Genetic investigation of Bisphosphonate-Related Osteonecrosis of Jaw (BRONJ) via whole exome sequencing and bioinformatics

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(Directed by Prof. Dong-hoo Han, D.D.S., M.S.D., Ph.D.)

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감사의 글

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오랜 시간 저를 지켜주시고 믿어주신 어머니, 장인, 장모께 감사를 드리며, 항상 마음의 안식처로 제 업을 지켜주며 무한한 신뢰와 격려를 아끼지 않아준 사랑하는 아내 혜련과 예쁘게 자라나는 모습이 너무나 감사한 말 지수에게 고마움과 사랑을 전하며 이 논문을 나누고자 합니다.

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Abstract

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Purpose: Prescription of Bisphosphonate (BP) has increased over the years along with the increase of complications associated with the use of BP. Bisphosphonate-related osteonecrosis of jaw (BRONJ) is one of the complications linked to the consumption of BP and has greatly affected patients with minor dental trauma resulting in a long healing period. Not all patients prescribed with BP experience BRONJ and it is multigenic disease possibly affected by both environmental and genetic factors reflecting distinctive phenotype. The purpose of this study is to discover genetic biomarkers associated with BRONJ via WES followed by statistical analysis and comparison with known genes.

Materials & Methods: 16 individuals who have been prescribed with bisphosphonate medication were chosen and each individual’s saliva sample was collected for whole exome sequencing (WES). Saliva sample was taken for massive sequencing and SnpEff, 1000 genomes project East Asian population, 126 healthy Korean randomized subsample originally recruited for thyroid cancer (GSK project), and Polyphen were used to filter out
common variants from 16 individuals’ whole exome sequencing data. Common variants with minor allele frequencies (MAF) >= 0.05 from all randomized datasets were eliminated and different impacts (high, moderate and loss of function) were used for comparison.

To examine the association between BRONJ and known genes from previous studies (VEGF, COL1A1, CYP2C8, FDPS, RBMS3, G20210A, PPARG, MMP9, RANKL, IL1B, LRP5, VDR, IGFBP7, ABCC4, MMP2, RANK, OPG, OPN, CYP19A1 and Absorption, distribution, metabolism and excretion (ADME) genes), gene lists were constructed for comparison with current study’s filtered gene lists.

**Results:** Total of 118,856 variants were detected and 2,180 which is equivalent to 1,866 genes was recovered after the filtering step. Bioinformatics study revealed possible gene sets related to risk of developing BRONJ. Known genes associated to BRONJ from previous studies have been tested for presence in current study and only RBMS3 was detected from current study. Comparison to ADME gene lists yielded several genes in current study’s results indicating their association with BRONJ.

**Conclusion:** Our results suggest that various genes and gene sets might have important role in developing BRONJ in patients with BP medication history. Also, the results confirmed the association between BRONJ and previously discovered genes such as RBMS3, ADME genes.

**Key words:** Bisphosphonate, BRONJ, Whole Exome Sequencing(WES), ADME genes, RBMS3
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I. Introduction

Bisphosphonates (BP) are commonly prescribed medication to treat bone metastases, multiple myeloma, osteoporosis and other bony diseases. No significant side effects of BP have been reported but patients who have been prescribed with BP for a long period of time tend to experience complications in healing period after minor trauma in dental
such as tooth extraction, periodontal operation, alveolar bone, and tooth operations. Bisphosphonate-related osteonecrosis of jaw (BRONJ) which can be derived from exposure and necrosis of alveolar bone, pain, infection and abscess formation was first reported in 2003⁴ and several other cases have followed over the years ⁵-⁸.

Many groups and societies have recently published recommendations or guidelines on the prevention, staging and management strategies for BRONJ ⁹-²⁰. Despite these recommendations there is still a lack of information concerning the incidence, pathogenesis, treatment strategies and prevention of BRONJ.

Diagnostic criteria for BRONJ is patient having exposed jaw bone that did not heal within 8 wk after identification by a health care provider, in a patient who was receiving or had been exposed to a bisphosphonate and had not had radiation therapy to the craniofacial region. The 8-wk duration is consistent with a time frame where most trauma, extractions, and oral surgical procedures would have resulted in soft tissue closure, and exposed bone would no longer be present³¹.

The mechanism of BP in biological system is known to inhibit osteoclastic bone resorption via attraction to and localization in, areas of the bone undergoing inflammation or resorption. BPs are subsequently phagocytozed and internalized by osteoclasts and these internalized BPs trigger apoptosis (cell death) of the osteoclasts, thus inhibiting osteoclast-mediated bone resorption ²²,²³.

Although previous studies have discussed suppression of bone remodeling, angiogenesis inhibition and infection as pathogenesis of BRONJ in the past ²⁴-²⁹, neither solid mechanism how BP sanctions and jaw necrosis are directly related nor any effective
treatment on BRONJ other than the least invasive treatment methods on sequestrum and maintenance of clean oral cavity have been reported to this date.

Several studies on BRONJ linked environmental risk factors such as the use of intravenous vs. oral BPs, concomitant use of chemotherapy, treatment with glucocorticoid or thalidomide, length of exposure to BP treatment, the presence of comorbid conditions such as obesity, alcohol and/or tobacco abuse and pre-existing dental or periodontal disease to the occurrence of BRONJ. Of all mentioned above, dental trauma such as tooth extraction is known to be the most common immediate precipitation risk factor.

The incidence of BRONJ in patients receiving bisphosphonates for osteoporosis is not known. Several reports were published but these cumulative reporting rates were different. In part, these different estimates may be related to underreporting, different durations of exposure in countries that have adopted bisphosphonates more recently, and/or differing definitions of the disease. The true incidence of BRONJ in patients with osteoporosis may be higher than noted in these estimates because of these potential confounders.

The incidence of the disease seems to be relatively low in patients receiving oral bisphosphonates for osteoporosis or Paget’s disease and considerably higher in patients with malignancy receiving high doses of intravenous bisphosphonates. The mean incidence after intravenous application was 7% and the overall incidence of BRONJ after oral bisphosphonate application was 0.12%. However, more information is needed on the true incidence of BRONJ and the other major risk factors for developing this complication. The task force recognizes that information on incidence of BRONJ is rapidly evolving, that continued surveillance will undoubtedly result in identification and
publication of more cases, and that estimates of the frequency of BRONJ may change for patients receiving bisphosphonates for both malignant and nonmalignant disease.21

From clinical investigation of BRONJ in patients with malignant tumors, BRONJ recurred at the same sites in 7 patients out of 20 patients (37%) and at the different sites in 3 patients (16%) 40. Not all patients receiving BP treatment experience BRONJ and clinical study showed an estimated risk of BRONJ between 0.8% and 12% 41. These varying statistical values imply that BRONJ is a multifactorial disease where several factors in combination would cause BRONJ among patients.

