OPG, TRAP, MMP-9, CtsK and RUNX2 were observed by qRT-PCR technique. The gene expression profiles of the edentulous bone samples and alveolar bone samples of impacted teeth were compared.

Cone beam computed tomography (CBCT)

There are several radiographic methods to achieve the anatomical data. In implant dentistry, the precise position and direction of the implant are necessary to avoid surgical complications such as nerve injury, bone and sinus perforations. 2-dimensional image from periapical or panoramic radiograph cannot represent 3-dimensional object and provide insufficient information for dental implant treatment plan. Cone beam computed tomography (CBCT) has been used in dentistry since 1999, CBCT is the technic using a scanner, a divergent conical shape radiation source with a sensor. The scanner rotates 360° around the region of interest resulting in hundreds of raw image data which are reconstructed into a digital volume and can be visualized by the computer software. CBCT gives 3-dimensional data which clinician can examine the cross sectional image of the bone at the implant site. Furthermore, this technique is less expensive and uses lower dose of radiation than medical CT (J. Gupta & Ali, 2013; Hatcher, 2010; Scarfe, Li, Aboelmaaty, Scott, & Farman, 2012). Since CBCT image was considered to be the most consistent with direct measurements (Peker, Alkurt, & Michcioglu, 2008), the gene expression of edentulous bones were also correlated to their skeletal bone profiles from the CBCT scans.

In this study, we used gene expression data combining with bone morphology data from CBCT scan to find a correlation between the genotype and the phenotype.

RESEARCH QUESTIONS

Are there any differences in the expressions of OPG, RUNX2, RANKL, CtsK, MMP9 and TRAP among the mandibular alveolar bones which have different residual ridge resorption levels and the impacted tooth alveolar bones?

Are there any associations between the gene expression levels and the skeletal bone profiles from the cone beam computed tomography scan (CBCT)?

RESEARCH OBJECTIVES

The objectives of this study were to examine the expressions of OPG, RUNX2, RANKL, CtsK, MMP9 and TRAP in mandibular alveolar bones which have different residual ridge resorption levels and impacted tooth alveolar bones by using the quantitative reverse transcription PCR (qRT-PCR) technique, and to determine the associations between the gene expressions and the skeletal bone profiles measured from the cone beam computed tomography (CBCT).

HYPOTHESES

H_0: There are no differences in the expressions of OPG, RUNX2, RANKL, CtsK, MMP9 and TRAP in mandibular alveolar bones which have different residual ridge resorption levels and impacted tooth alveolar bones.

There are no associations between the gene expression levels and the skeletal bone profiles from the cone beam computed tomography scan (CBCT).

H_A: There are differences in in the expressions of OPG, RUNX2, RANKL, CtsK, MMP9 and TRAP in mandibular alveolar bones which have different residual ridge resorption levels and impacted tooth alveolar bones.

There are associations between the gene expression levels and the skeletal bone profiles from the cone beam computed tomography scan (CBCT).

EXPECTED BENEFIT

This study is the first in vivo study that reveals the gene expression of marker genes associated with bone remodeling in RNA level which is specific to bone site. This will provide the genetic information of the alveolar bone resorption. It could be used as the additive information to predict the pathologic bone resorption before having optimum prosthetic treatment plan, especially when the plan has implant involved.

CHAPTER III RESEARCH METHODOLOGY

Bone sample

Eighteen bone samples from edentulous area of the posterior mandibular regions from first premolar to second molar which had been extracted for more than 6 months, and seven bone samples from the lower third molar alveolar regions were collected. The posterior mandibular alveolar bone was collected during the dental implant placement surgery. The lower third molar alveolar bone was collected during the surgical removal of the lower third molar impaction. All the subject had signed the consent forms. The inclusion criteria for the subjects are male or female with the age over 25 years, the tooth was no medical history of hypertension, diabetes mellitus or medical conditions which affect the bone metabolism, no history of periodontitis, no drug use that influence bone metabolism.

The exclusion criteria for the subjects are subjects that have previous trauma to the jaw and the subjects that have worn any removable prostheses.

After collecting the bone from surgical sites, the bone particles were stored in cryotubes and freezed in liquid nitrogen immediately to preserve the RNA (Figure 5).

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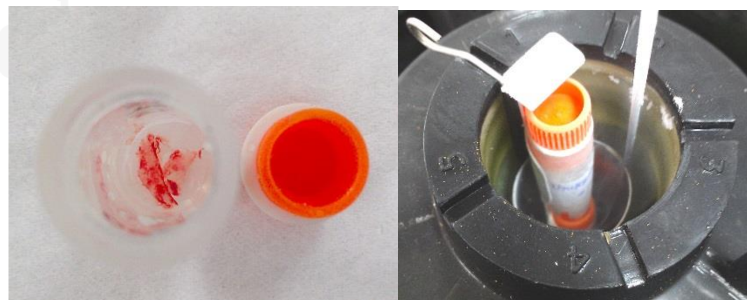

Figure 5 The bone particles

The bone particles collected from the surgical procedure and stored in the cryotube before submerged in liquid nitrogen.

Bone homogenization and cell lysis

To isolate total RNA, 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, Kilroy, Gimble, & Floyd, 2012). The Lysis reagent was also pre-chilled on ice. One ml of the pre-chilled lysis reagent was added to the microtube containing bone particles and beads. The bone was homogenized in homogenizer by 3,500 RPM speed, 2 x 45 sec. number of cycles x time and 30 sec. pause between cycles.

Figure 6 Homogenization

The 2.38 mm metal beads in the 2.0 ml impact resistant plastic tube. The bone particles were transferred into the tube and homogenized within the lysis reagent by the bead-based homogenizer.

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RNA isolation

mRNA was extracted by the spin-column based method (PureLink® RNA Mini Kit, Life Technologies, Inc., USA). Followed the manufacturer protocol.

Phase Separation

After the bone homogenization and cell lysis, the RNA was isolated using the following protocol:

1. Incubate the lysate with lysis reagent at room temperature for 5 minutes to allow complete dissociation of nucleoprotein complexes.
2. Add 0.2 ml chloroform per 1 ml lysis reagent used. Shake the tube vigorously by hand for 15 seconds.
3. Incubate at room temperature for 2–3 minutes.
4. Centrifuge the sample at 12,000 x *g* for 15 minutes at 4°C.

After centrifugation, the mixture separates into a lower, red phenol–chloroform phase, an interphase, and a colorless upper aqueous phase which contains the RNA. The volume of the aqueous upper phase is ~600 μl.

5. Transfer ~600 μl of the colorless, upper phase containing the RNA to a fresh RNase-free tube.
6. Add an equal volume of 70% ethanol to obtain a final ethanol concentration of 35%. Mix well by vortexing.
7. Invert the tube to disperse any visible precipitate that may form after adding ethanol. Proceed to binding, washing and elution steps.

Binding, Washing and Elution

1. Transfer up to 700 μl of sample prepared above to a Spin Cartridge (with a Collection Tube).
2. Centrifuge at 12,000 × *g* for 15 seconds at room temperature. Discard the flow-through and re-insert the Spin Cartridge into the same Collection Tube.
3. Repeat Steps 1–2 until the entire sample has been processed.
4. Add 700 μl Wash Buffer I to the Spin Cartridge. Centrifuge at 12,000 × *g* for 15 seconds at room temperature. Discard the flow-through and the Collection Tube. Insert the Spin Cartridge into a new Collection Tube.
5. Add 500 μl Wash Buffer II with ethanol to the Spin Cartridge.

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6. Centrifuge at 12,000 × *g* for 15 seconds at room temperature. Discard the flow-through, and reinsert the Spin Cartridge into the same Collection Tube.
7. Repeat Steps 5–6 once.
8. Centrifuge the Spin Cartridge and Collection Tube at 12,000 × *g* for 1 minute at room temperature to dry the membrane with attached RNA. Discard the Collection Tube and insert the Spin Cartridge into a Recovery Tube.
9. Add 30 μl – 3 × 100 μl (3 sequential elutions with 100 μl each) RNase-Free Water to the center of the Spin Cartridge.
10. Incubate at room temperature for 1 minute.
11. Centrifuge the Spin Cartridge with the Recovery Tube for 2 minutes at ≥12,000 × *g* at room temperature. Discard the Spin Cartridge. Store the purified RNA on ice if used within a few hours. For long-term storage, store the purified RNA at –80°C.
12. Proceed to qRT-PCR step.

