

An insertion/deletion polymorphism within the 3'-untranslated region of COL1A2 confers susceptibility to osteoporosis

ZHEN-SONG JIANG¹ and ZHEN-HAI HAO²

Departments of ¹Spinal Surgery and ²Trauma Orthopedics,
Provincial Hospital Affiliated to Shandong University, Jinan, Shandong 250014, P.R. China

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Abstract. Polymorphisms located in microRNA (miRNA) binding sites may interfere with the interaction between miRNAs and mRNAs, and thereby alter the expression of genes. The current study aimed to investigate the association between an insertion/deletion (INS/DEL) polymorphism in the 3'-untranslated region (3'-UTR) of COL1A2 and the risk of developing osteoporosis. In the present study, COL1A2 was identified as a target gene of let-7g in osteoblast cells obtained from patients, using a luciferase reporter system. This was further confirmed by the observation that exogenous overexpression of let-7g in the osteoblast cells downregulated the expression of COL1A2 in the cells in the INS/INS group, however not in the DEL/DEL group. In addition, a total of 487 subjects were enrolled in the present study and their bone mineral density (BMD) was measured. The BMD at the four tested sites, the femoral neck, total left hip, L1-L4 and intertrochanteric areas, were significantly reduced in the INS/DEL or DEL/DEL group compared with the INS/INS group. Furthermore, the levels of COL1A2 and let-7g were measured in the primary osteoblasts obtained from 48 patients with osteoporosis. While the let-7g levels were comparable between each genotype group, the expression level of COL1A2 in the DEL/DEL and INS/DEL group was significantly greater compared with the INS/INS group. In conclusion, the present study demonstrated that the INS/DEL polymorphism in the 3'-UTR of COL1A2 is able to interfere with the interaction between miRNA and mRNA. In addition, it is the first study, to the best of our knowledge, to indicate that the minor allele (Del) is associated with a reduced risk of developing osteoporosis.

Introduction

Osteoporosis is a common disease which is characterized by the deterioration of the micro-architecture of bone tissue, reduced bone mineral density (BMD) and an increased risk of fragility fracture (1). Previous studies conducted in families and twins have indicated that BMD is strongly controlled by genes (2-6). It is important to identify the genetic variants that result in interindividual differences in bone traits, as this may lead to the identification of the cellular pathways that impact bone. Furthermore, this may lead to the formulation of novel treatments for bone diseases and aid in the identification of populations at risk of osteoporosis. Genetic variation serves a role in the regulation of BMD, with a range of genetic loci observed to contribute. In addition, certain gender-specific effects of genes and loci upon BMD have been observed (7), as indicated by gene-by-gene interactions (8). Collagen type I is present at high levels in connective tissue and is required for the normal functioning of bone and blood vessels (9). Approximately 90% of the bone matrix protein is collagen type I, which is important for the framework for mineralization and the tensile strength that gives bone elasticity (9). The collagen type I triple helix includes two $\alpha 1(I)$ chains and one $\alpha 2(I)$ chain, encoded by the genes COL1A1 and COL1A2, respectively. Mutations in these genes result in diseases such as osteogenesis imperfecta and Ehler-Danlos syndrome, which are characterized by low BMD, moderate to severe bone fragility and an increased tendency for bruising and bleeding (10,11).

MicroRNAs (miRNAs) are small highly conserved noncoding RNA species that serve important roles in numerous cellular activities (12). The dysregulation of miRNA expression has been associated with a variety of human medical conditions (13). miRNAs recognize their targets predominantly via base-pairing interactions between the 5' end of miRNA and complementary sequences in the 3'-untranslated regions (3'-UTRs) of the target mRNAs (12). The binding of miRNA to mRNA is important for the regulation of mRNA and protein expression. It has been demonstrated that genetic polymorphisms in the 3'-UTR of mRNAs targeted by miRNAs alters the strength of miRNA binding, impacting upon the regulation of target genes, and as a result impacting the individual's susceptibility to disease (14-16).

