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SIRT1 Gene SNP rs932658 Is Associated With Medication-Related Osteonecrosis of the Jaw

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Abstract

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PEER REVIEW

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Medication-related osteonecrosis of the jaw (MRONJ) is a rare but serious adverse drug reaction. Our previous whole-exome sequencing study found *SIRT1* intronic region single-nucleotide polymorphism (SNP) rs7896005 to be associated with MRONJ in cancer patients treated with intravenous (iv) bisphosphonates (BPs). This study aimed to identify causal variants for this association. In silico analyses identified three SNPs (rs3758391, rs932658, and rs2394443) in the *SIRT1* promoter region that are in high linkage disequilibrium ($r^2 > 0.8$) with rs7896005. To validate the association between these SNPs and MRONJ, we genotyped these three SNPs on the germline DNA from 104 cancer patients of European ancestry treated with iv BPs (46 cases and 58 controls). Multivariable logistic regression analysis showed the minor alleles of these three SNPs were associated with lower odds for MRONJ. The odds ratios (95% confidence interval) and *p* values were 0.351 (0.164–0.751; *p* = 0.007) for rs3758391, 0.351 (0.164–0.751; *p* = 0.007) for rs932658, and 0.331 (0.157–0.697; *p* = 0.0036) for rs2394443, respectively. In the reporter gene assays, constructs containing rs932658 with variant allele A had higher luciferase activity than the reference allele, whereas constructs containing SNP rs3758391 and/or rs2394443 did not significantly affect activity. These results indicate that the promoter SNP rs932658 regulates the expression of *SIRT1* and presumably lowers the risk of MRONJ by increasing *SIRT1* expression.

Keywords

ANTIRESORPTIVES; BONE MODELING AND REMODELING; OSTEOBLASTS; OSTONECROSIS OF THE JAW; PHARMACOGENOMICS

Introduction

Medication-related osteonecrosis of the jaw (MRONJ) is diagnosed as the exposure of the jaw bone (mandible, maxilla, or both) with slow healing for >8 weeks or no healing. (1) MRONJ is painful and affects the quality of life. MRONJ occurs as an infrequent adverse drug reaction of treatment with various therapeutic drugs, the most prominent being nitrogen-containing bisphosphonates (BPs) and the receptor activator of NF- κ B ligand (RANKL) inhibitor denosumab. (2) BPs are widely used to treat or prevent osteoporosis or skeletal-related events (SREs) in cancer patients (3–5) and are the most common agents for osteoporosis treatment. (6) BPs have also shown high efficacy in preventing bone loss in patients with metastatic cancer, such as multiple myeloma (MM), breast cancer, and prostate cancer. (5) The first MRONJ case was reported by Marx RE in 2003. (7) Since then, the reported incidence of MRONJ ranges from 0.001% to 0.15% in osteoporosis patients (1,8–11) and 0.5% to 12% in patients with metastatic cancer. (12–18) Although many mechanisms for MRONJ have been proposed, (19,20) the pathogenesis of MRONJ remains largely undetermined. Many studies have suggested that there is a genetic component for MRONJ, (21–26) and the incidences of MRONJ vary in genetically diverse populations. (27)

Our previous whole-exome sequencing (WES) results identified a single-nucleotide polymorphism (SNP), rs7896005, within an intronic region of *SIRT1* locus to be associated with MRONJ. (28) *SIRT1* is a protein-coding gene, which encodes a member of the sirtuin family of conserved NAD⁺-dependent protein deacetylase. As a protein deacetylase, SIRT1 plays a vital role in multiple important biological processes, such as energy metabolism,

inflammation, tumorigenesis, longevity, cell differentiation, and bone remodeling.^(29–37) Based on the results of in silico analyses, there is no evidence indicating that previously identified WES index SNP rs7896005 was a functional SNP. The purpose of this study was to identify functional SNPs in high linkage disequilibrium (LD) with the index SNP that might be causal SNPs for this association in a retrospective case–control study.

