ABERRANT INCREASE IN ACTIVE TGFβ1 IS ASSOCIATED WITH OSTEOARTHRITIS PROGRESSSION IN HUMAN TIBIAL SUBCHONDRAL BONE

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ABSTRACT

Osteoarthritis is a highly prevalent joint disorder with no effective disease-modifying treatment. Animal studies demonstrate that increased TGFβ1 activation in the subchondral bone induces osteoarthritis, whereas inhibition of TGFβ1 prevents disease progression. However, the understanding of this mechanism in human subchondral bone is still limited. This study aims to investigate the role of TGFβ1 in human osteoarthritic subchondral bone at the tissue, cellular, and molecular level. Tibial plateaus were collected from 35 osteoarthritic patients undergoing total knee replacement surgery (15 males, aged 68 ± 10 years; and 20 females, aged 71 ± 8 years). Subchondral bone tissue was sampled from regions with less and more degenerated cartilage (mild and severe groups). Tissue sections were stained with Safranin O Fast Green and graded using OARSI grading system. They were also stained with Haematoxylin and Eosin to quantify osteocyte and vascular density, and with Tartrate-Resistant Acid Phosphatase for $TRAP^+$ cells density. ELISA was then performed to determine TGFβ1 concentrations. Additionally, TGFβ1 and bone marker gene expressions were determined by RT-PCR technique. A significant increase in the OARSI score ($p < 0.0001$), osteocytes ($p = 0.012$), vascular ($p = 0.003$), $TRAP^+$ osteoclasts (($p = 0.002$) and total $TRAP^+$ cells (($p = 0.006$) density were observed in this study. The concentration of active TGFβ1 and RANK/OPG mRNA expression were also increased in the severe group ($p = 0.031$ and $p < 0.0001$, respectively). Furthermore, OARSI scores revealed positive correlations with active/total TGFβ1 (r = 0.34, $p = 0.006$), total $TRAP^+$ cell (r = 0.34, $p = 0.005$), $TRAP^+$ osteoclast cell (r = 0.30, $p = 0.014$), osteocyte (r = 0.29, $p = 0.018$), and vascular (r = 0.36, $p = 0.003$) density. Together, this study demonstrates increased in TGFβ1 activation is associated with changes seen in the human osteoarthritic subchondral bone as disease progressed.

(300 words)

INTRODUCTION

Osteoarthritis is a highly prevalent joint disorder that affects the entire structure of the joint, including articular cartilage and subchondral bone[1, 2]. It presents with pain, stiffness and structural deformation of the joint making it a leading cause of disability among elderly worldwide[3, 4]. Despite the known risk factors for osteoarthritis, such as genetics, aging, obesity and joint injury[4], the underlying mechanism behind the development of osteoarthritis remains unknown, as a result, no effective disease-modifying treatment currently available for osteoarthritis.

In recent years, increasing number of studies suggest that transforming growth factor (TGF)β1 is implicated in the pathogenesis of osteoarthritis[5, 6]. TGFβ is a cytokine that belongs to the TGF superfamily. TGFβ exist in three different isoforms: TGFβ1, TGFβ2 and TGFβ3[7]. Amongst them, TGFβ1 is the most abundant growth factor in the bone[8]. TGFβs mainly stored as a latent complex in the extracellular matrix[9]. Released of TGFβ1 from the bone matrix during osteoclastic activity transduces signal through type I and II receptor serine/threonine kinase and subsequently activating the phosphorylation of SMAD2/3 pathway[10, 11]. TGFβ signalling regulates cellular processes, including proliferation, differentiation, migration and apoptosis, as well as production and degradation of extracellular matrix[12, 13]. In healthy cartilage, TGFβ/SMAD2/3 signalling pathway is responsible for the maintenance of articular cartilage homeostasis. TGFβ signalling stimulates chondrogenesis and inhibits chondrocytes terminal differentiation[14]. However, inhibition of TGFβ signalling in a SMAD3 knockout mice model demonstrates a chondrocyte hypertrophic, thereby leading to the degeneration of cartilage[14, 15].

TGFβ1 also has a critical role in maintaining homeostasis of the bone. During subchondral bone remodelling, TGFβ1 acts as a coupling factor for bone resorption and formation[16]. An osteoclast precursor receptor activator of nuclear factor-kB ligand (RANKL) expressed by preosteoblast cells bind to its receptor, RANK, on the surface of osteoclast cells[17]. The interaction of RANKL/RANK enhances differentiation of osteoclast precursor and activation of mature osteoclast to dissolve bone mineral and degrade bone matrix[18]. Soluble receptor osteoprotegerin (OPG), which is also secreted by osteoblast cells, can block the action of RANKL by binding with RANK to suppress osteoclastic bone resorption and stimulate bone formation[17]. The expression of RANKL and OPG is regulated by TGFβ1[18, 19]. Therefore, interruption in TGFβ1 signalling causes an imbalance in the RANKL/OPG ratio leading to abnormal bone metabolism and damaged subchondral bone[20].

