Original Communication

Osteoarthritis improvement effect of *Chrysanthemum zawadskii* var. *latilobum* extract in relation to genotype

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Abstract: *Objectives:* To determine whether SNPs of osteoarthritis (OA)-related genes predict the effect of *Chrysanthemum zawadskii* var. *latilobum* (CZ) extract in OA patients with OA. *Subjects/methods:* To analyze correlations between CZ extract effects in humans and their genotypes, 121 Korean patients with OA were recruited. Patients ingested 600 mg/day of the CZ extract GCWB106 (one tablet daily), including 250-mg CZ, or placebo (one tablet daily) for 12 weeks. Twenty SNPs were genotyped in 11 genes associated with OA pathogenesis, including tumor necrosis factor-alpha (TNF-α) and matrix metalloproteinases (MMPs), and 9 genes involved in OA-related dietary intervention. The Visual Analogue Scale (VAS) and Korean Western Ontario and McMaster Universities (K-WOMAC) were measured as indicators of GCWB106 effect. Statistical comparisons were performed using Kruskal-Wallis tests to identify associations between these scales and genotyped loci in patients with OA. *Results:* Three SNPs (*PPARG* rs3856806, *MMP13* rs2252070, and *ZIP2* rs2234632) were significantly associated with the degree of change in VAS pain score. Homozygous CC genotype carriers of rs3856806, G allele carriers (GA or GG) of rs2252070, and T allele carriers (GT or TT) of rs2234632 showed lower VAS score (i.e., less severe symptoms) in the GCWB106 group (n=53) than the placebo group (n=57) (p=0.026, p=0.009, and p=0.025, respectively). Gene–gene interaction effects on GCWB106-mediated pain relief were then examined, and it was found that the addition of each genotype resulted in a greater decrease in VAS pain score in the GCWB106 group (p=0.0024) but not the placebo group (p=0.7734). *Conclusions:* These novel predictive markers for the pain-relieving effects of GCWB106 may be used in the personalized treatment of patients with OA.

Keywords: Knee osteoarthritis, genetic testing, genetic markers, SNPs, precision medicine

Introduction

Description of osteoarthritis and its mechanism of development

Osteoarthritis (OA), which is the most common form of arthritis, is a joint pathology characterized by the progressive breakdown of cartilage. In modern times, OA is a common disease. Due to changes in the eating habits of Koreans resulting in increased obesity, the age group affected by OA has become younger. Main symptoms of OA include pain, inflammation, stiffness, and loss of mobility in joints. An imbalance between anabolism and catabolism of extracellular matrix (ECM) components is the central feature of cartilage destruction.

Inflammation is also involved in the development and progression of OA, even in early stages of the disease [1]. Proinflammatory cytokines, such as IL-1β, IL-6, and TNF-α, are mediators of disturbed metabolism and enhanced catabolism of joint tissue [2]. Previous studies have shown that cartilage destruction in OA results from the inflammation-induced activation of matrix metalloproteinases (MMPs) [3, 4]. The MMP family consists of 23 neutral enzymes in mammals, which are zinc (Zn)- and calcium-dependent [5]. MMPs are thought to be the major proteolytic enzymes that facilitate ECM turnover and breakdown in physiological and pathological situations [6, 7, 8]. Of proteins in the MMP family, MMP13 functions as an ECM breakdown enzyme in OA because it is significantly overexpressed in the articular cartilage [9, 10]. MMP13 cleaves type II collagen, which is the major structural component of cartilage ECM, and acts as a central node in the cartilage degradation network [11, 12, 13]. Several factors, including cytokines, diet, obesity, and mechanical forces, are also

known pathogenic risk factors of knee OA [14, 15]; however, the exact pathological mechanisms of OA are not yet fully understood.

There has been significant research and clinical efforts to control OA. However, no fundamental treatment has been developed. The therapeutic aim for arthritis is to reduce the inflammation and pain in joints and prevent joint deformation. Medications like acetaminophen and nonsteroidal anti-inflammatory drugs an relieve OA symptoms, primarily pain; however, these medications can cause serious side effects, including stomach upset, liver and kidney damage, cardiovascular problems, and gastrointestinal bleeding [16].