Materials and Methods

In silico analysis

LDlink,⁽³⁸⁾ an in silico tool provided by the National Cancer Institute (NCI) Division of Cancer Epidemiology and Genetics, was used to identify all SNPs from phase 3 (version 5) of the 1000 Genomes Project that were in high LD ($r^2 > 0.8$) with the previously discovered WES index SNP rs7896005 (including rs7896005). The Genotype-Tissue Expression (GTEx) portal⁽³⁹⁾ and single nucleotide polymorphism annotator (SNiPA)⁽⁴⁰⁾ were used to identify expression quantitative trait loci (eQTL) SNPs for *SIRT1*. UCSC Genome Browser,⁽⁴¹⁾ SNPnexus,⁽⁴²⁾ SNiPA,⁽⁴⁰⁾ and Haploreg V4.1⁽⁴³⁾ were used to identify locus-related information for the SNPs. Based on the location of the SNPs, different in silico tools were used to predict the potential functional effects of the SNPs. Briefly, Encyclopedia of DNA Elements (ENCODE),^(44,45) UCSC Genome Browser, Haploreg V4.1, SNPnexus, RegulomeDB,⁽⁴⁶⁾ and Ensembl⁽⁴⁷⁾ were used to identify SNPs in regulatory elements. miRWalk,⁽⁴⁸⁾ TargetScan,⁽⁴⁹⁾ PolymiRTS,⁽⁵⁰⁾ miRBase,⁽⁵¹⁾ and miRDB^(52,53) were used to predict SNPs that may potentially affect microRNA (miRNA) binding. Human Splicing Finder⁽⁵⁴⁾ and Alternative Splice Site Predictor (ASSP)⁽⁵⁵⁾ were used to predict the SNPs that potentially affect splicing (Fig. 1).

Patients

Genomic DNA samples were obtained from 104 cancer patients of European ancestry treated with intravenous (iv) BPs, including 46 patients who developed MRONJ (cases) and 58 who did not develop MRONJ (controls) from multiple institutions. All patients regardless of cancer stages or concomitant diseases who were treated with iv bisphosphonates (zoledronate or pamidronate) coming to Dr Moreb's clinic or Dr Katz's clinic at the University of Florida between 2016 and 2018 were approached for study participation. All patients who agreed to participate, signed consent, and provided genomic DNA were included in this study. Patients who developed MRONJ and confirmed by Dr Katz were included as MRONJ cases; those who did not develop MRONJ after at least 12 months of iv BP treatment were included as controls. Additional MRONJ cases were also obtained from Semmelweis University of Medical School and Dental School in Budapest, Hungary, and the University of Bologna in Bologna, Italy. All patients signed informed consent at each study site. This study was approved by the Institutional Review Board at the University of Florida (IRB nos. IRB201501186 and IRB201800934).

Genotyping

Seven SNPs identified from in silico analyses or our previous WES were genotyped in the 104 samples. Genomic DNA was isolated from buffy coat using the FlexiGene DNA kit (Qiagen, Valencia, CA, USA). SNPs rs7896005, rs3758391, rs3740051, and rs35706870

were genotyped using TaqMan Genotyping on QuantStudio 12 K Flex Real-Time PCR System (Applied Biosystems, Waltham, MA, USA). All Taqman probes were ordered from Thermo Fisher Scientific (Winooski, VT, USA). SNPs with no existing Taqman probed (rs12778366, rs932658, and rs2394443) were genotyped using pyrosequencing. Pyrosequencing assays were designed using pyrosequencing assay design software.⁽⁵⁶⁾ The PCR and sequencing primer information is shown in Supplemental Table S1. All primers for the pyrosequencing assay were ordered from Integrated DNA Technologies, Inc (Coralville, IA, USA).

Based on the genotyping results, SNPs were selected for further investigation. The potential function of promoter region SNPs was validated using the luciferase assay.

In vitro luciferase reporter assay

Cell culture: The human osteoblast cell line (hFOB 1.19) purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA) was cultured in complete 1:1 mixture of Ham's F12 Medium Dulbecco's Modified Eagle's Medium (DMEM) (without phenol red) (Thermo Fisher) supplemented with fetal bovine serum (Thermo Fisher) (10%), 2.5 mM L-glutamine, 0.3 mg/mL G418 (Thermo Fisher). Cells were grown at 34°C in a humidified atmosphere containing 5% CO₂.

Plasmid constructs: PCR and appropriate primers (IDT) were used to amplify two fragments of the *SIRT1* promoter of different lengths (Promoter_short [1.13 kb] and Promoter_long [1.54 kb]) using human genomic DNA as a template. Briefly, PCR was performed in a maximum volume of 25 µL: 12.5 µL of CloneAmp HiFi PCR Premix (Takara Bio, Mountain View, CA, USA), 1 µL of forward primer, 1 µL of reverse primer, 8.5 µL of RNA free water, and 2 µL of DNA template. The parameters for the amplification reaction were the following: 95°C for 15 minutes, which was followed by 40 cycles of 98°C for 10 seconds, 60°C for 30 seconds, and 72°C for 1 to 2 minutes based on the length of DNA fragment. The primers were designed using the Takara In-Fusion primer design tool. All promoter fragments were purified using a DNA purification kit (Takara Bio). The primers are listed as 1.54 kb-NheI-forward-5' - ACCTGAGCTCGCTAGCAGGCTTCTAGGACTGGAGATG-3', 1.13 kb-NheI-forward-5' - ACCTGAGCTCGCTAGCGGTAC CCCTCGTTTTTACATCTGG-3', and HinIII-reverse-5' - CCGGATTGC CAAGCTTTGCGGAGCGGCTCCCCGG-3'.