In a previous study, COL1A2 was identified as a target gene of the miRNA, let-7g, in liver cells, and that an

Correspondence to: Dr Zhen-Hai Hao, Department of Trauma Orthopedics, Provincial Hospital Affiliated to Shandong University, 9677 Jingshi Road, Jinan, Shandong 250014, P.R. China
E-mail: zhenhai_a@163.com

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insertion/deletion (INS/DEL) polymorphism in the 3'-UTR of COL1A2 interferes with the interaction between let-7g and COL1A2 (17). The rs3917 polymorphism consists of two alleles with the wild-type being an insertion (INS) and the minor allele being a deletion (DEL). Considering the fact that COL1A2 and let-7 serve important roles in regulating osteogenesis, the present study hypothesized that an INS/DEL polymorphism in the 3'-UTR of COL1A2 may compromise the physiological inhibition of COL1A2 by let-7g, and that this may represent a potential molecular mechanism underlying the interindividual variation in susceptibility to osteoporosis in the population.

Materials and methods

Subjects. A total of 487 participants (42-77 years old) including 155 women and 332 men were recruited at the Provincial Hospital Affiliated to Shandong University (Jinan, China). Subjects with a history of hip fracture and metabolic bone disease were excluded from the study. In addition, subjects who were treated with bisphosphonates, calcitonin, fluoride or hormone replacement therapy were excluded from the study. A total of 48 underwent surgery, during which peripheral blood was obtained with the use of Lymphocyte Separation Medium (Human; Applygen Technologies, Inc., Beijing, China). The study was conducted with approval from the Clinical Research Ethics Committee of the Provincial Hospital Affiliated to Shandong University (Shandong, China), and informed consent was obtained from all subjects prior to the study.

Determination of BMD. BMD was determined using a Hologic QDR 2000 dual-energy X-ray densitometer (Hologic, Inc., Waltham, MA, USA). Measurements were taken from the proximal hip (trochanter left, total left hip and femoral neck), and the lumbar spine (L1-L4).

DNA extraction and genotyping. A DNA extraction kit (Qiagen GmbH, Hilden, Germany) was used to extract the DNA from peripheral blood in accordance with the manufacturer's protocol. The COL1A2 forward primer, 5'-CTGTGG AACCATGGAAGAAG-3' and reverse primer, 5'-GTATTG AGTTGTATCGTGTGG-3' (Takara Biotechnology Co., Ltd., Dailan, China), were used for amplification of DNA fragments containing the polymorphism. Polymerase chain reaction (PCR) was conducted in 37.5 μ l of mixed solution containing 0.5 mmol/l each primer, 1.5 mmol/l $MgCl_2$, 0.25 mmol/l dNTPs (Qiagen GmbH), 3.75 ml 10X PCR buffer (Bio-Rad Laboratories, Inc., Hercules, CA, USA), 1.5 units Taq DNA polymerase and 100 ng genomic DNA. The cycling conditions were as follows: 94°C for 5 min, 34 cycles of 30 sec at 94°C, followed by 30 sec at 60°C, and 30 sec at 72°C, and finally 72°C for 5 min. The PCR products were then sent for direct sequencing (Provincial Hospital Affiliated to Shandong University, Jinan, China), and rs3917 genotypes were measured by the peak chromatograph.

Isolation and culture of primary human osteoblasts. Of the 477 study participants, primary human osteoblasts were isolated from 48 participants, from left-over surgically removed bone and its use was approved by the Human Ethics