Chrysanthemum zawadskii var. *latilobum* (CZ), which has traditionally been used as an ingredient for oriental tea in Asia, contains natural flavonoids, which have antioxidant and anti-inflammatory activities [17]. Extracts from CZ flowers possess anti-inflammatory activities [18], and CZ leaf extracts inhibit inflammation by decreasing levels of inflammatory mediators in lipopolysaccharide treated macrophages and the differentiation and formation of osteoclasts from bone marrow cells [19, 20]. Further, CZ extracts protect mice against rheumatoid arthritis through the suppression of nuclear factor kappa B (NF-κB)-mediated inflammation [21], and a previous study has shown that GCWB106, which is an ethanol extract of CZ, reduces levels of MMP2 and MMP13 in a monoiodoacetate (MIA)-induced OA rat model [22]. In addition, GCWB106 possesses a protective effect on OA-induced cartilage damage by suppressing the expression of proapoptotic molecules, while inducing the expression of autophagosome- and autolysosome-related molecules [22]. The main compounds of GCWB106 are linarin, chlorogenic acid, and isochlorogenic acid. Previously, it was reported that the marker compound of CZ extract, linarin, decreases the catabolism of articular cartilage via the downregulation of ECM-degrading enzymes (MMPs and ADAMTSs) in a rat model of OA and human chondrosarcoma cell line [23].

This study investigated the relationship between genetic variants implicated in OA pathogenesis or OA-related dietary intervention and the effect of GCWB106 on symptoms of OA and discover prediction markers for the effect of GCWB106 in patients with OA.

Materials and methods

Study design

Subjects aged between 40 and 75 years who had Grade I or Grade II knee OA based on the Kellgren-Lawrence classification with a baseline functional assessment of overall pain of at least 30 mm on a 100 mm Visual Analogue Scale (VAS) were enrolled. Subjects (n=121) were instructed to consume 600 mg/day of GCWB106 (one tablet daily), including 250-mg CZ, or placebo (one tablet daily) for 12 weeks. GCWB106 was obtained from Green Cross Wellbeing Corporation (Seoul, Korea). GCWB106 was extracted from stems and leaves of CZ. The end product was dispensed and packaged according to regulatory guidelines for a clinical study. This study was approved by the Ethics Committee of Korea Inje University Seoul Paik Hospital (IRB number: PAIK 2017-09-004) and the Korean Clinical Research Information Service (number: KCT0004238). All subjects signed the written informed consent before participating in this study.

Changes in Korean Western Ontario and McMaster Universities (K-WOMAC) scores were checked every 6 weeks from baseline to week 12. For K-WOMAC scoring, pain, stiffness, function subscales, and total scores were assessed with a five-point Likert scale, in which a lower number indicates less severe symptoms. The VAS is a subjective measurement that a subject reports on a 10 cm horizontal line, where 0 indicates no pain and 10 indicates the worst pain. The VAS is particularly useful for assessing changes in the pain of individuals receiving therapy.

SNP selection

To identify genetic markers associated with GCWB106 efficacy in patients with mild OA, 20 SNPs with a known correlation with symptoms of OA were selected based on a review of the research literature (Table E1 in Electronic Supplementary Material 1). Among them, we included 10 functional variants associated with inflammatory response, six of which change the expression of MMP-encoding genes and four that affect TNF-α. In addition, ten variants of genes known to interact with dietary metabolism were included due to a high correlation between the development of OA and obesity.

Genotyping

Venous blood samples (3 mL) were aseptically collected from each subject. Genomic DNA was extracted from standard EDTA-preserved whole blood using a Chemagen DNA blood kit (PerkinElmer, Baesweiler, Germany). PCR primers were designed based on sequences of 20 genes using PrimerQuest Tool (Integrated DNA Technologies, Inc., Coralville, Iowa, USA). Primers used for RT-PCR are shown in Table E2 in ESM 1. TaqMan genotyping was performed using 15 ng of DNA mixed with THUNDERBIRD™ Probe qPCR Mix (TOYOBO, Osaka, Japan) and custom TaqMan SNP assays (Thermo Fisher Scientific, Waltham, MA, USA) following manufacturers' instructions.

