JBMR®

SIRT1/HERC4 Locus Associated With Bisphosphonate-Induced Osteonecrosis of the Jaw: An Exome-Wide Association Analysis

Guang Yang,[1*] Issam S Hamadeh,[1,2*] Joseph Katz,[3] Alberto Riva,[4] Peter Lakatos,[5] Bernadett Balla,[6] Janos Kosa,[5,6] Mihaly Vaszilko,[7] Gian Andrea Pelliccioni,[8] Noa Davis,[9] Taimour Y Langaee,[1] Jan S Moreb,[10] and Yan Gong[1]

[1]Department of Pharmacotherapy and Translational Research and Center for Pharmacogenomics, College of Pharmacy, University of Florida, Gainesville, FL, USA
[2]Cancer Pharmacology Department, Levine Cancer Institute, Charlotte, NC, USA
[3]Department of Oral Medicine, College of Dentistry, University of Florida, Gainesville, FL, USA
[4]Bioinformatics Core, Interdisciplinary Center for Biotechnology Research, University of Florida, Gainesville, FL, USA
[5]1st Department of Medicine, Semmelweis University Medical School, Budapest, Hungary
[6]PentaCore Laboratory, Budapest, Hungary
[7]Department of Oro-Maxillofacial Surgery and Stomatology, Semmelweis University Dental School, Budapest, Hungary
[8]Department of Biomedical and Neuromotor Sciences, Section of Dentistry, Alma Mater Studiorum, Università di Bologna, Bologna, Italy
[9]Micromedic Technologies Ltd., Tel Aviv, Israel
[10]Department of Medicine, College of Medicine, University of Florida, Gainesville, FL, USA

ABSTRACT

Osteonecrosis of the jaw (ONJ) is a rare, but serious drug side effect, mainly associated with the use of intravenous (iv) bisphosphonates (BPs). The purpose of this study was to identify genetic variants associated with ONJ in patients of European ancestry treated with iv BPs using whole-exome sequencing (WES). The WES phase 1 included 44 multiple myeloma patients (22 ONJ cases and 22 controls) and WES phase 2 included 17 ONJ patients with solid tumors. Multivariable logistic regression analysis was performed to estimate the odds ratios (ORs) and 95% confidence intervals (CI), adjusting for age, sex, and principal components for ancestry. Meta-analysis of WES phase 1 and 2 was performed to estimate the combined ORs. In silico analyses were then performed to identify expression quantitative loci (eQTL) single-nucleotide polymorphisms (SNPs) that are in high linkage disequilibrium (LD) with the top SNPs. The associations of the potentially functional SNPs were replicated and validated in an independent case-control study of 48 patients of European ancestry treated with iv BPs (19 ONJ cases and 29 controls). The top SNPs in the exome-wide association meta-analysis were two SNPs on chromosome 10: *SIRT1* SNP rs7896005 and *HERC4* SNP rs3758392 with identical OR of 0.07 (0.01–0.46; $p = 3.83 \times 10^{-5}$). In the in silico functional analyses, two promoter region SNPs (rs7894483 and rs3758391) were identified to be in high LD with the index SNPs and are eQTLs for *SIRT1* gene in whole blood in the GTEx database. The ORs were 0.30 (0.10–0.88), 0.26 (0.12–0.55), and 0.26 (0.12–0.55) for the WES top SNP rs7896005 and two promoter SNPs rs7894483 and rs3758391, respectively, in the replication sample. In summary, we identified the *SIRT1/HERC4* locus on chromosome 10 to be associated with iv BP-induced ONJ and two promoter SNPs that might be the potential genetic markers for this association. © 2017 The Authors. *Journal of Bone and Mineral Research* Published by Wiley Periodicals Inc.

KEY WORDS: OSTEONECROSIS OF THE JAW; WHOLE-EXOME SEQUENCING; *SIRT1* GENE; BISPHOSPHONATES; PHARMACOGENOMICS

Introduction

Osteonecrosis of the jaw (ONJ), a rare but serious drug-induced adverse effect, is one of the most feared and debilitating side effects of anti-resorptive agents such as nitrogen-containing bisphosphonates (BPs) and receptor activator of NF-κB ligand (RANKL) inhibitors. The main hallmark of ONJ is the presence of area(s) of exposed bone in the mandible and/or maxilla for at least 8 weeks, which show(s) no signs of healing. Drug-induced ONJ was first reported in 2003, less than

Received in original form May 31, 2017; revised form August 20, 2017; accepted August 28, 2017. Accepted manuscript online August 30, 2017.

Address correspondence to: Yan Gong, PhD, Department of Pharmacotherapy and Translational Research, University of Florida, PO Box 100486, 1600 SW Archer Road, Gainesville, FL 32610-0486, USA. E-mail: gong@cop.ufl.edu

*GY and ISH contributed equally to this manuscript.

Journal of Bone and Mineral Research, Vol. 33, No. 1, January 2018, pp 91–98
DOI: 10.1002/jbmr.3285

1 year after the approval of intravenous (iv) BPs such as zoledronate and pamidronate for the prevention of skeletal-related events (SREs) in the setting of metastatic bone tumors.(1,2) Because ONJ was initially recognized as a serious complication of BPs, the term "bisphosphonate-related osteonecrosis of the jaw (BRONJ)" was introduced to distinguish this side effect from other spontaneous cases of ONJ.(3) With the advent of new classes of drugs or pharmacotherapies targeting signaling pathways that are essential for bone remodeling or osteoclast activation and proliferation, new cases of non-BP-induced ONJ started to emerge. In the updated guidelines from the American Association of Oral and Maxillofacial Surgeons (AAOMS), the term "medication-related osteonecrosis of the jaw (MRONJ)"(4) was introduced in light of the large body of evidence linking ONJ to non-BP drugs indicating that ONJ is no longer considered a class adverse effect of BPs. ONJ has been associated with other classes of drugs, including RANKL inhibitors, vascular endothelial growth factor (VEGF) inhibitors, as well as mammalian target of rapamycin (m-TOR) inhibitors,(5) and more recently, adalimumab (an inhibitor for tumor necrosis factor α, TNF-α) has also been found to be associated with ONJ.(6)