Following injury in an acute cruciate ligament transection (ACLT) murine model, an increase in subchondral bone resorption is associated with increase active TGFβ1 expression in that region[21]. Elevated TGFβ1 activity through SMAD2/3 pathway stimulates the formation of bone marrow lesion as a sign of early osteoarthritis[21.] Bone cells exposed to a high level of active TGFβ1 induce clustering of nestin^{+} MSCs, which leads to the formation of bone marrow osteoid islets and accompanied by increase angiogenesis[21]. The expression of osterix^{+} osteoprogenitor, a marker for osteoprogenitor cells, also increases in the subchondral bone suggesting that nestin^{+} MSCs differentiate into osteoblast cells for the development of new bone cells[21]. These findings were consistent with recent studies in animal models of spontaneous osteoarthritis and Camurati-Engelmann disease (CED)[22, 23]. Furthermore, overexpression of active TGFβ1 in leads to the development of subchondral bone sclerosis and degeneration of cartilage in a transgenic mice[21]. However, inhibition of TGFβ1 activity using an inhibitor reduced uncoupled bone remodelling and inhibited aberrant angiogenesis in the

subchondral bone, which results in improved quality of the bone[21]. Therefore, TGFβ1 may potentially emerge as a novel therapeutic target for osteoarthritis through inhibition.

The role of TGFβ1 has been well established in animal models. However, due to the limitation in tissues samples obtained from human osteoarthritis are exclusively at the late stage of the disease makes the understanding of the mechanism and prediction of disease development a challenging task. Therefore, it is difficult to distinguish whether the role of TGFβ1 observed in the preclinical animal model of osteoarthritis is translatable to humans in order to find an effective treatment for human osteoarthritis. This study proposes, for the first time, the use of paired bone samples from mild and severe osteoarthritis regions of the same joint.

The hypothesis of this study is increased activity of TGFβ1 in the subchondral bone is associated with the progression of osteoarthritis. To investigate this, the expression and activation of TGFβ1 were measured in the mild and severe stages of osteoarthritis. Changes in the tissue, cellular and gene expression involved in bone remodelling at the different stage of disease were also investigated.

MATERIALS AND METHODS

Human bone samples

With the approval from the Royal Adelaide Hospital and The University of Adelaide Human Research Ethics Committees, fresh tibial plateau samples were obtained from 35 individuals presenting with late stage of osteoarthritis that were undergoing total knee replacement surgery. Written informed consent was obtained from patients. In the medial tibia compartment, subchondral bone tissues were extracted from the underlying regions of less and more

degenerated cartilage (Fig. 1). These tissues were processed for ELISA, real-time PCR and histological examination. The characteristics of patients are indicated in Table 1.

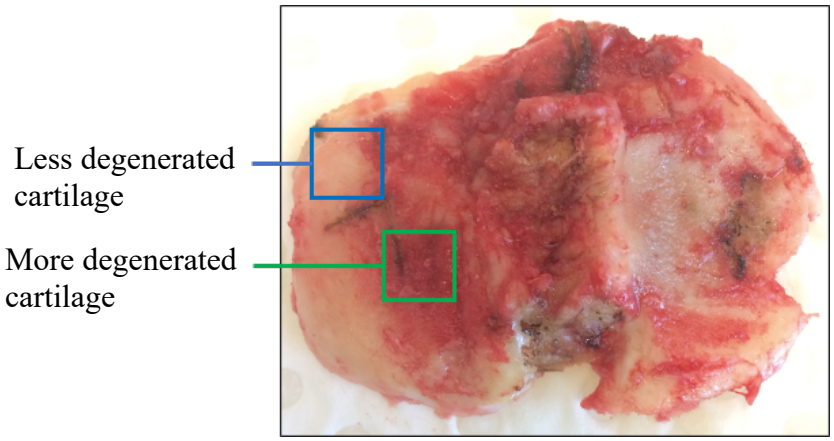


Figure 1 Gross image of a female osteoarthritic tibial plateau, age 67 years, showing regions with less and more degenerated cartilage in the medial compartment.

Table 1

Basic characteristics of patients

	Male	Female	*p*-value
Sample size (n)	15	20	
Age (years)	68 ± 10	71 ± 8	0.402

Values presented as mean ± standard deviation.

Histological staining and histomorphometry

For histological evaluation, regions of the medial tibia with less and more degenerated cartilage were sectioned with a low-speed diamond wheel saw (Model 660, South Bay Technology) to produce 10cm by 10cm by 10cm cuboidal cartilage-bone tissue blocks. Blocks of tissue were fixed with 10% neutral buffered formalin for 4 days. Then the samples were decalcified with

10% EDTA for 5 to 11 weeks. After radiologic confirmation of bone decalcification, the samples were processed and embedded in paraffin, and 5μm of sections were cut using a microtome (Leica RM2235, Leica Biosystems). The sections were stained with Safranin O Fast Green, Haematoxylin and Eosin (H&E) and Tartrate-Resistant Acid Phosphatase (TRAP). All stained slide sections were scanned using the Nanozoomer Digital Pathology slide scanner (C13239-01, Hamamatsu Photonics, Japan) and viewed using the NanoZoomer Digital Pathology system (U12388-01, Hamamatsu Photonics, Japan).

Safranin O Fast Green stain and osteoarthritis scoring

Sections were hydrated with distilled water 3 times for 1 minute each, stained with fast green solution for 20 minutes, and differentiated with 2 quick dipped into 1% acetic acid. After that, the sections were stained with safranin O solution for 5 minutes, rehydrated with ethanol in increasing concentrations, 95% and 100%, and dehydrated with xylene twice.