Other predisposing factors for BRONJ were age, race, smoking, obesity, cancer diagnosis, and poor oral health yet they have only explained a small percentage of the entire risk 33,36,42. Hence, patients with BP medication would have similar biological effect due to the intake of BP and considering the fact that only a small number of BP users experience BRONJ, it can be hypothesized that genetic susceptibility may be conferred by multiple genes regulating the metabolism of BP or skeletal homeostasis with small variations 43. Therefore, BRONJ, like many other complex trait diseases, can be caused by combination of environmental and genetic risk factors.

Previous genetic association studies of BRONJ found various genes such as vascular endothelia growth factor (VEGF), collagen Type 1 A 1 (COL1A1), cytochrome P450 subfamily 2 polypeptide 8 (CYP2C8), fatsesyl disphosphate synthase gene, Matrix metalloproteinase-9 (MMP9), and peroxisome proliferator-activated receptor gamma (PPARG) 44-53 to be associated with risk of developing BRONJ. Until 2004, genetic research has been grown through advanced technologies and case control study was primarily conducted identifying only small number of variants related to BRONJ. Case
specific approaches have been attempted to accommodate small case number and the first genome-wide association study (GWAS) reported rs1934951 (CYP2C8) single nucleotide polymorphism (SNP) was associated with BRONJ in multiple myeloma (MM) \(^{54}\). However, two other studies reported that such SNP did not show correlation with jaw osteonecrosis in patients suffering from prostate cancer and both research groups were unable to confirm a significant association between polymorphisms in the CYP2C8 gene and the risk of developing osteonecrosis of the jaw in patients with MM receiving treatment with BP in an independent series \(^{55,56}\). Another previous genetic study reported that genetic susceptibility plays a role in the pathophysiology of BRONJ discovering with RBMS3 having a significant effect in the risk \(^{57}\).

As for now, no definite gene has been recognized as risk factor despite the number studies through GWAS. This represents the limit of GWAS in representing SNPs where only five-thousand to one million bases out of three billion human base pairs are analyzed. New discoveries of genetic indicators showed limits in GWAS and there have been many discussions regarding the cause and solution to missing heritability in GWAS. Hence, next generation sequencing (NGS) has been developed to overcome limitations arose from previous genetic study methods.

NGS technology changed genetic research from where candidate genes were known first to reveal the mutations of the gene to where discovering the candidate gene by comparing case and control. Of all NGS technologies, whole exome sequencing (WES) which analyzes exome regions that changes every gene could be considered as the most effective method. Targeting exome, mutations in non-synonymous, splice site, coding indel can be identified especially focusing on non-synonymous mutation where changes
in amino acids affect protein function. To investigate genes known to be associated with the pathogenesis of BRONJ, gene lists were formed via their biological function for examination.

The objective of this study is to discover genetic biomarkers associated with BRONJ via WES followed by statistical analysis and comparison with known genes.
II. Materials and Methods

1. Ethics Statement

All research involving human subjects or human data was approved by the Institutional review board of Yonsei University College of Dentistry. All clinical investigation was performed in accordance with the Declaration of Helsinki. Written informed consent was obtained from all participants before participating in this study.

2. Patient Selection

Sixteen individuals aged between 55 and 90 with BRONJ were analyzed by massively parallel sequencing in this study (1 male, 15 female) (Table I).

Sixteen individuals had tooth extraction or implant surgery in the Implant Clinic of Yonsei University Dental hospital from 2008 to 2013. These patients had history of bisphosphonate medication with varying duration, presence of exposed bone in the maxillofacial region for more than eight weeks and no history of radiation therapy to the jaws. Purpose of BP prescription to these patients was osteoporosis and seven out of sixteen individuals stopped taking medication after the surgical procedure whereas other seven individuals stopped taking medication before the surgical procedure. Medication history of two individuals was unknown.
| Sample ID | Gender | Age | Smoking | Prescribed bisphosphonate | Duration of Prescription |
|-----------|--------|-----|---------|--------------------------|-------------------------|
| Exp1      | Female | 71  | NO      | Fosamax*, Bonviva**       | 7 years                 |
| Exp2      | Female | 68  | NO      | Tybonweekly*              | 5 years                 |
| Exp3      | Female | 79  | NO      | Fosamax*                 | 4 years                 |
| Exp4      | Female | 82  | NO      | Fosamax*                 | 8 months               |
| Exp5      | Female | 66  | NO      | Ostol***                 | 3 years                 |
| Exp6      | Female | 85  | NO      | Fosamax*, Bonviva**       | 5 years                 |
| Exp7      | Female | 71  | NO      | Aidbone*                 | 1 year                  |
| Exp8      | Female | 78  | NO      | Fosamax*                 | 6 months               |
| Exp9      | Female | 72  | NO      | Fosamax*                 | 1 year                  |
| Exp10     | Female | 73  | NO      | Bonviva**                | 10 years               |
| Exp11     | Female | 90  | N/A     | Fosamax*                 | 6 years                 |
| Exp12     | Female | 55  | NO      | Alenmax*                 | 2 years                 |
| Exp13     | Female | 67  | NO      | Risenex-Plus***          | 7 years                 |
| Exp14     | Male   | 79  | NO      | Alendronate              | 4 years                 |
| Exp15     | Female | 71  | NO      | Fosamax*                 | N/A                     |
| Exp16     | Female | 75  | NO      | Fosamax*                 | 3 years                 |

* refer to Alendronate, ** Zoledronic Acid, and *** Risedronate
3. Comparing Data Set

dbSNP137, the 1000 Genomes Project East Asian population and 126 Korean randomized subsamples from the GSK project aged between 23 and 46 (109 male, 17 female) were used as comparing data set. Common variants derived from all data sets were used to filter out common variants from case population’s WES data. The 126 Korean randomized subsamples from reference population, relatively healthy Koreans regardless of gender and age, had originally been recruited for a thyroid cancer study (GSK project).

4. Sample Collection

To obtain DNA data, all sixteen individuals participated in this study were asked to collect 2 mL of saliva in the tube of an Oragene DNA Self-Collection kit (DNA GenoTek, Ottawa, Ontraio, Cat. #OG-500) containing 2 mL of DNA-preserving solution. After collecting patient’s saliva, the lid was closed to release DNA-preserving solution to mix with the saliva. Collection of genomic DNA, extraction of DNA, and further analysis were completed by DNA link Inc. (Seoul, South Korea).
5. Whole Exome Sequencing on HISEQ 2500 using SureSelect All Exon kit 50Mb

To check the quality of DNA, 1% agarose gel electrophoresis and PicoGreen® dsDNA Assay (Invitrogen) were used. With an OD260/280 ratio of 1.8-22, DNA should be as intact as possible.