In-Fusion cloning: The pGL4.10 [luc2] vector (Promega, Madison, WI, USA) was linearized by digestion with the NheI and HinIII restriction enzymes (New England BioLabs, Ipswich, MA, USA) and then purified using a DNA purification kit (Takara Bio). Two DNA fragments (Promoter_short and Promoter_long) from the *SIRT1* promoter containing different combinations of candidate SNPs were then cloned into pGL4.10 vector using the In-Fusion cloning kit (Takara Bio). Clones were screened with restriction enzyme digestion, and DNA fragments were sequenced (Eton Bioscience, San Diego, CA, USA) to ensure no random mutations. All primers used for sequencing are listed in Supplemental Table S2. Plasmid DNA was prepared using the PureYield Plasmid Midiprep System (Promega).

Dual-luciferase reporter gene assay: HFOB 1.19 cells were plated in 96-well plates with 10,000 cells in 100 μ L per well. After 24 hours, 10 μ L of FuGENE 6 reagent (Promega)/plasmid DNA (DNA/fuGENE6 ratio to 1:3) mixture was added to each well of cells for transfection. pGL4.74 (hRluc/TK) expressing Renilla luciferase was used as an internal control. Empty pGL4.10 [luc2] basic vector was used as a negative control. Forty-eight hours post-transfection, luciferase activities of the transfected cells were measured using the dual-luciferase reporter assay system (Promega) on the BioTek Synergy HT Reader (Thermo Fisher). All experiments were repeated independently in triplicate for three times. The firefly luciferase activity normalized by Renilla luciferase activity represents the transcriptional activities of the *SIRT1* promoter. The luminescence ratio was represented by the ratio of the firefly luciferase activity and Renilla luciferase activity. The relative luciferase activity is the normalized ratio of the luminescence ratio, which normalized the results from each experimental sample to control samples repeated on each plate.

Statistical analysis

Multivariable logistic regression was performed to estimate the odds ratios (ORs) and 95% confidence interval (CI) of each potential candidate SNP with MRONJ adjusting for age and sex. To account for multiple comparisons, SNPs with p values <0.0125 (0.05/4) were considered statistically significant. Luciferase reporter assay results are represented as mean \pm SE and analyzed with Student's t test. A p value <0.0125 was considered statistically different. All statistical analyses were performed using SAS 9.4 (Cary, NC, USA).

Results

Three *SIRT1* promoter SNPs were associated with MRONJ

A total of 184 SNPs in high LD ($r^2 > 0.8$) with index SNP rs7896005 in the European population in the 1000 Genome Project were extracted using LDlink 3.8. After excluding SNPs that were not eQTLs for *SIRT1* based on the GTEx database, 181 SNPs were classified into four groups: intergenic (110), 5'-upstream of *SIRT1* (37), intronic (33), and 3'-downstream (1) (Fig. 1).

According to the UCSC Genome Browser, 20 of 181 SNPs were located at five potential enhancer/promoter elements of *SIRT1* (Supplemental Fig. S1A; Supplemental Table S3). The RegulomeDB rank was represented in Supplemental Table S3. The rank of RegulomeDB refers to different available data types for a single coordinate, which gives a prediction of the potential function of these 20 SNPs. After evaluating Ensembl, ENCODE, and the *Homo sapiens SIRT1* promoter region (LOC107832851) from National Center for Biotechnology Information (NCBI), SNPs rs3758391, rs932658, and rs2394443 were selected as candidate functional SNPs, all of which were located in the promoter region (GH10J067883) of *SIRT1* gene (Supplemental Table S4; Supplemental Fig. S1B). The UCSC Genome Browser showed that rs932658 and rs2394443 were in a DNase I hypersensitivity signal peak, enriched for H3K27Ac and near CpG island, which mostly occurs in the promoter region (Supplemental Fig. S1B). The DNase I hypersensitivity signal peak identified the potential regulatory region, which is sensitive to cleavage by DNase H3K27Ac was defined as an enhancer mark, which was mostly found nearby the

transcription start site. SNP rs3758391 was also in the promoter region and showed the perfect LD with rs932658. The UCSC Genome Browser also reported the interactions between this promoter region (GH10J067883) and other enhancer regions (GH10J067837, GH10J067848, GH10J067855). The 3D Genome Browser Hi-C data^(57,58) predicted these SNPs are located in a topologically associated domain (TAD) in the ENCODE data using human mesenchymal stem cells line (H1-MSC) (Supplemental Fig. S1C). Based on the annotation by chromHMM, these SNPs are located at an active TSS (red). According to virtual 4C data, there is a potential interaction between this active TSS and the SIRT1 enhancer (orange). This linkage is also supported by DNase I hypersensitive site (DHS) linkage. This region is also located at a H3K4me3 peak region, which is a promoter mark (Supplemental Fig. S1D). The allele frequency for all three promoter region candidate SNPs and the index SNP rs7896005 were shown in Supplemental Table S5.