The stained sections were then used to assess the severity of degenerated cartilage using the Osteoarthritis Research Society International (OARSI) grading system by two assessors[24]. The OARSI grades ranged from 1 to 6.5, where 1 indicates intact cartilage and 6.5 indicates a total loss of cartilage with affected underlying subchondral bone (Fig. 2).

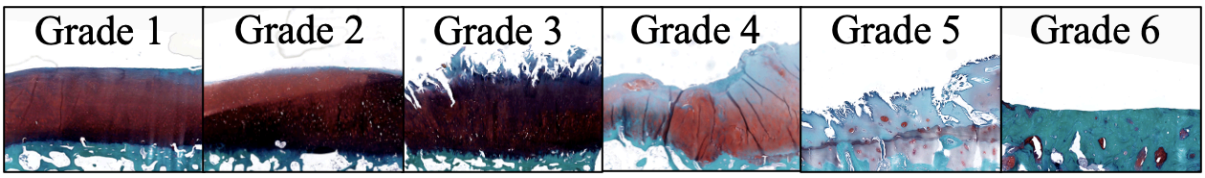


Figure 2 Representative images of OARSI histological grades observed in human tibial osteoarthritis samples from grade 1 to 6.

Haematoxylin and eosin (H&E) stain and histoquantitative analaysis for osteocyte lacunar and vascular density

Sections were stained with haematoxylin for 10 minutes and washed with acetate buffer for 5 seconds. Then they were dipped into 0.1% acid alcohol for 30 seconds and stained with eosin for 5 minutes. After that, the sections underwent the rehydration step with ethanol in increasing concentrations, 95% and 100% and dehydrated with xylene twice.

The H&E sections were used to quantify osteocyte lacunar density in the trabecular bone and vascular density in the subchondral bone marrow. In osteocyte lacunar density quantification, three regions of interest within the trabecular bone surface from each section were captured using NanoZoomer under 20x magnification (Fig. 3A). The area of the trabecular bone surface and the number of osteocytes occupied and empty lacunae were calculated using ImageJ software. The average values were obtained from each specimen. Occupied lacunae were defined as the presence of osteocyte nuclei in the lacunae (Fig. 3B). The density of osteocyte occupied and empty lacunae were expressed as the number of lacunae per unit area (mm^2) of tissue.

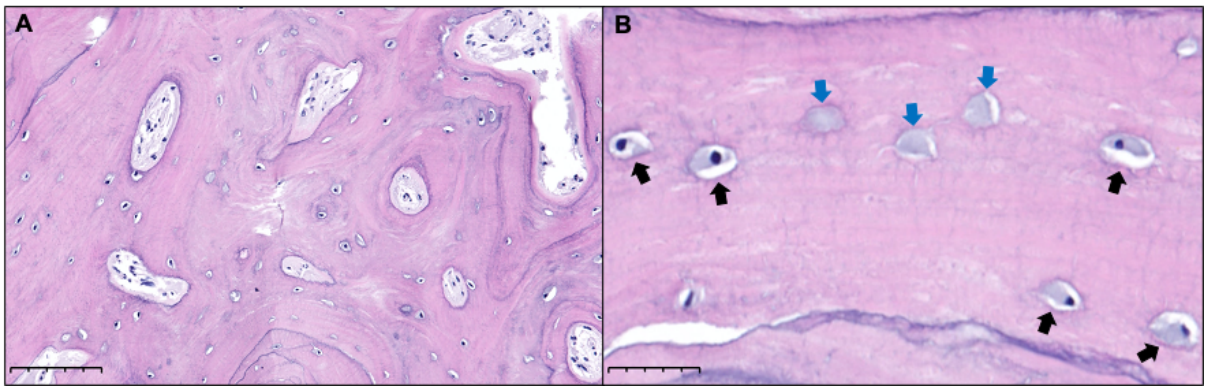


Figure 3 H&E staining of osteocyte lacunar in the trabecular bone. (A) A region of interest of osteocyte lacunar at 20x magnification. Scale bar: 100µm; (B) Osteocyte lacunar at 80x

magnification. Black arrows indicate osteocyte occupied lacunae and the blue arrows indicate empty lacunae. Scale bar: 25μm.

In vascular density quantification, image of the sections was captured using NanoZoomer at 1x magnification to enable measurement of the whole subchondral bone area. The number of thick-walled blood vessels present in the subchondral bone marrow was counted using NanoZoomer (Fig. 4A and 4B), and the area of the bone was measured using ImageJ. The vascular density was expressed as the number of blood vessels per unit area (mm^2) of tissue.