Table 1. Average changes of variables for evaluating the effect in humans in GCWB106 and placebo groups (n=110)

	Mean ΔVAS	Mean ΔK-WOMAC (min, max)			
Group	(min, max)	Total	Pain	Stiffness	Function
GCWB106 (n=53)	−12.7925 (−38, 37)	−8.3774 (−27, 31)	−1.7736 (−9, 6)	−0.6792 (−5, 2)	−5.9245 (−21, 23)
Placebo (n=57)	−6.0877 (−22, 3)	−4.6667 (−56, 32)	−1.0351 (−9, 6)	−0.5439 (−6, 2)	−3.0877 (−41, 25)
P-value between Groups*	*P*<0.0001	0.0424	0.2162	0.5856	0.0157

*P-values were derived from Wilcoxon rank sum test between groups.

Statistical analysis

Before tests of statistical association, quality control steps were conducted of both genotype and phenotype data. SNPs with a missing call rate greater than 5%, a minor allele frequency less than 0.01, or significant deviation from Hardy–Weinberg equilibrium ($p<0.00001$) were excluded. The Kruskal-Wallis test was conducted with R version 3.4.4 (R Foundation for Statistical Computing, Vienna, Austria). P-values were adjusted using the Benjamini–Hochberg false discovery rate (FDR) method. Allele frequency differences in these 20 SNPs between our cohort and the Korean Reference Genome Database (KRGDB) were compared by Fisher's exact test.

Preparation and high-performance liquid chromatography analysis of CZ extract

CZ extracts were prepared and analyzed using a 1260 Infinity II LC system (Agilent, Santa Clara, CA, USA) according to a previously published method (21, 23). A 70% ethanol extract of CZ (code name: GCWB106) was supplied by Green Cross WellBeing Corporation. The active ingredients were extracted from dried whole plant CZ using 70% ethanol at 50 °C in a water bath and concentrated using a rotary evaporator at 50 °C. The concentrated extract showed a yield of roughly 20% with respect to the weight of raw material. CZ extract was dissolved in distilled 50% ethanol and then analyzed by HPLC. The CZ extract was separated using a Phenomenex Kinetex column C18 (4.6 × 250 mm, 5 μm) at a flow rate of 1.0 mL/min. To detect the chromatogram, the mobile phase was composed of 0.025 M KH_2PO_4 (pH 3.0, phosphoric acid) in water (solution A) and 100% acetonitrile (solution B), with a gradient of 0–12 min (15–25% solution B), 12–20 min (25–41% solution B), and 20–30 min (41–90% solution B), and then equilibrated with 15% solution B for 5 min. Components of CZ extract verified by HPLC were chlorogenic acid, isochlorogenic acid A, isochlorogenic acid B, isochlorogenic acid C, and linarin (Table E5 in ESM 1). Standard compounds for HPLC analysis were used. Chlorogenic acid (96.67% purity, Cat. No. 0050-05-90) was obtained from the HWI Group (Rülzheim, Germany), whereas isochlorogenic acid A (98.0% purity, Cat. No. BP0056), isochlorogenic acid B (98.0% purity, Cat. No. BP0055), and linarin (97.63% purity, Cat. No. BP0115) were obtained from Chengdu Biopurify Phytochemicals (Chengdu, China). Isochlorogenic acid C (98% purity, Cat. No. CFN90354) was purchased from Chemfaces (Wuhan, China).

Results

Improvement effect of GCWB106 in patients with OA

To determine whether there was evidence of an association between GCWB106 effect and genotype in OA, we recruited 121 patients (108 female and 13 male) with mild OA of Kellgren–Lawrence Grades 1 and 2. Recruited patients were randomly assigned to either the GCWB106 (n=60) or placebo group (n=61), and for 12 weeks, they were examined for changes in effect-evaluating variables. Eleven patients were excluded due to dropout and undercompliance during the 12-week administration period. Thus, data from a total of 110 patients (97 female and 13 male) were analyzed. The 13 males were similarly distributed between the GCWB106 (n=7) and placebo (n=6) groups.

K-WOMAC and VAS were used as variables to evaluate effects of GCWB106 in our patient cohort. The total score of K-WOMAC and scores of subcategory items (pain, stiffness, and physical function) were used as individual indices. To examine OA improvement effects of GCWB106, K-WOMAC and VAS scores were converted to measures of change (Δ) (last visit value – baseline value) and used for analysis. Note that when the score of an evaluation variable decreases, reflecting improvement or less severe symptoms, the degree of change (Δ) becomes more negative.