Ensembl Genome Browser showed a total of six *SIRT1* transcripts in the human genome (Supplemental Fig. S2), containing 11 exons and 10 introns. Based on the GTEx portal, there are 11 splice junctions. Intron 1–7 could potentially modify the SIRT1 transcript expression by affecting splicing. However, human splice finder (HSF) and alternative splice site predictor (ASSP) results showed that none of the intron SNPs affect splicing (Supplemental Table S6).

MicroRNAs (miRNAs) are small non-coding RNAs, which affect mRNA stability or translation by base-pairing with complementary sequences within target mRNAs. miRNAs can also regulate protein expression via binding to the 3' UTR region of mRNA. Studies have identified that miRNA can also bind to intron regions or promoter TATA-box motifs to affect mRNA stability and alternative expression.^(59,60) We used miRWalk, TargetScan 7.2, and miRDB to extract all potential miRNA and their binding sites. None of the target SNPs are predicted to be located at a miRNA binding site.

After in silico analyses, a total of three *SIRT1* promoter region SNPs (rs3758391, rs932658, and rs2394443) were identified as potential functional SNPs. The GTEx expression data for SNPs rs3758391, rs932658, and rs2394443 showed that the minor allele for each of these three SNPs in our study had increased *SIRT1* mRNA expression in whole blood, with *p* values of 9.3×10^{-46} , 9.3×10^{-46} , and 4.2×10^{-33} , respectively (Supplemental Fig. S3).

Genotyping validated three promoter SNPs associated with MRONJ

To further validate these three SNPs, we performed an independent genotyping study of 104 cancer patients of European ancestry treated with iv BPs, including 58 controls and 46 MRONJ cases. The demographics of the samples are shown in Table 1. The mean age of the patients was 64 years. For MRONJ cases, the mean duration of BP treatment was 26.55 months before the development of MRONJ. For controls, the mean duration of treatment was 33.06 months. There was no statistically significant difference in the duration of iv BP treatment between the two groups ($p = 0.194$). Multivariable logistic regression analysis found that the WES index SNP and the three candidate SNPs were associated with lower odds for MRONJ: OR = 0.331 and 95% CI 0.157–0.697 ($p = 0.0036$) for index SNP rs7896005, OR = 0.351 and 95% CI 0.164–0.751 ($p = 0.007$) for rs3758391, OR =

0.351 and 95% CI 0.164–0.751 ($p = 0.007$) for rs932658, and OR = 0.331 and 95% CI 0.157–0.697 ($p = 0.0036$) for rs2394443, respectively (Fig. 2).

SNP rs932658 affected promoter activity of SIRT1—To assess the effects of these SNPs on transcriptional activity, we performed luciferase reporter gene assays using DNA fragments derived from the promoter region of the *SIRT1* gene. The *Homo sapiens SIRT1* promoter region (LOC107832851) from NCBI is 2958 bp and includes 11 SNPs (Fig. 3A). We generated two DNA constructs: Promoter_short is a 1.13 kb DNA fragment, which included SNPs rs35706870, rs3740051, rs932658, rs3740053, and rs2394443; Promoter_long is a 1.54 kb DNA fragment, which included SNPs rs12778366, rs3758391, rs35706870, rs3740051, rs932658, rs3740053, and rs2394443 (Fig. 3B). The sequence information of the two DNA fragments is shown in Supplemental Table S7. After genotyping, samples with homozygous T/T, A/A, and A/A for rs12778366, rs35706870, and rs3740051 were selected for further studies. The LD plot showed complete LD between rs3740051 and rs3740053, so SNP rs3740053 homozygous A/A were also identified in these samples. From these samples, heterozygous T/C, A/C, G/C for SNPs rs3758391, rs932658, and rs2394443 were selected for cloning.

Three different haplotypes of the target SNPs are reported in human populations for both of the *SIRT1* DNA fragments (Supplemental Fig. S2), all of which were identified in our samples (Fig. 3C). The construct Promoter_short containing the rs932658 variant A allele showed significantly higher luciferase activity compared with those containing the reference C allele ($p = 0.0052$), whereas SNP rs2394443 did not affect reporter gene activity (Fig. 4A, B). Similar results were obtained with the Promoter_long constructs, showing higher luciferase activity only when containing the variant A allele of rs932658 (Fig. 4C, D). Moreover, the two constructs with different lengths had similar luciferase activities (A-G versus T-A-G) ($p = 0.93$) (Fig. 4E, F). Therefore, the addition of SNP rs3758391 did not affect luciferase activity. These results demonstrate that the promoter SNP rs932658 increases transcriptional activity in reporter gene assays.