The quality check of nucleic acid was performed using a spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific, Inc., USA), the samples that had the ratio of A260/280 less than 1.8 were excluded from the study.

Quantitative RT-PCR (qRT-PCR)

The two steps quantitative RT-PCR (qRT-PCR; real-time RT-PCR) was used, first with the reverse transcription and then the PCR. Bio-Rad CFX96^{TM} real time RT-PCR system (Bio-Rad laboratories, Inc., USA) was used to observe gene expressions of RANKL, OPG, TRAP, MMP-9, CtsK and RUNX2. We designed primers for the genes by using primer designing tool (Primer3 and BLAST, http://www.ncbi.nlm.nih.gov/tools/primer-blast/), (Table 1).

GAPDH was used as a reference gene for normalization and was amplified in

the separated tube (Bustin and Nolan, 2004). Isolated RNA was reverse transcribed to cDNA. Then the cDNA was used as a template for real time PCR by using the reverse transcription kit (Sensiscript®, Qiagen, Inc., USA) with the real time PCR Master Mix (SYBR® FAST Universal kit, Kapa Biosystems, Inc., USA). The protocols were set according to the manufacturer's instructions.

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

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Reverse-transcription protocol

1. Thaw template RNA, Oligo dT primer solutions, dNTP Mix, 10x Buffer RT, RNase-free water, and place on ice. Mix thoroughly before use.
2. Prepare a master mix according to Table 2 The reaction mix contains all the components except the template RNA. Prepare a volume of reaction mix.
3. Mix the reaction mix gently by vortexing for no more than 5 s. Dispense 19.0 µl into PCR tubes.
4. Add template RNA 1.0 µl to the individual PCR tubes.

. 5. Incubate for 60 min at 37°C in the thermal cycler.

6. Place the reverse-transcription reactions on ice and proceed directly with PCR.

Table 2 Reverse transcription reaction components by using Sensiscript® reverse transcription kit.

Component	Volume/reaction	Final concentration
10x Buffer RT	2.0 μl	1x
dNTP Mix (5mM each dNTP)	2.0 μl	0.5mM each dNTP
Oligo-dT primer (10 μM)	2.0 μl	1 μM
Sensiscript Reverse Transcriptase	1.0 μl	
RNase-free water	12.0 μl	-
Template RNA	1.0 μl	<50 ng (per reaction)
Total volume	20.0 μl	-

PCR protocol

1. Prepare a PCR master mix consisting of the appropriate volumes of KAPA SYBR® FAST qPCR Master Mix according to the table 3. Mix the reaction mix gently.
2. Transfer 19.0 μl PCR master mix, 1.0 μl cDNA template (previous step) to each well of a PCR tube.
3. Run the qPCR protocol as described in table 4.

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Table 3 qPCR reaction components by using KAPA SYBR® FAST qPCR Master Mix

Component	Volume/reaction	Final concentration
PCR-grade water	8.2 μl	N/A
2x KAPA SYBR® FAST qPCR Master Mix Universal	10 μl	1x
10 μM Forward Primer	0.4 μl	200 nM
10 μM Reverse Primer	0.4 μl	200 nM
Template DNA	1.0 μl	<20 ng
Total volume	20 μl	-

Table 4 The protocol of quantitative RT-PCR using KAPA SYBR® FAST qPCR Master Mix Universal kit

Step	Temperature	Duration	Cycles
Enzyme activation	95°C	3 min	Hold
Denature	95°C	1-3 sec	40
Anneal/extend	60°C	≥ 20 sec+ Plate Read	
Dissociation (Melt curve)	60.0-95.0 °C,	5 sec + Plate Read	increment 0.5 °C until reach 95.0 °C

During the PCR cycle, the fluorescence detection data would be plotted as described in Figure 7 (a) and (b)

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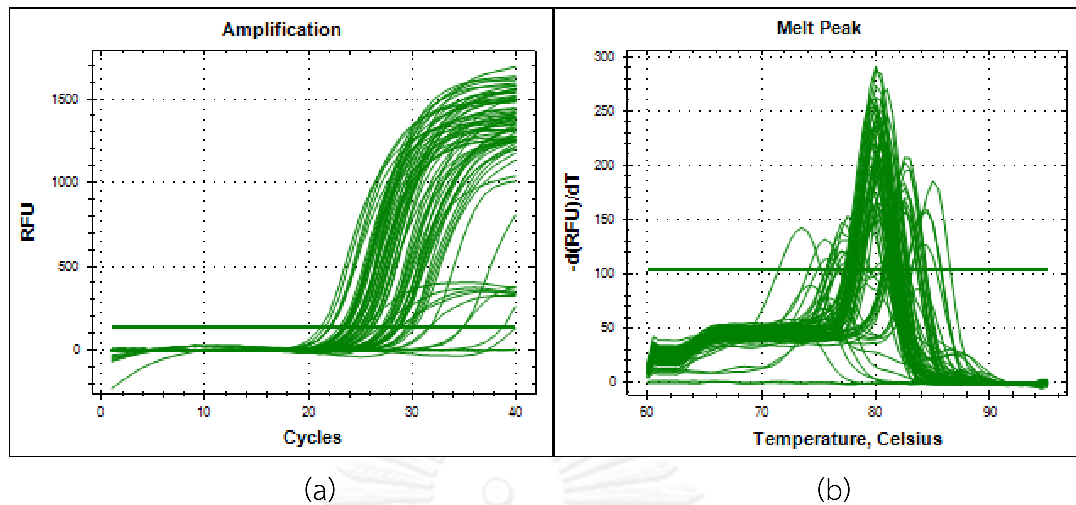

(a) (b)

Figure 7 Real time amplification plot (a) and melting curve (b)

(a) The linear scale amplification plot of the fluorescence detection during the PCR cycle. The baseline is the noise level in early cycles, the baseline threshold is adjusted to a value above the background and placed within the linear region of the amplification curve, which represents the detectable log-linear range of the PCR. The cycle that the fluorescence detection reaches the baseline threshold is described as a threshold cycle (C_T). (b) Melting curve is an assessment of the dissociation-characteristics of double-stranded DNA during heating. The samples with the same primer should have approximately the same peak.

Gene expression analysis

The expressions of six target genes were normalized with the reference gene, GAPDH. The relative quantifications were performed according to the delta delta C_T from the C_T value for any samples normalized to the reference genes (Livak & Schmittgen, 2001), by using the gene expression analysis software (CFX Manager^{TM}, Bio-Rad, USA).

The relative quantity (ΔCq) for any sample (GOI) is calculated with this formula:

$$\textbf{Relative Quantity}_{\text{sample (GOI)}} = \mathrm{E}_{\mathrm{GOI}}^{(\mathrm{C}_{\mathrm{q(MIN)}} - \mathrm{C}_{\mathrm{q(sample)}})}$$

Where:

• E = Efficiency of primer and probe set. This efficiency is calculated with the formula (% Efficiency * 0.01) + 1, where 100% efficiency = 2

• Cq (MIN) = Average Cq for the Sample with the lowest average Cq for GOI

• Cq (sample) = Average Cq for the Sample

• GOI = Gene of interest (one target)

The calculation for normalized expression is described in the following formula, which uses the calculated Relative Quantity (RQ) calculation:

$$\textbf{Normalized Expression}_{\text{sample (GOI)}} = \frac{\mathrm{RQ}_{\text{sample (GOI)}}}{(\mathrm{RQ}_{\text{sample (REf}_n)})^{\frac{1}{n}}}$$

Where:

• RQ = Relative Quantity of a sample

• Ref = Reference target in a run that includes one or more reference targets in each sample

• GOI = Gene of interest (one target)

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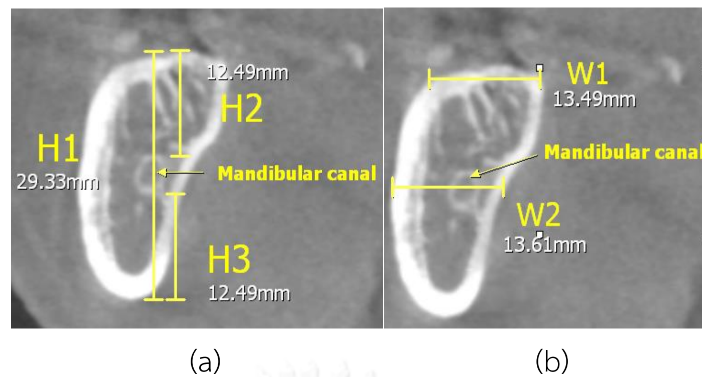
Bone measurement

The CBCT scan of the edentulous areas at the dental implant sites (n=18) were examined. The examinations were performed with Cone beam computed tomography (CBCT, CB MercuRay, Hitachi medico Technology Corp., Japan). The data were manipulated in CB work version 2.12 software then the images were reconstructed. At the implant site, the cross-sectional image of the mandible was examined as described in Figure 8. To test the precision, the measurements were repeated 3 times with no reference to the original data.