The results of our analysis of all evaluation variables in our 110 patients with OA showed that the degree of reduction in Δ was greater in the GCWB106 group than the placebo group after 12 weeks, verifying the OA improvement effect of GCWB106 (Table 1). In the placebo group, all evaluation variables tended to improve (i.e., negative Δ value), indicating that OA improved in the placebo group during the study period. This change reflects a placebo effect as the subjective judgment of patients cannot be excluded in

Table 2. Results of analysis of 20 SNPs for 110 patients selected for this trial

		Patients					Genotype		
Gene	rs number	Common homo	Hetero	Rare homo	MAF	HWE-p	Common homo	Hetero	Rare homo
MMP-1	rs1799750	58	45	7	0.268	0.658	2G2G	1G2G	1G1G
MMP-2	rs243865	88	20	2	0.109	0.498	CC	CT	TT
MMP-3	rs3025058	86	23	1	0.114	0.691	6A6A	5A6A	5A5A
MMP-9	rs3918242	83	21	6	0.150	0.008	CC	CT	TT
MMP-12	rs2276109	109	1	–	0.005	0.962	AA	AG	GG
MMP-13	rs2252070	27	64	19	0.464	0.075	GG	GA	AA
TNF	rs1799964	67	38	5	0.218	0.895	TT	TC	CC
TNF	rs1800630	76	30	4	0.173	0.632	CC	CA	AA
TNF	rs1799724	77	29	4	0.168	0.545	CC	CT	TT
TNF	rs1800629	95	15	0	0.068	0.443	GG	GA	AA
MTHFR	rs1801133	37	54	19	0.418	0.926	CC	CT	TT
FTO	rs9939609	80	30	0	0.136	0.098	TT	TA	AA
MTNR1B	rs10830963	35	54	21	0.436	0.984	CC	CG	GG
PPARG	rs1801282	99	10	1	0.055	0.214	CC	CG	GG
PPARG	rs3856806	75	31	4	0.177	0.723	CC	CT	TT
APOA5	rs662799	50	44	16	0.345	0.226	TT	TC	CC
TCF7L2	rs12255372	109	1	–	0.005	0.962	GG	GT	TT
GCKR	rs1260326	27	59	24	0.486	0.441	TT	TC	CC
ADIPOQ	rs182052	22	57	31	0.459	0.649	AA	AG	GG
ZIP2	rs2234632	54	49	7	0.286	0.346	GG	GT	TT

MAF: minor allele frequency; HWE-p: p-value for Hardy-Weinberg Equilibrium test.

VAS and K-WOMAC evaluations as they are questionnaire-based.

Genotype analysis of recruited subjects

To determine whether there are correlations between individual genotypes and effects of GCWB106 in patients with OA, genotyping was performed for 20 genetic variations functionally related to degenerative arthritis or associated with dietary metabolism in our cohort (Table 2). Fisher's exact test was performed to examine differences in allele frequencies of these 20 variants between our cohort of 110 subjects and the Korean population using KRGDB. None of these 20 variants showed a significant difference in allele frequency between the two groups (Table E3 in ESM 1). In addition, analyzed variants underwent Hardy–Weinberg equilibrium testing in our cohort. For 19 SNPs, we found a normal distribution of genotype frequencies (all $p>0.05$), except for *MMP9* rs3918242 (Table 2). To determine whether there was a possibility of an error when analyzing *MMP9* genotypes, rs3918242 was genotyped in 30 normal samples. These samples were found to have a normal distribution, consisting of 20 common homozygotes, 9 heterozygotes, and 1 rare homozygote ($p=0.992$) (Table E4 in ESM 1). Therefore, we determined that *MMP9* rs3918242 is out of Hardy–Weinberg equilibrium due to characteristics of our patient cohort and the limited number of recruited subjects rather than genotyping error and was excluded from further analysis. Additionally, *MMP-12* rs2276109 and *TCF7L2* rs12255372 are rare variants with a minor allele frequency less than 0.02. As they were not found in subjects who were randomly assigned to the GCWB106 group, these genotypes were also not further analyzed in our investigation.

Associations between evaluation variables and genotypes in GCWB106 and placebo groups

Nonparametric tests were conducted because variables for evaluating effects in humans do not have normal distributions. Thus, the Kruskal–Wallis test was performed to examine correlations between effects of GCWB106 and genotypes in patients. To examine genetic effects, correlations were analyzed using additive, dominant, and recessive models. Further, SNPs that showed a significant difference in evaluation variables in relation to genotypes in the GCWB106 group ($p<0.05$) versus the placebo group ($p>0.05$) were selected as putative biomarkers for predicting effects of GCWB106 in our study.