To further confirm that rs932658 is the causal SNP, we performed a conditional logistic regression analysis in the 104 samples. After adjusting for rs932658, the index SNP was no longer associated with MRONJ (OR = 0.263 and 95% CI 0.026–2.674; $p = 0.26$).

Discussion

In this study, we identified three promoter region SNPs, rs3758391 (C > T), rs932658 (C > A), and rs2394443 (C > G) in the *SIRT1* gene that were associated with lower odds of developing MRONJ among cancer patients who were treated with iv BPs for the prevention of SREs. All three SNPs were in high LD ($r^2 > 0.8$) with our previous WES study index SNP rs7896005. In addition, in silico analyses using the GTEx portal provided evidence that the T allele of rs3758391, the A allele of rs932658, and the G allele of rs2394443 were associated with higher expression of *SIRT1*. Luciferase reporter gene assays showed that allele A of SNP rs932658 was associated with higher promoter activity of *SIRT1*, which leads to higher expression of *SIRT1* mRNA. These results indicate that the promoter SNP

rs932658 is the functional variant regulating the expression of *SIRT1* and protects against MRONJ by increasing *SIRT1* expression.

SIRT1 plays an important role in bone remodeling, especially by promoting bone formation.⁽⁶⁾ *SIRT1* could regulate the Wnt signaling pathway by deacetylating β -catenin,⁽⁶¹⁾ causing β -catenin to accumulate in the nucleus and induce mesenchymal stem cell (MSC) differentiation to osteoblasts, rather than adipogenesis.^(61,62) Low expression of *SIRT1* also inhibits the *RUNX2* pathway, which plays an important role in osteoblast differentiation.⁽⁶³⁾ *SIRT1* also deacetylates the forkhead box proteins (FOX) O1, FOXO3, and FOXO4, which inhibit osteoclast differentiation and proliferation.⁽³⁴⁾ Based on these studies, *SIRT1* may regulate bone remodeling by both promoting osteoblast differentiation while inhibiting osteoclast formation. *in vivo* experiments have shown that *SIRT1* knockout (KO) animals develop defects in bone formation^(64–66) and that deletion of *SIRT1* in osteoblasts or osteoclasts induced loss of bone mass.^(6,67) Furthermore, previous studies showed that *SIRT1* stimulates MSC proliferation and differentiation into mature osteoblasts.^(63,68) *SIRT1* is associated with inflammation.⁽⁶⁹⁾ *SIRT1* deacetylates the p65 protein, which is a subunit of the NF- κ B complex and deacetylation of p65 leads to the inhibition of NF- κ B signaling,⁽³²⁾ thereby reducing the immune response and inducing the inflammation process through the NF- κ B pathway.⁽⁷⁰⁾ *SIRT1* also promotes angiogenesis by regulating the MAPK pathway.⁽⁷¹⁾ Inflammation and anti-angiogenesis are both risk factors of MRONJ based on the updated 2014 guidelines from the American Association of Oral and Maxillofacial Surgeons (AAOMS).⁽¹⁾ Therefore, *SIRT1* may reduce the risk of MRONJ by inhibiting inflammation and inducing angiogenesis, improving healing of oral cavity wounds. Combined with the above results, the *SIRT1* SNP rs932658 was associated with decreased risk of MRONJ induced by BPs by increasing the expression of *SIRT1*. Overall, increased expression of *SIRT1* due to pharmacogenomic markers, such as polymorphism A of rs932658 identified in this study, may lower the risk of patients developing MRONJ when treated with BPs.

It is important to recognize that MRONJ is a complex disease that is likely driven by many genetic factors, environmental factors, and lifestyle. The genetic factors are just one factor. If a patient is homozygous or heterozygous for the risk allele, that does not mean that they will get MRONJ if they are treated with BPs. Rather it means they are at higher risk for MRONJ. There are other genetic factors that could contribute to the risk, as well as other environmental and lifestyle factors. Because MRONJ is rare, the only appropriate study design is a case–control study design. We found that the minor allele frequency of SNP rs932658 was 22% in MRONJ cases, but 39% in controls. So the appropriate interpretation of our result would be that patients with the minor allele A of the SNP rs932658 were at lower odds for MRONJ when treated with BPs than those who were homozygotes for major allele C of this SNP.