Although more than a decade has passed since the first reported cases of ONJ, the pathophysiology of this disease remains elusive despite all ongoing efforts aimed at not only deciphering its pathogenesis but also identifying the patients at risk of developing ONJ when treated with relevant medications. The fact that ONJ develops in a minority of patients receiving the aforementioned drug classes suggests that genetics may contribute to or has a role in its pathogenesis. The majority of the published genetic association studies on ONJ were candidate gene studies, including our own,(7) that reported genetic association of different polymorphisms/genes with ONJ such as *COL1A1, RANK, MMP2, OPG, OPN*,(7,8) *PPARG*,(9) aromatase polymorphism,(10) and MHC class II polymorphisms.(11) Two genome-wide association studies of ONJ have been published so far. Neither of the two genes discovered (*CYP2C8* and *RBMS3*) has been replicated or functionally validated.(12,13) So far, there have been no replicated or validated genetic variants associated with risk of ONJ with compelling evidence. The purpose of this study was to identify functional genetic variants associated with ONJ in patients treated with iv BPs using a two-phased whole-exome sequencing (WES) approach followed by replication/validation in an independent study. Our hypothesis is that genetic variants play a significant role in BP-induced ONJ in cancer patients.

Materials and Methods

Study design and patient selection

The inclusion criteria of our study is adult patients of any age, sex, and race who have been treated with iv BPs. The exclusion criterion is radiation therapy. ONJ cases were adjudicated by at least two investigators. Because ONJ is a rare adverse effect, the best study design is the case-control study design. ONJ cases were defined according to the AAOMS guideline.(4) The controls were selected to match the cases based on age, sex, and race among the patients who were treated with iv BPs but had not developed ONJ after 24 months of treatment. These controls were monitored to ascertain their control status. To identify genetic variants associated with ONJ in an unbiased manner, we have performed a two-phased WES on germline genomic DNA samples of a case-control study of 61 patients of European ancestry treated with iv BPs. In phase 1, we sequenced the exons and intron/exon junctions of 44 multiple myeloma patients treated with iv BPs including 22 ONJ cases and 22 age- and sex-matched controls who did not develop ONJ after at least 24 months of iv BP treatment. In phase 2, we sequenced 17 ONJ cases who were patients with solid cancers (12 breast cancer, 3 prostate cancer, 1 cervix cancer, and 1 lung cancer). These patients were recruited from the University of Florida in Gainesville, FL, USA (IRB#: IRB201501186), Semmelweis University Medical School and Dental School in Budapest, Hungary;(14) and University of Bologna, Italy.(15) This study was conducted according to principles of the Declaration of Helsinki. All patients signed an informed consent at the study site.

Whole-exome sequencing and bioinformatics analysis

Genomic DNA isolated from these patients was used for WES at Otogenetics Corporation (Norcross, GA, USA) using Human Agilent V5, 51 Mb exome kit, and Illumina Hiseq2500 sequencing platform. Bioinformatics analysis was then performed at University of Florida Bioinformatics Center. Briefly, the sequence reads were aligned to the human genome (version GRCh37/hg19) using Bowtie, version 2.2.3.(16) Sequence alignment/map tool (SAMtools) version 1.4(17,18) was used to process the alignments (eg, sorting and indexing), and variant calling was performed using the Genome Analysis Toolkit (GATK)(19) haplotype calling pipeline in multi-sample mode, according to GATK best practices.(20,21) Identified variants were then annotated for their functional effects using Annovar.(22)

Exome-wide association analysis

Variants genotype data from WES went through additional quality control (QC) steps that were designed to identify both problematic variants and problematic samples before association analysis. The QC procedure included the following: sample call rate (>95%), SNP call rate (>95%), sex/gender check, identity by state/identity by descendant check for relatedness, principal component analysis (PCA) using EIGENSTRAT software(23,24) compared with self-identified race/ethnicity, and Hardy Weinberg Equilibrium (HWE). To control for known confounders, all association analyses were adjusted for covariates such as age,(25–30) sex,(31) and principle components for ancestry. Multivariable logistic regression was performed to estimate the odds ratio (OR) and 95% confidence interval (CI) of each variant for the development of ONJ (yes/no) using PLINK(32) in the phase 1 and the phase 2 separately. Meta-analysis was then performed using METAL(33) to estimate the combined ORs of the two phases. The I^2 statistics and Cochran Q test were used to assess the heterogeneity of the studies for each meta-analysis. SNPs with $p < 10^{-6}$ was considered statistically significant, whereas SNPs with $p < 10^{-4}$ were considered suggestive. Power calculation indicates that at alpha level of 10^{-6}, minor allele frequency of 30%, we have 80% power to detect ORs of $\geq$6.5 or $\leq$0.15, assuming additive mode of inheritance.

In silico analyses

In silico analyses were performed to identify expression quantitative loci (eQTL) SNPs that are in high linkage disequilibrium (LD) with the top SNPs. LocusZoom(34) was used to create regional plots of the top SNPs. HaploReg v4.1,(35) regulomeDB(36) (Version 1.1), and Genotype-Tissue Expression project (GTEx) database(37) were used to select the most functionally important SNPs in high LD with top SNPs and to check if the selected SNPs are eQTL for certain genes.

Replication/validation study

The association of top SNPs and additional SNPs identified from in silico analyses with iv BP-induced ONJ were evaluated further using TaqMan Genotyping Assay. The top SNP was genotyped in the WES phase 1 and 2 samples using Taqman as validation for the WES genotypes and in an independent case-control study of 48 patients of European ancestry treated with iv BPs (19 ONJ cases and 29 controls) as independent replication. The additional SNPs identified using in silico analysis were not captured in the WES; therefore, genotypes in all samples were analyzed as a validation analysis of the in silico analysis. The genotyping was performed on QuantStudio 12K Flex RealTime PCR System according to the company's recommendations (Applied Biosystems, ThermoFisher, Waltham, MA, USA). Genotype concordance of WES and Taqman genotyping was evaluated when appropriate.

In the replication/validation phase, multivariable logistic regression was performed to estimate the ORs and 95% CI of each SNP adjusting for age and sex using SAS 9.4 (Cary, NC, USA). SNPs with $p < 0.05$ and the same direction of effect with the WES analysis were considered statistically significant. Meta-analysis was then performed using METAL(33) to estimate the combined ORs of top SNPs based on results of the three phases.