PPARG rs3856806, *MMP2* rs243865, and *MTNR1B* rs10830963 were associated with stiffness, as measured by K-WOMAC, although no evidence of a clinical significance

Table 3. Markers verified to show correlations with variables for evaluating effects of GCWB106 in humans through Kruskal-Wallis test

Variable	Gene	rs number	Genetic effect	P-value		Note
				GCWB106	Placebo	
VAS	MMP-13	rs2252070	Additive	0.011	0.115	
			Recessive	0.009	0.119	
	ZIP2	rs2234632	Additive	0.025	0.167	GCWB106 group TT (n=3)
			Dominant	0.017	0.167	
	PPARG	rs3856806	Additive	0.042	0.881	Placebo group TT (n=1)
			Dominant	0.026	0.881	
K-WOMAC Stiffness	PPARG	rs3856806	Recessive	0.032	0.687	Placebo group TT (n=1)
	MMP-2	rs243865	Dominant	0.030	0.910	
	MTNR1B	rs10830963	Dominant	0.019	0.268	

GCWB106 p-value <0.05, Placebo p-value >0.05.

was found between GCWB106 effect and stiffness (Table 1).

Except for stiffness, other K-WOMAC indexes such as function subscales and pain were not significantly associated with any evaluated genetic marker. For VAS, our analysis showed correlations between the pain index and a single SNP in three genes, namely, *MMP13* rs2252070, *ZIP2* rs2234632, and *PPARG* rs3856806. We also found that *PPARG* rs3856806 and two other SNPs, *MMP2* rs243865 and *MTNR1B* rs10830963, were correlated with K-WOMAC stiffness (Table 3). However, these results did not undergo further analysis. Although K-WOMAC and VAS are used together, VAS is particularly useful for assessing changes in pain for individuals who are given treatment. Furthermore, in a previous study, which was conducted without genetic information, the GCWB106 group did not significantly improve its K-WOMAC stiffness scores after 6 and 12 weeks compared to the placebo group [24]. Therefore, although there was evidence of an association between *MMP2* and *MTNR1B* with stiffness in our cohort, the two genes were excluded from further analysis in this study.

For the three SNPs correlated with pain measured by VAS, evidence of a correlation was observed not only in dominant or recessive models, but also in an additive model. However, for *ZIP2* rs2234632 and *PPARG* rs3856806, the number of patients with the TT genotype was small. Therefore, cases were analyzed in the T allele group (dominant model) to increase the statistical power. Additionally, because the number of TT homozygous patients was small (n=3 and n=1 for *ZIP2* rs2234632 in the GCWB106 group and *PPARG* rs3856806 in the placebo group, respectively), *ZIP2* rs2234632 GT + TT and *PPARG* rs3856806 CT + TT patients were combined into a single T allele group to improve the statistical power. Therefore, effects of *ZIP2* and *PPARG* genotypes on variables were analyzed mainly for two groups (GG vs. T allele and CC vs. T allele, respectively) in our study.

Next, the three SNPs that showed a statistically significant correlation with ΔVAS in either a recessive (*MMP13* rs2252070) or dominant (*ZIP2* rs2234632 and *PPARG* rs3856806) model were selected as putative markers for predicting the pain-relieving effect of GCWB106. We found that the *MMP13* rs2252070 AA genotype showed no significant difference in ΔVAS between the GCWB106 and placebo groups. However, for cases possessing a G allele (GA or GG genotype), a greater decrease in the VAS index was observed in the GCWB106 group than the placebo group (Figure 1A). For cases with at least one *ZIP2* rs2234632 T allele, we found a larger decrease in the VAS index in the GCWB106 group compared to those with a GG genotype, suggesting a greater pain-relieving effect of GCWB106 in the presence of the T allele (Figure 1B). Finally, for *PPARG* rs3856806, we found no significant difference in ΔVAS in relation to genotype in the placebo group, whereas a greater decrease in the VAS index was found in patients with a CC genotype in the GCWB106 group (Figure 1C).