Committee at the Provincial Hospital Affiliated to Shandong University. The bone was sectioned into 1-mm³ pieces and washed using phosphate-buffered saline (PBS). Bone pieces were digested using 0.02% trypsin (Sigma-Aldrich, St. Louis, MO, USA) at 37°C for 90 min. Following digestion, the cells were cultured in complete α -minimum essential medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 100 U/ml penicillin and streptomycin (Gibco; Thermo Fisher Scientific, Inc.), 10% (v/v) heat-inactivated fetal calf serum (Gibco; Thermo Fisher Scientific, Inc.) at 37°C in a humid atmosphere with 5% CO₂. Only passage 2 or 3 human osteoblast cells were used in the current study. When the cells had reached 80% confluence, cells were transfected with 50 nM hsa-let-7g mimics (Shanghai GenePharma Co., Ltd., Shanghai, China) and 50 nM COL1A2 short interfering RNA (siRNA) (anti-COL1A2 siRNA) (Shanghai GenePharma Co., Ltd.) using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) in accordance with the manufacturer's instructions. Three independent experiments were conducted.

Reverse transcription-quantitative PCR (RT-qPCR). Following culture for 48-72 h, the cells were washed with ice-cold PBS following the removal of the medium. TRIzol reagent (Sigma-Aldrich) was added and RNA was precipitated in isopropanol (Sigma-Aldrich). Following centrifugation at 12,000 \times g for 20 min at 4°C, RNA pellets were washed in 70% ethanol. Subsequently, diethylpyrocarbonate-H₂O (Invitrogen; Thermo Fisher Scientific, Inc.) was used to dissolve the RNA and a NanoDrop spectrophotometer (Thermo Fisher Scientific, Inc.) was used to quantify the concentration of RNA present in the samples. A QuantiTect ReverseTranscription kit (Qiagen GmbH) was used to synthesize first-strand cDNA from 1 μ g RNA according to the manufacturer's instructions. The specific Taqman microRNA Assays (Applied Biosystems, Germany) was used to perform let-7g RT-qPCR based on the instructions by supplier. A QuantiTect SYBR Green PCR kit (Qiagen GmbH) was used for COL1A2 qPCR in accordance with the manufacturer's protocol. Rotor-Gene 6000 (Qiagen GmbH) was used to analyze the expression levels of COL1A2 and let-7g. The housekeeping gene U6 was used to normalize relative gene expression. Data Assist software, version 2.0 (Applied Biosystems; Thermo Fisher Scientific, Inc.) was used to perform the statistical analysis of microRNA-let-7g expression, and the $2^{-\Delta\Delta C_q}$ method was used to calculate expression levels. The primer sequences were: COL1A2, forward 5'-TGA GGTAGTTTGTACAGTT-3' and reverse 5'-TCAGTCCAA TGGGTACGGT-3'; U6, forward 5'-TCAGTTTGCTGTTC TGGGTG-3' and reverse 5'-CGGTTGGCTGGAAAGGAG -3'. The cycling conditions were as follows: 95°C for 5 min followed by 30 cycles of 30 sec at 94°C, 50 sec at 55°C or 60°C, 60 sec at 72°C and a final elongation step for 5 min at 72°C.

Protein extraction and western blot analysis. Following culture for 48-72 h, the cells were lysed in radioimmunoprecipitation assay lysis buffer (Invitrogen; Thermo Fisher Scientific, Inc.), and a bicinchoninic acid assay kit (Pierce Biotechnology, Inc., Rockford, IL, USA) was used to measure protein concentration. A total of 10 μ g of each protein sample containing 4X sample buffer (Invitrogen; Thermo Fisher Scientific, Inc.) was heated at 70°C for 10 min. Subsequently, the samples were separated

Table I. The demographic and clinical characteristics of the study participants.