Results

Exome-wide association analysis

After the WES bioinformatics quality control and variant calling procedures, an average of 3,667,001 SNPs per sample (min = 3,441,884, max = 4,048,829) were identified. After additional quality-control steps, 427,367 variants remained for further analysis. Two samples did not cluster with the rest of samples of European descent in the PCA analysis and therefore were excluded in the exome-wide association analysis.

The characteristics of the 61 patients included in the WES analysis are summarized in Table 1. Overall, the mean age of these patients was 64 years and 56% were females. Seventy-two percent of these patients were treated with iv BPs because of multiple myeloma (45% stage IIA, 42% in stage IIIA, and 12% stage IIIB based on the Durie/Salmon staging system(38)) and 19.6% because of metastatic breast cancer. According to the AAOMS staging criteria, 47%, 33%, and 20% of the ONJ cases were categorized as stage 1, 2, and 3, respectively. There was no statistically significant difference in age and type of iv BPs between those with and without ONJ ($p = 0.14$) (Table 1).

The 20 top SNPs with the $p < 5 \times 10^{-3}$ in WES phase 1 were selected to be tested in WES phase 2 and only 4 SNPs (*SIRT1* SNP rs7896005, *HERC4* SNP rs3758392, *SV2C* SNP rs10070440, and *TBCK* SNP rs11938792) showed significant *p* values (<0.05) (Table 2). The meta-analysis of WES phase 1 and phase 2 identified SNPs rs7896005 and rs3758392 to be associated with lower odds of iv BP-induced ONJ with meta-analysis OR of 0.07 (95% CI 0.02–0.26; $p = 3.8 \times 10^{-5}$) (Table 2). Regional association plots for these two top-ranked SNPs created using LocusZoom indicated that rs7896005 is located in the *SIRT1* gene and rs3758392 in the *HERC4* gene (Fig. 1). These two SNPs had identical minor allele frequencies and *p* values and therefore were both included in the following in silico analyses.

In silico analyses identified two promoter region *SIRT1* SNPs (rs3758391 and rs7894483) in high LD with *SIRT1* SNP rs7896005 and *HERC4* SNPrs3758392 ($r^2 > 0.8$, $D' > 0.9$). The GTEx expression data for *SIRT1* SNPs rs7896005, rs3758391, and rs7894483 showed that these three SNPs are all eQTLs for SIRT1 gene such that the minor allele for each SNP is associated with increased *SIRT1* gene expression in whole blood, with a *p* value of 5.1×10^{-15}, 2.8×10^{-21}, and 2.8×10^{-21}, respectively (Fig. 2*A–C*). The *HERC4* SNP rs3758392 is also an eQTL SNP for the *SIRT1* gene in the GTEx database ($p = 1.8 \times 10^{-15}$) (Fig. 2*D*). These SNPs are all in high LD, and we opted to genotype three *SIRT1* SNPs (rs7896005, rs3758391, and rs7894483) in the independent validation sample.

Replication/validation of SNPs associated with BP-induced ONJ

To further validate our findings from the WES phase 1 and 2, we genotyped the top SNPs in the WES phase 1 and 2 samples ($n = 61$) in addition to an independent sample of 48 cancer patients of European ancestry using TaqMan assays. For the 61 patients with both WES genotypes and TaqMan genotypes, the results from these two platforms were 100% concordant.

The allele frequencies of these SNPs in our study were comparable to those in the 1000 Genomes Project, and LD between the SNPs in our patients are summarized in Table 3. The

Table 1. Whole-Exome Sequencing Samples' Demographics of the Discovery and Validation Phases

Characteristics	Total ($N = 61$)	ONJ ($n = 39$)	Non-ONJ ($n = 22$)
Age (years) (mean ± SD)	64.0 ± 9.5	65.3 ± 9.5	61.6 ± 9.1
Female (%)	55.74%	58.97%	50%
White race	100%	100%	100%
Cancer type			
Multiple myeloma	44	22	22
Breast cancer	12	12	0
Cervix cancer	3	3	0
Prostate cancer	1	1	0
Renal cancer	1	1	0
Intravenous bisphosphonate used[a]			
Zoledronate	68.85%	68.42%	69.57%
Pamidronate	29.51%	29.14%	30.43%

[a] When more than one type of bisphosphonate was used, the one with the longest exposure was presented here.

Table 2. Top SNPs in the Exome-Wide Association Analysis

					Discovery			Replication			
SNP	Nearest gene	CHR	A1	A2	MAF	OR (95%CI)	p	MAF	OR (95%CI)	*p*	meta *p*
rs7896005	*SIRT1*	10	A	G	0.35	0.07 (0.01–0.40)	2.52E–03	0.35	0.07 (0.01–0.46)	0.0052	3.83E–05
rs3758392	*HERC4*	10	T	C	0.35	0.07 (0.01–0.40)	2.52E–03	0.35	0.07 (0.01–0.46)	0.0052	3.83E–05
rs10070440	*SV2C*	5	G	A	0.21	13.62 (2.47–75.04)	2.71E–03	0.16	11.68 (1.64–83.36)	0.0143	7.19E–05
rs11938792	*TBCK*	4	C	A	0.19	59.42 (4.70–752.10)	1.61E–03	0.15	20.38 (1.28–324.20)	0.0328	1.79E–04
rs7931681	*ACAT1*	11	C	A	0.24	0.11 (0.03–0.49)	3.53E–03	0.29	0.18 (0.03–1.02)	0.0522	3.12E–04
rs2252518	*ACVR1B*	12	A	C	0.29	10.75 (2.28–50.60)	2.66E–03	0.20	4.56 (0.97–21.37)	0.0544	3.21E–04
rs11074355	*TMC7*	16	A	G	0.46	0.08 (0.02–0.36)	9.84E–04	0.40	3.61 (0.83–15.72)	0.0877	3.38E–04
rs2304645	*ICD*	15	C	G	0.46	5.98 (1.92–18.64)	2.06E–03	0.35	3.5 (0.88–13.91)	0.0751	4.48E–04
rs6696772	*PPFIA4*	1	C	G	0.21	0.04 (0.01–0.27)	9.00E–04	0.29	0.31 (0.10–1.01)	0.0528	6.94E–04
rs4692549	*TBC1D19*	4	T	C	0.40	0.15 (0.04–0.53)	2.97E–03	0.49	0.33 (0.09–1.22)	0.0961	7.46E–04
rs17854511	*TMC7*	16	G	A	0.47	0.08 (0.02–0.39)	1.56E–03	0.39	2.90 (0.79–10.62)	0.1090	7.74E–04
rs867583	*NDRG4*	16	A	G	0.25	10.55 (2.36–47.19)	2.06E–03	0.16	3.41 (0.64–18.09)	0.1495	1.13E–03
rs3809594	*SETD6*	16	T	G	0.27	12.99 (2.58–65.55)	1.90E–03	0.18	3.26 (0.63–16.95)	0.1599	1.21E–03
rs8054895	*CNOT1*	16	G	A	0.27	12.99 (2.58–65.55)	1.90E–03	0.18	3.26 (0.63–16.95)	0.1599	1.21E–03
rs28583298	*TMC7*	16	G	A	0.45	0.06 (0.01–0.33)	1.17E–03	0.40	2.49 (0.64–9.69)	0.1879	1.41E–03
rs12051270	*TMC7*	16	G	A	0.46	0.09 (0.02–0.41)	1.94E–03	0.40	2.59 (0.69–9.65)	0.1564	1.47E–03
rs12068167	*SPATA17*	1	A	G	0.17	21.88 (3.18–150.60)	1.72E–03	0.09	3.51 (0.36–34.00)	0.2778	1.90E–03
rs2306413	*ANXA5*	4	C	T	0.43	11.2 (2.70–46.46)	8.78E–04	0.35	2.14 (0.65–7.05)	0.2124	1.98E–03
rs647630	*ARRB1*	11	C	T	0.47	12.3 (2.40–62.97)	2.60E–03	0.38	2.22 (0.65–7.58)	0.2050	4.23E–03
rs788827	*LGR6*	1	C	T	0.42	9.74 (2.17–43.79)	2.99E–03	0.33	1.81 (0.51–6.41)	0.3578	6.92E–03