Pain-relieving effects of GCWB106 in relation to additive effects of predictive markers

To examine whether the three predictive markers for GCWB106-mediated pain relief acted independently or were interrelated, ΔVAS was examined in relation to the number of predictive markers and extent of pain relief. As the number of predictive markers increased, we found that the pain index decreased further (p=0.002) in the GCWB106 group; however, there was no significant difference in the reduction of the pain index in the placebo group in relation to the number of predictive markers (p=0.773) (Figure 2). Therefore, not only are the three predictive markers individually associated with GCWB106-mediated pain relief, but there is also a synergy among them in our cohort. In other words, determining the number of predictive markers through genotyping can be used to predict the pain-relieving effects of GCWB106 administration.

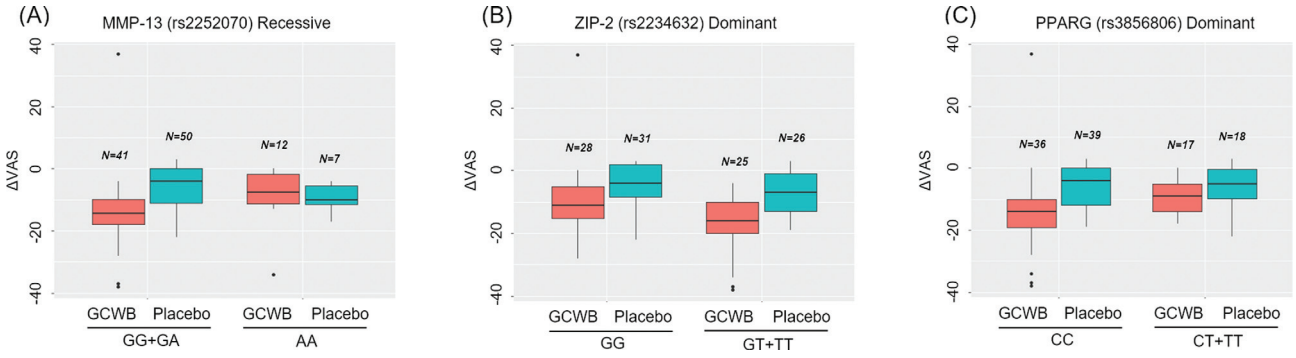

Figure 1. Boxplots of ΔVAS of (A) *MMP13* rs2252070, (B) *ZIP2* rs2234632, and (C) *PPARG* rs3856806. N is the number of participants with each genotype.

Discussion

Role of MMP13 in OA

MMP13 encodes a Zn^{2+}-dependent enzyme known as collagenase 3 that cleaves type II collagen. It is a major gene that influences the physiological turnover of cartilage associated with OA [25, 26]. Zn plays a role in the growth and maturation of bone and cartilage by stimulating metallothionein synthesis and regulating the activity of vitamin D [27]. MMP13 is not found in normal adult tissues, although it is expressed in the joints and articular cartilage of patients with OA [13]. The expression level of MMP13 is significantly higher in chondrocytes of late-stage OA cartilage than in early OA or normal knee cartilage [28]. Further, serum MMP13 is negatively associated with cartilage volume but positively associated with Kellgren–Lawrence Grade [29].

Previously, it was reported that individuals with the *MMP13* rs2252070 AA genotype, compared to carriers of the G allele, have higher levels of MMP13 mRNA and a higher risk of knee OA (adjusted odds ratio=1.361; $p<0.001$). Additionally, as the Kellgren–Lawrence Grade increases, the frequency of the AA genotype increases [30]. In a previous study using a MIA-induced rat OA model, GCWB106 reduced OA-induced damage to the cartilage by inhibiting MMP13 mRNA expression [22]. *MMP13* rs2252070 is associated with the regulation of MMP13 expression, and MMP13 plays a known role in OA progression. Thus, the inhibition of MMP13 expression may be a mechanistic effect of GCWB106 and differences in the pain-relieving effect of GCWB106 may be due to differences in the expression level of MMP13 caused by different genotypes.