Category	INS/INS (n=378)	INS/DEL+DEL/DEL (n=109)	P-value
Age (years)	62.3±12.7	60.6±13.3	NS
Gender (M/F)	265/113	67/42	NS
BMI (kg/cm ²)	26.6±6.8	26.3±7.8	NS
BMD (g/cm ²)			
Femoral neck left	0.85±0.11	0.92±0.13	0.018
Total left hip	0.95±0.18	1.02±0.21	0.028
Trochanter left	0.82±0.14	0.93±0.16	0.018
Lumbar spine (L1-L4)	1.12±0.19	1.23±0.22	0.025

INS, insertion; DEL, deletion; NS, not significant; BMI, body mass index; BMD, bone mineral density.

by 12% SDS-PAGE (Invitrogen; Thermo Fisher Scientific, Inc.) and transferred to a polyvinylidene fluoride membrane (EMD Millipore, Billerica, MA, USA). The membrane was then washed using Tris-buffered saline with Tween-20 (TBS-T; 20 mM Tris-HCl, pH 7.6 and 137 mM NaCl). The membrane was blocked in TBS-T containing 1% bovine serum albumin (Gibco, Thermo Fisher Scientific, Inc.) at room temperature for 1 h, prior to incubation with the primary antibodies [anti-COL1A2 antibody (ab208638; 1:4,000) and anti- β -actin (ab8227; 1:10,000) antibody; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA] at 4°C overnight. Following three washes, the membrane was incubated with the secondary antibody (Santa Cruz Biotechnology Inc.) at room temperature for 1 h. Chemiluminescence (GE Healthcare Life Sciences, Chalfont, UK) was used to detect protein bands, which were visualized using a Bio-Rad ChemiDoc MP Imaging system (Bio-Rad Laboratories, Inc.).

COL1A2 3'-UTR luciferase assay. A DNA extraction kit (Qiagen GmbH) was used to extract DNA from peripheral blood in accordance with the manufacturer's protocol. The forward PCR primer, 5'-CAGTCGTATGCGCGTATAGC-3' and reverse primer, 5'-CGTAGTCGTAGCTAGCTAGAGA-3' were used for amplification of the full-length of the human COL1A2 3'-UTR. The cycling conditions were as follows: 95°C for 30 sec, then 40 cycles of 30 sec at 95°C, 2 min at 58°C and 30 sec at 68°C, followed by 72°C for 5 min, using a PTC-100 thermocycler (Bio-Rad Laboratories, Inc.). A TA Cloning kit (Invitrogen; Thermo Fisher Scientific, Inc.) was used to clone the PCR product. Sanger sequencing was used to determine the accuracy of the insert. Subsequently, an additional allele of the polymorphism was introduced using a QuikChange XL Site-Directed Mutagenesis kit (Agilent Technologies, Inc., La Jolla, CA, USA). The generated wild-type and mutant 3'-UTR of COL1A2 were used to substitute the 3'-UTR of *Renilla* luciferase in the pRL-SV40 vector (Promega Corporation, Madison, WI, USA). Human primary osteoblasts were cultured at a density of 1×10^5 cells/well in 24-well plates. Following incubation for 12 h, Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) was used to transfect the cells according to the manufacturer's instructions. A total of 100 pmol let-7g

Hsa-let-7g target COL1A2

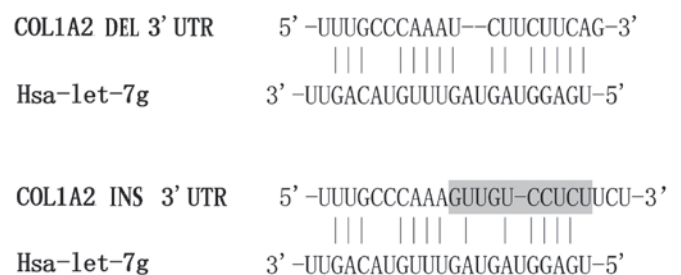


Figure 1. Comparison of the sequences of hsa-let-7g and the 3'-UTR of COL1A2 with the DEL or INS genotype. UTR, untranslated region; COL1A2, collagen type 1 $\alpha 2$; DEL, deletion; INS, insertion.