SNP = single-nucleotide polymorphism; CHR = chromosome; A1 = minor allele; A2 = major allele; MAF = minor allele frequency; OR = odds ratio; CI = confidence interval; *p* = *p* value of association analyses.

allele frequencies of the SNPs in our patients of European ancestry were similar to those reported in the 1000 Genomes Project. Based on the 1000 Genomes data and our own data, the original *SIRT1* SNP rs7896005 is in high LD with the two promoter region SNPs rs3758391 and rs7894483 in individuals of European ancestry. The 1000 Genomes Project data also indicate that these three SNPs are in high LD in Asians and intermediate LD in admixed Americans but low LD ($r^2 < 0.2$) in individuals of African ancestry (Table 3).

The characteristics of the validation sample of 48 cancer patients of European ancestry including 19 ONJ cases and 29 non-ONJ controls treated with iv BPs are summarized in Table 4.

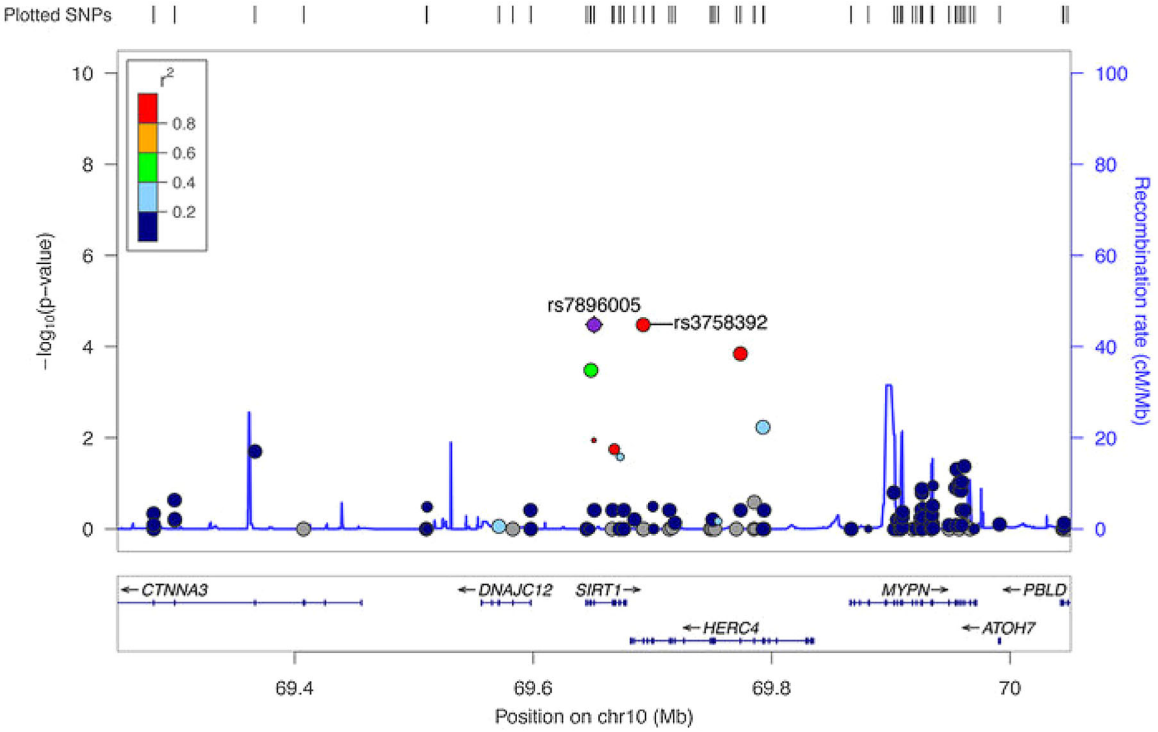

Fig. 1. The regional plot of SIRT1/HERC4 SNPs associated with bisphosphonate-induced ONJ in exome-wide association analysis.