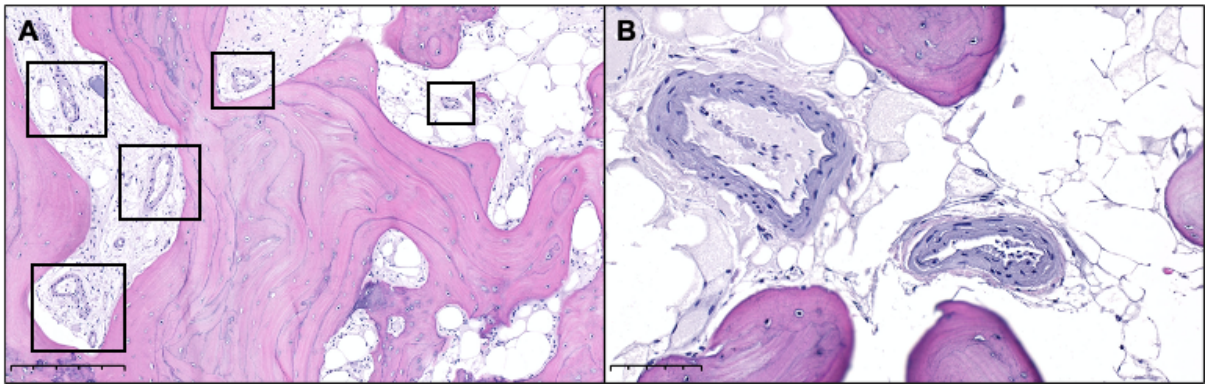


Figure 4 H&E staining of blood vessels in the subchondral bone marrow. (A) Blood vessels at 10x magnification indicated by black squares. Scale bar: 250μm; (B) Blood vessels at 20x magnification. Scale bar: 100μm.

Tartrate-Resistant Acid Phosphatase (TRAP) stain and $TRAP^+$ cell quantification

Sections were hydrated with distilled water for 2 minutes, incubated in HCL solution for 1 hour at 37°C, and washed with distilled water for 2 minutes. The sections were then stained in freshly prepared acid phosphatase solution for 1 hour at 37°C and washed with distilled water for 1 minute. After that, the sections were dipped 4 times in haematoxylin stain and washed again with distilled water for 1 minute. Then the sections were dipped 4 times in lithium carbonate stain, and washed with distilled water for 1 minute. The sections were then

rehydrated with ethanol in increasing concentrations, 95% and 100% and dehydrated with xylene twice.

The stained sections were imaged using NanoZoomer at 1x magnification and used to quantify $TRAP^+$ cells in the trabecular bone. The number of $TRAP^+$ osteoclast cells and the total number of $TRAP^+$ cells that comprised both $TRAP^+$ osteoclasts and $TRAP^+$ osteocyte cells were counted by two assessors. The area of the trabecular bone was measured using Bone J program in ImageJ.

Enzyme-linked immunosorbent assay (ELISA)

Subchondral bone tissues extracted from the samples were crushed in PEB solution and spin in a centrifuge at 13000g for 15 minutes. Bone tissue homogenates obtained were used to determine the concentration of active and total TGFβ1 by human TGFβ1 Quantikine ELISA kit (Cat. No: DB100B, R&D Systems, USA) following the manufacturer's instructions. The total TGFβ1 was activated by adding 20µl of 1 N HCl into 100µl of the homogenate, incubated for 10 minutes at room temperature and neutralised by adding 20µl of 1.2 N NaOH. A total of 50µl homogenate was added into the wells on ELISA plate and incubated for 2 hours. Then the wells were washed 4 times with 400µl wash buffer using microplate washer. After that, 100µl of TGFβ1 conjugate was added to each well and incubated for 2 hours at room temperature. Then the wells were washed again for 4 times, added with 100µl of substrate solution and incubated for 30 minutes with a cover protected from the light. Then 100µl stop solution were added to each well. The absorbance value of the samples was recorded using a microplate reader at a wavelength of 450nm.

Reverse transcription polymerase chain reaction (RT-PCR)

Subchondral bones extracted from human tibial plateaus were washed with PBS solution to remove blood and fat cells. Bone was crushed using approximately 20ml of liquid nitrogen and placed into 1ml of cold Trizol (Thermo Fisher Scientific). Specifically, samples were added with 0.2ml of chloroform and centrifuge for 20 minutes at 12000rcf at 4ºC. RNA was precipitated from the samples with 0.5ml of isopropanol and stored at -20ºC overnight. Then the RNA precipitates were washed 3 times in 75% ethanol. The yield and purity of the RNA were tested using Nanodrop One Microvolume Spectrophotometer (Thermo Fisher Scientific) and then they were stored in -80ºC until use. RNA was reverse transcribed using Superscript™ IV Reverse Transcriptase (Thermo Fisher Scientific). RT-PCR quantification of TGFβ1, SMAD3, TRAP, RANKL, OPG, DMP1, ALP, OCN and COL1 was performed in triplicate for each specimen using SYBR Green Mastermix (Cat. No: 330513, Qiagen, USA). 18S rRNA was considered as an internal control of the other mRNAs. Data were normalised using the $2^{-(\Delta Ct)}$ method for analysis[25]. $\Delta Ct = Ct_{target\ gene} - Ct_{18S}$. Primer sequences are listed in Table 2.