(a) (b)

Figure 8 Bone measurements

(a) For measurements at the implant site, total bone height (H1) is the distance from the alveolar crest to the lower border of the mandible parallel to the mid-sagittal plane. Alveolar bone height (H2) is the distance from the alveolar crest to the superior border of mandibular canal. Basal bone height (H3) is 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) are the bucco-lingual bone width at superior portion of trabeculae bone and at mandibular canal level respectively.

Data analysis

The ratios of total bone heights (H1) and basal bone heights (H3) of the edentulous areas (n=18) were calculated and grouped into a high resorption group (H1/H3 < 3.0) and a low resorption group (H1/H3 > 3.0). The differences of the gene expressions among the impacted tooth alveolar bone (n=7), the high resorption group and the low resorption group were analyzed by Kruskal-Wallis test, p-value of <0.05 was considered significant. Correlations between the gene expressions of edentulous areas (n=18) and the radiographic measurements were analyzed by Pearson's correlation analysis.

CHAPTER IV RESULTS

Bone measurements (n = 18) were described in the table 5: Mean value of the total bone height (H1) was 32.62 mm, the alveolar bone height (H2) was 17.85 mm and the basal bone height (H3) was 11.15 mm. The mean value of crestal bone width (W1) was 6.44 mm and total bone width (W2) was 13.96 mm.

The ratios of H1 to H3 were calculated and the data were plotted (Figure 9), then the bone sample which has $H1/H3 < 3.0$ was considered to be in a high resorption group (n=9) and the bone which has $H1/H3 > 3.0$ was in a low resorption group (n=9).

There was significant difference ($p<0.01$) of the H1/H3 ratios between the groups (Table 6).

Table 5 Descriptive statistics of the radiographic measurements

	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
W1(mm)	2.76	16.19	6.4419	2.89423
W2(mm)	5.62	27.53	13.9585	5.10329

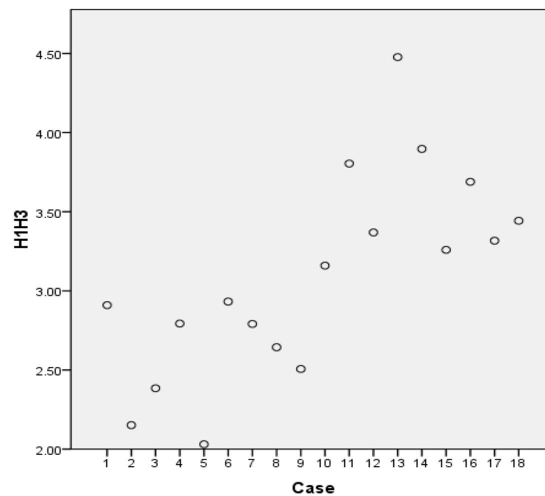


Figure 9 The value plots of H1to H3 ratios of edentulous bone samples

The bone sample which has H1/H3 < 3.0 was considered to be in a high resorption group (n=9) and the bone which has H1/H3 > 3.0 was in a low resorption group (n=9).

Table 6 Independent T-Test shows significant difference ($p<0.01$) of the H1/H3 ratios between the high and low resorption groups.

Resorptions	n	Mean	Std. Deviation	Mean difference	Sig.(2-tail)
High resorption (H1/H3 < 3.0)	9	2.5715	0.32703	-1.03019	0.000
Low resorption (H1/H3 > 3.0)	9	3.6017	0.41455		

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Gene expression of OPG, RUNX2, RANKL, CtsK, MMP9 and TRAP

Figure 10 shows the relative quantity gene expression among 25 samples; 18 mandibular alveolar bone, 7 impacted tooth alveolar bone. The data was not normally distributed. Therefore non-parametric data was used and the analyses were done by Kruskal-Wallis test and Mann-Whitney U test. There were significant differences in expressions ($p<0.05$) among the high resorption bone, low resorption bone and impacted tooth alveolar bone group as described in table 7.

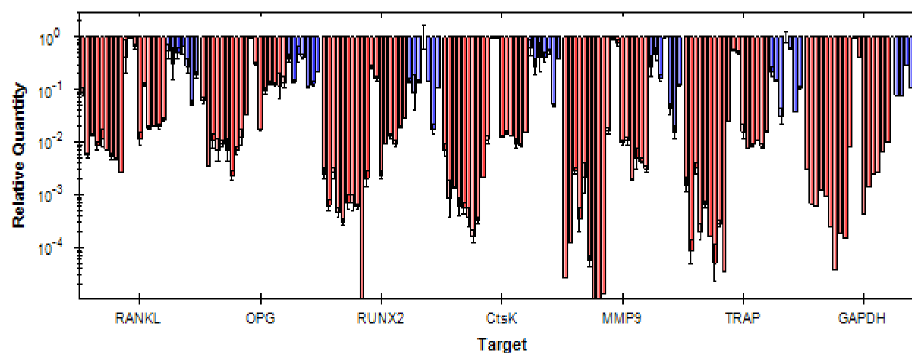

Figure 10 The relative quantity gene expressions of the samples (n=25)

The red bars represent the edentulous bones and the blue bars represent the impacted tooth alveolar bones.

Table 7 Medians of relative expression of OPG, RUNX2, RANKL, CtsK, MMP9, TRAP and RANKL/OPG expression from impacted tooth alveolar bone, high resorption bone (H1/H3 < 3.0) and low resorption bone (H1/H3>3.0). All target gene expressions were normalized to GAPDH. Kruskal Wallis test showed significant difference among the groups (p<0.05).

	Impacted tooth alveolar bone (n= 7)	High resorption bone (n=9)	Low resorption bone (n=9)	Sig. (2-tailed)
OPG	0.2238	0.0107	0.0945	0.017
RUNX2	0.1426	0.0007	0.0092	0.011
RANKL	0.3162	0.0119	0.0197	0.028
CtsK	0.4048	0.0014	0.0095	0.012
MMP9	0.1672	0.0021	0.0031	0.009
TRAP	0.1477	0.0007	0.0084	0.006
RANKL/OPG	1.3360	1.2893	0.6672	0.033

The differences of target gene expressions between each group were analyzed by Mann-Whitney U test (Figure 11-17).

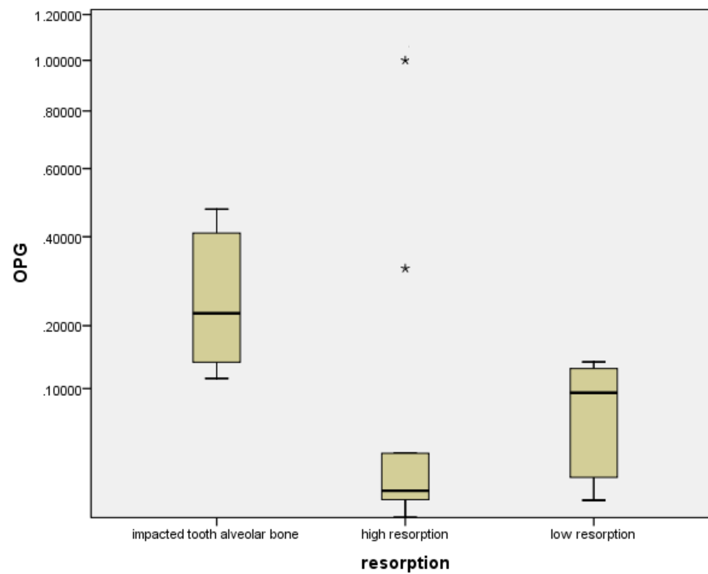

Figure 11 Expression level of OPG in impacted tooth alveolar bone, high resorption bone and low resorption bone, Outlier (*) are excluded from the analysis.