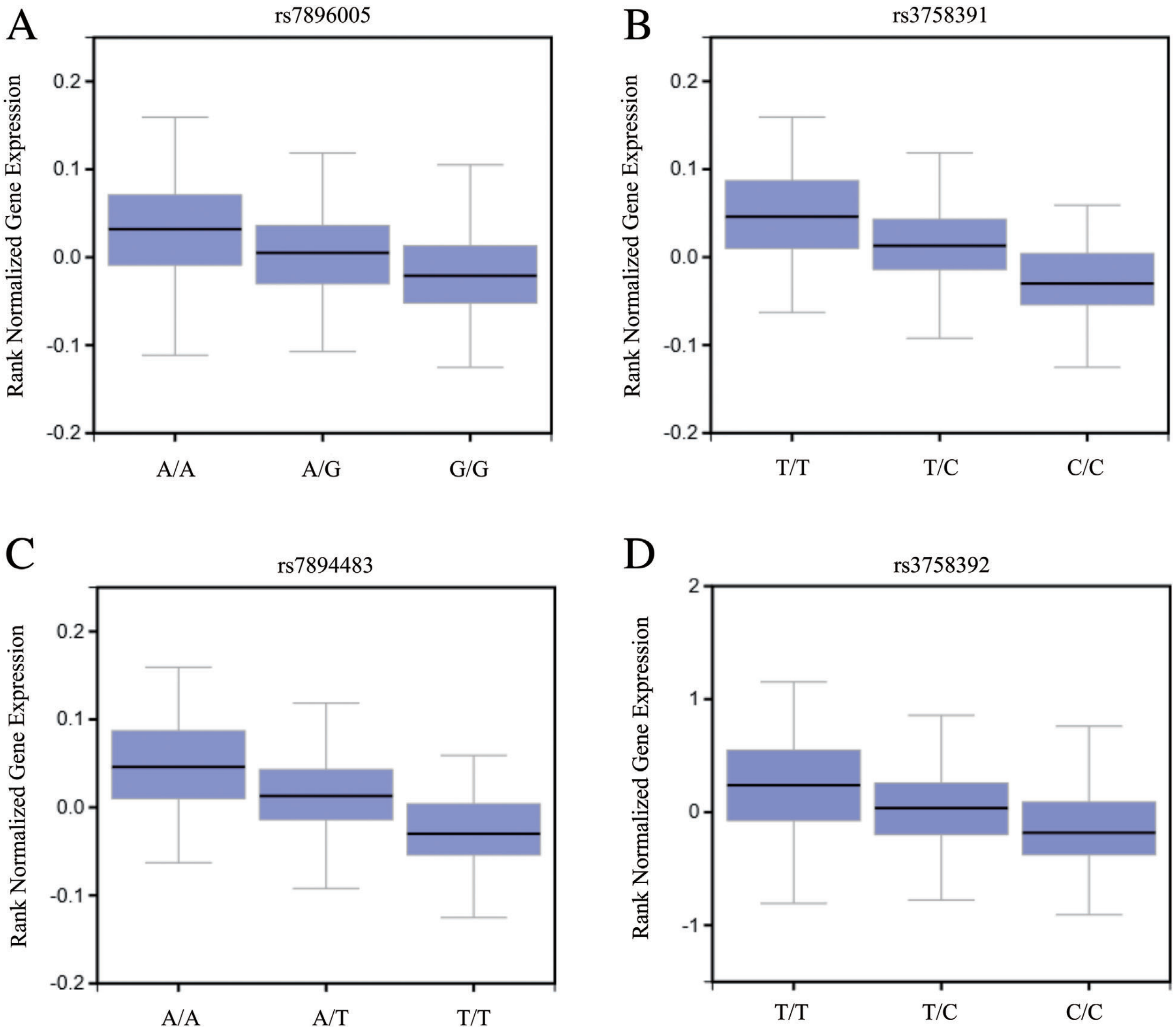

Fig. 2. GTEx gene expression data show that the minor allele A of *SIRT1* SNP rs7896005 (*A*), minor allele T of *SIRT1* SNP rs3758391 (*B*), minor allele A of *SIRT1* SNP rs7894483 (*C*), and minor allele T of *HERC4* SNP rs3758392 (*D*) had increased expression of *SIRT1* gene in a dose-dependent manner.

The indication for iv BPs included multiple myeloma, breast cancer, cervix cancer, prostate cancer, and renal cancer (Table 4). Multivariable logistic regression analysis showed that the top *SIRT1* SNP rs7896005 was associated with lower odds for ONJ with the OR of 0.3 (0.10–0.88; $p = 0.003$) in the validation sample (Fig. 3*A*). The combined OR of SNP rs7896005 in three phases was 0.16 (0.07–0.37; $p = 3.9 \times 10^{-7}$) (Fig. 3*A*).

The two promoter region *SIRT1* SNPs (rs3758391 and rs7894483) were identified by in silico analysis but not captured in the WES analysis, therefore their associations with ONJ were evaluated based on Taqman genotyping results in all samples including WES phase 1 and 2 and the validation sample ($n = 109$). Logistic regression showed that both of these SNPs were also associated with lower odds for ONJ with identical ORs

Table 3. Allele Frequencies and Linkage Disequilibrium of the *SIRT1* SNPs

			A1 allele frequency								LD (r^2) with rs7896005				
SNPs	A1	A2	Our sample			1000 Genomes Project					Our sample	1000 Genomes Project			
			EUR	AFR	Other	EUR	AFR	AMR	EAS	SAS	EUR	EUR	AFR	AMR	EAS
rs7896005	A	G	0.32	0.87	0.5	0.35	0.94	0.53	0.84	0.58	1	1	1	1	1
rs3758391	T	C	0.29	0.29	0.5	0.33	0.35	0.47	0.84	0.57	0.84	0.87	<0.2	0.69	0.95
rs7894483	A	T	0.29	0.29	0.5	0.33	0.35	0.46	0.84	0.57	0.84	0.88	<0.2	0.69	0.93

SNP = single-nucleotide polymorphism; A1 = allele 1, minor allele in European; A2 = the other allele; LD = linkage disequilibrium; EUR = European; AFR = African; AMR = Admixed American; EAS = East Asian; SAS = South Asian.

Table 4. Demographics of the Replication Samples

Characteristics	Total (*N* = 48)	ONJ (*n* = 19)	Non-ONJ (*n* = 29)
Age (years) (mean ± SD)	65.9 ± 8.6	67.1 ± 9.8	65.1 ± 7.9
Female (%)	41.67%	68.42%	24.14%
White race	48 (100%)	19 (100%)	29 (100%)
Cancer type			
Multiple myeloma	28	3	25
Breast cancer	12	11	1
Cervix cancer	1	1	0
Prostate cancer	6	3	3
Renal cancer	1	1	0

and *p* values: OR of 0.26 (0.12–0.55; $p = 0.0004$) (Fig. 3*B*). The ORs were identical due to complete LD between these two SNPs ($r^2 = 1$) in our samples of European descent.