Use of a Bravo automated liquid handler, SureSelect sequencing libraries were prepared following the manufacturer’s instructions. In 120 mL EB buffer, 1 ug of genomic DNA was fragmented to a median size of 150 bp using a Covaris-S2 (Covaris) and following settings were used: duty cycle 10%, intensity 5, cycles per burst 200, and mode frequency sweeping for 360 s at 4°C. Evaluation of the efficiency of the fragmentation was done using Capillary electrophoresis on DNA 100 chips (Bioanalyzer, Agilent). With the manufacturer’s protocol (Agilent), secunging adapter were ligated on the DNA fragments and PCR was used to amplify the adapter ligated DNA. The quality of the PCR products was checked using Capillary electrophoresis (Bioanalyzer, Agilent) and following instructions were used: Reagents #1, #2, #3, and #4 (Agilent) were mixed to prepare the hybridization buffer and the amplified DNA fragments were concentrated to 500 ng in 3.4 ul. SureSelect block reagents #1, #2, and #3 (Agilent) were mixed with 500 ng of DNA. At 95°C, the hybridization buffer and DNA blocker mix were incubated for 5 and at 65°C, incubation was done for 10 min in a thermal cycler. Rnase block (Agilent) was added to the SureSelect oligo capture library (Agilent) and the capture library was incubated for 2 min at 65°C. The mixture of the hybridization buffer and
DNA blocker was added to the capture library and was incubated for 24 hours at 65 °C in a thermal cycler. 200mL of SureSelect binding buffer (Agilent) was used to wash Fifty ul of streptavidin coating the Dynal MyOne Streptavidin T1 (Invitrogen) three times. The hybridization mixture was incubated for 30 min after the addition to the bead suspension for 30 min at room temperature with mixing. Following solution and time instruction was used to wash beads: 500 mL SureSelect wash buffer #1 (agilent) for 15 min at room temperature followed by three times wash with 500 mL SureSelect buffer #2 (Agilent) for 10 min at 65 °C. DNA was eluted with 50 mL SureSelect elution buffer (Agilent) for 10 min at room temperature. 50 mL of SureSelect neutralization buffer (Agilent) was added to the eluted DNA. Purification of the reaction product was done using the AMPure XP bead (Beckman). The capture library was amplified to add index tags and capillary electrophoresis (Bioanalyzer, Agilent), and to verify the quality of the amplified libraries using Herculase II Fusing DNA Polymerase (Finnzymes). The 6 libraries, index tagged in equimolar amounts in the pool, were combined after GPCR using SYBR Green PCR Master Mix (Applied Biosystems). On the cBot automated cluster generation system (Illumina), cluster generation appeared in the flow and the flow cell was loaded on the HISEQ 2500 sequencing system (Illumina) for sequencing with 2x101bp read length.

6. Whole Exome Sequencing and variant analysis

An average of 6.77 gagabases of raw sequence was generated per sample achieving an average of 80.36x coverage of the WES target regions (80 megabases). Screening of 16
individuals’ raw data was done to eliminate common artifacts prior to comparison with comparing data sets. Following was considered selecting and filtering SNP variants: variants were considered to be common if present in the 1000 Genomes Project East Asian database. Variants present in 1000 Genomes Project East Asian Population, Polyphen, dbSNP137, and 126 Korean Population data were filtered out from case variants. Variants were eliminated if they had minor allele frequencies (MAF) $\geq 0.05$ from all comparing datasets. Effect, impact (high or moderate), and loss of function in WES were established with SnpEff v3.3h (http://snpeff.sourceforge.net/). To building the reference genome, B37 was used and only reads mapping to a unique position in the reference genomes were used for prediction of variants (variant calling). Taking into account the single nucleotide polymorphisms obtained from the Single Nucleotide Polymorphism Database (dbSNP, National Center for Biotechnology Information) and from the 1000 Genomes project, genome Analysis Toolkit (GATKv2.7-1) software was used to identify variants Annotated, non-synonymous variants found in affected individuals were compared to variants present in the non-affected comparing sets. Variants present in affected individuals but not in comparing data were ranked based on this analysis to generate a list of candidate genes.

7. **Statistical analysis and comparison to known genes**

   Sequence Kernel Association Test (SKAT) which can evaluate the cumulative effect of rare and common variants in genetic study was applied for the current study. With the use
of SKAT, multiple regression test of phenotype on genotypes for all variants in the region is possible \(^5\). All values in SKAT including magnitudes and directionality of the associations are based on estimates made from raw data set. In current study, results were categorized by p-value and used for further analysis. Known genes list from the previous studies was constructed to investigate the association between candidate genes from the current and previous studies known to be associated with BRONJ. Because BRONJ occurs after taking bisphosphonate medication, absorption, distribution, metabolism, and excretion (ADME) gene list was constructed to see the association between BRONJ and drug response. Candidate genes from previous studies included \textit{VEGF, COLIA1, CYP2C8, FDPS, RBMS3, G20210A, PPARG, MMP9, RANKL, IL1B, LRP5, VDR, IGFBP7, ABCC4, MMP2, RANK, OPG, OPN}, and \textit{CYP19A1}. ADME gene list consisted of genes determined to be associated with drug metabolism (www.pharmaADME.org). Filtered gene list of case population was compared to gene lists mentioned above.

To investigate the association between ADME genes discovered from the current study and BRONJ pathogenesis, gene set enrichment analysis (GSEA) was applied.
III. Results

The mean age of the 16 individuals recruited for current study was 73.90 ± 8.37. All individuals had history of BP medication with varying duration. 7 individuals discontinued their BP medication before the surgery where as 7 other individuals’ discontinuation of BP drugs was after the surgery. Specific history of BP medication history of remaining 2 individuals was not available at the time of sample collection. Information regarding all 16 individuals is provided in Table I. Quality Control (QC) run ensured the samples condition for WES. An average of 67,035,644 reads and 6,771 megabases were obtained from the sixteen individual’s WES results. An average of 4,138,925,783.75 total bases was aligned with mean coverage depth of 80.36. All information regarding number of reads, sample coverage and sequencing depth, as well as the data quality, are summarized in Table II. A total of 118,856 variants were established and variants were annotated via SnpEff to eliminate the ones not altering protein sequence as well as to predict effects of each variant on genes. Total number of 32,160 variants remained after filtering via SnpEff where impact of each gene was used to eliminate the ones with low effect (High, Moderate, Low, Modifier). Ones with high and moderate impact only remained after SnpEff filtering. Common variants from 1000 Genomes project East Asian population and 126 Korean randomized subsamples were used to filter out common variants in 16 individuals’ WES result where variants with p-value greater than 0.05 was categorized as common variant. The result showed 15,633 and 11,144 remaining variants respectively in each steps. Filtering against Polyphen
database finalized the filtering process and 2,180 variants remained (Table III). Variants were then translated into genes and number of genes remaining after all filtering step was 1,866. SKAT was utilized to enhance statistical significance of all genes in current study which had relatively small number of cases. Gene transcripts were aligned by p-value and 998 gene transcripts remained after ranking them by p-value (p-value < 0.05).
Table II. Summary of number of reads and coverage