In OPG, Impacted tooth alveolar bone had the highest median value of OPG which significantly higher than the high resorption group (p=0.031) and the low resorption groups (p=0.005). The low resorption group had the median value of OPG higher than the high resorption group (p=0.297) (Figure 11).

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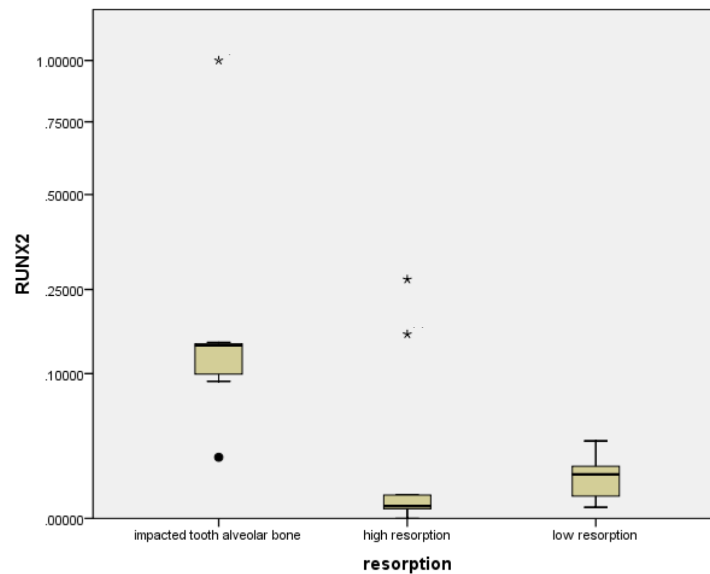

Figure 12 Expression level of RUNX2 in impacted tooth alveolar bone, high resorption bone and low resorption bone, Outlier (*) are excluded from the analysis.

In RUNX2, Impacted tooth alveolar bone had the highest median value of RUNX2 which significantly higher than the high resorption group (p=0.042) and the low resorption groups (p=0.001). The high resorption group had median value of OPG higher than the low resorption group (p=0.258) (Figure 12).

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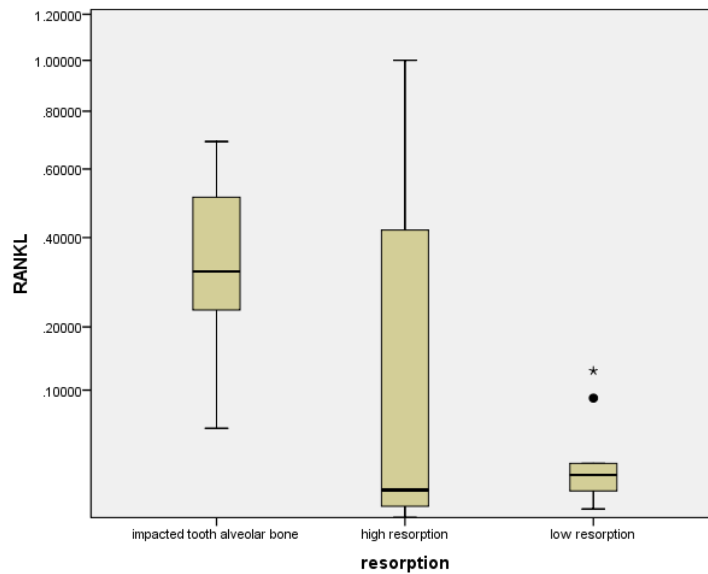

Figure 13 Expression level of RANKL in impacted tooth alveolar bone, high resorption bone and low resorption bone, Outlier (*) are excluded from the analysis.

In RANKL, Impacted tooth alveolar bone expressed had the highest median value of RANKL which higher than the high resorption group (p=0.142) and significantly higher than the low resorption group (p=0.001). The low resorption group had median value of RANKL higher than the high resorption group (p=0.796) (Figure 13).

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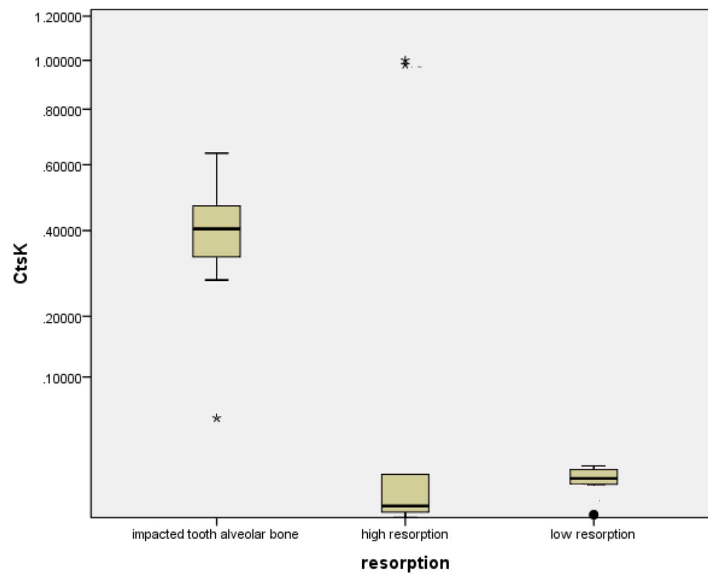

Figure 14 Expression level of CtsK in impacted tooth alveolar bone, high resorption bone and low resorption bone, Outlier (*) are excluded from the analysis.

In CtsK, Impacted tooth alveolar bone had the highest median value of CtsK which higher than the high resorption group (p=0.071) and significantly higher than the low resorption group (p=0.000). The low resorption group had median value of CtsK higher than the high resorption group (p=0.546) (Figure 14).

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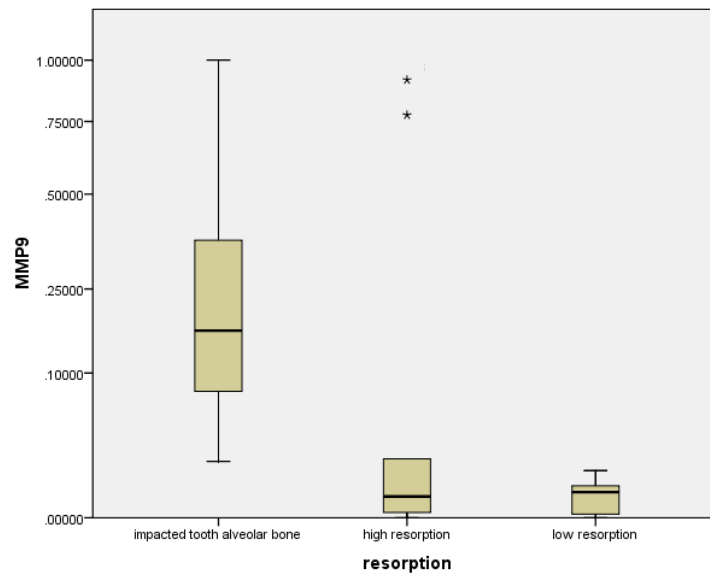

Figure 15 Expression level of MMP9 in impacted tooth alveolar bone, high resorption bone and low resorption bone, Outlier (*) are excluded from the analysis.

In MMP9, Impacted tooth alveolar bone had the highest median value of MMP9 which higher than the high resorption group (p=0.055) and significantly higher than the low resorption group (p=0.000). The low resorption group had median value of MMP9 higher than the high resorption group (p=0.863) (Figure 15).