Table 2

List of genes and primer sequences used for RT-PCR

Gene	Primer sequence (5' – 3')
18S	F – GCG TTG ATT AAG TCC CTG CC
	R – CAC CTA AGG AAA CCT TGT TAC GAC
TGF-β1	F – GAC ACC AAC TAT TGC TTC AG
	R – AGA AGT TGG CAT GGT AGC CC
SMAD3	F – TTC AAC AAC CAG GAG TTC GC
	R – TAC TGG TCA CAG TAT GTC TC

Table 2 (continued)

TRAP	F – GTG CAG ACT TCA TCC TGT CTC TA
	R – AAT ACG TCC TCA AAG GTC TCC
RANKL	F – TCA GCC TTT TGC TCA TCT CAC TAT
	R – CCA CCC CCG ATC ATG GT
OPG	F – GTC CAC AAG AAC AGA CTT TCC AG
	R – CTG TTT TCA CAG AGG TCA ATA TCT T
OCN	F – TGA GAG CCC TCA CAC TCC TC
	R – ACC TTT GCT GGA CTC TGC AC
DMP1	F – GAT CAG CAT CCT GCT CAT GTT
	R – AGC CAA ATG ACC CTT CCA TTC
ALP	F – TGC TCC CAC GCG CTT GTG CCT GGA
	R – CTG GCA CTA AGG AGT TAG TAA G
COL1	F – AGG CCT CCA ACG AGA TCG AGA TCC G
	R – TAC AGG AAG CAG ACA GGG CCA ACG TCG

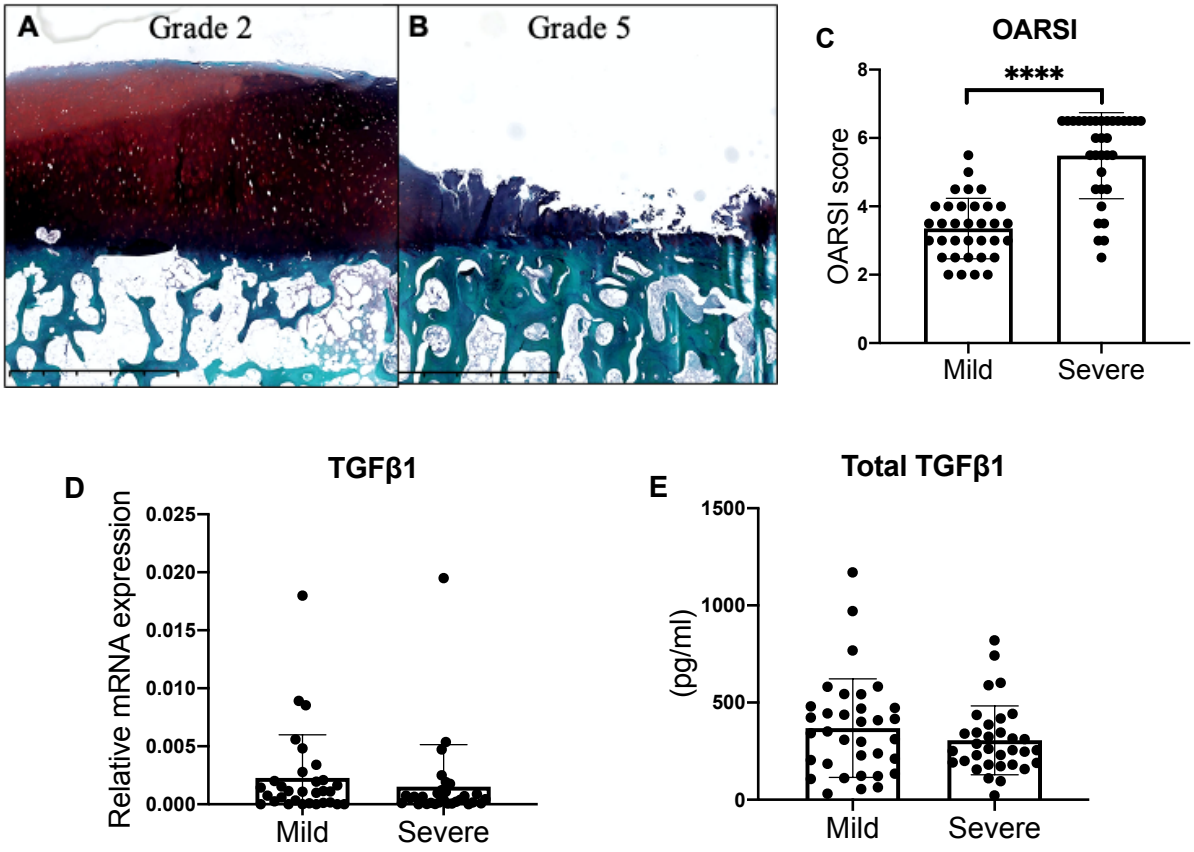
Statistical analysis

Shapiro-Wilk test was performed to assess the distribution of data. Comparisons between groups were evaluated using paired *t*-test for parametric data or Wilcoxon signed-rank test for non-parametric data, followed by Spearman rank to test for correlation. These analyses were performed using GraphPad Prism Software (GraphPad Prism, San Diego, CA, USA). Parametric data are presented as mean ± standard deviation (SD), and non-parametric data as median ± interquartile range. Statistical significance was set at $p < 0.05$.

RESULTS

Elevated TGFβ1 activation with increasing severity of osteoarthritis

To determine the expression of TGFβ1 in osteoarthritis progression, subchondral bone tissues from the medial tibial plateau were extracted in regions with less and more degenerated cartilage categorised as mild and severe osteoarthritis groups (Fig. 5A and 5B). OARSI scores revealed the degeneration of cartilage in the severe group was significantly higher than in the mild group, indicating more progressive osteoarthritis (Fig. 5C). The expression of TGFβ1 gene and concentration of total TGFβ1 protein in the subchondral bone was not different between mild and severe groups (Fig 5D and 5E). However, the concentration of active TGFβ1 and the ratio of active/total TGFβ1 was significantly higher in the severe than in the mild group (Fig. 5F and 5G).



