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# Clinical and genetic factors associated with radiographic damage in patients with ankylosing spondylitis

Bora Nam ,[1,2] Sungsin Jo ,[2] So-Young Bang ,[1,2] Youngho Park,[3] Ji Hui Shin,[1] Ye-Soo Park,[4] Seunghun Lee,[5] Kyung Bin Joo,[2] Tae-Hwan Kim [1,2]

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[1]Department of Rheumatology, Hanyang University Hospital for Rheumatic Diseases, Seoul, South Korea
[2]Hanyang University Institute for Rheumatology Research (HYIRR), Seoul, South Korea
[3]Department of Big Data Application College of Smart Convergence, Hannam University, Daejeon, South Korea
[4]Department of Orthopaedic Surgery, Hanyang University College of Medicine, Guri Hospital, Guri, South Korea
[5]Department of Radiology, Hanyang University Hospital for Rheumatic Diseases, Seoul, South Korea

**Correspondence to**
Dr Tae-Hwan Kim, Department of Rheumatology, Hanyang University Hospital for Rheumatic Diseases, Seoul 04763, South Korea; thkim@hanyang.ac.kr

BN and SJ contributed equally.

BN and SJ are joint first authors.

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## ABSTRACT

**Objectives** To identify clinical and genetic factors associated with severe radiographic damage in patients with ankylosing spondylitis (AS).

**Methods** We newly generated genome-wide single nucleotide polymorphism data (833K) for 444 patients with AS. The severity of radiographic damage was assessed using the modified Stoke Ankylosing Spondylitis Spinal Score (mSASSS). To identify clinical and genetic factors associated with severe radiographic damage, multiple linear regression analyses were performed. Human AS-osteoprogenitor and control-osteoprogenitor cells were used for functional validation.

**Results** The significant clinical factors of final mSASSS were baseline mSASSS ($\beta$=0.796, p=3.22×10$^{-75}$), peripheral joint arthritis ($\beta$=−0.246, p=6.85×10$^{-6}$), uveitis ($\beta$=0.157, p=1.95×10$^{-3}$), and smoking ($\beta$=0.130, p=2.72×10$^{-2}$) after adjusting for sex, age and disease duration. After adjusting significant clinical factors, the *Ryanodine receptor 3* (*RYR3*) gene was associated with severe radiographic damage (p=1.00×10$^{-6}$). For pathway analysis, the PI3K-Akt signalling pathway was associated with severe radiographic damage in AS (p=2.21×10$^{-4}$, false discovery rate=0.040). Treatment with rhodamine B, a ligand of RYR3, dose-dependently induced matrix mineralisation of AS osteoprogenitors. However, the rhodamine B-induced accelerated matrix mineralisation was not definitive in control osteoprogenitors. Knockdown of RYR3 inhibited matrix mineralisation in SaOS2 cell lines.

**Conclusions** This study identified clinical and genetic factors that contributed to better understanding of the pathogenesis and biology associated with radiographic damage in AS.

### WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Ankylosing spondylitis (AS) is an inflammatory rheumatic disease characterised by progressive spinal damage.
⇒ The severity of spinal damage is highly variable among individuals and can be affected by both clinical and genetic factors.
⇒ Most genome-wide association studies for AS have only focused on disease susceptibility rather than radiographic damage.

### WHAT THIS STUDY ADDS

⇒ We identified the *RYR3* gene as a novel candidate gene for severe radiographic damage in AS.

### HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ Our results can provide new insights on the underlying pathogenesis of spinal damage in AS and open new avenues for better personalised medicine by identifying high-risk patients of severe structural damage.