Association between ZIP2 and OA

There are 14 members in the ZIP family of Zn^{2+} importers in mammals (ZIP1–ZIP14) that promote Zn^{2+} influx from the extracellular or luminal side into the cytoplasm. The dysregulation of certain Zn transporters can result in impaired Zn homeostasis and the development of Zn imbalance-related diseases, such as cancer, diabetes, and OA [31, 32]. In addition, Zn^{2+} is a cofactor of MMPs [33], and Zn^{2+} insufficiency inhibits chondrocyte proliferation and induces chondrocyte apoptosis [34]. Thus, Zn^{2+} level may serve as a biomarker for OA progression. In fact, a clinical study has reported significantly increased levels of serum Zn^{2+} in patients with OA [35]. Further, ZIP8-mediated Zn^{2+} influx upregulates expression levels of several MMPs, namely, MMP3, MMP9, MMP12, and MMP13, as well as ADAMTS5 in chondrocytes [31]. The *ZIP2* rs2234632 c.128T>G polymorphism results in an amino acid substitution of leucine (Leu) to arginine (Arg) [36]. ZIP2 mRNA levels and plasma levels of IL-6, TNF-α, and RANTES as inflammation transducers are increased in individuals with Arg (GG or GT genotype) instead of Leu (TT genotype) [37]. In addition, research has shown that RANTES as a chemokine increases the expression of MMP1 and MMP13 and induces collagen degradation [38].

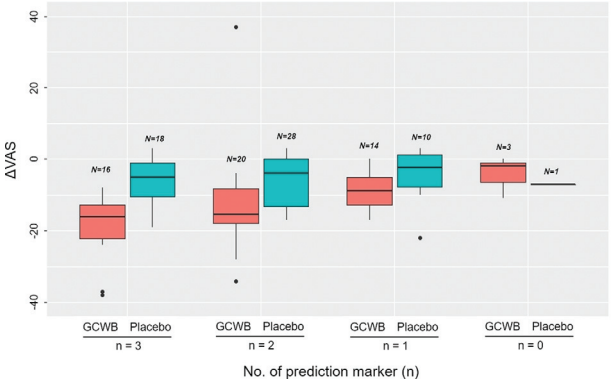

Figure 2. Boxplot of ΔVAS according to the number of predictive markers (*MMP13* rs2252070, *ZIP2* rs2234632, and *PPARG* rs3856806), where n is the number of predictive markers. The sample size (N) of each group is as follows: GCWB (N=3): 16, placebo (N=3): 18, GCWB106 (N=2): 20, placebo (N=2): 28, GCWB106 (N=1): 14, placebo (N=1): 10, GCWB106 (N=0): 3, and placebo (N=0): 1.

The *ZIP2* rs2234632 polymorphism is not only associated with ZIP2 expression but also OA development by regulating levels of Zn^{2+} (a cofactor of MMPs) and proinflammatory molecules. Based on these findings, we considered that differences in the pain-relieving effect of GCWB106 in patients with OA depend on the *ZIP2* rs2234632 genotype.

Role of PPARG in OA

PPARG belongs to the peroxisome proliferator activated receptor family of ligand-activated transcription factors [39]. PPARG regulates fatty acid storage and glucose metabolism; genes activated by PPARG play a role in the stimulation of lipid uptake and adipogenesis by fat cells. PPARG also possesses an immune and inflammation suppressive function [40, 41]. Patients with OA have elevated levels of the proinflammatory cytokine IL-1β in synovial fluid, synovial membrane, cartilage, and subchondral bone layer [42, 43, 44, 45]. Further, PPARG inhibits osteoblast differentiation and bone formation [46].

Human cartilage predominantly expresses PPARG isoform 1. The expression level of this isoform is reduced in patients with OA than in those with normal cartilage. In human OA chondrocytes, treatment with IL-1β decreases PPARG protein expression [47]. Moreover, *PPARG* knockout mice exhibit an accelerated OA phenotype with increased apoptosis of chondrocytes, cartilage degradation, and overproduction of OA inflammatory factors with suppression of key autophagy markers [48]. This is similar to the mechanistic effects of CZ extract protecting the cartilage from damage by inhibiting inflammation, suppressing the expression of proapoptotic molecules, and inducing the expression of autophagy-related molecules. Therefore, PPARG may be involved in the mechanisms of OA development and GCWB106 action.

Liu et al. have showed that CC homozygotes of *PPARG* rs3856806 have higher MMP9 or TNF-α levels than T allele carriers [49]. Further, *PPARG* rs3856806 T allele carriers have significantly lower levels of serum osteoprotegerin (OPG), which is an inhibitor of osteoclastogenesis [50, 51]. Treatment with OPG decreases pain behavior in a MIA-induced rat OA model [52]. Thus, observed differences in the pain-relieving effect of GCWB106 may be due to differences in inflammatory molecules and cartilage degradation depending on the *PPARG* rs3856806 genotype.