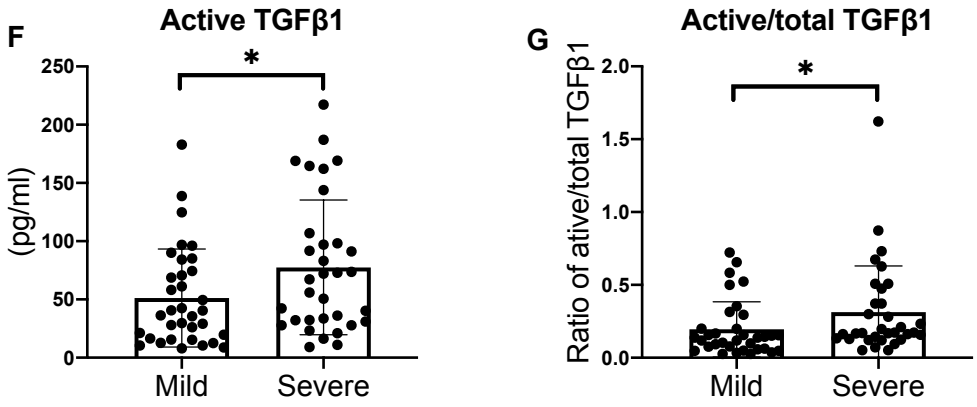


Figure 5 Expression of TGFβ1 at the gene and protein level between mild and severe osteoarthritis. Safranin O Fast Green staining of sections from (A) mild and (B) severe cartilage degeneration in the same tibial plateau. Scale bar: 2.5mm. (C) OARSI scores; (D) TGFβ1 relative mRNA expression; (E) Concentration of total TGFβ1; (F) Concentration of active TGFβ1; (G) Ratio of active/total TGFβ1 between groups. Each point represents an individual sample. Graphs show median ± interquartile range. * $p < 0.05$, **** $p < 0.0001$.

Upregulated osteoclastic activity in severe osteoarthritis

The activity of osteoclast cells in the trabecular bone was observed using TRAP staining (Fig. 6A). The result shows that the number of TRAP^{+} osteoclast cells and total TRAP^{+} cell density was increased significantly in the severe relative to the mild group (Fig. 6B and 6C).

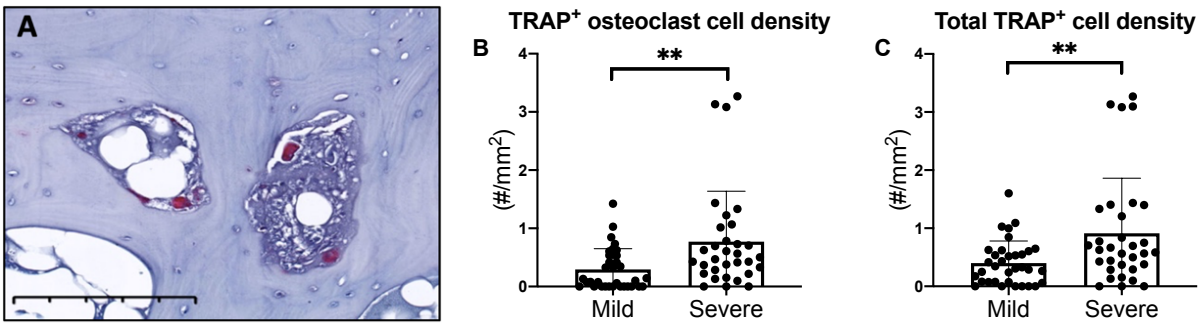


Figure 6 TRAP activity in human osteoarthritic trabecular bone (A) TRAP staining of TRAP^{+} osteoclast cells (pink cytoplasmic stained). Scale bar: 250μm. Quantitative analysis of (B)

$TRAP^+$ osteoclast cell density; (C) Total $TRAP^+$ cell density between groups. Each point represents an individual sample. Graphs show median ± interquartile range. ** $p < 0.01$.

High level of active TGFβ1 induces osteoblast differentiation and angiogenesis in the subchondral bone

Next, the study examines the effect of elevated active TGFβ1 on osteocyte lacunar parameters in the trabecular bone. The result shows that there is a significant increase in osteocyte cells density in the severe compare to the mild group, suggesting an increase in osteoblast differentiation into osteocyte cells. However, the empty lacunar density was significantly decreased in the severe group (Table 3).

Table 3

Osteocyte and lacunar morphometric parameters in human osteoarthritis subchondral trabecular bone

	Mild	Severe	*p* value
Osteocyte density (#/mm^2)	167.5 ± 41.2	204.8 ± 80.0	**0.012**
Empty lacunar density (#/mm^2)	108.8 ± 39.8	84.6 ± 28.8	**0.01**
Total lacunar density (#/mm^2)	276.4 ± 48.5	289.4 ± 95.5	0.457
Empty/total lacunae (%)	39.2 ± 11.9	30.2 ± 9.6	**0.002**

Values are presented as mean ± SD.

This study also investigated the effect of TGFβ1 on the density of blood vessels in the subchondral bone marrow as an increase in angiogenesis is one of the hallmarks of osteoarthritis[26]. Vascular density in the subchondral bone marrow was significantly elevated in the severe compared to the mild group (Fig. 7).