## INTRODUCTION

Ankylosing spondylitis (AS) is a heritable inflammatory disease eventually leading to spinal fusion.[1] A genome-wide association study (GWAS) is a hypothesis-free approach to identify genetic variants underlying disease. GWAS has led to valuable insights into the complex genetic background of autoimmune disease.[2] Since the first GWAS for AS was published,[3] aminopeptidases such as *endoplasmic reticulum aminopeptidase (ERAP) 1* and *ERAP2*, and genes in the tumour necrosis factor and interleukin (IL)-23 pathways have been identified as AS-associated genetic variants through subsequent GWAS and Immunochip studies.[4 5]

However, most previous genetic studies for AS have only focused on disease susceptibility. Genetic studies targeting radiographic damage remain limited. Only a few candidate gene studies showed meaningful results, including *HLA-B*4100, DRB1*0804, DQA1*0401, DQB1*0603, DPB1*0202, ADRB1* and *NELL1*.[6 7] *LMP2* was associated with baseline damage, but not radiographic progression.[8] Previous study including 1537 AS cases identified two candidate single nucleotide polymorphisms (SNPs) (rs8092336 and rs1236913) which lies within RANK and PTGS1 (prostaglandin-endoperoxide synthase 1).[9] However, unfortunately, there is no GWAS-scaled study that can suggest candidate loci involved in radiographic severity in AS.

Therefore, we conducted GWAS to identify both clinical and genetic factors associated with radiographic damage in Korean patients with AS.

## METHODS

### GWAS participants

All AS cases who satisfied the 1984 modified New York criteria were recruited from Hanyang University Hospital for Rheumatic Diseases and all patients provided informed consent.[10] Since we focused on radiographic damage followed by long-term disease course, only patients having available at least two complete sets of spine radiographs and

**Table 1** Demographics of patients and clinical factors associated with radiographic damage in ankylosing spondylitis (AS)

| | Patients with AS (n=444) | Factors associated with final mSASSS*† | | |
|---|---|---|---|---|
| | | Estimate | SE | P value |
| Age at enrolment, years | 31.4 (25.5–37.2) | 0.007 | 0.003 | $4.13\times10^{-2}$ |
| Male sex | 401 (90.3) | 0.138 | 0.094 | 0.142 |
| Symptom duration at enrolment, years | 8.5 (4.0–14.0) | 0.010 | 0.004 | $1.14\times10^{-2}$ |
| Follow-up duration, years | 9.6 (7.9–11.3) | 0.038 | 0.009 | $2.14\times10^{-5}$ |
| HLA-B27 positivity | 427 (96.2) | 0.087 | 0.130 | 0.504 |
| Family history of AS | 30 (6.8) | 0.078 | 0.097 | 0.424 |
| Uveitis | 189 (42.6) | 0.157 | 0.050 | $1.95\times10^{-3}$ |
| Peripheral joint involvement | 199 (44.8) | −0.246 | 0.054 | $6.85\times10^{-6}$ |
| Inflammatory bowel disease | 4 (0.9) | 0.164 | 0.258 | 0.526 |
| Psoriasis | 15 (3.4) | −0.016 | 0.135 | 0.903 |
| Ever smoker† | 292 (66.1) | 0.130 | 0.059 | $2.72\times10^{-2}$ |
| Use of anti-TNF | 277 (62.4) | 0.056 | 0.053 | 0.287 |
| Use of NSAIDs | 439 (98.9) | 0.220 | 0.232 | 0.343 |
| Baseline mSASSS | 7.7 (5.5–16.8) | 0.796 | 0.035 | $3.22\times10^{-75}$ |

Results are expressed as median (IQR) and n (%).
*Log (mSASSS+1) value was used.
†Smoking data were available for 442 patients.
HLA, human leucocyte antigen; mSASSS, modified stoke ankylosing spondylitis spinal score; NSAIDs, non-steroidal anti-inflammatory drug; TNF, tumour necrosis factor.

at least a 5-year time interval between baseline and the last radiograph sets were enrolled. The severity of structural damage on radiography was assessed using the modified Stoke Ankylosing Spondylitis Spinal Score (mSASSS).[11]

We defined the index date as the first date of spine radiographs taken. All patients were observed until their last day of having complete set of spine radiographs or assessed as the highest possible mSASSS; 72. Radiographic progression rate was calculated as an increase in mSASSS units per year, and the final mSASSS was defined as the highest mSASSS during observational period.