Our study had some limitations that need to be acknowledged. Because of the low rate of this drug-related adverse event, we used a case–control study design, which has some inherent limitations. One major limitation was that we could not ascertain that the controls who did not develop MRONJ at the time of analyses were truly controls. Nevertheless, in the literature, the median duration of treatment before the development of MRONJ was 15.6

months for zoledronate, 26.4 months for pamidronate, and 28.8 months for alendronate.⁽⁷²⁾ When we compared the duration of iv BP treatment between MRONJ cases and controls (26.55 versus 33.06 months), there were no statistical differences between the two groups. So the controls in our study were likely to be real controls. The other limitation was that because of the low event rate of MRONJ and therefore small sample size of our study, we could not match MRONJ cases and controls within each cancer type. This might not be an issue because the germline genetic polymorphisms associated with MRONJ is likely not cancer specific.

In conclusion, this study validated our previous WES results⁽²⁸⁾ and also suggested a causal variant for this association. These results indicate that the promoter SNP rs932658 regulates the expression of *SIRT1* and presumably lowers the risk of MRONJ by increasing *SIRT1* expression. SNPs rs932658 may be the potential pharmacogenomic marker for a personalized approach to regulate the use of BPs for the prevention of SREs. Based on our data, patients carrying the major allele C in rs932658 may be at higher risk of MRONJ and therefore may potentially benefit from lower exposure or less frequent administration of a BP agent. Further investigations are warranted before such findings can be translated into clinic settings.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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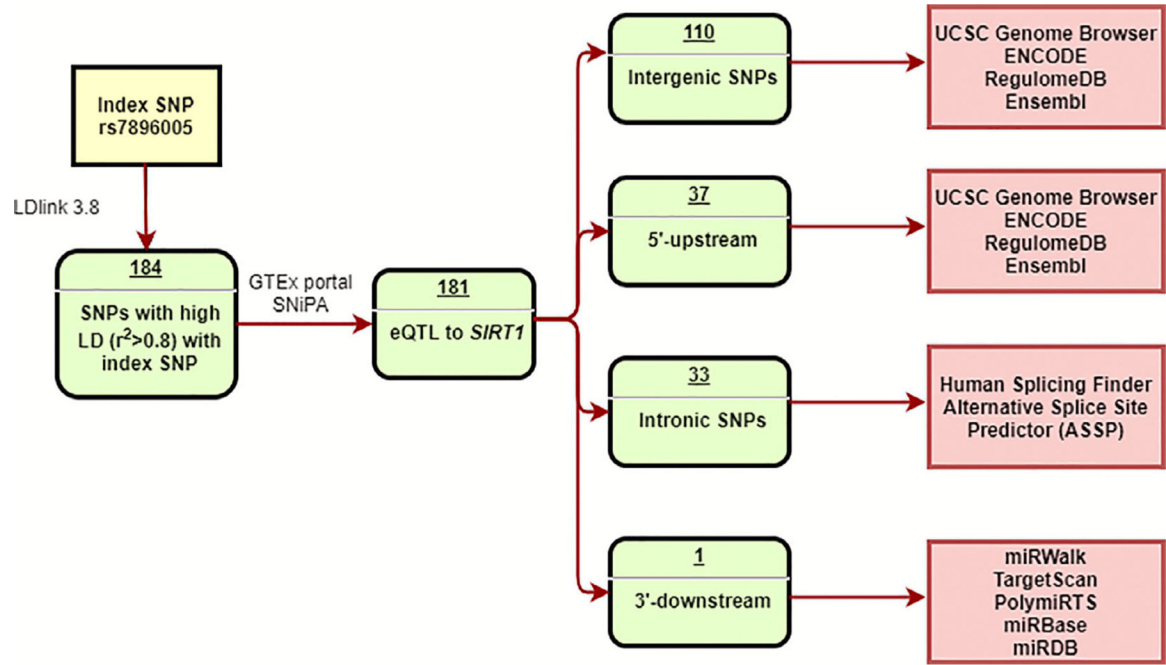


Fig 1.
Flow chart for in silico analyses.

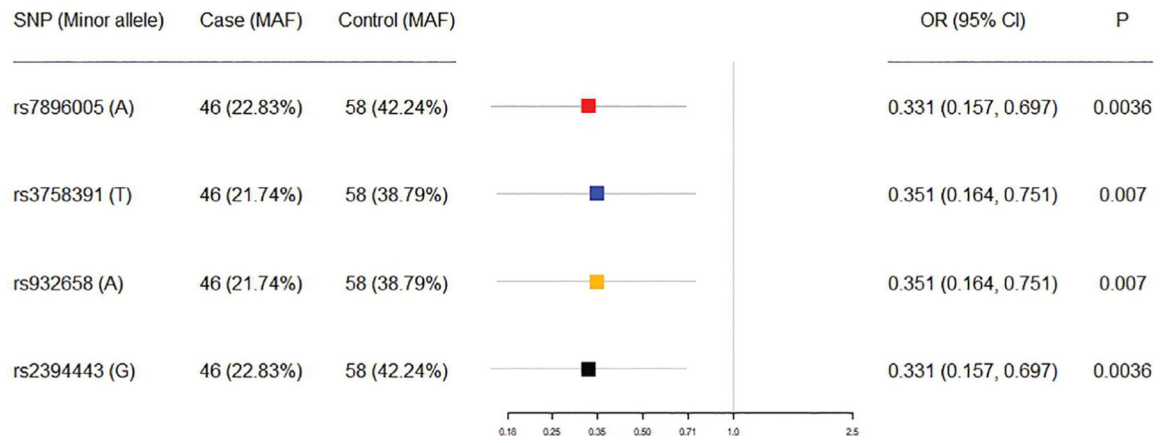


Fig 2. Odds ratio (OR) of three promoter region SNPs. MAF = minor allele frequency; CI = confidence interval.