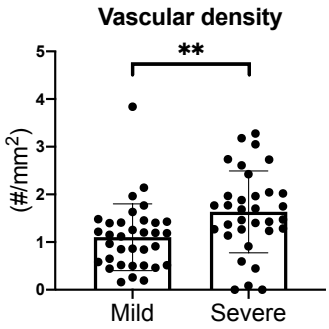


Figure 7 Quantitative analysis of vascular density in the human osteoarthritic subchondral bone marrow between the mild and severe groups. Each point represents an individual sample. Graph shows median ± interquartile range. ** $p < 0.01$.

Association between TGFβ1 activity and bone parameters with increasing severity of osteoarthritis

Correlation analysis of active/total TGFβ1 and bone parameters measured at the tissue level with OARSI score were all positively correlated in the combined cohort consisting of both mild and severe groups (Table 4). This indicates that active TGFβ1 and bone parameters are associated with increased progression of osteoarthritis.

Table 4

Correlations (r) with OARSI score

	Mild	Severe	All
Active/total TGFβ1	r = -0.03, p = 0.871	r = 0.28, p = 0.127	**r = 0.34, p = 0.006**
Total $TRAP^+$ cell density	r = 0.18, p = 0.3	r = 0.15, p = 0.425	**r = 0.34, p = 0.005**
$TRAP^+$ osteoclast cell density	r = -0.03, p = 0.854	r = 0.14, p = 0.453	**r = 0.30, p = 0.014**
Osteocyte density	r = 0.14, p = 0.428	r = 0.22, p = 0.209	**r = 0.29, p = 0.018**
Vascular density	r = 0.31, p = 0.07	r = -0.01, p = 0.965	**r = 0.36, p = 0.003**

Differential gene expression for bone remodelling markers in human subchondral bone

To investigate the molecular mechanism underlying the progression of osteoarthritis in human subchondral bone, the gene expressions of TGFβ1, SMAD3 and bone marker genes associated with bone resorption (TRAP, RANKL and OPG) and formation (DMP1, ALP, OCN and COL1) between the mild and severe osteoarthritis were examined. There was no significant difference in the mRNA expression of TGFβ1, SMAD3, TRAP, RANKL, OPG, DMP1, ALP, OCN and COL1 between the groups (data not shown). However, this study observed a significant increase in RANKL/OPG ratio in severe osteoarthritis, indicating an abnormal bone remodelling (Fig. 8).

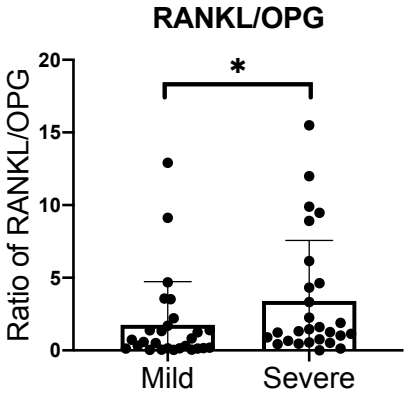


Figure 8 Bone remodelling marker RANKL/OPG gene expression ratio between mild and severe group. Each point represents an individual sample. Graph shows median ± interquartile range. * $p < 0.05$.

Relationships between bone marker gene expressions

The correlation between gene expressions was also examined to identify their relationship in co-regulating bone remodelling in the mild and severe osteoarthritis. The relationships between these genes are summarised in Table 5. TGFβ1, SMAD3 and DMP1 mRNA expressions were positively correlated in mild and severe osteoarthritis. TGFβ1, SMAD3 and DMP1 mRNA

expressions were also correlated positively with all bone marker genes except for OCN, a late osteoblast differentiation marker[27]. Both TGFβ1 and SMAD3 mRNA expression were positively correlated with OCN in mild osteoarthritis, while, no significant correlation observed between DMP1 and OCN in both groups.

TRAP mRNA expression was positively correlated with RANKL, OPG, OCN and COL1 in the mild and severe osteoarthritis. However, an early osteoblast marker, ALP, was correlated positively with TRAP only in the severe group[27]. Further, ALP, RANKL and OPG mRNA expressions were correlated positively in the mild and severe osteoarthritis.

Another early osteoblast differentiation marker, COL1, was positively correlated with RANKL, OCN and ALP in the mild osteoarthritis[27]. Meanwhile, COL1 and OCN mRNA expressions were correlated positively in both groups.

Table 5

Correlations between bone marker gene expression in mild and severe osteoarthritis

	Mild	Severe
TGFβ1 vs RANKL	$r = 0.70$, $p < 0.0001$	$r = 0.71$, $p < 0.0001$
TGFβ1 vs OPG	$r = 0.87$, $p < 0.0001$	$r = 0.83$, $p < 0.0001$
TGFβ1 vs TRAP	$r = 0.86$, $p < 0.0001$	$r = 0.60$, $p = 0.001$
TGFβ1 vs OCN	$r = 0.55$, $p = 0.002$	$r = 0.23$, $p = 0.214$ (ns)
TGFβ1 vs COL1	$r = 0.74$, $p < 0.0001$	$r = 0.51$, $p = 0.004$
TGFβ1 vs ALP	$r = 0.41$, $p = 0.037$	$r = 0.66$, $p = 0.001$
TGFβ1 vs SMAD3	$r = 0.85$, $p < 0.0001$	$r = 0.71$, $p < 0.0001$