## Gene data

Enrolled patients were genotyped with Korea Biobank Array (KoreanChip) which is optimised for the Korean population. The KoreanChip comprises>833 000 markers including>247 000 rare or functional variants, derived from sequencing data for over 2500 Koreans.[12] The genotyping data were filtered based on the quality control requirements that showed a high call rate (≥ 99%), no excessive heterozygosity, no cryptic first-degree relatives and sex consistency. SNPs were removed based on the following criteria: Hardy-Weinberg equilibrium$<10^{-6}$, genotype call rates<95% and minor allele frequency<0.5%.

**Table 2** The top 10 SNPs associated with higher final mSASSS score (n=442*†)

| SNP | Chr:Position | Beta | Gene | Function | P value |
|---|---|---|---|---|---|
| rs191573523 | 15:33 922 208 | 1.127 | RYR3 | Exonic | $1.00\times10^{-6}$ |
| rs7518201 | 1:203 773 096 | −0.149 | ZC3H11A | Intronic | $1.00\times10^{-5}$ |
| rs76789191 | 3:55 614 320 | 0.580 | ERC2 | Intronic | $1.21\times10^{-5}$ |
| rs117416665 | 2:44 606 325 | 0.611 | CAMKMT | Intronic | $2.36\times10^{-5}$ |
| rs139358058 | 15:49 888 627 | 0.976 | FAM227B | Intronic | $2.78\times10^{-5}$ |
| rs7502336 | 17:78 281 770 | −0.160 | RNF213 | Intronic | $2.78\times10^{-5}$ |
| rs144589250 | 9:140 218 962 | 0.438 | EXD3 | Intronic | $3.17\times10^{-5}$ |
| rs16915331 | 9:101 135 201 | 0.897 | GABBR2 | Intronic | $3.28\times10^{-5}$ |
| rs11686946 | 2:128 394 955 | 0.182 | MYO7B | Exonic | $4.08\times10^{-5}$ |
| rs76838622 | 1:183 839 856 | 0.727 | RGL1 | Intronic | $4.36\times10^{-5}$ |

*Smoking data were available for 442 patients.
†Log(mSASSS+1) value was used.
Chr, chromosome; mSASSS, modified Stoke Ankylosing Spondylitis Spinal Score; SNP, single nucleotide polymorphism.

## Statistical analysis

To identify clinical factors contributing to a higher final mSASSS, multiple linear regression analyses were performed. The analyses were performed using the SAS V.9.2 statistical software (SAS Institute). P<0.05 was considered statistically significant. To identify genetic factors, multiple linear regression analyses were performed after adjusting for clinical factors. $p<5.00\times10^{-8}$ and $p<1.00\times10^{-5}$ were considered as the genome-wide significance threshold and suggestive level of association, respectively.

A nominal p<0.01 was used to filter SNPs from the GWAS analysis for pathway analysis after excluding intergenic genes. We used the DAVID online method based on the KEGG reference database (p<0.05; false discovery rate (FDR) < 0.05).

## Functional validation

### Isolation of osteoprogenitor cells and differentiation

Human spine specimens were collected from 11 male patients who underwent spinal surgery. Seven of the patients satisfied the 1984 modified New York criteria for the classification of AS (median age 45.1 (28–61) years).[10] Four patients without inflammatory disease were enrolled as control group (median age 65.5 (56–73) years). Osteoprogenitors were isolated using an outgrowth method and stimulated to induce osteoblast differentiation, as described previously.[13]

### Assessment of osteoblast differentiation

To investigate the impact of rhodamine B on osteoblast differentiation, AS-osteoprogenitor cells and control-osteoprogenitor cells were treated with vehicle or rhodamine B solution (Sigma, 02558) at the indicated dose (1 and 2 μM/mL) during osteoblast differentiation. For the assessment of the matrix maturation phase, alkaline phosphates (ALP) and collagen deposition were assessed while Alizarin red staining (ARS), von Kossa (VON) and hydroxyapatite (HA) staining were used for the matrix mineralisation.