| Sample ID | Yield (Mbases) | # Reads   | % of >= Q30 Bases (PF) | Mean Quality Score (PF) |
|-----------|----------------|-----------|------------------------|-------------------------|
| Exp1      | 7,928          | 78,492,298| 91.67                  | 35.77                   |
| Exp2      | 5,323          | 52,704,436| 91.52                  | 35.72                   |
| Exp3      | 6,661          | 65,954,782| 91.65                  | 35.76                   |
| Exp4      | 5,794          | 57,370,070| 91.45                  | 35.69                   |
| Exp5      | 6,466          | 64,019,308| 92.54                  | 36.03                   |
| Exp6      | 7,150          | 70,789,362| 92.4                   | 35.97                   |
| Exp7      | 7,183          | 71,115,752| 92.37                  | 35.98                   |
| Exp8      | 6,999          | 69,295,136| 92.33                  | 35.96                   |
| Exp9      | 6,879          | 68,105,898| 92.26                  | 35.94                   |
| Exp10     | 6,330          | 62,677,148| 92.41                  | 35.97                   |
| Exp11     | 6,570          | 65,050,110| 92.25                  | 35.92                   |
| Exp12     | 6,317          | 62,549,018| 92.11                  | 35.86                   |
| Exp13     | 6,939          | 68,700,752| 92.19                  | 35.91                   |
| Exp14     | 8,307          | 82,242,770| 92.13                  | 35.91                   |
| Exp15     | 6,736          | 66,694,634| 92.26                  | 35.93                   |
| Exp16     | 6,748          | 66,808,832| 92.31                  | 35.95                   |
Table III. Number of variants/genes common to the 16 individuals in each filtering step

| Filtering Step                        | Number of Variants | Number of Genes |
|---------------------------------------|--------------------|-----------------|
| Total Gene Transcripts                 | 118,856            | 21,581          |
| Impact                                | 32,160             | 11,369          |
| 1000 Genomes East Asian               | 15,633             | 7,512           |
| Korean randomized subsample           | 11,144             | 6,768           |
| Polyphen                              | 2,180              | 1,866           |
Known genes associated with BRONJ from previous studies were tested for their presence in current study (Table IV). VEGF, COL1A1, CYP2C8, FDPS, RBMS3, G20210A, PPARG, MMP9, RANKL, IL1B, LRP5, VDR, IGFBP7, ABCC4, MMP2, RANK, OPG, OPN and CYP19A1 were all examined and only RBMS3 was detected in the current study.
**Table IV.** Number of known variants/genes present in each filtering step

| Filtering Step                | Number of Variants | Number of Genes |
|-------------------------------|--------------------|-----------------|
| Total Gene Transcripts        | 88                 | 12              |
| Impact                        | 21                 | 9               |
| 1000 Genomes East Asian       | 9                  | 6               |
| Korean                        | 6                  | 4               |
| Polyphen                      | 1*                 | 1*              |

*refers to gene RBMS3
A list of 4,564 SNPs compiled for pharmacogenetics studies related to ADME genes were examined and total of 38 out of 299 genes were detected from current study’s filtered gene lists. This result was then categorized by function and the number of samples affected by ADME gene and the result are shown in Table V.

GSEA was applied to see the association between 38 ADME genes from the current study result and BRONJ pathogenesis. The result of GSEA was significant with the p-value of 2.2857x10^{-7} (Table VI).
**Table V.** Number of ADME genes in BRONJ case population and ADME gene list categorized by function and the number of affected samples

| ADME | Number of genes : 38 |
|------|----------------------|
| Gene | Class  | Count | Gene | Class | Count |
| ADH1A | Phase I | 1 | ABCB11 | Transporter | 1 |
| AOX1 | Phase I | 1 | ABCC10 | Transporter | 1 |
| CES1 | Phase I | 1 | ABCC11 | Transporter | 1 |
| CYP1A1 | Phase I | 1 | ABCC2 | Transporter | 1 |
| CYP27B1 | Phase I | 1 | ABCC5 | Transporter | 1 |
| CYP3A5 | Phase I | 1 | ABCG2 | Transporter | 1 |
| CYP51A1 | Phase I | 1 | SLC10A1 | Transporter | 1 |
| CYP7A1 | Phase I | 1 | SLC13A2 | Transporter | 1 |
| DHRS1 | Phase I | 1 | SLC22A1 | Transporter | 1 |
| DHRS2 | Phase I | 1 | SLC22A1 | Transporter | 1 |
| HSD17B14 | Phase I | 1 | SLC28A1 | Transporter | 1 |
| PON3 | Phase I | 1 | SLC28A2 | Transporter | 1 |
| CYP21A2 | Phase I | 2 | SLC29A2 | Transporter | 1 |
| CYP2A7 | Phase I | 2 | SLC2A5 | Transporter | 1 |
| CYP2B6 | Phase I | 2 | SLCO2B1 | Transporter | 1 |
| ALDH3B2 | Phase I | 3 | SLC15A1 | Transporter | 2 |
| CHST8 | Phase II | 1 | ABCB6 | Transporter | 3 |
| GSTZ1 | Phase II | 1 | ABCA4 | Transporter | 4 |
| TPMT | Phase II | 1 | CFTR | Modifier | 4 |

**Table VI.** ADME gene’s Gene Set Enrichment Analysis Result

| ADME | Likelihood score | P-value |
|------|-----------------|---------|
|      | 2.74823225      | 2.2857x10^{-7} |
IV. Discussion

BP is widely used drug for osteoporosis. According to the statistics, it is prescribed at 73 percent of physician visit for osteoporosis in the United States \(^{59}\). It is difficult to determine the exact prevalence of BRONJ and the statistics varies in multiple studies. For example, intravenous application reported the prevalence to be 0-27.5\% where as oral application reported 0-4.3\% of prevalence. Despite all statistics regarding BRONJ and BP prescriptions given to osteoporosis patients, the effect of BP use needs to be ascertained classifying any complications that might arise in any medical procedure. There are few BRONJ diagnostic methods and serodiagnosis such as serum CTX (carboxy-terminal collagen crosslinks) or measuring the level of osteocalcin has been used to diagnose the risk of developing BRONJ. Radiologic examination such as bone scintigraphy and MRI are often used in current clinical practice despite the inaccuracy of tests because there is no better diagnostic tool to measure the risk of developing BRONJ at this point \(^{60,61}\). Hence, developing innovative diagnostic tool for BRONJ is essential for improvement.