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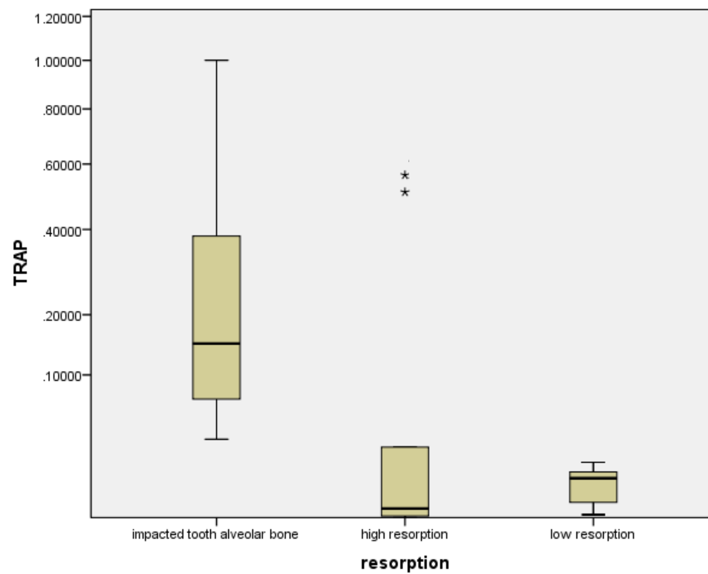

Figure 16 Expression level of TRAP in impacted tooth alveolar bone, high resorption bone and low resorption bone, Outlier (*) are excluded from the analysis.

In TRAP, Impacted tooth alveolar bone had the highest median value of TRAP which significantly higher than the high resorption group (p=0.023) and the low resorption group (p=0.000). The low resorption group had median value of TRAP higher than the high resorption group (p=0.546) (Figure 16).

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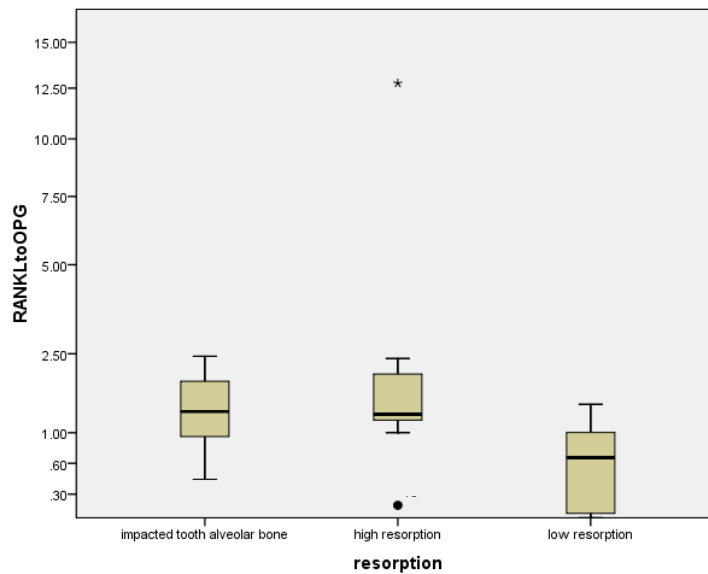

Figure 17 Ratio of RANKL to OPG expression in impacted tooth alveolar bone, high resorption bone and low resorption bone, Outlier (*) are excluded from the analysis.

RANKL/OPG, Impacted tooth alveolar bone had the highest median value of ratio between RANKL and OPG expressions which higher than the high resorption group (p=0.918) and significantly higher than the low resorption group (p=0.019). High resorption group also had significantly higher median value than the low resorption group (p=0.042) (Figure 17).

Correlation of bone morphology and gene expression between the data

There are significant correlations between the bone morphology measurements and the gene expression:

Total bone heights (H1) have significant positive correlations to OPG, RUNX2, CtsK, MMP9, TRAP ($p<0.05$) and RANKL ($p<0.01$).

Alveolar bone heights (H2) have significant positive correlations to OPG, RUNX2 and RANKL ($p<0.05$).

Basal bone heights (H3) have significant positive correlations to OPG, RUNX2, CtsK, MMP9, TRAP ($p<0.05$) and RANKL ($p<0.01$).

The ratios of total bone height to basal bone height (H1/H3) have no significant correlation with any target genes.

Crestal bone widths (W1) have significant positive correlations to the ratios of RANKL to OPG ($p<0.01$).

Total bone widths (W2) have no significant correlation with any target genes.

The results of correlations are shown in the table 8.

Table 8 The relationships between the measurement data and the gene expression profiles

		OPG	RUNX2	RANKL	CtsK	MMP9	TRAP	RANKL/OPG
H1	Correlation	.573*	.570*	.653**	.512*	.541*	.538*	.353
	Sig. (2-tailed)	.013	.014	.003	.030	.020	.021	.150
H2	Correlation	.547*	.485*	.540*	.396	.426	.424	.227
	Sig. (2-tailed)	.019	.041	.021	.104	.078	.080	.366
H3	Correlation	.522*	.544*	.641**	.510*	.541*	.531*	.402
	Sig. (2-tailed)	.026	.020	.004	.031	.020	.023	.098
H1/H3	Correlation	-.130	-.191	-.278	-.240	-.253	-.241	-.281
	Sig. (2-tailed)	.606	.447	.265	.337	.311	.335	.259
W1	Correlation	.053	.120	.331	.169	.166	.180	.633**
	Sig. (2-tailed)	.833	.635	.179	.503	.509	.475	.005
W2	Correlation	.168	.193	.146	.180	.192	.194	-.099
	Sig. (2-tailed)	.505	.442	.563	.474	.445	.442	.697

(*) Correlation is significant at the 0.05 level (2-tailed). (**) Correlation is significant at the 0.01 level (2-tailed).

There are significant positive correlations among all target genes ($p<0.01$) The results of correlations are shown in the table 9.

Table 9 The relationships among the target gene expressions

		OPG	RUNX2	RANKL	CtkK	MMP9	TRAP
OPG	Correlation	1	.538**	.880**	.823**	.777**	.613**
	Sig. (2-tailed)		.006	.000	.000	.000	.001
RUNX2	Correlation	.538**	1	.646**	.474*	.790**	.875**
	Sig. (2-tailed)	.006		.000	.017	.000	.000
RANKL	Correlation	.880**	.646**	1	.886**	.878**	.764**
	Sig. (2-tailed)	.000	.000		.000	.000	.000
CtsK	Correlation	.823**	.474*	.886**	1	.797**	.728**
	Sig. (2-tailed)	.000	.017	.000		.000	.000
MMP9	Correlation	.777**	.790**	.878**	.797**	1	.848**
	Sig. (2-tailed)	.000	.000	.000	.000		.000
TRAP	Correlation	.613**	.875**	.764**	.728**	.848**	1
	Sig. (2-tailed)	.001	.000	.000	.000	.000	

(**) Correlation is significant at the 0.01 level (2-tailed)

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CHAPTER V DISCUSSION

The healing of tooth extraction consists of 2 parts; the soft tissue healing and the bone healing. The soft tissue healing seems to complete within the first month, while the bone healing, both formation and resorption, is extend to 6 months. In clinical observation, the most dimensional change of alveolar bone occurs within the first 3 months (Johnson, 1969; Schropp et al., 2003), resulting in loss in bone width and height. After 6 months, the bone remodeling due to the healing process is approximately constant, but we can observed the slightly dimensional change throughout the life. In this study, we collected the bone samples from 6 months after tooth extraction to eliminate the resorptive activity that had occurred during the initial healing process.

To date, no single factor alone has been stated that contributes the resorption of the bone. It was proved clearly that the functional factor, the metabolic factor and the prosthetic factor strongly affect to the amount of bone loss. On the other hand, the genetic factor that has been interested since the last few decades still has incomplete information. Our strategies were to examine the gene expression associated with the alveolar bone resorption after tooth extraction, and to compare the gene expressions among the groups of mandibular alveolar bone with different resorption levels and the alveolar bone from impacted tooth. Furthermore, the correlations between the gene expressions and the bone dimensions were also investigated. However, in this study, we examined only in the mandibular bone.