Discussion

In this study, we identified a locus on chromosome 10 including *SIRT1* SNP rs7896005 and *HERC4* SNP rs3758392 to be associated with lower odds of developing ONJ among cancer patients of European ancestry who were treated with iv BPs for prevention of SREs. Two promoter region *SIRT1* SNPs (rs3758391 and rs7894483) were identified using in silico analysis and, more importantly, were validated in an independent sample. In silico analyses using GTEx database indicated that these SNPs are eQTL SNPs regulating the expression of the *SIRT1* gene in whole blood where the presence of the variant alleles resulted in reduced *SIRT1* expression. Furthermore, LD analysis of the *SIRT1* SNP rs7896005 and *HERC4* SNP rs3758392 indicated these two SNPs are in fact different markers for the same signal or locus. To our knowledge, there are currently no studies reported in the literature linking the *SIRT1/HERC4* locus to BP-induced ONJ.

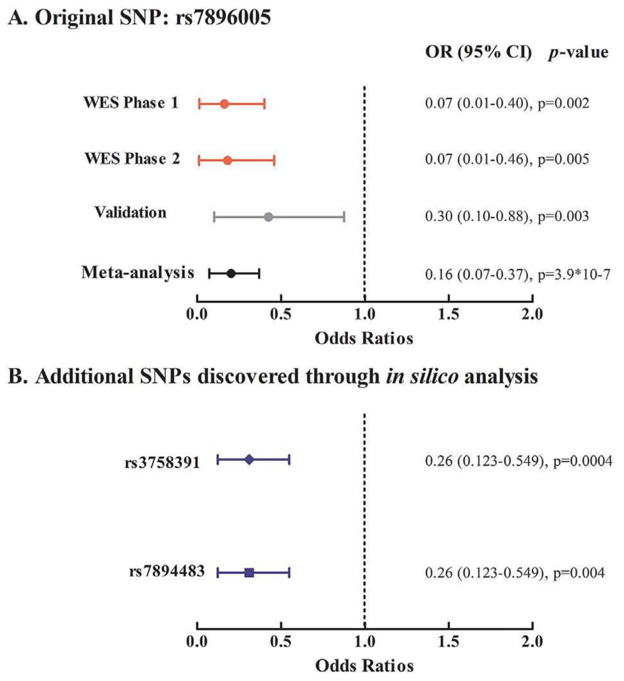

Fig. 3. Association of *SIRT1* SNPs and bisphosphonate-induced ONJ in cancer patients of European ancestry. (*A*) ORs (95% CI) of rs7896005 in whole-exome sequencing (WES) phase 1, WES phase 2, and replication/validation phase. Combined OR (95% CI) of rs7896005 in three phases was calculated by meta-analysis. (*B*) ORs (95% CI) of two promoter region *SIRT1* SNPs (rs3758391 and rs7894483) in all samples.

From a functional perspective, the *SIRT1* protein product, Sirtuin 1 (Sirt1), belongs to a family of enzymes that collectively mediate NAD dependent deacetylation of proteins, histones, and transcription factors, thereby regulating a wide array of biological processes that are pivotal for cell growth and differentiation. However, the main question that has to be addressed is whether Sirt1 plays any role in promoting bone formation. Without delving into the intricacies of this process, it is important to point out that it is a well-established fact that the Wnt signaling pathway is integral to bone formation.(39) Findings from several studies have demonstrated that there are several ways by which Sirt1 potentiates Wnt signaling, particularly in the bones.(40–43) To illustrate, Sirt1 via epigenetic silencing, downregulates the expression of Wnt antagonists such as the secreted frizzled related proteins (SFRPs) and dickkopf (Dkk) protein, leading to increased availability of the receptors for Wnt ligands and subsequently stimulation of the downstream signaling pathways. Within this context, studies have shown that inhibition of Sirt1 resulted in reduced intracellular levels of dishevelled (dvl) proteins, which are responsible for relaying the Wnt signal upon receptor stimulation to the downstream intracellular signaling components such as the beta-catenins. Sirt1-mediated deacetylation of β-catenin promotes the accumulation of the latter in the nucleus and activation of several transcription factors that are involved in bone formation. Activation of *Sirt1* as demonstrated in the study by Shakibaei and colleagues(44) resulted in suppression of osteoclastogenesis (osteoclast development) secondary to inhibition of the RANK/RANK signaling pathway. Simultaneously, Sirt1 promoted osteogenesis or bone growth through direct association with the Cbfa-1 transcription factor, thereby promoting its activation (Cbfa-1) and production of bone-specific collagen type I. A recent study demonstrated that *SIRT1* is a positive regulator of the master osteoblast transcription factor *RUNX2* and treatment with a *SIRT1* agonist promotes osteoblast differentiation.(45) Finally, therapy of teriparatide, a recombinant form of parathyroid hormone (PTH), was shown to result in healing of ONJ patients.(46) This is particularly interesting because Sirt1 may play a role in PTH regulation of osteoblasts(47) and therefore osteoclasts. All of these evidence underscores the importance of the *SIRT1* gene and provides a plausible explanation as to why inhibition of Sirt1 or the reduced expression of Sirt1 due to polymorphisms such as rs7896005, rs3758391, or rs7894483 as noted in our study may predispose patients to develop ONJ with BPs or other anti-resorptive drugs in general. Although the *SIRT1* seems to be the obvious functional candidate in this association study, the *HERC4* gene might also be relevant. *HERC4* belongs to the HERC family of ubiquitin ligases and encodes HECT and RLD domain containing E3 ubiquitin protein ligase 4.(48) A recent mouse study demonstrated that ubiquitin E3 ligases, involved in protein degradation, regulate osteoblast function.(49) Therefore, it is plausible that *HERC4* might be also important in the pathophysiology of ONJ. Nonetheless, further analysis is warranted to pinpoint the functional variant for this association. Most importantly, knock-out and functional studies should be conducted to further elucidate the role of *SIRT1* in bone formation and understand how bisphosphonates can perturb this process and subsequently predispose patients to development of this debilitating complication that could negatively affect the patients' quality of life.