The occurrence of the disease in human can be distinguished via the difference in individual susceptibility to the disease caused by genetic variants of genes determined according to the type of genetic disease association\(^ {29} \). Two types of genetic diseases are often discussed in genetic studies and they are monogenic disorders and multigenic disorders. In pathogenesis of both monogenic and multigenic disorders, changes in normal protein sequence play the major role and the frequency of these protein sequence changes has varieties. Early studies on BRONJ focused on finding candidate genes and
using those candidate genes to see association of genetic variants and risk of developing BRONJ. Such method was developed based on that the number of BRONJ patients who participated in the experiment was small and selection process of candidate genes seemed contrived. By 2005, researchers were able to use haplotype map of human genome (HapMap) information and researchers could genotype multiple genes at the same time which allowed them to perform genome wide association study (GWAS) where selection of candidate gene is not necessary in the beginning of the study compared to studies on polymorphism on candidate genes.

After Frederick Sanger developed Sanger Sequencing, genetic information on various species has been revealed throughout the years. This led to completion of Human Genome Project in 2003 which was to develop the genome map and the project is still going on sequencing animals, plants and microorganisms all over the world. However, there are cost and time limitations in pursuing Whole genome sequencing to reveal genetic variants related to various diseases in clinical studies. In previous genetic association study of BRONJ, conventional genetic sequencing method such as candidate gene analysis, GWAS and target sequencing have been used to link specific genes with BRONJ. Nonetheless, none of previous researches identified specific gene(s) related to risk of developing BRONJ in patients taking BP.

Next generation sequencing (NGS) has been widely discussed over the years in genetic association studies. Of all NGS method, WES in particular is cost effective covering up to 80 percent of coding region. Hence, this particular sequencing method is the most powerful approach in sequencing and the most effective way to identify genes associated with clinical research through functional sequence variations. Using already developed
human genome database, WES can build case database and eliminate common variants detected from the control group in case WES data revealing possible variants associated with disease. Such approach was taken for current study narrowing down number of variants to 2,180 from total of 118,856 variants initially called from the case groups. In the past, most genetics studies utilized microarray data or metabolic pathway databases to find candidate genes associated with certain disease. Known genes from previous BRONJ genetic studies have been explored in current study for their presence and only RBMS3 was detected from 16 patients’ WES data. This particular result showed uncertainty of previously revealed genes known to be associated with BRONJ. The presence of RBMS3 from the current study is significant in comparison to that of previous BRONJ genetic studies. RBMS3 was an intron detection from previous BRONJ studies but its presence in the current study was found in axon which could imply its significance in BRONJ pathogenesis.

RBMS3 is a binding protein for Prx1, a homeobox transcriptional factor that upregulates collagen type I in fibroblasts. Type I collagen, coded by the COL1A gene family, is the main part of the bone matrix. Mutations in those genes produce genetic bone disorders characterized by fragile bones such as osteogenesis imperfecta. Variations in RBMS3 and COL1A have previously been associated with a decrease in bone mass and osteoporotic fractures, linking both genes with bone turnover. However, COL1A gene was not present in the current study. RBMS3 on the other hand is known to be associated with cell proliferation, angiogenesis inhibition and apoptosis induction. One of the possible BRONJ etiopathogenic mechanisms assumes that it can be caused by BP-associated suppressed bone turnover that leads to decreased
blood flow, bone cell necrosis, and apoptosis. Recently it was also shown that BPs downregulated collagen type I synthesis in human gingival fibroblasts and osteoblasts.

BRONJ only occurring on patients with BP medication could be explained by ADME gene association. Such hypothesis was tested via ADME gene list association analysis and total of 38 genes were detected. Following four categories apply to ADME genes: Phase I metabolism enzymes, responsible for the modification of functional groups; Phase II metabolism enzymes responsible for the conjugation with endogenous moieties; transporters, responsible for the uptake and excretion of drugs in and out of cells; and modifiers, that can either alter the expression of other ADME genes or affect the biochemistry of ADME enzymes. Four categories take 43%, 23%, 26%, and 8% respectively in the whole ADME gene list and ADME genes associated with transport showed the highest percentage in the current study. Most of transport genes detected in the current study are in charge of multi-drug resistance as well as having protective role in biological system such as bone marrow. Considering their role in drug delivery, a mutation in transporter genes could aggravate the adverse drug reaction. Further analysis via GSEA elucidated the significant association to BRONJ. Previous study on pharmacogenetics of BRONJ described few aspects related to adverse drug reactions and pointed out that the absence of human leukocyte antigen (HLA) variant which is associated with adverse drug reactions that have an immune-related pathogenesis could imply that the adverse drug reaction of BP could be a toxic adverse drug reaction. With limitations in mind, the current study could imply that mutations on ADME genes might enlarge intrinsic toxic effects of BP drugs based on the types of ADME genes and RBMS3 SNP found in the current study along with the absence of HLA variation.
The limitation of the current study was that the case population was relatively small to obtain specific genes associated with BRONJ. The better study design with a bigger study population including the ones without BRONJ even though they have been prescribed with BP medication in the past as the control population would provide more precise results along with the genetic explanation to BRONJ pathogenesis.
V. Conclusion

Previous analyses of BRONJ focused on identifying single candidate gene and its polymorphisms possibly associated with the risk of developing BRONJ. Even though the case population was small, WES result confirmed the presence of genes from previous studies implying that several genes are involved in the pathogenesis of BRONJ. In the current study, *RBMS3* and ADME genes are thought to play a pivot role in developing BRONJ. Further study with more case and control population would ascertain the pathogenesis and early diagnosis of BRONJ in patients with BP prescription history.
References

1. Carter G, Goss AN, Doecke C. Bisphosphonates and avascular necrosis of the jaw: a possible association. *The Medical journal of Australia*. Apr 18 2005;182(8):413-415.

2. Drake MT, Clarke BL, Khosla S. Bisphosphonates: mechanism of action and role in clinical practice. *Mayo Clinic proceedings. Mayo Clinic*. Sep 2008;83(9):1032-1045.

3. Greenberg MS. Intravenous bisphosphonates and osteonecrosis. *Oral surgery, oral medicine, oral pathology, oral radiology, and endodontology*. Sep 2004;98(3):259-260.

4. Wang J, Goodger NM, Pogrel MA. Osteonecrosis of the jaws associated with cancer chemotherapy. *Journal of oral and maxillofacial surgery : official journal of the American Association of Oral and Maxillofacial Surgeons*. Sep 2003;61(9):1104-1107.

5. Capsoni F, Longhi M, Weinstein R. Bisphosphonate-associated osteonecrosis of the jaw: the rheumatologist’s role. *Arthritis research & therapy*. 2006;8(5):219.

6. Conte P, Guarnieri V. Safety of intravenous and oral bisphosphonates and compliance with dosing regimens. *The oncoologist*. 2004;9 Suppl 4:28-37.

7. Markiewicz MR, Margarone JE, 3rd, Campbell JH, Aguirre A. Bisphosphonate-associated osteonecrosis of the jaws: a review of current knowledge. *J Am Dent Assoc*. Dec 2005;136(12):1669-1674.