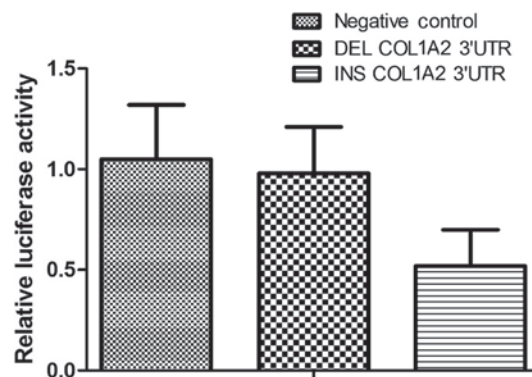


Figure 2. A luciferase assay was conducted in primary osteoblast cells, and the cells transfected with the COL1A2 3'-UTR of the INS genotype exhibited reduced luciferase activity compared with the negative control. COL1A2, collagen type 1 $\alpha 2$; UTR, untranslated region; INS, insertion; DEL, deletion.

mimics or negative control (Ambion; Thermo Fisher Scientific, Inc.) was used to co-transfect 500 ng of the wild-type or mutant construct and 50 ng pGL3 control vector. Following transfection for 24 h, passive lysis buffer (Invitrogen; Thermo Fisher Scientific, Inc.) was added to harvest cells. The Dual-Luciferase Report Assay system (Promega Corporation) was used to determine the luciferase activity in the cell lysates in a Turner BioSystems TD-20/20 luminometer (Promega Corporation).

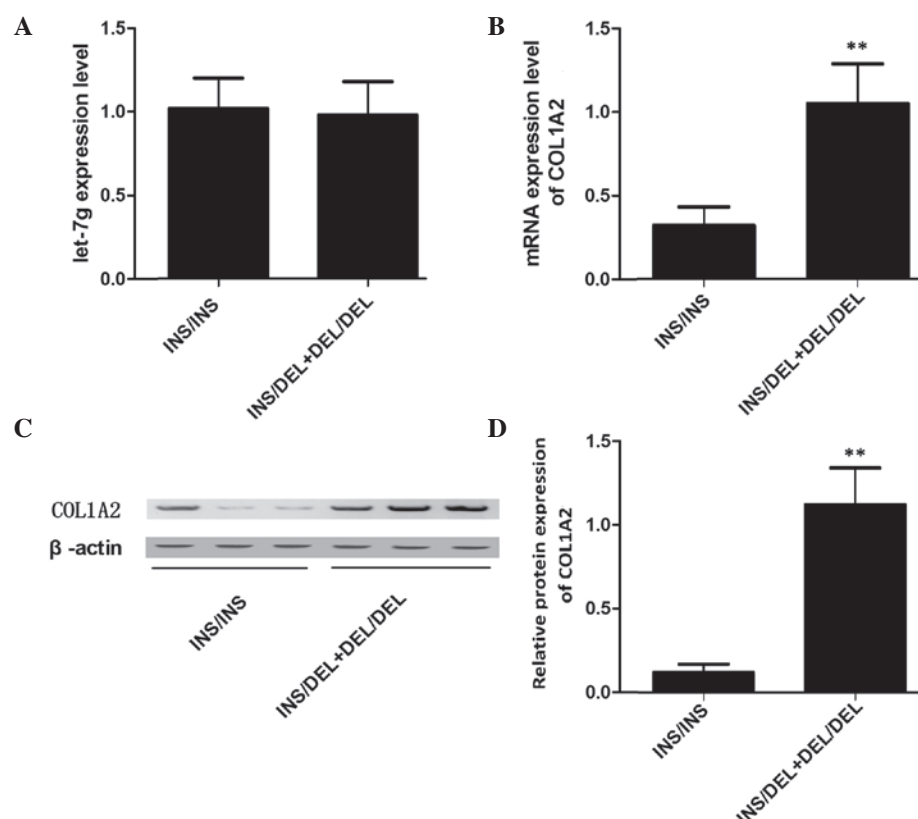


Figure 3. Expression levels of (A) let-7g and COL1A2 (B) mRNA and (C and D) protein. COL1A2, collagen type 1 α 2; UTR, untranslated region; INS, insertion; DEL, deletion.