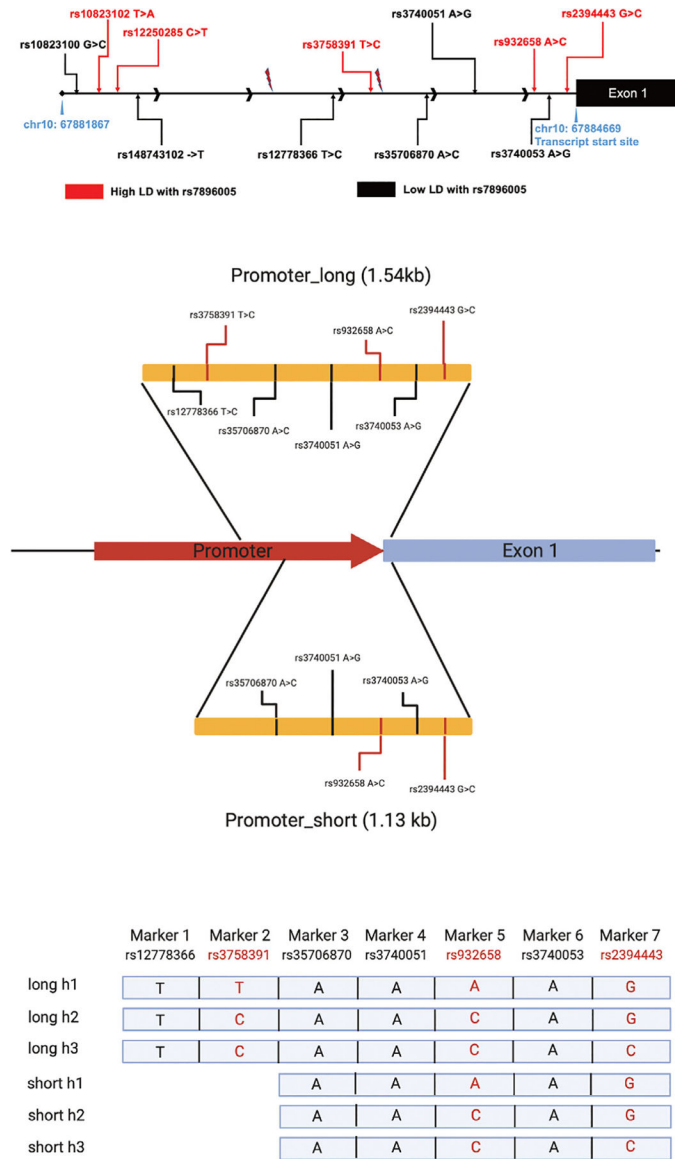


Fig 3. SNPs at *SIRT1* promoter region. (A) Eleven SNPs are located in the *SIRT1* promoter region according to the 1000 Genome Project. (B) We designed two promoter region constructs with different lengths: Promoter_short included SNPs rs35706870, rs3740051, rs932658, rs3740053, and rs2394443; Promoter_long included SNPs rs12778366, rs3758391, rs35706870, rs3740051, rs932658, rs3740053, and rs2394443. (C) The six possible haplotypes were used to perform luciferase reporter gene assay.