8. Ruggiero SL, Drew SJ. Osteonecrosis of the jaws and bisphosphonate therapy. *Journal of dental research*. Nov 2007;86(11):1013-1021.

9. Advisory Task Force on Bisphosphonate-Related Osteonecrosis of the Jaws AAoO, Maxillofacial S. American Association of Oral and Maxillofacial Surgeons position paper on bisphosphonate-related osteonecrosis of the jaws. *Journal of oral and maxillofacial surgery : official journal of the American Association of Oral and Maxillofacial Surgeons*. Mar 2007;65(3):369-376.

10. American Dental Association Council on Scientific A. Dental management of patients receiving oral bisphosphonate therapy:
expert panel recommendations. J Am Dent Assoc. Aug 2006;137(8):1144-1150.

11. Bagan J, Blade J, Cozar JM, et al. Recommendations for the prevention, diagnosis, and treatment of osteonecrosis of the jaw (ONJ) in cancer patients treated with bisphosphonates. Medicina oral, patología oral y cirugía bucal. Aug 2007;12(4):E336-340.

12. Edwards BJ, Hellstein JW, Jacobsen PL, et al. Updated recommendations for managing the care of patients receiving oral bisphosphonate therapy: an advisory statement from the American Dental Association Council on Scientific Affairs. J Am Dent Assoc. Dec 2008;139(12):1674-1677.

13. Khan AA, Sandor GK, Dore E, et al. Canadian consensus practice guidelines for bisphosphonate associated osteonecrosis of the jaw. The Journal of rheumatology. Jul 2008;35(7):1391-1397.

14. Khosla S, Burr D, Cauley J, et al. Bisphosphonate-Associated Osteonecrosis of the Jaw: Report of a Task Force of the American Society for Bone and Mineral Research. J Bone Miner Res. 2007;22(10):1479-1491.

15. McLeod NM, Patel V, Kusanale A, Rogers SN, Brennan PA. Bisphosphonate osteonecrosis of the jaw: a literature review of UK policies versus international policies on the management of bisphosphonate osteonecrosis of the jaw. The British journal of oral & maxillofacial surgery. Jul 2011;49(5):335-342.

16. MIGLIORATI CA, CASIGLIA J, EPSTEIN J, JACOBSEN PL, SIEGEL MA, WOO S-B. Managing the care of patients with bisphosphonate-associated osteonecrosis: An American Academy of Oral Medicine position paper. The Journal of the American Dental Association. December 1, 2005 2005;136(12):1658-1668.

17. Ruggiero S, Gralow J, Marx RE, et al. Practical Guidelines for the Prevention, Diagnosis, and Treatment of Osteonecrosis of the Jaw in Patients With Cancer. Journal of Oncology Practice. January 1, 2006 2006;2(1):7-14.

18. Ruggiero SL, Dodson TB, Assael LA, et al. American Association of Oral and Maxillofacial Surgeons position paper on bisphosphonate-related osteonecrosis of the jaws—2009 update. Journal of oral and
maxillofacial surgery: official journal of the American Association of Oral and Maxillofacial Surgeons. May 2009;67(5 Suppl):2-12.

19. Tubiana-Hulin M, Spielmann M, Roux C, et al. Physiopathology and management of osteonecrosis of the jaws related to bisphosphonate therapy for malignant bone lesions. A French expert panel analysis. Critical reviews in oncology/hematology. Jul 2009;71(1):12-21.

20. Weitzman R, Sauter N, Eriksen EF, et al. Critical review: updated recommendations for the prevention, diagnosis, and treatment of osteonecrosis of the jaw in cancer patients—May 2006. Critical reviews in oncology/hematology. May 2007;62(2):148-152.

21. Khosla S, Burr D, Cauley J, et al. Bisphosphonate-associated osteonecrosis of the jaw: report of a task force of the American Society for Bone and Mineral Research. Journal of bone and mineral research: the official journal of the American Society for Bone and Mineral Research. Oct 2007;22(10):1479-1491.

22. Borromeo GL, Tsao CE, Darby IB, Ebeling PR. A review of the clinical implications of bisphosphonates in dentistry. Australian dental journal. Mar 2011;56(1):2-9.

23. Zavras AI. The impact of bisphosphonates on oral health: lessons from the past and opportunities for the future. Annals of the New York Academy of Sciences. Feb 2011;1218:55-61.

24. Allen MR, Burr DB. The pathogenesis of bisphosphonate-related osteonecrosis of the jaw: so many hypotheses, so few data. Journal of oral and maxillofacial surgery: official journal of the American Association of Oral and Maxillofacial Surgeons. May 2009;67(5 Suppl):61-70.

25. Bamias A, Kastritis E, Bamia C, et al. Osteonecrosis of the jaw in cancer after treatment with bisphosphonates: incidence and risk factors. Journal of clinical oncology: official journal of the American Society of Clinical Oncology. Dec 1 2005;23(34):8580-8587.

26. Bi Y, Gao Y, Ehirchiou D, et al. Bisphosphonates cause osteonecrosis of the jaw-like disease in mice. The American journal of pathology. Jul 2010;177(1):280-290.
27. Kim JH, Park YB, Li Z, et al. Effect of alendronate on healing of extraction sockets and healing around implants. *Oral diseases.* Oct 2011;17(7):705-711.

28. Santini D, Vincenzi B, Tonini G, Scarpa S, Baldi A. Zoledronic acid exhibits inhibitory effects on osteoblastic and osteolytic metastases of prostate cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research.* Aug 1 2003;9(8):3215; author reply 3216.

29. Wood J, Bonjean K, Ruetz S, et al. Novel antiangiogenic effects of the bisphosphonate compound zoledronic acid. *The Journal of pharmacology and experimental therapeutics.* Sep 2002;302(3):1055-1061.

30. Bilezikian JP. Osteonecrosis of the jaw - Do bisphosphonates pose a risk? *New Engl J Med.* Nov 30 2006;355(22):2278-2281.

31. Schwartz HC. Osteonecrosis of the jaws: a complication of cancer chemotherapy. *Head & neck surgery.* Jan-Feb 1982;4(3):251-253.

32. Zervas K, Verrou E, Teleioudis Z, et al. Incidence, risk factors and management of osteonecrosis of the jaw in patients with multiple myeloma: a single-centre experience in 303 patients. *British journal of haematology.* Sep 2006;134(6):620-623.

33. Badros A, Weikel D, Salama A, et al. Osteonecrosis of the jaw in multiple myeloma patients: clinical features and risk factors. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology.* Feb 20 2006;24(6):945-952.

34. Bagan J, Scully C, Sabater V, Jimenez Y. Osteonecrosis of the jaws in patients treated with intravenous bisphosphonates (BRONJ): A concise update. *Oral oncology.* Jul 2009;45(7):551-554.

35. Dimopoulos MA, Kastritis E, Anagnostopoulos A, et al. Osteonecrosis of the jaw in patients with multiple myeloma treated with bisphosphonates: evidence of increased risk after treatment with zoledronic acid. *Haematologica.* Jul 2006;91(7):968-971.