Since the study in 1974 has proposed that the basal bone from the lower edge of mental foramen to the lower border of mandible was about one-third in proportion to the total mandibular bone height (Wical & Swoope, 1974), many studies referred to this proportion to evaluate the degree of mandibular bone resorption (Packota,

Hoover, & Neufeld, 1988). In addition, the recent study using cone beam computed tomography reported that the alveolar bone height of the posterior mandible was relatively constant from premolar to molar (Braut, Bornstein, Lauber, & Buser, 2012). From the previous studies, to eliminate the differences from the jaw size, we measured the distance from the mandibular canal instead of the mental foramen since we collected the alveolar bone from the first premolar to second molar. We postulated that the bone which had the ratio of total bone height to basal bone height less than 3.0 could represent the higher bone resorption than another group, the low resorption group, which had the ratio more than 3.0. The data analysis had confirmed that the expression of RANKL and the value of RANKL/OPG which were the markers for high resorptive activity were significantly higher in the high resorption than the low resorption group.

From the results, the highest median expression values of target gene including RANKL, RUNX2, OPG, CtsK, MMP9, TRAP and also RANKL/OPG ratio were from the impacted tooth alveolar bones. From 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 the alveolar bone cells (osteoclasts and osteoblasts) (Wise et al., 2002). At the specific time, the reduction of OPG in dental follicle allows for osteoclast formation in alveolar bone (Wise, Lumpkin, Huang, & Zhang, 2000) then the RANKL/OPG and other signaling cascades regulate the bone deposition and resorption during eruption. In addition, the areas involved in impacted third molars usually have some degrees of inflammation. It has been observed that the inflammation is correlated to the histological alveolar bone loss, especially in the adult (Yamaoka et al., 2009).

Our data has indicated that the well-known bone resorption markers including TRAP, RANKL, CtsK, MMP9 and especially the ratio of RANKL to OPG were highest in the impacted tooth. It might be postulated that the resorption activity of the impacted

tooth alveolar bones were more than that in the edentulous bone. However, it wouldn't be stated that any gene alone represented the result of remodeling activity. The higher TRAP marker could only informed that the numbers of multinucleated cells were increased, but the markers which confirmed the higher resorption activity were CtsK, MMP9 and RANKL/OPG levels. Because osteoclast without RANKL could not function properly, and the higher in CtsK and MMP9 meant there was higher proteolytic enzyme in the resorption lacunae (Michael, 2006).

Although, the high resorption bone had lower median value for all target genes than the low resorption bone, but the ratio of RANKL to OPG was significantly higher in the high resorption bone. This result showed that the balance between RANKL and OPG expression was probably more powerful to represent the resorptive activity than individual gene expression. The large amount of RANKL in the edentulous bone could be explained by the mechanical stimuli to osteocyte which had lost after tooth extraction, resulting in increasing RANKL expression by osteocyte. Furthermore, the reduction of Ca^{2+} level in the non-functional bone can stimulate parathyroid hormone that regulates the expression of RANKL from osteoblast (Bronner et al., 2003). The differences in these pathways might cause the differences of RANKL levels between the groups.

RUNX2 was expressed higher in the low resorption bone than the high resorption bone. It had been clearly discussed that RUNX2 promoted the differentiation of osteoblast in the bone formation. This could be the factor that decreased bone loss in the low resorption bone even there was the study speculated that the more RUNX2 might resulted in the more RANKL expression because osteoblast was an essential source of RANKL. However, the same study concluded that osteoblasts were preferentially respond to hormone or other stimuli, so RANKL was not directly required RUNX2 in transcription (O'Brien, 2010).

The correlation analysis revealed the significant positive correlation among the target genes. TRAP, RANKL, MMP9 and CtsK have been proved to be the marker genes of bone resorption. However, individual gene expression must be interpreted along with the other genes as a result of their functional relations. There was a study that examined the bone resorption occurred under the different treatment conditions. The study treated mononuclear cells with M-CSF which functioned as a promoter of osteoclast differentiation, prevented osteoclast apoptosis and enhanced osteoclast motility (Fuller et al., 1993). The number of osteoclasts was increased and the high level of TRAP can be seen, but without resorptive activity. When RANKL was added, osteoclasts were positive for F-actin ring and NFAT protein that augmented MMPs and CtsK and indicated the ability of osteoclast (Michael, 2006) .

For correlations, There were significant positive correlations ($p<0.05$) between the target gene expressions to the total bone height and the basal bone height. However, there was no significant correlation between the ratio of the total bone height to the basal bone height and the gene expressions.

It has been noticed that the ratio of RANKL to OPG did not significantly correlate to bone height, but to crestal bone width. It might be concluded that six months after tooth extraction, the more RANKL was overexpressed, the more alveolar crest width was reduced. Previous studies had shown that residual ridge resorbed from labial and lingual aspects, resulting in narrow and knife-edged. As the process continues, the knife-edge becomes shorter, leaving a low well-rounded or flat ridge (A. Gupta, Tiwari, Goel, & Shekhawat, 2010) . Afterall, the crest of the residual ridge and the sharp edges of the alveolar processes are reduced (Douglas Allen Atwood, 1963; Enlow, Bianco, & Eklund, 1976; Pietrokovski & Massler, 1967). There was no significant correlation between the total edentulous bone width and the gene expression. It might be postulated that the major loss in bone width occurred in the superior part to the mandibular canal.

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

In this study, all bone samples were collected from the minimally to moderate resorbed edentulous jaw (McGarry et al., 2002) as a result of the limitation in collecting bone from severe resorbed edentulous jaw. We suggested that this study should be extended in the future to collect the samples from 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.

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CHAPTER VI CONCLUSION

This study gave the descriptive data of the gene expression profiles of the edentulous bone and the alveolar bone of impacted tooth. The impacted tooth alveolar bone expressed RANKL, RUNX2, OPG, CtsK, MMP9 and TRAP significantly higher than edentulous bone. The RANKL/OPG ratios were highest in the impacted tooth alveolar bone then the high resorption bone group which had the ratio of total bone height to basal bone height >3.0 and the low resorption bone which had the ratio of total bone height to basal bone height <3.0, respectively. There is significant positive correlation between the crestal bone width and RANKL/OPG ratio. It might be recommended that the patients with high level of RANKL/OPG expression were possible candidates for implant placement or for maintaining root or natural teeth to preserve bone.

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APPENDIX

จุฬาลงกรณ์มหาวิทยาลัย
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Bone measurements from CBCT scan

Sample	Procedure	sex	age		Bone height(mm)				Bone width (mm)	
					H1	H2	H3	H1/H3	W2	W1
1	36 implant	F	62		16.94	9.57	5.44		5.79	2.86
					17.06	9.21	5.5		5.71	2.78
					17.03	9.56	5.21		5.35	2.64
				mean	17.01	9.45	5.38	3.16	5.62	2.76
2	47 implant	F	55		33.57	13.17	16.31		20.79	7.15
					33.79	13.38	16.28		20.99	6.02
					33.68	13.38	17.17		21.08	6.36
				mean	33.68	13.31	16.59	2.03	20.95	6.51
3	46 implant	F	61		26.75	15.75	9.00		8.50	4.25
					27.25	15.75	9.50		8.50	4.25
					26.75	15.50	9.25		8.50	4.00
				mean	26.92	15.67	9.25	2.91	8.50	4.17
4	45 implant	F	28		29.50	12.75	13.75		12.00	8.50
					29.50	12.75	13.75		12.00	8.50
					29.75	12.75	13.75		12.00	8.75
				mean	29.58	12.75	13.75	2.15	12	8.58
5	45 implant	F	69		26.00	11.75	11.00		12.25	7.00
					25.75	11.25	10.75		12.00	7.25
					25.75	12.00	10.75		12.50	7.00
				mean	25.83	11.67	10.83	2.38	12.25	7.08
6	36 implant	F	32		31.75	20.00	8.50		11.50	4.25
					31.25	20.75	8.00		11.50	4.00
					31.50	20.25	7.75		11.50	4.25
				mean	31.50	20.33	8.08	3.90	11.50	4.17