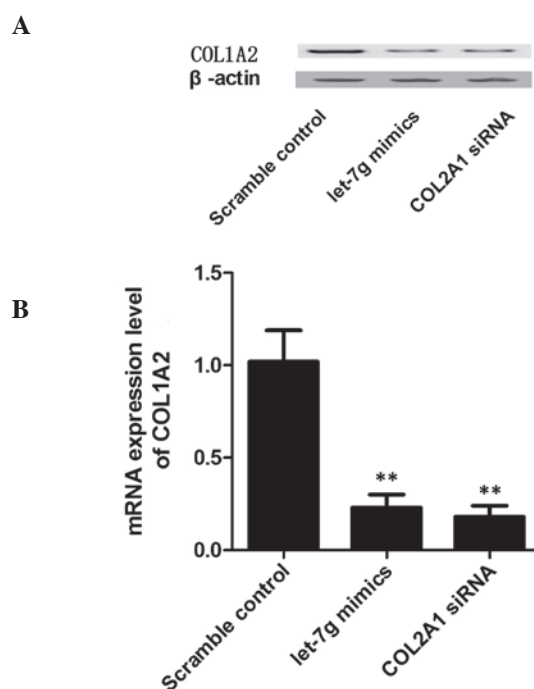


Figure 4. In primary osteoblast cells of the INS/INS genotype, the let-7g mimics and COL1A2 siRNA significantly reduced the (A) protein and (B) mRNA expression levels of COL1A2. INS, insertion; COL1A2, collagen type 1 α 2; siRNA, short interfering RNA.

gender, age and body mass index (BMI), was analyzed using covariance analysis. Student's t-test was used to compare between two groups and one-way analysis of variance was used to compare three groups. SPSS software, version 19.0 (IBM SPSS, Armonk, NY, USA) was used for the statistical analysis. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

BMD is significantly associated with the rs3917 genotype. A total of 487 subjects were recruited in the current study, and the age, gender and BMI stratified by the COL1A2 3'-UTR rs3917 genotypes are presented in Table I. There was no statistically significant difference in age, gender or BMI between the two genotype groups. When the BMD was compared between the three rs3917 genotype groups, the BMD was comparable between the INS/DEL and DEL/DEL groups (data not shown), therefore the two genotype groups were combined, and compared with the INS/INS genotype group. This indicated that the BMD was increased at the four tested sites, the femoral neck ($P = 0.018$), total left hip ($P = 0.028$), L1-L4 (0.025) and intertrochanteric ($P = 0.018$) areas, in the INS/DEL or DEL/DEL group compared with the INS/INS group, as presented in Table I. The statistically significant differences remained following adjustment for age, gender and BMI.

Statistical analysis. The χ^2 test was used to analyze the genotype distribution for the Hardy-Weinberg equilibrium. The correlation between the polymorphism and BMD, adjusted for

rs3917 genotypes are associated with the expression of COL1A2 by compromising its binding to let-7g in human osteoblast cells. To investigate how the rs3917 genotypes