36. Wessel JH, Dodson TB, Zavras AI. Zoledronate, smoking, and obesity are strong risk factors for osteonecrosis of the jaw: a case-control study. *Journal of oral and maxillofacial surgery : official journal of the American Association of Oral and Maxillofacial Surgeons.* Apr 2008;66(4):625-631.
37. Marx RE, Sawatari Y, Fortin M, Broumand V. Bisphosphonate-induced exposed bone (osteonecrosis/osteopetrosis) of the jaws: risk factors, recognition, prevention, and treatment. *Journal of oral and maxillofacial surgery : official journal of the American Association of Oral and Maxillofacial Surgeons*. Nov 2005;63(11):1567-1575.

38. Mehrotra B, Ruggiero S. Bisphosphonate complications including osteonecrosis of the jaw. *Hematology / the Education Program of the American Society of Hematology. American Society of Hematology. Education Program*. 2006:356-360, 515.

39. Kuhl S, Walter C, Acham S, Pfeffer R, Lambrecht JT. Bisphosphonate-related osteonecrosis of the jaws--a review. *Oral oncology*. Oct 2012;48(10):938-947.

40. Kim S-KK, Tea-Geon. Clinical investigation of bisphosphonate-related osteonecrosis of the jaws in patients with malignant tumors. *Journal of the Korean association of oral and maxillofacial surgeons*. 2012;38(NO.3):152-159.

41. Saia G, Blandamura S, Bettini G, et al. Occurrence of bisphosphonate-related osteonecrosis of the jaw after surgical tooth extraction. *Journal of oral and maxillofacial surgery : official journal of the American Association of Oral and Maxillofacial Surgeons*. Apr 2010;68(4):797-804.

42. Hoff AO, Toth B, Hu M, Hortobagyi GN, Gagel RF. Epidemiology and risk factors for osteonecrosis of the jaw in cancer patients. *Annals of the New York Academy of Sciences*. Feb 2011;1218:47-54.

43. De Gobbi M, Viprakasit V, Hughes JR, et al. A regulatory SNP causes a human genetic disease by creating a new transcriptional promoter. *Science (New York, N.Y.).* May 26 2006;312(5777):1215-1217.

44. Arduino PG, Menegatti E, Scoletta M, et al. Vascular endothelial growth factor genetic polymorphisms and haplotypes in female patients with bisphosphonate-related osteonecrosis of the jaws. *Journal of oral pathology & medicine : official publication of the International Association of Oral Pathologists and the American Academy of Oral Pathology*. Jul 2011;40(6):510-515.
45. Ashford RU, Luchetti M, McCloskey EV, et al. Studies of bone density, quantitative ultrasound, and vertebral fractures in relation to collagen type I alpha 1 alleles in elderly women. *Calcified tissue international*. Jun 2001;68(6):348-351.

46. Balla B, Vaszilko M, Kosa JP, et al. New approach to analyze genetic and clinical data in bisphosphonate-induced osteonecrosis of the jaw. *Oral diseases*. Sep 2012;18(6):580-585.

47. Basi DL, Hughes PJ, Thumbigere-Math V, et al. Matrix metalloproteinase-9 expression in alveolar extraction sockets of Zoledronic acid-treated rats. *Journal of oral and maxillofacial surgery : official journal of the American Association of Oral and Maxillofacial Surgeons*. Nov 2011;69(11):2698-2707.

48. Katz J, Gong Y, Salmasinia D, et al. Genetic polymorphisms and other risk factors associated with bisphosphonate induced osteonecrosis of the jaw. *International journal of oral and maxillofacial surgery*. Jun 2011;40(6):605-611.

49. Massart F, Brandi ML. Genetics of the bone response to bisphosphonate treatments. *Clinical cases in mineral and bone metabolism : the official journal of the Italian Society of Osteoporosis, Mineral Metabolism, and Skeletal Diseases*. Jan 2009;6(1):50-54.

50. Nuttall JM, Hettema EH, Watts DJ. Farnesyl diphosphate synthase, the target for nitrogen-containing bisphosphonate drugs, is a peroxisomal enzyme in the model system Dictyostelium discoideum. *The Biochemical journal*. Nov 1 2012;447(3):353-361.

51. Olmos JM, Zarrabeitia MT, Hernandez JL, Sanudo C, Gonzalez-Macias J, Riancho JA. Common allelic variants of the farnesyl diphosphate synthase gene influence the response of osteoporotic women to bisphosphonates. *The pharmacogenomics journal*. Jun 2012;12(3):227-232.

52. Vairaktaris E, Vassiliou S, Avgoustidis D, Stathopoulos P, Toyoshima T, Yapijakis C. Bisphosphonate-induced avascular osteonecrosis of the mandible associated with a common thrombophilic mutation in the prothrombin gene. *Journal of oral and maxillofacial surgery : official journal of the American Association of Oral and Maxillofacial Surgeons*. Sep 2009;67(9):2009-2012.
Zhong DN, Wu JZ, Li GJ. Association between CYP2C8 (rs1934951) polymorphism and bisphosphonate-related osteonecrosis of the jaws in patients on bisphosphonate therapy: a meta-analysis. *Acta haematologica*. 2013;129(2):90-95.

Sarasquete ME, Garcia-Sanz R, Marin L, et al. Bisphosphonate-related osteonecrosis of the jaw is associated with polymorphisms of the cytochrome P450 CYP2C8 in multiple myeloma: a genome-wide single nucleotide polymorphism analysis. *Blood*. Oct 1 2008;112(7):2709-2712.

English BC, Baum CE, Adelberg DE, et al. A SNP in CYP2C8 is not associated with the development of bisphosphonate-related osteonecrosis of the jaw in men with castrate-resistant prostate cancer. *Therapeutics and clinical risk management*. 2010;6:579-583.

Such E, Cervera J, Terpos E, et al. CYP2C8 gene polymorphism and bisphosphonate-related osteonecrosis of the jaw in patients with multiple myeloma. *Haematologica*. Oct 2011;96(10):1557-1559.

Nicoletti P, Cartsos VM, Palaska PK, Shen Y, Floratos A, Zavras AI. Genomewide pharmacogenetics of bisphosphonate-induced osteonecrosis of the jaw: the role of RBMS3. *The oncologist*. 2012;17(2):279-287.

Wu MC, Lee S, Cai T, Li Y, Boehnke M, Lin X. Rare-variant association testing for sequencing data with the sequence kernel association test. *American journal of human genetics*. Jul 15 2011;89(1):82-93.

Stafford RS, Drieling RL, Hersh AL. National trends in osteoporosis visits and osteoporosis treatment, 1988-2003. *Archives of internal medicine*. Jul 26 2004;164(14):1525-1530.