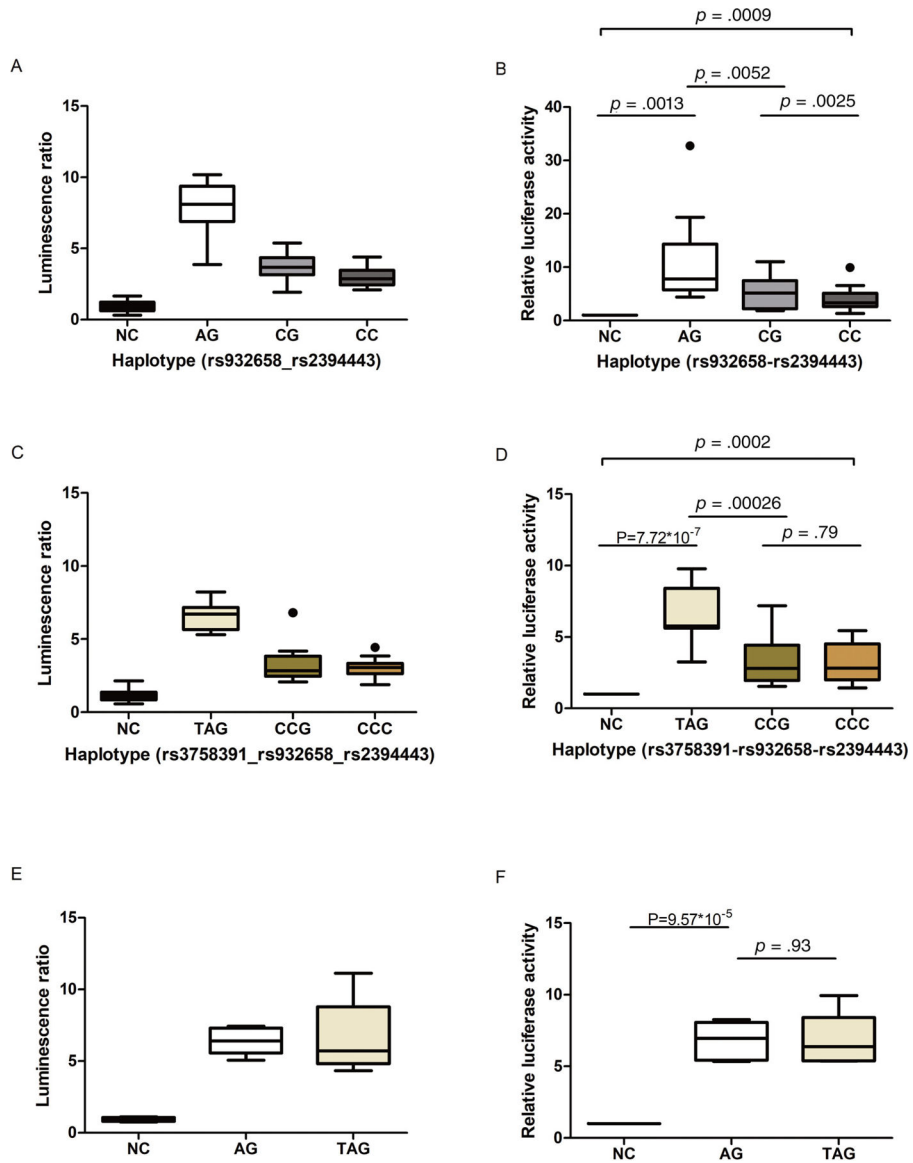


Fig 4. The effect of different haplotypes in luciferase reporter assays. Luminescence activity for different haplotypes is shown in a relative luminescence activity plot. (A, C, E) Luminescence ratio is represented by the ratio of the firefly luciferase activity and Renilla luciferase activity. (B, D, F) Box plots of the normalized ratios of the luminescence ratios, which normalized the results from each experimental sample to control samples repeated on each plate. (A–D) Polymorphism A of rs932658 increased promoter activity of *SIRT1*. rs2394443 did not affect promoter activity significantly. (E, F) There was no significant difference between haplotype A-G and haplotype T-A-G. Box plot key: line within box = median; upper boundary of box = third quartile (Q3); lower boundary of box = first quartile (Q1); vertical length of box = interquartile range (IQR); upper whisker = smaller value of the maximum and $Q3 + 1.5 \times IQR$; lower whisker = larger value of the minimum and $Q1 - 1.5 \times IQR$. Dots indicate outliers outside of the boundaries defined by whiskers.

Table 1.
Demographic Table of Patients Included in the Logistic Regression Analysis

Characteristics	Total (n = 104)	MRONJ (n = 46)	Non-MRONJ (n = 58)	p Value
Age (years) (mean ± SD)	63.92 ± 9.93	65.13 ± 10.47	62.97 ± 9.46	0.4
Female (%)	50.96%	69.57%	36.21%	0.0008
Duration of treatment (mean ± SD)	29.94 ± 4.99	26.55 ± 7.36	33.06 ± 6.82	0.194
Race				
European ancestry	104 (100%)	46 (100%)	58 (100%)	
Cancer type				
Multiple myeloma	68 (65.38%)	15 (32.61)	53 (91.38%)	0.0001
Breast cancer	26 (25.00%)	24 (52.17)	2 (3.45%)	
Lung cancer	1 (0.96%)	1 (2.17%)	0 (0%)	
Prostate cancer	6 (5.77%)	3 (6.52)	3 (5.17%)	
Renal cancer	2 (1.92)	2 (4.35)	0 (0%)	
Cervix cancer	1 (0.96%)	1 (2.17%)	0 (0%)	

MRONJ = medication-related osteonecrosis of the jaw.