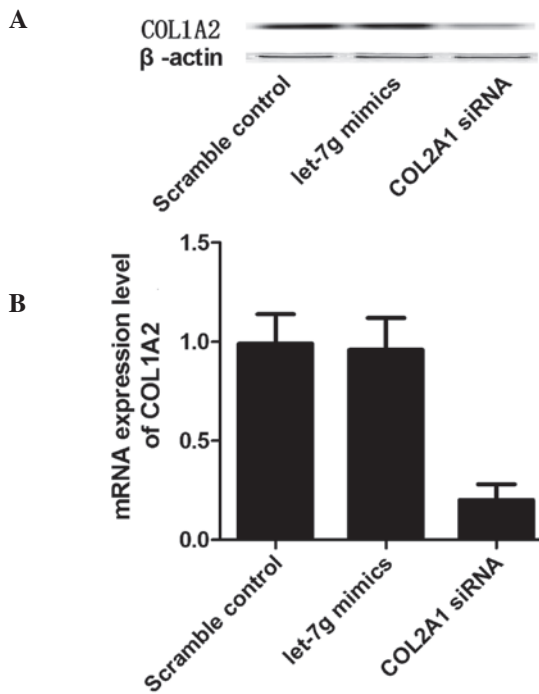


Figure 5. In primary osteoblast cells of the DEL/DEL genotype, COL1A2 siRNA was able to reduce the (A) protein and (B) mRNA expression levels of COL1A2, while the let-7g mimics had no effect on the expression levels of COL1A2. DEL, deletion; COL1A2, collagen type 1 α 2; siRNA, short interfering RNA.

affect the expression of COL1A2, the 3'-UTR of a *Renilla* luciferase reporter gene was replaced with the full-length COL1A2 3'-UTR containing either allele of rs3917 (Fig. 1). In transiently transfected human osteoblast cells, compared with the constructs containing the wild-type allele, the luciferase activity in the cells containing the deletion allele was significantly higher in the presence of let-7g (Fig. 2). This result indicates that the transcription of COL1A2 is negatively influenced by the presence of the rs3917 deletion allele, which is suggested to affect the binding of let-7g the COL1A2 transcript.

Association of rs3917 genotypes with the expression levels of COL1A2 in human primary osteoblasts. Three different genotypes of primary osteoblasts, INS/INS (32 samples), INS/DEL (12 samples) and DEL/DEL (4 samples), were used to further investigate the effect of rs3917 on the transcription of COL1A2. Using RT-qPCR, the expression levels of let-7g were observed to be similar between the INS/INS and the INS/DEL + DEL/DEL groups (Fig. 3A). In addition, the mRNA expression levels of COL1A2 were measured, and the expression levels of COL1A2 mRNA were observed to be reduced in the INS/INS group compared with the INS/DEL + DEL/DEL group (Fig. 3B). Consistent with this, western blot analysis indicated that the protein expression levels of COL1A2 were reduced in the INS/INS group compared with the INS/DEL + DEL/DEL group (Fig. 3C and D).

Exogenous expression of let-7g suppresses the expression of COL1A2 in primary osteoblasts with the INS/INS genotype,

however not the DEL/DEL genotype. Hsa-let-7g mimics and anti-COL1A2 siRNA together with scramble control were transfected into the osteoblast cells of the INS/INS or DEL/DEL genotypes, and the mRNA and protein expression levels of COL1A2 were measured in osteoblast cells. In the primary osteoblast cells of the INS/INS genotype, the let-7g mimics and the COL1A2 siRNA markedly reduced the mRNA and protein expression levels of COL1A2 (Fig. 4). In the primary osteoblast cells of the DEL/DEL genotype, whilst COL1A2 siRNA was able to reduce the mRNA and protein expression levels of COL1A2, the let-7g mimics had no effect on the expression levels of COL1A2 (Fig. 5).

Discussion

Osteoporosis is a common disease, characterized by micro-architectural deterioration of bone tissue, reduced bone mass and an increased risk of fragility fractures (1). Genetic factors have been observed to serve an important role in the pathogenesis of osteoporosis via multiple mechanisms associated with variation in several genes involved in the regulation of bone geometry, quality and mineral density (18). Following the identification of miRNAs around 20 years ago, numerous studies (>20,000) have been published on miRNAs (19). Certain studies (>600) have focused on the effects of miRNAs on bone and cartilage tissues. Numerous reviews have discussed the various roles of miRNA in skeletal development and disease (20-25), including regulatory roles in the growth, differentiation and function of osteoblasts, osteoclasts, chondrocytes and other mesenchymal cell types (for example, adipocytes and myoblasts) (26). The miRNA, let-7g, has been demonstrated to be associated with the regulation of ontogenesis (27). In the current study, the BMD at the four tested sites, the femoral neck, total left hip, L1-L4 and intertrochanteric areas, was reduced in the COL1A2 3'-UTR rs3917 INS/DEL or DEL/DEL group compared with the INS/INS (Table I). This statistically significant difference remained following adjustment for age, gender and BMI.