### Knockdown of the RYR3 gene

Small interfering RNA (siRNA) against human *RYR3* (siRYR3) or control (siCON) were synthesised by Genolution (Seoul, South Korea). The siRYR3 or siCON was transduced into SaOS2 cells for 48 hours using lipofectamine 3000 (Invitrogen, L3000008). Gene knockdown efficiency of transduced cells was analysed by reverse transcription-PCR (RT-PCR), immunoblotting and immunofluorescence. The siRNA sequences are shown as follows: RYR3 #1 sense, CCUUCAUCUCUCAGUAUCAUU and RYR3 #1 antisense UGAUACUGAGAGAUGAAGGUU; RYR3 #2 sense, CAUCUACAGACCAGAAUGAUU and RYR3 #2 antisense UCAUUCUGGUCUGUAGAUGUU; RYR3 #3 sense, GCAUUGACCGCUUAAAUGUUU and RYR3 #3 antisense ACAUUUAAGCGGUCAAUGCUU; RYR3 #4 sense, GGUA CUUCGAGCUGAUUAUUU and RYR3 #4 antisense AUAA UCAGCUCGAAGUACCUU; RYR3 #5 sense, CCUUCGAU CUGGUUUCUAUUU and RYR3 #5 antisense AUAGAAAC CAGAUCGAAGGUU; Control sense, CCUCGUGCCGUU CCAUCAGGUAGUU and Control antisense, CUACCUGA UGGAACGGCACGAGGUU.

**Table 3** Pathway analysis using DAVID

| KEGG pathway | Count / Genes | P value | Benjamini | FDR |
|---|---|---|---|---|
| PI3K-Akt signalling pathway (hsa04151) | 52 | $2.21\times10^{-4}$ | 0.042 | 0.040 |
| | *FLT1, LAMC3, TNC, LAMC2, FGF1, FGF2, IFNA8, IGF1R, GHR, TNN, CREB3L2, TNR, JAK2, MAGI1, PDGFRB, CHUK, HGF, MAGI2, PRKCA, PRLR, CCNE2, COL4A1, ITGA8, COL6A3, SGK2, IFNAR1, LAMA1, LAMA4, LAMA3, PDGFB, LPAR1, FOXO3, EGFR, GNG2, RXRA, ERBB4, PPP2R3C, GNG7, PDGFC, ANGPT2, PTK2, CDK6, PPP2R2C, IL7, CDK4, ITGA11, RPS6KB2, BCL2, GNB3, PKN1, TEK, FGFR2* | | | |

Akt, α serine/threonine-protein kinase; FDR, false discovery rate; KEGG, kyoto encyclopaedia of genes and genomes; PI3K, phosphoinositide 3-kinase.

### Reverse transcription-PCR

Total RNA of transduced SaOS2 cells was extracted by TRIzol (Invitrogen, 10296028) and complementary DNA (cDNA) was amplified to perform RT-PCR following the manufacturer's protocol.[14] The following specific primers were used for RT-PCR: GAPDH, forward 5'- GTCAGTGGTGGACCTGACCT-3' and reverse 5'-AGGGGTCTACATGGCAACTG-3'; *RYR3*, forward 5'- GGACTTGGGAATCGCCTGTG-3' and reverse 5'-GCTC TGACAGATAGGGACTGTTC-3'.

### Immunoblotting and immunofluorescence

Transduced SaOS2 cells were carried out immunoblotting and immunofluorescence as described.[14] Anti-RYR3 (Invitrogen, PA5-77718) and anti-vinculin (Abclonal, A2752) were used. Immunoblotting and immunofluorescent images were visualised by the Uvitech System (Cambridge, UK) and Leica confocal microscopy (Wetzlar, Germany), respectively.

## RESULTS

### Patient characteristics

As shown in table 1, a total of 444 AS cases (male 90.3%) were included. The median patient age and symptom duration at enrolment were 31.4 (25.5–37.2) and 8.5 (4.0–14.0) years, respectively. Baseline mSASSS was 7.7 (5.5–16.8). Patients were observed for 9.6 (7.9–11.3) years. Within this period, mSASSS increased to 14.0 (7.0–36.8). The median age of patients was 40.8 (35.9–47.3) years when they have highest mSASSS during observational period. The median mSASSS progression rate was 0.43 (0.03–1.35) unit/year after excluding 8 patients having possible highest mSASSS; 72 at enrolment.