It is also important to recognize that the minor allele frequencies of and LD between these three *SIRT1* SNPs vary across race/ethnicity groups based on data from the 1000 Genomes Project. The minor allele (A) for rs7896005, which is associated with lower odds of ONJ, has the lowest allele frequency in individuals of European descent (35%), highest frequency (94%) in Africans, and intermediate frequencies in admixed Americans (53%), South Asians (58%), and East Asians (84%), respectively. Based on these allele frequency differences and that the minor allele was associated with lower risk for ONJ, we would expect highest incidence of ONJ in patients of European ancestry and lowest in those of African ancestry, and this is exactly what we and others have observed.(7,50)

Our study has some limitations that need to be recognized. To minimize false-positive findings potentially caused by population stratification, we chose to analyze patients of European ancestry alone, which further decreased our sample size and limited the generalizability of our study result. Hence, the association between the *SIRT1* SNPs identified herein and the development of ONJ with BPs in other racial/ethnic groups remains elusive or unknown and warrants further investigation. Because the haplotype structures tend to vary between races, it is imperative that all three *SIRT1* SNPs and not only rs7896005 be interrogated particularly in patients with African ancestry where the SNPs are in low LD ($r^2 < 0.2$). Also, because of our small sample size, we did not have enough patients to evaluate the genetic association with ONJ within each cancer population such as multiple myeloma or breast cancer.

As for the implications of our findings, we believe that our data may set the groundwork for a personalized approach with regards to the use of BPs in the setting of SREs prevention. Hence, to circumvent the incidence of ONJ, patients carrying the G polymorphism for rs7896005, C polymorphism for rs3758391, or T polymorphism for rs7894483 in *SIRT1* may potentially benefit from lower exposure or less frequent administration of these drugs. Nonetheless, the efficacy of this strategy requires further validation in a clinical study to ensure that the efficacy of these drugs is not compromised. With the ever-increasing number of pharmacotherapies causing ONJ, including denosumab, VEGF inhibitors, and others, it is imperative that more studies be conducted to uncover the genetic determinants of drug-induced ONJ and understand how these offending agents disrupt the signaling or biological pathways that lead to its pathogenesis.

Disclosures

YG, JK, TYL, and JSM received grant support from Micromedic Inc., Isreal for this project. Other authors declare no conflict of interest.

Acknowledgments

This project was funded by Micromedic Inc, Israel. However, the funding agency has no influence on the analysis of this manuscript.

Authors' roles: Study design: YG, JK, JSM, TYL, ND, and IH. Data collection: JK, JSM, TYL, PL, BB, JK, MV, and GAP. Data analysis: YG, GY, and AR. Data interpretation: YG, IH, and JSM. Drafting manuscript: GY, IH, and YG. Revising manuscript content: all coauthors. Approving final version of manuscript: YG takes responsibility for the integrity of the data analysis.

References

1. Marx RE. Pamidronate (Aredia) and zoledronate (Zometa) induced avascular necrosis of the jaws: a growing epidemic. J Oral Maxillofac Surg. 2003;61(9):1115–7.
2. Ruggiero SL, Mehrotra B, Rosenberg TJ, Engroff SL. Osteonecrosis of the jaws associated with the use of bisphosphonates: a review of 63 cases. J Oral Maxillofac Surg. 2004;62(5):527–34.
3. Advisory Task Force on Bisphosphonate-Related Ostenonecrosis of the Jaws AeAoOaMS. American Association of Oral and Maxillofacial Surgeons position paper on bisphosphonate-related osteonecrosis of the jaws. J Oral Maxillofac Surg. 2007;65(3):369–76.
4. Ruggiero SL, Dodson TB, Fantasia J, et al. American Association of Oral and Maxillofacial Surgeons position paper on medication-related osteonecrosis of the jaw—2014 update. J Oral Maxillofac Surg. 2014;72(10):1938–56.
5. Hamadeh IS, Ngwa BA, Gong Y. Drug induced osteonecrosis of the jaw. Cancer Treat Rev. 2015;41(5):455–64.
6. Cassoni A, Romeo U, Terenzi V, et al. Adalimumab: another medication related to osteonecrosis of the jaws? Case Rep Dent. 2016;2016:2856926.
7. Katz J, Gong Y, Salmasinia D, et al. Genetic polymorphisms and other risk factors associated with bisphosphonate induced osteonecrosis of the jaw. Int J Oral Maxillofac Surg. 2011;40(6):605–11.
8. 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. J Oral Pathol Med. 2011;40(6):510–5.
9. Di Martino MT, Arbitrio M, Guzzi PH, et al. A peroxisome proliferator-activated receptor gamma (PPARG) polymorphism is associated with zoledronic acid-related osteonecrosis of the jaw in multiple myeloma patients: analysis by DMET microarray profiling. Br J Haematol. 2011;154(4):529–33.
10. La Ferla F, Paolicchi E, Crea F, et al. An aromatase polymorphism (g.132810C>T) predicts risk of bisphosphonate-related osteonecrosis of the jaw. Biomark Med. 2012;6(2):201–9.
11. Stockmann P, Nkenke E, Englbrecht M, et al. Major histocompatibility complex class II polymorphisms are associated with the development of anti-resorptive agent-induced osteonecrosis of the jaw. J Craniomaxillofac Surg. 2013;41(1):71–5.
12. 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. Oncologist. 2012;17(2):279–87.
13. Sarasquete ME, García-Sanz R, Marín 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. 2008;112(7):2709–12.