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Sample	Procedure	sex	age		Bone height(mm)				Bone width (mm)	
					H1	H2	H3	H1/H3	W2	W1
7	36 implant	F	64	mean	28.25	15.00	10.25	2.79	10.25	5.00
					28.00	15.00	10.00		10.25	5.25
					28.25	15.00	10.00		10.25	5.25
					28.17	15.00	10.08		10.25	5.17
8	36 implant	M	53		29.00	18.25	7.50		12.75	6.50
					29.25	18.00	7.50		12.50	6.00
					29.25	18.00	8.00		12.25	6.50
				mean	29.17	18.08	7.67	3.80	12.50	6.33
9	37 implant	F	66		38.59	21.08	12.94		19.85	5.20
					38.37	20.74	13.16		19.75	5.35
					38.49	20.52	13.27		19.74	5.80
				mean	38.48	20.78	13.12	2.93	19.78	5.45
10	36 implant	F	61		46.31	23.81	17.62		27.42	5.56
					46.18	24.08	17.34		27.68	5.30
					46.20	24.00	17.50		27.50	5.40
				mean	46.23	23.96	17.49	2.64	27.53	5.42
11	35 implant	F	63		53.41	28.09	21.12		14.63	8.81
					52.86	28.09	21.11		14.80	8.98
					52.90	27.83	21.28		14.63	8.98
				mean	53.06	28.00	21.17	2.51	14.69	8.92
12	46 implant	M	59		36.55	17.73	13.21		17.88	6.86
					35.99	17.86	12.68		18.00	6.60
					36.00	17.80	13.00		18.00	6.80
				mean	36.18	17.80	12.96	2.79	17.96	6.75
13	37 implant	M	37		37.58	19.63	11.71		14.50	16.18
					37.92	19.63	11.49		14.05	16.20
					37.92	19.85	11.60		14.16	16.20
				mean	37.81	19.70	11.60	3.26	14.24	16.19

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Sample	Procedure	sex	age		Bone height(mm)				Bone width (mm)	
					H1	H2	H3	H1/H3	W2	W1
14	37 implant	F	68		30.86	19.42	8.49		12.92	6.90
					31.05	19.50	8.22		12.92	7.20
					31.05	19.27	8.49		12.86	6.80
				mean	30.99	19.40	8.40	3.69	12.90	6.97
15	45 implant	M	49		34.00	20.25	10.25		12.50	4.00
					34.00	20.00	10.25		12.50	3.75
					34.00	20.00	10.25		12.50	3.75
				mean	34.00	20.08	10.25	3.32	12.50	3.83
16	47 implant	M	56		27.00	18.00	7.00		10.50	5.00
					27.00	17.25	7.00		10.50	5.75
					16.75	17.25	7.00		10.25	5.75
				mean	23.58	17.50	7.00	3.37	10.42	5.50
17	46 implant	M	46		40.37	23.53	11.49		16.30	5.69
					40.93	23.75	12.04		16.73	5.58
					40.82	23.75	11.94		16.73	5.91
				mean	40.71	23.68	11.82	3.44	16.59	5.73
18	36 implant	F	53		24.25	14.00	5.25		11.25	6.00
					24.25	14.50	5.75		11.00	6.75
					24.25	14.00	5.25		11.00	6.50
				mean	24.25	14.17	5.42	4.48	11.08	6.42

Kruskal-Wallis Test

Ranks

	resorption	N	Mean Rank
OPG	impacted tooth alveolar bone	7	19.57
	high resorption	9	9.33
	low resorption	9	11.56
	Total	25	
RUNX2	impacted tooth alveolar bone	7	20.00
	high resorption	9	9.33
	low resorption	9	11.22
	Total	25	
RANKL	impacted tooth alveolar bone	7	19.29
	high resorption	9	11.00
	low resorption	9	10.11
	Total	25	
CtsK	impacted tooth alveolar bone	7	20.00
	high resorption	9	10.22
	low resorption	9	10.33
	Total	25	
MMP9	impacted tooth alveolar bone	7	20.14
	high resorption	9	11.17
	low resorption	9	9.28
	Total	25	
TRAP	impacted tooth alveolar bone	7	20.57
	high resorption	9	9.78
	low resorption	9	10.33
	Total	25	
RANKLtoOPG	impacted tooth alveolar bone	7	15.57
	high resorption	9	16.11
	low resorption	9	7.89
	Total	25	

Test Statistics[a,b]

	OPG	RUNX2	RANKL	CtsK	MMP9	TRAP	RANKLtoOPG
Chi-Square	8.161	9.091	7.157	8.796	9.458	10.315	6.803
df	2	2	2	2	2	2	2
Asymp. Sig.	.017	.011	.028	.012	.009	.006	.033

a. Kruskal Wallis Test

b. Grouping Variable: resorption

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Mann-Whitney U test between the gene expressions of impacted tooth alveolar bone and high resorption bone

Ranks

	resorption	N	Mean Rank	Sum of Ranks
OPG	impacted tooth alveolar bone	7	11.43	80.00
	high resorption	9	6.22	56.00
	Total	16		
RUNX2	impacted tooth alveolar bone	7	11.29	79.00
	high resorption	9	6.33	57.00
	Total	16		
RANKL	impacted tooth alveolar bone	7	10.57	74.00
	high resorption	9	6.89	62.00
	Total	16		
CtsK	impacted tooth alveolar bone	7	11.00	77.00
	high resorption	9	6.56	59.00
	Total	16		
MMP9	impacted tooth alveolar bone	7	11.14	78.00
	high resorption	9	6.44	58.00
	Total	16		
TRAP	impacted tooth alveolar bone	7	11.57	81.00
	high resorption	9	6.11	55.00
	Total	16		
RANKLtoOPG	impacted tooth alveolar bone	7	8.29	58.00
	high resorption	9	8.67	78.00
	Total	16		

Test Statistics[b]

	OPG	RUNX2	RANKL	CtsK	MMP9	TRAP	RANKLtoOPG
Mann-Whitney U	11.000	12.000	17.000	14.000	13.000	10.000	30.000
Wilcoxon W	56.000	57.000	62.000	59.000	58.000	55.000	58.000
Z	-2.170	-2.064	-1.535	-1.852	-1.958	-2.276	-.159
Asymp. Sig. (2-tailed)	.030	.039	.125	.064	.050	.023	.874
Exact Sig. [2*(1-tailed Sig.)]	.031[a]	.042[a]	.142[a]	.071[a]	.055[a]	.023[a]	.918[a]

a. Not corrected for ties.

b. Grouping Variable: resorption

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Mann-Whitney U test between the gene expressions of impacted tooth alveolar bone and low resorption bone

Ranks

	resorption	N	Mean Rank	Sum of Ranks
OPG	impacted tooth alveolar bone	7	12.14	85.00
	low resorption	9	5.67	51.00
	Total	16		
RUNX2	impacted tooth alveolar bone	7	12.71	89.00
	low resorption	9	5.22	47.00
	Total	16		
RANKL	impacted tooth alveolar bone	7	12.71	89.00
	low resorption	9	5.22	47.00
	Total	16		
CtsK	impacted tooth alveolar bone	7	13.00	91.00
	low resorption	9	5.00	45.00
	Total	16		
MMP9	impacted tooth alveolar bone	7	13.00	91.00
	low resorption	9	5.00	45.00
	Total	16		
TRAP	impacted tooth alveolar bone	7	13.00	91.00
	low resorption	9	5.00	45.00
	Total	16		
RANKLtoOPG	impacted tooth alveolar bone	7	11.29	79.00
	low resorption	9	6.33	57.00
	Total	16		

Test Statistics[b]

	OPG	RUNX2	RANKL	CtsK	MMP9	TRAP	RANKLtoOPG
Mann-Whitney U	6.000	2.000	2.000	.000	.000	.000	12.000
Wilcoxon W	51.000	47.000	47.000	45.000	45.000	45.000	57.000
Z	-2.699	-3.123	-3.123	-3.334	-3.334	-3.334	-2.064
Asymp. Sig. (2-tailed)	.007	.002	.002	.001	.001	.001	.039
Exact Sig. [2*(1-tailed Sig.)]	.005[a]	.001[a]	.001[a]	.000[a]	.000[a]	.000[a]	.042[a]

a. Not corrected for ties.

b. Grouping Variable: resorption

Mann-Whitney U test between the gene expressions of high resorption and low resorption bone