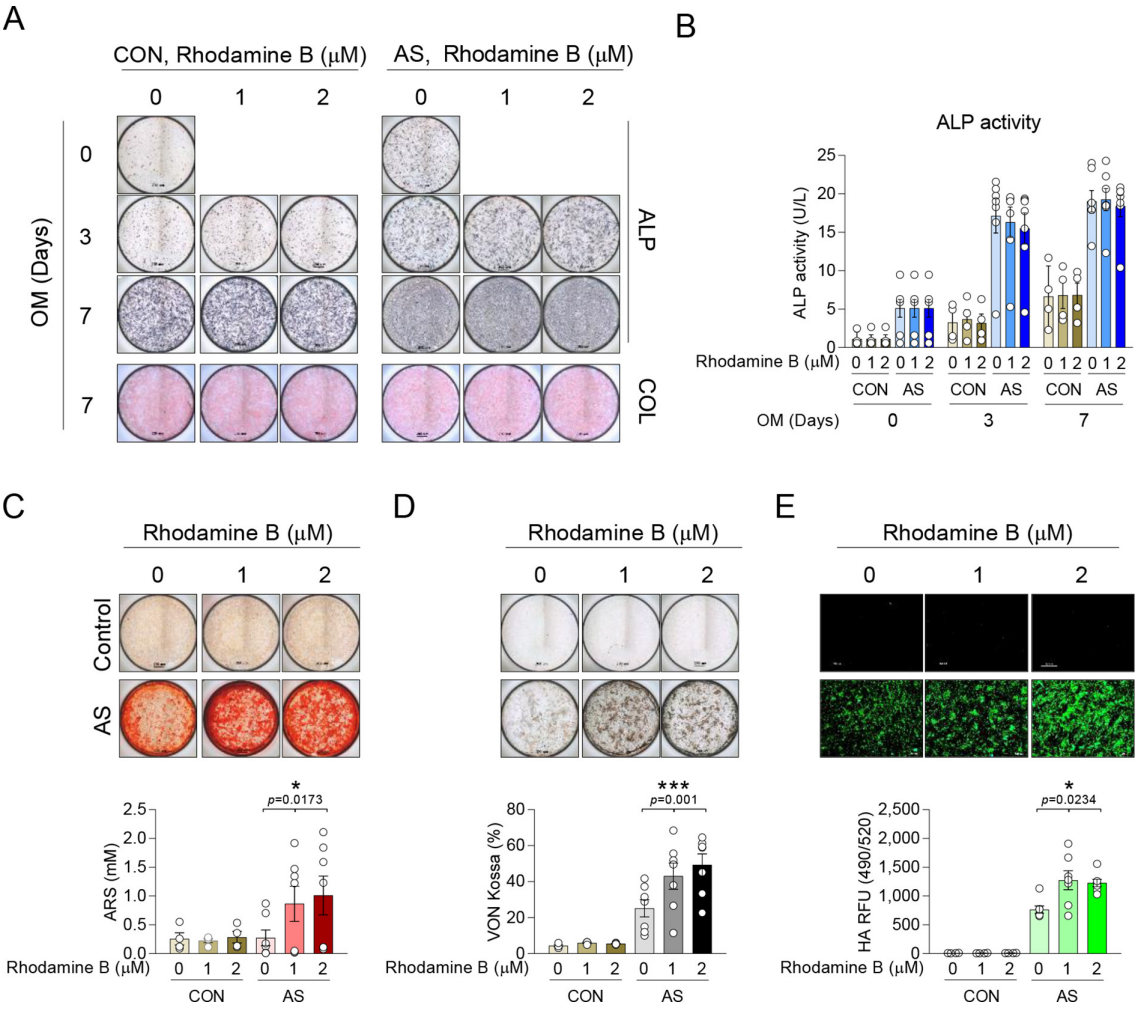


**Figure 1** Rhodamine B, a ligand of RYR3, promotes the matrix mineralisation in AS osteoprogenitors. Control (CON) and AS osteoprogenitors were differentiated into osteoblasts and continually stimulated by vehicle or rhodamine B with indicated dose during osteoblast differentiation. Osteogenic differentiation activity was assessed by (A) alkaline phosphates (ALP) and collagen (COL) staining, (B) ALP activity of (A), (C) Alizarin red staining (ARS) and ARS quantification, (D) von Kossa staining (VON) and mineralisation area (%), (E) hydroxyapatite (HA) staining and HA quantification.

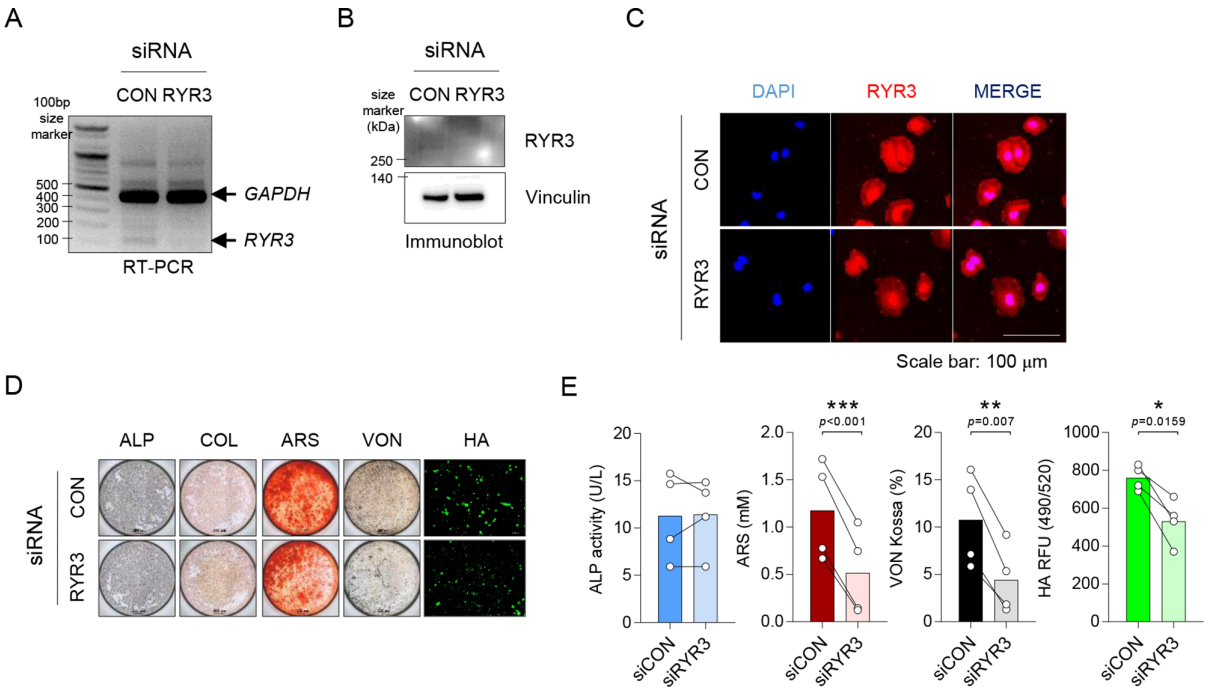


**Figure 2** RYR3 knockdown inhibits matrix mineralisation in SaOS2 cells. SaOS2 cells were transfected with siRNA against human RYR3 and control (CON), incubated for 48 hours, and differentiated for day 7. The stimulated cells were subjected to (A) RT-PCR, (B) immunoblotting, and (C) immunofluorescence. (D) Osteogenic differentiation activity was assessed by alkaline phosphates (ALP), collagen (COL), alizarin red staining (ARS), von Kossa staining (VON), and hydroxyapatite (HA). (E) Quantification data of figure D (n=4). Representative images are shown.