14. Balla B, Vaszilko M, Kósa JP, et al. New approach to analyze genetic and clinical data in bisphosphonate-induced osteonecrosis of the jaw. Oral Dis. 2012;18(6):580–5.
15. Moretti F, Pelliccioni GA, Montebugnoli L, Marchetti C. A prospective clinical trial for assessing the efficacy of a minimally invasive protocol in patients with bisphosphonate-associated osteonecrosis of the jaws. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2011;112(6):777–82.
16. Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. Nat Methods. 2012;9(4):357–9.
17. Li H, Handsaker B, Wysoker A, et al. The Sequence Alignment/Map format and SAMtools. Bioinformatics. 2009;25(16):2078–9.
18. Li H. A statistical framework for SNP calling, mutation discovery, association mapping and population genetical parameter estimation from sequencing data. Bioinformatics. 2011;27(21): 2987–93.
19. McKenna A, Hanna M, Banks E, et al. The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. Genome Res. 2010;20(9):1297–303.
20. DePristo MA, Banks E, Poplin R, et al. A framework for variation discovery and genotyping using next-generation DNA sequencing data. Nat Genet. 2011;43(5):491–8.
21. Van der Auwera GA, Carneiro MO, Hartl C, et al. From FastQ data to high confidence variant calls: the Genome Analysis Toolkit best practices pipeline. Curr Protoc Bioinformatics. 2013;43(10):1–33.
22. Yang H, Wang K. Genomic variant annotation and prioritization with ANNOVAR and wANNOVAR. Nat Protoc. 2015;10(10):1556–66.
23. Price AL, Patterson NJ, Plenge RM, Weinblatt ME, Shadick NA, Reich D. Principal components analysis corrects for stratification in genome-wide association studies. Nat Genet. 2006;38(8):904–9.
24. Reich D, Price AL, Patterson N. Principal component analysis of genetic data. Nat Genet. 2008;40(5):491–2.
25. Bamias A, Kastritis E, Bamia C, et al. Osteonecrosis of the jaw in cancer after treatment with bisphosphonates: incidence and risk factors. J Clin Oncol. 2005;23(34):8580–7.
26. Badros A, Weikel D, Salama A, et al. Osteonecrosis of the jaw in multiple myeloma patients: clinical features and risk factors. J Clin Oncol. 2006;24(6):945–52.
27. 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. 2006;91(7):968–71.
28. 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. Br J Haematol. 2006;134(6):620–3.
29. Corso A, Varettoni M, Zappasodi P, et al. A different schedule of zoledronic acid can reduce the risk of the osteonecrosis of the jaw in patients with multiple myeloma. Leukemia. 2007;21(7):1545–8.
30. Hoff AO, Toth BB, Altundag K, et al. Frequency and risk factors associated with osteonecrosis of the jaw in cancer patients treated with intravenous bisphosphonates. J Bone Miner Res. 2008;23(6):826–36.
31. Zhang X, Hamadeh IS, Song S, et al. Osteonecrosis of the jaw in the United States Food and Drug Administration's Adverse Event Reporting System (FAERS). J Bone Miner Res. 2016;31(2):336–40.
32. Purcell S, Neale B, Todd-Brown K, et al. PLINK: a tool set for whole-genome association and population-based linkage analyses. Am J Hum Genet. 2007;81(3):559–75.
33. Willer CJ, Li Y, Abecasis GR. METAL: fast and efficient meta-analysis of genomewide association scans. Bioinformatics. 2010;26(17):2190–1.
34. Pruim RJ, Welch RP, Sanna S, et al. LocusZoom: regional visualization of genome-wide association scan results. Bioinformatics. 2010;26(18):2336–7.
35. Ward LD, Kellis M. HaploReg: a resource for exploring chromatin states, conservation, and regulatory motif alterations within sets of genetically linked variants. Nucleic Acids Res. 2012; 40(Database issue): D930–4.
36. Boyle AP, Hong EL, Hariharan M, et al. Annotation of functional variation in personal genomes using RegulomeDB. Genome Res. 2012;22(9):1790–7.
37. Consortium G. Human genomics. The Genotype-Tissue Expression (GTEx) pilot analysis: multitissue gene regulation in humans. Science. 2015;348(6235):648–60.
38. Spasov E, Goranova V. Prognostic assessment of the Durie and Salmon staging system in patients with multiple myeloma. Folia Med (Plovdiv). 1998;40(3B Suppl 3):121–3.
39. Zhou Y, Song T, Peng J, et al. SIRT1 suppresses adipogenesis by activating Wnt/β-catenin signaling in vivo and in vitro. Oncotarget. 2016;7(47):77707–20.
40. Feng G, Zheng K, Song D, et al. SIRT1 was involved in TNF-α-promoted osteogenic differentiation of human DPSCs through Wnt/β-catenin signal. In Vitro Cell Dev Biol Anim. 2016;52(10): 1001–11.
41. Subramaniyan B, Jagadeesan K, Ramakrishnan S, Mathan G. Targeting the interaction of Aurora kinases and SIRT1 mediated by Wnt signaling pathway in colorectal cancer: A critical review. Biomed Pharmacother. 2016;82:413–24.
42. Zhou Y, Zhou Z, Zhang W, et al. SIRT1 inhibits adipogenesis and promotes myogenic differentiation in C3H10T1/2 pluripotent cells by regulating Wnt signaling. Cell Biosci. 2015;5(61):1–12.
43. Abed É, Couchourel D, Delalandre A, et al. Low sirtuin 1 levels in human osteoarthritis subchondral osteoblasts lead to abnormal sclerostin expression which decreases Wnt/β-catenin activity. Bone. 2014;59:28–36.
44. Bourguignon LY, Xia W, Wong G. Hyaluronan-mediated CD44 interaction with p300 and SIRT1 regulates beta-catenin signaling and NFkappaB-specific transcription activity leading to MDR1 and Bcl-xL gene expression and chemoresistance in breast tumor cells. J Biol Chem. 2009;284(5):2657–71.
45. Zainabadi K, Liu CJ, Guarente L. SIRT1 is a positive regulator of the master osteoblast transcription factor, RUNX2. PLoS One. 2017;12(5): 1–14.
46. Cheung A, Seeman E. Teriparatide therapy for alendronate-associated osteonecrosis of the jaw. N Engl J Med. 2010;363(25):2473–4.
47. Fei Y, Shimizu E, McBurney MW, Partridge NC. Sirtuin 1 is a negative regulator of parathyroid hormone stimulation of matrix metalloproteinase 13 expression in osteoblastic cells: role of sirtuin 1 in the action of PTH on osteoblasts. J Biol Chem. 2015;290(13):8373–82.
48. Hochrainer K, Mayer H, Baranyi U, Binder B, Lipp J, Kroismayr R. The human HERC family of ubiquitin ligases: novel members, genomic organization, expression profiling, and evolutionary aspects. Genomics. 2005;85(2):153–64.
49. Liu J, Li X, Zhang H, et al. Ubiquitin E3 ligase Itch negatively regulates osteoblast function by promoting proteasome degradation of osteogenic proteins. Bone Joint Res. 2017;6(3):154–61.
50. Badros A, Weikel D, Salama A, et al. Osteonecrosis of the jaw in multiple myeloma patients: clinical features and risk factors. J Clin Oncol. 2006;24(6):945–52.