Predictive markers for OA pain

Statistical analysis showed that the three polymorphisms, *MMP13* rs2252070, *ZIP2* rs2234632, and *PPARG* rs3856806, were significantly correlated with the pain-relieving effect on the administration of GCWB106 in patients with mild OA. The G allele of rs2252070, T allele of rs2234632, and CC genotype of rs3856806 were selected as putative markers for predicting pain-relieving effects. To determine whether the effects of these three polymorphisms in GCWB106-mediated pain relief were independent or interrelated, ΔVAS was examined according to the number of predictive markers. As a result, a positive correlation was identified between the degree of pain relief (ΔVAS) and the number of predictive markers (0–3).

Since the Kruskal–Wallis test showed that all three variants were related not only in dominant or recessive models, but also in an additive model, it was expected that the ΔVAS can be predicted by combining the three markers. When ΔVAS was examined according to the combination of these three markers, the VAS pain index progressively decreased with the addition of more markers; although there was a difference in the pain-relieving effect in cases with two markers (Figure E1 in ESM 1). However, the number of samples was small (n<3) among five (placebo: −/−/−; both groups: −/−/+; placebo: +/−/−; and GCWB106: +/−/+) of eight groups according to genotype combinations of the three markers (*PPARG*, *MMP13*, and *ZIP2*, respectively). As a limitation, this study was conducted on individuals with mild OA having Kellgren–Lawrence Grades of I and II. Therefore, further study of participants with severe OA is needed to confirm whether the effect of GCWB106 differs by genotype. Additionally, further studies with more samples to increase the statistical power are warranted. Although a limitation of this study is the small number of samples in each group, statistical significance was found in some groups. Conventionally for nonparametric analysis, at least 15 people in a group are required; therefore, we will continue efforts to recruit additional participants and increase our cohort size. Moreover, as this study was conducted in Korea with a high percentage of women, which is a relatively homogenous population in terms of genetics and ethnicity, further research is warranted to determine the applicability of our findings in other ethnic groups and populations. Nevertheless, GCWB106 is effective in relieving knee pain of patients with OA, and based on our results, it is possible to develop a detailed model for predicting pain-relieving effects according to individual genotypes of these three polymorphisms. Importantly, the identification of novel predictive markers for the pain-relieving effects of GCWB106 may be used in the personalized treatment of patients with OA.

Conclusions

In our cohort of patients with mild OA, the effect of GCWB106 administration on pain relief was verified. There was a significant difference in GCWB106-mediated pain

relief in patients depending on the genotypes of *MMP13* rs2252070, *ZIP2* rs2234632, and *PPARG* rs3856806. In particular, the pain-relieving effects of GCWB106 were prominent in patients possessing the G allele of rs2252070, T allele of rs2234632, or CC genotype of rs3856806. In addition, the extent of GCWB106-mediated pain relief increased with the number of predictive markers. These findings may contribute to the development of a tool to predict the pain-relieving effect of GCWB106.

Electronic supplementary material

The electronic supplementary material (ESM) is available with the online version of the article at https://doi.org/10.1024/0300-9831/a000745

ESM 1. 20 SNPs known to change MMPs and TNF gene expression and interact with dietary metabolism (Table E1), Primers used for RT-PCR of the 20 SNPs (Table E2), Allelic frequencies of 20 variants analyzed between 110 subjects and the Korean Reference Genome Database (KRGDB) (Table E3), MMP-9 genotype validation in normal sample (n=30) (Table E4), Components of CZ extract verified by HPLC (Table E5), ΔVAS values according to the combination of predictive markers for pain-relieving effect (Figure E1).

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Conflict of interest
The authors declare that there are no conflicts of interest.

Authorship
The authors' contributions to the work are as follows: T. Lee: formal analysis and visualization; C. Na: investigation, validation, and writing of the original draft; D. Kim: investigation and methodology; E. Cho: resources and project administration; K. Park: investigation and methodology; H. J. Han: resources and project administration; and J. Yun: data curation and resources. T. Lee and C. Na contributed equally to this work.

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