### Clinical and genetic factors associated with severe radiographic damage in patients with AS

The most influential clinical factor of final mSASSS was a higher baseline mSASSS (β=0.796, $p=3.22\times10^{-75}$). Older age at enrolment, longer symptom duration and follow-up duration, uveitis and smoking were also related (β=0.007, $p=4.13\times10^{-2}$; β=0.010, $p=1.14\times10^{-2}$; β=0.038, $p=2.14\times10^{-5}$; β=0.157, $p=1.95\times10^{-3}$, β=0.130, $p=2.72\times10^{-2}$, respectively). Peripheral joint involvement was associated with less severe radiographic damage (β=−0.246, $p=6.85\times10^{-6}$) (table 1).

After adjusting for clinical factors, we identified two novel loci reaching the suggestive level of association: one exonic SNP of *Ryanodine receptor 3* (*RYR3*) (rs191573523) (β=1.127, $p=1.00\times10^{-6}$) and intronic SNP of *ZC3H11A* (rs7518201) (β=−0.149, $p=1.00\times10^{-5}$). The top 10 SNPs associated with severe radiographic damage are available in table 2.

Pathway analyses highlighted the phosphoinositide 3-kinase (PI3K)-RAC-α serine/threonine-protein kinase (Akt) signalling pathway ($p=2.21\times10^{-4}$, FDR=0.040) (table 3).

### Rhodamine B, a ligand of RYR3, promoted the matrix mineralisation in AS osteoprogenitors

For functional validation, human control osteoprogenitors and AS osteoprogenitors were induced to mature osteoblasts treated with vehicle or rhodamine B (figure 1). Collagen staining and ALP staining and activity were not affected by rhodamine B in both control and AS osteoprogenitors. And the ALP activity of AS osteoprogenitors was higher than that of control osteoprogenitors (figure 1A,B). However, we observed dose-dependent increases in intensity of ARS and VON stain, as well as ARS concentration and mineralisation area in human AS osteoprogenitors (figure 1C,D). Treatment of rhodamine B also increased HA in AS osteoprogenitors (figure 1E). Collectively, rhodamine B increased matrix mineralisation, but not matrix maturation during osteoblast differentiation in AS osteoprogenitors. However, the rhodamine B-induced accelerated matrix mineralisation was not definitive in control osteoprogenitors (figure 1C–E).

### Knockdown of RYR3 inhibits matrix mineralisation

We established RYR3 knockdown in the SaOS2 cell line (figure 2). Knockdown of RYR3 was confirmed by RT-PCR, immunoblotting and immunofluorescence (figure 2A–C). RYR3 knockdown cells and control SaOS2 cells were stimulated to induce osteoblast differentiation. There was no significant difference in the intensity of collagen and ALP staining and ALP activity between RYR3 knockdown cells and control cells. However, the intensity of ARS and VON stain and HA were significantly decreased in RYR3 knockdown cells (figure 2D,E). These results suggest that the RYR3 knockdown has no effect on matrix maturation but inhibits matrix mineralisation during osteoblast differentiation.

## DISCUSSION

The present study identified both clinical and genetic factors associated with severe radiographic damage in AS. The clinical factors were largely comparable with those of previous studies, for example, baseline mSASSS, uveitis, smoking and peripheral joint involvement.[8 15–19] HLA-B27, the most important genetic risk factor for AS but repeatedly shown not be linked with radiographic damage,[9 20] is not related to radiographic severity in this study. The most highlighted part of our study is the *RYR3* gene extracted from GWAS data as a novel candidate gene for severe radiographic damage in AS. Further, rhodamine B (a ligand of RYR3)-induced matrix mineralisation was confirmed using human AS-osteoprogenitor cells. And knockdown of RYR3 inhibits matrix mineralisation in SaOS2 cell lines. In addition, PI3K-Akt signalling pathway was identified as candidate pathway of severe radiographic damage in AS.

