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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고용재 드림

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Abstract

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

Yong Jae Ko D.D.S., M.S.D.

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

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, COLIAI, 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 improtant 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 [1-3]. 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 2003 [4] 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 present[21].

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 [30], concomitant use of chemotherapy [31], treatment with glucocorticoid [9] or thalidomide [32], length of exposure to BP treatment [33-35], the presence of comorbid conditions such as obesity [36,37], 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 [38].

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[21].

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%[39]. 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 (*COLIAI*), cytochrome P450 subfamily 2 polypeptide 8 (*CYP2C8*), fatnesyl 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.

Table I. Patient and prescribed bisphosphonate drug information

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	Fosaqueen*	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	Fosaqueen*	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℃. Evaluation of the efficiency of the fragmentation was done using Capillary electrophoresis on DNA 100 chips (Bioanalyzer, Agilent). With the manufactruer'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℃, the hybridization buffer and DNA blocker mix were incubated for 5 and at 65℃, 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℃. The mixture of the hybridization buffer and

DNA blocker was added to the capture library and was incubated for 24 hours at 65℃ 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℃. 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) >= 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 [58]. 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 *VEGF, COLIAI, CYP2C8, FDPS, RBMS3, G20210A, PPARG, MMP9, RANKL, IL1B, LRP5, VDR, IGFBP7, ABCC4, MMP2, RANK, OPG, OPN*, and *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, COLIAI, 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 2.2857×10^{-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	*SLC22A11*	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	2.74823225
P-value	2.2857×10^{-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 [44,54,57]. 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 [62-65]. Of all NGS method, WES in particular is cost effective covering up to 80 percent of coding region [66]. 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 pervious 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 [67]. 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 [68]. Variations in *RBMS3* and *COL1A* have previously been associated with a decrease in bone mass and osteoporotic fractures, linking both genes with bone turnover [69,70].[69,70]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 [71]. 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 [72]. Recently it was also shown that BPs downregulated collagen type I synthesis in human gingival fibroblasts and osteoblasts [73].

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 [57]. 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.

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국문요약

전체 엑솜 염기서열 분석과 생물정보학을 통한 비스포스포네이트 관련 악골괴사의 유전적 연구

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고 용 재

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 질환으로 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 BRONJ 와 관련된 원인 유전자군을 밝히는 것이다.

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

BRONJ 와 이전 연구들을 통하여 알려진 유전자들과의 연관성을 알아보기 위하여 ADME(absorption, distribution, metabolism and excretion) 유전자 목록들, *VEGF, COLIAI, CYP2C8, FDPS, RBMS3, G20210A, PPARG, MMP9, RANKL, IL1B, LRP5, VDR, IGFBP7, ABCC4, MMP2, RANK, OPG, OPN, CYP19A1* 등의 유전자 목록들과 본 연구에서 추출된 유전자목록들을 비교하였다.

결과: 총 118,856 개의 변이들이 발견되었고 1,866 개의 유전자에 해당하는 2,180 개의 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 BRONJ 를 발생시키는데 중요한 역할을 한다는 것을 제안하고 있다. 또한 결과를 통하여 선행연구에서 언급된 *RBMS3*, ADME 와 같은 유전자들과 BRONJ 와의 연관성을 확인할 수 있었다.

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