The COL1A2 gene encodes the pro- α 2 chain of type I collagen, whose triple helix consists of two α 1 chains and one α 2 chain. Type I collagen is a fibril-forming collagen, is present in the majority of connective tissues, and is highly expressed in dermis, bone, cornea and tendon tissue. A previous study demonstrated that COL1A2 is a direct target of let-7g in certain tumor cells (28). In addition, the levels of let-7g and COL1A2 were inversely associated with hepatocellular carcinoma clinical specimens (28). In the current study, the expression levels of let-7g were observed to be similar between all the three rs3917 genotype groups (Fig. 3A). In addition, the mRNA expression levels of COL1A2 were measured and observed to be similar between the INS/DEL and DEL/DEL groups, with the levels greater than in the INS/INS group (Fig. 3B). The correlation between single nucleotide polymorphisms (SNPs) in COL1A2 and BMD, and additional surrogate phenotypes (29-32) has been investigated in numerous studies to date. A previous study by Lindahl *et al* (30), conducted in older men from Sweden, Hong Kong and the United Kingdom (n=2004), identified an association between the COL1A2 gene and BMD. The study indicated that rs42524 was significantly associated

with BMD in older men, with those carrying a CC or GG genotype exhibiting a higher BMD compared with men with the CG genotype (30). However, it was demonstrated that the same SNP was not associated with BMD or the risk for osteoporotic fracture in postmenopausal women. As reported by Lau *et al* (29), the SNPs *PvuII* and *EcoRI* in the COL1A2 gene were associated with BMD in older men in Hong Kong, however, no association between these two SNPs and BMD was observed in 450 postmenopausal women. The study by Lei *et al* (31) investigated the association between *MspI* in the COL1A2 gene and osteoporosis according to bone size, and reported that this SNP was associated with femoral neck bone size in a Chinese population. The majority of studies regarding COL1A2 have focused on two nucleotide substitutions within the gene: G to C substitution at nucleotide 19713 in intron 24, which created a *PvuII* restriction site; and C to T substitution at nucleotide 14589 at the 5' end of intron 12, which created an *EcoRI* restriction site (GenBank sequence, accession no. AF004877). However, there is no evidence that the *EcoRI* and *PvuII* loci have any function (33). Therefore, it has been suggested that there may be additional regulatory mechanisms involved, including miRNA, methylation and linkage equilibrium. In the current study, the mRNA let-7g was observed to bind and negatively regulate the transcription of COL1A2, with this regulation negatively influenced by the presence of the rs3917 deletion allele. To further investigate the regulation of COL1A2 by let-7g, primary osteoblast cells of the INS/INS or DEL/DEL genotypes were transfected with let-7g mimics and COL1A2 siRNA. In the INS/INS genotype, let-7g mimics and anti-COL1A2 siRNA reduced the mRNA and protein expression of COL1A2 (Fig. 4). However, in the primary osteoblast cells of the DEL/DEL genotype, COL1A2 siRNA was able to reduce the mRNA and protein expression levels of COL1A2, while let-7g mimics had no effect on the expression levels of COL1A2 (Fig. 5).

The current study has limitations, as although a large cohort demonstrated statistically significant correlations, this is based on associations in an observational trial, with the correlation was evidenced supported by a preliminary functional analysis. Further *in vivo* experiments of this allele are required to support the results of the current study. In addition, the participants in the current study were selected from a single hospital, therefore selection bias may have occurred, and so further studies in a wider population are required to support the observations of the current study.

In conclusion, the results of the current study suggest that rs3917 is associated with BMD. In addition, the present study suggests that the rs3917 polymorphism may interfere with the interaction between let-7g and COL1A2, and that the presence of the minor allele releases the physiological inhibition of the target gene, which may represent a novel therapeutic or preventive target in osteoporosis.

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