Kwon YD, Kim DY, Ohe JY, Yoo JY, Walter C. Correlation between serum C-terminal cross-linking telopeptide of type I collagen and staging of oral bisphosphonate-related osteonecrosis of the jaws. *Journal of oral and maxillofacial surgery : official journal of the American Association of Oral and Maxillofacial Surgeons*. Dec 2009;67(12):2644-2648.

O'Ryan FS, Khoury S, Liao W, et al. Intravenous bisphosphonate-related osteonecrosis of the jaw: bone scintigraphy as an early
indicator. *Journal of oral and maxillofacial surgery : official journal of the American Association of Oral and Maxillofacial Surgeons.* Jul 2009;67(7):1363-1372.

62. Chiu RW, Lo YM. Non-invasive prenatal diagnosis by fetal nucleic acid analysis in maternal plasma: the coming of age. *Seminars in fetal & neonatal medicine.* Apr 2011;16(2):88-93.

63. Link DC, Schuettelpelz LG, Shen D, et al. Identification of a novel TP53 cancer susceptibility mutation through whole-genome sequencing of a patient with therapy-related AML. *JAMA : the journal of the American Medical Association.* Apr 20 2011;305(15):1568-1576.

64. Lupski JR, Reid JG, Gonzaga-Jauregui C, et al. Whole-genome sequencing in a patient with Charcot-Marie-Tooth neuropathy. *The New England journal of medicine.* Apr 1 2010;362(13):1181-1191.

65. Sobreira NL, Cirulli ET, Avramopoulos D, et al. Whole-genome sequencing of a single proband together with linkage analysis identifies a Mendelian disease gene. *PLoS genetics.* Jun 2010;6(6):e1000991.

66. Kahvejian A, Quackenbush J, Thompson JF. What would you do if you could sequence everything? *Nature biotechnology.* Oct 2008;26(10):1125-1133.

67. Fritz D, Stefanovic B. RNA-binding protein RBMS3 is expressed in activated hepatic stellate cells and liver fibrosis and increases expression of transcription factor Prx1. *Journal of molecular biology.* Aug 17 2007;371(3):585-595.

68. Rauch F, Glorieux FH. Osteogenesis imperfecta. *Lancet.* Apr 24 2004;363(9418):1377-1385.

69. Kiel DP, Demissie S, Dupuis J, Lunetta KL, Murabito JM, Karasik D. Genome-wide association with bone mass and geometry in the Framingham Heart Study. *BMC medical genetics.* 2007;8 Suppl 1:S14.

70. Qureshi AM, Herd RJ, Blake GM, Fogelman I, Ralston SH. COLIA1 Sp1 polymorphism predicts response of femoral neck bone density to cyclical etidronate therapy. *Calcified tissue international.* Mar 2002;70(3):158-163.
71. Chen J, Kwong DL, Zhu CL, et al. RBMS3 at 3p24 inhibits nasopharyngeal carcinoma development via inhibiting cell proliferation, angiogenesis, and inducing apoptosis. *PloS one.* 2012;7(9):e44636.

72. Rizzoli R, Burlet N, Cahall D, et al. Osteonecrosis of the jaw and bisphosphonate treatment for osteoporosis. *Bone.* May 2008;42(5):841-847.

73. Simon MJ, Niehoff P, Kimmig B, Wiltfang J, Acil Y. Expression profile and synthesis of different collagen types I, II, III, and V of human gingival fibroblasts, osteoblasts, and SaOS-2 cells after bisphosphonate treatment. *Clinical oral investigations.* Feb 2010;14(1):51-58.
국문요약

전체 엑솜 염기서열 분석과 생물정보학을 통한 비스포스포네이트 관련 악골괴사의 유전적 연구

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목적: 비스포스포네이트는 골다공증, 악성종양 환자에서 널리 사용되고 있는 약제이다. 2003 년부터 이 약제의 부작용을 치과의사가 보고하기 시작하였다. 비스포스포네이트 관련 악골괴사(BRONJ)는 비스포스포네이트 관련 합병증들 중 가장 심각한 합병증이라 할 수 있으며 이는 주로 사소한 외과적 치과 치료 후 장시간 동안 치유되지 않는 형태로 광범위한 악골의 손실을 야기한다. 주목할 점은 비스포스포네이트를 처방 받은 모든 환자에게 BRONJ 가 나타나는 것은 아니며, 다양한 환경적 요인들과 유전적인 요인들의 영향을 받는 점으로 여겨진다. 본 연구의 목적은 전체 엑솜 염기서열 분석법(Whole Exome Sequencing:...
WES)을 통해 얻은 자료를 토대로 통계 분석 및 선행 연구에서 알려진 유전자들과의 비교를 통해 BRONJ와 관련된 원인 유전자군을 밝히는 것이다.

방법: 전체 액손 염기서열 분석법을 위하여 비스포스포네이트 약제를 처방 받은 16명의 환자들의 타액을 채취하였다. 타액 표본들에 대해 전체 액손 염기서열 분석을 시행하였고 그 자료는 SnpEff, 동아시아 인구의 1000 유전체 프로젝트, 무작위로 표본추출 된 126명의 건강한 한국인 유전체(GSK project), Polyphen을 이용하여 일반적인 변이들을 제거하였다.

BRONJ와 이전 연구들을 통하여 알려진 유전자들과의 연관성을 알아보기 위하여 ADME(absorption, distribution, metabolism and excretion) 유전자 목록들, VEGF, COLIA1, CYP2C8, FDP5, RBMS3, G20210A, PPARG, MPP9, RANKL, IL1B, LRP5, VDR, IGFBP7, ABCC4, MMP2, RANK, OPG, OPN, CYP19A1 등의 유전자 목록들과 본 연구에서 추출된 유전자목록들을 비교하였다.

결과: 총 118,856개의 변이들이 발견되었고 1,866개의 유전자에 해당하는 2,180개의 변이들이 필터링 과정을 통하여 얻어졌다. 생물정보학을 통하여 BRONJ 유발과 관련이 있을 수 있는 유전자 집합체를 찾아냈다. 비스포스포네이트 약제의 기능과 연관 있다고 알려진 유전자들과 본 연구에서 발견한 유전자들과의 비교를 통하여서는 가장 최근 GWAS 연구를 통해 알려진 RBMS3 유전자가 확인되었다. 또한 ADME 유전자들이 BRONJ 발생과 관련이 있음을 확인하였다.
결론: 본 연구는 여러 유전자들이 비스포스포네트를 복용한 환자들에게 있어서 BRONJ를 발생시키는데 중요한 역할을 한다는 것을 제안하고 있다. 또한 결과를 통하여 선행연구에서 언급된 RBMS3, ADME 와 같은 유전자들과 BRONJ와의 연관성을 확인할 수 있었다.

핵심되는 말: 비스포스포네트, BRONJ, 악골괴사, 전체 엑솜 염기서열 분석법 (WES), 생물정보학, RBMS3, ADME