RYR3 encoded by the *RYR3* gene is a calcium release channel protein and regulates intracellular calcium homeostasis. The

Genotype Tissue Expression project from 17 382 samples and, 948 donors showed that *RYR3* gene is mainly expressed in musculoskeletal tissues (online supplemental figure 1). Although no previous study has investigated the relationship between RYR3 and AS, there have been several studies that may support our results, including RYR3 is related to calcification in patients with breast cancer and fibro-calcific aortic valve disease.[21 22] Moreover, transforming growth factor-beta (TGFβ) reduces RYR3 and inhibits matrix mineralisation in osteoblast differentiation.[23 24] Considering that TGFβ plays a role in inflammation and AS acting on the formation/repair of cartilage and bone, which are the major targets of AS,[25] our results might provide a clue to elucidate the underlying pathogenesis of radiographic damage in AS.

The PI3K-Akt signalling pathway, an intracellular signal transduction pathway involved in cell cycle and growth, is one of five significant signalling pathways in patients with AS compared with controls.[26] IL-8 and IL-37, which are closely related to AS, promote osteogenic differentiation via the PI3K-Akt signalling pathway.[27 28] Moreover, the PI3K-Akt signalling pathway was reported to promote inflammation and fibroblastic ossification in AS and has many downstream effects including activating NF-κB.[13 29] However, further study is required to identify the particular stage related to AS pathogenesis since this pathway participate in numerous biological processes.

The present study has a few limitations. First, the study population was relatively small and our study remains cross sectional focusing final mSASSS during observation period. Further longitudinal study using a large multiethnic cohort with longer follow-up period is needed since that the radiographic damage in very elderly patients might be fully reflect genetic factors. Second, the underlying mechanism of rhodamine B-induced accelerated matrix mineralisation and the link between inflammation and RYR3 are not elucidated, which require further investigation. Lastly, though the functional study was conducted using human control-osteoprogenitor and AS-osteoprogenitor cells and RYR3 knockdown SaOS2 cells. And the results can suggest that RYR3 may play a role in spine ankylosis in AS. However, whether they have relevant *RYR3* SNP and its impact on RYR3 protein are not confirmed in AS. We are planning further experiments on RYR3 knockout cell line using clustered regular interspaced short palindromic repeats for more confirmative functional study.

To conclude, the radiographic damage in AS is affected by both clinical and genetic factors. We discovered *RYR3* as a novel candidate gene for severe radiographic progression in AS. Future effort should look closely into RYR3 and RYR3-related mechanism on bone mineralisation to solve pathogenesis of spinal ankylosis in AS. Our results may pave a way for better personalised medicine by identifying patients at high risk of severe structural damage, but also open up new opportunities for drug development for radiographic damage in AS.

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**Contributors** BN, SJ, S-YB and T-HK contributed to the study conception and design. BN and JHS assisted in identification and characterisation of patients. SL and KBJ performed the blinded reading of the radiographs. YP performed the statistical analyses. Y-SP contributed to collect human bone specimens. SJ is responsible for the experiments. BN and SJ drafted the manuscript. S-YB and T-HK reviewed and edited original draft. And all authors revised the paper and approved the final version of the manuscript. T-HK is responsible for the overall content as the guarantor.

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**Competing interests** None declared.

**Patient and public involvement** Patients and/or the public were not involved in the design, or conduct, or reporting, or dissemination plans of this research.

**Patient consent for publication** Consent obtained directly from patient(s)

**Ethics approval** This study was carried out in accordance with the Declaration of Helsinki and approved by the Institutional Review Board (IRB) of Hanyang University Hospital (IRB file No. 2014-05-002 and 2019-09-013). Participants gave informed consent to participate in the study before taking part.

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**Data availability statement** Data are available upon reasonable request.



**ORCID iDs**
Bora Nam http://orcid.org/0000-0003-0215-3855
Sungsin Jo http://orcid.org/0000-0003-3034-5029
So-Young Bang http://orcid.org/0000-0003-4699-9350
Tae-Hwan Kim http://orcid.org/0000-0002-3542-2276

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