Ranks

	resorption	N	Mean Rank	Sum of Ranks
OPG	high resorption	9	8.11	73.00
	low resorption	9	10.89	98.00
	Total	18		
RUNX2	high resorption	9	8.00	72.00
	low resorption	9	11.00	99.00
	Total	18		
RANKL	high resorption	9	9.11	82.00
	low resorption	9	9.89	89.00
	Total	18		
CtsK	high resorption	9	8.67	78.00
	low resorption	9	10.33	93.00
	Total	18		
MMP9	high resorption	9	9.72	87.50
	low resorption	9	9.28	83.50
	Total	18		
TRAP	high resorption	9	8.67	78.00
	low resorption	9	10.33	93.00
	Total	18		
RANKLtoOPG	high resorption	9	12.44	112.00
	low resorption	9	6.56	59.00
	Total	18		

Test Statistics[b]

	OPG	RUNX2	RANKL	CtsK	MMP9	TRAP	RANKLtoOPG
Mann-Whitney U	28.000	27.000	37.000	33.000	38.500	33.000	14.000
Wilcoxon W	73.000	72.000	82.000	78.000	83.500	78.000	59.000
Z	-1.104	-1.192	-.309	-.662	-.177	-.662	-2.340
Asymp. Sig. (2-tailed)	.270	.233	.757	.508	.860	.508	.019
Exact Sig. [2*(1-tailed Sig.)]	.297[a]	.258[a]	.796[a]	.546[a]	.863[a]	.546[a]	.019[a]

a. Not corrected for ties.

b. Grouping Variable: resorption

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Pearson's correlation test

Correlations

		H1	H2	H3	H1H3	C1	C2	W1	W2	OPG	RUNX2	RANKL	CtsK	MMP9	TRAP	RANKLtoOPG
H1	Pearson Correlation	1	.870**	.851**	-.283	.243	.741**	.718**	.352	.573*	.570*	.653**	.512*	.541*	.538*	.353
	Sig. (2-tailed)		.000	.000	.255	.332	.000	.001	.152	.013	.014	.003	.030	.020	.021	.150
	N	18	18	18	18	18	18	18	18	18	18	18	18	18	18	18
H2	Pearson Correlation	.870**	1	.525*	.121	.292	.744**	.498*	.189	.547*	.485*	.540*	.396	.426	.424	.227
	Sig. (2-tailed)	.000		.025	.632	.240	.000	.035	.453	.019	.041	.021	.104	.078	.080	.366
	N	18	18	18	18	18	18	18	18	18	18	18	18	18	18	18
H3	Pearson Correlation	.851**	.525*	1	-.713**	.214	.530*	.704**	.317	.522*	.544*	.641**	.510*	.541*	.531*	.402
	Sig. (2-tailed)	.000	.025		.001	.394	.024	.001	.200	.026	.020	.004	.031	.020	.023	.098
	N	18	18	18	18	18	18	18	18	18	18	18	18	18	18	18
H1H3	Pearson Correlation	-.283	.121	-.713**	1	-.044	.015	-.349	-.127	-.130	-.191	-.278	-.240	-.253	-.241	-.281
	Sig. (2-tailed)	.255	.632	.001		.862	.954	.155	.614	.606	.447	.265	.337	.311	.335	.259
	N	18	18	18	18	18	18	18	18	18	18	18	18	18	18	18
C1	Pearson Correlation	.243	.292	.214	-.044	1	.509*	.166	-.329	.301	.316	.348	.288	.296	.294	.156
	Sig. (2-tailed)	.332	.240	.394	.862		.031	.510	.182	.225	.202	.157	.246	.233	.237	.535
	N	18	18	18	18	18	18	18	18	18	18	18	18	18	18	18
C2	Pearson Correlation	.741**	.744**	.530*	.015	.509*	1	.349	.332	.710**	.738**	.703**	.693**	.709**	.706**	-.021
	Sig. (2-tailed)	.000	.000	.024	.954	.031		.156	.178	.001	.000	.001	.001	.001	.001	.936
	N	18	18	18	18	18	18	18	18	18	18	18	18	18	18	18
W1	Pearson Correlation	.718**	.498*	.704**	-.349	.166	.349	1	.158	.053	.120	.331	.169	.166	.180	.633**
	Sig. (2-tailed)	.001	.035	.001	.155	.510	.156		.531	.833	.635	.179	.503	.509	.475	.005
	N	18	18	18	18	18	18	18	18	18	18	18	18	18	18	18
W2	Pearson Correlation	.352	.189	.317	-.127	-.329	.332	.158	1	.168	.193	.146	.180	.192	.194	-.099
	Sig. (2-tailed)	.152	.453	.200	.614	.182	.178	.531		.505	.442	.563	.474	.445	.442	.697
	N	18	18	18	18	18	18	18	18	18	18	18	18	18	18	18

		H1	H2	H3	H1H3	C1	C2	W1	W2	OPG	RUNX2	RANKL	CtsK	MMP9	TRAP	RANKLtoOPG
OPG	Pearson Correlation	.573*	.547*	.522*	-.130	.301	.710**	.053	.168	1	.538**	.880**	.823**	.777**	.613**	-.102
	Sig. (2-tailed)	.013	.019	.026	.606	.225	.001	.833	.505		.006	.000	.000	.000	.001	.626
	N	18	18	18	18	18	18	18	18	25	25	25	25	25	25	25
RUNX2	Pearson Correlation	.570*	.485*	.544*	-.191	.316	.738**	.120	.193	.538**	1	.646**	.474*	.790**	.875**	-.012
	Sig. (2-tailed)	.014	.041	.020	.447	.202	.000	.635	.442	.006		.000	.017	.000	.000	.955
	N	18	18	18	18	18	18	18	18	25	25	25	25	25	25	25
RANKL	Pearson Correlation	.653**	.540*	.641**	-.278	.348	.703**	.331	.146	.880**	.646**	1	.886**	.878**	.764**	.253
	Sig. (2-tailed)	.003	.021	.004	.265	.157	.001	.179	.563	.000	.000		.000	.000	.000	.223
	N	18	18	18	18	18	18	18	18	25	25	25	25	25	25	25
CtsK	Pearson Correlation	.512*	.396	.510*	-.240	.288	.693**	.169	.180	.823**	.474*	.886**	1	.797**	.728**	-.009
	Sig. (2-tailed)	.030	.104	.031	.337	.246	.001	.503	.474	.000	.017	.000		.000	.000	.966
	N	18	18	18	18	18	18	18	18	25	25	25	25	25	25	25
MMP9	Pearson Correlation	.541*	.426	.541*	-.253	.296	.709**	.166	.192	.777**	.790**	.878**	.797**	1	.848**	.006
	Sig. (2-tailed)	.020	.078	.020	.311	.233	.001	.509	.445	.000	.000	.000	.000		.000	.978
	N	18	18	18	18	18	18	18	18	25	25	25	25	25	25	25
TRAP	Pearson Correlation	.538*	.424	.531*	-.241	.294	.706**	.180	.194	.613**	.875**	.764**	.728**	.848**	1	.039
	Sig. (2-tailed)	.021	.080	.023	.335	.237	.001	.475	.442	.001	.000	.000	.000	.000		.854
	N	18	18	18	18	18	18	18	18	25	25	25	25	25	25	25
RANKLtoOPG	Pearson Correlation	.353	.227	.402	-.281	.156	-.021	.633**	-.099	-.102	-.012	.253	-.009	.006	.039	1
	Sig. (2-tailed)	.150	.366	.098	.259	.535	.936	.005	.697	.626	.955	.223	.966	.978	.854	
	N	18	18	18	18	18	18	18	18	25	25	25	25	25	25	25

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

VITA

Miss Tawanchai Wangsai was born on March 26, 1985 in Bangkok, Thailand. She recieved the Degree of Doctor surgery (D.D.S.) from the Faculty of Dentistry, Chulalongkorn University in 2009. After graduation, she enrolled as a member in the Maxillofacial Prosthodontic clinic, Dental Hospital, Faculty of Dentistry, Chulalongkorn University from 2009-2011. She started her post-graduated study in 2011 for the Master of Science Program (M.Sc.) in Prosthodontics at the Department of Prosthodontics Faculty of Dentistry, Chulalongkorn University. At present, She works in the private clinic and the special clinic, Faculty of Dentistry, Chulalongkorn University.

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