Table 5 (continued)

TGFβ1 vs DMP1	$r = 0.85$, $p < 0.0001$	$r = 0.75$, $p < 0.0001$
SMAD3 vs RANKL	$r = 0.71$, $p < 0.0001$	$r = 0.43$, $p = 0.031$
SMAD3 vs OPG	$r = 0.89$, $p < 0.0001$	$r = 0.68$, $p < 0.0001$
SMAD3 vs TRAP	$r = 0.80$, $p < 0.0001$	$r = 0.68$, $p = 0.0002$
SMAD3 vs OCN	$r = 0.43$, $p = 0.023$	$r = 0.14$, $p = 0.448$ (ns)
SMAD3 vs COL1	$r = 0.73$, $p < 0.0001$	$r = 0.50$, $p = 0.007$
SMAD3 vs ALP	$r = 0.46$, $p = 0.025$	$r = 0.49$, $p = 0.038$
SMAD3 vs DMP1	$r = 0.89$, $p < 0.0001$	$r = 0.76$, $p < 0.0001$
DMP1 vs RANKL	$r = 0.61$, $p = 0.0002$	$r = 0.47$, $p = 0.012$
DMP1 vs OPG	$r = 0.88$, $p < 0.0001$	$r = 0.68$, $p < 0.0001$
DMP1 vs TRAP	$r = 0.68$, $p < 0.0001$	$r = 0.58$, $p = 0.001$
DMP1 vs COL1	$r = 0.78$, $p < 0.0001$	$r = 0.75$, $p < 0.0001$
DMP1 vs ALP	$r = 0.44$, $p = 0.021$	$r = 0.42$, $p = 0.049$
TRAP vs RANKL	$r = 0.71$, $p < 0.0001$	$r = 0.48$, $p = 0.015$
TRAP vs OPG	$r = 0.79$, $p < 0.0001$	$r = 0.73$, $p < 0.0001$
TRAP vs OCN	$r = 0.55$, $p = 0.001$	$r = 0.47$, $p = 0.012$
TRAP vs COL1	$r = 0.73$, $p < 0.0001$	$r = 0.65$, $p = 0.0002$
TRAP vs ALP	$r = 0.29$, $p = 0.143$ (ns)	$r = 0.56$, $p = 0.017$
COL1 vs RANKL	$r = 0.60$, $p = 0.0003$	$r = 0.37$, $p = 0.056$ (ns)
COL1 vs OPG	$r = 0.80$, $p < 0.0001$	$r = 0.52$, $p = 0.003$
COL1 vs OCN	$r = 0.48$, $p = 0.006$	$r = 0.30$, $p = 0.101$ (ns)
COL1 vs ALP	$r = 0.39$, $p = 0.047$	$r = 0.34$, $p = 0.126$ (ns)

Table 5 (continued)

ALP vs RANKL	$r = 0.74$, $p < 0.0001$	$r = 0.86$, $p < 0.0001$
ALP vs OPG	$r = 0.64$, $p = 0.0005$	$r = 0.66$, $p = 0.002$
RANKL vs OPG	$r = 0.74$, $p < 0.0001$	$r = 0.61$, $p = 0.0007$

Only statistically significant correlations in one or both cohorts are shown.

DISCUSSION

Findings of the present study demonstrated that severe osteoarthritic subchondral bone exhibits several outcomes of abnormal bone remodelling, including increased TGFβ1 activation, osteoclastic bone resorption, osteoblast differentiation and angiogenesis. These observations were associated with increasing severity of osteoarthritis. The expression of RANKL/OPG ratio was also elevated in the severe osteoarthritis, implying uncoupled bone remodelling. Therefore, this study highlights the role of TGFβ1 in the regulation of subchondral bone homeostasis and suggest that uncoupled bone remodelling enhanced the progression of osteoarthritis in the human subchondral bone.

TGFβ1 has been widely known for its critical role in maintaining bone homeostasis by regulating osteoclast and osteoblast activity in the bone[7]. Similar to that observed in ACLT murine model, the activity of osteoclast and the level of active TGFβ1 were elevated in the severe osteoarthritis, indicating high concentrations of TGFβ1 were activated during osteoclastic bone resorption activity[21]. The aberrant release of active TGFβ1 disrupts the temporospatial relationship of bone resorption and formation resulting in further recruitment of nestin^{+} MSCs to the bone resorption site[7, 16]. The activity of MSCs to differentiate into

osteoblast cells is often accompanied by angiogenesis. In the severe osteoarthritic subchondral bone marrow, there was an increase in the density of blood vessels. Previous study reported that TGFβ1 signalling in endothelial progenitor cells promote angiogenesis[28]. Therefore, increase angiogenesis in human subchondral bone marrow suggest that the hallmark of disease progression is a result of aberrant TGFβ1 activation.

Osteocytes are terminally differentiated osteoblasts that become embedded in the bone matrix important for mineralisation of the bone[29, 30]. It forms dendritic processes that extend out of their cell bodies into the canaliculi forming lacuno-canalicular networks with the neighbouring osteocytes, osteoblast, osteoclast cells and vasculature[29]. Previous study revealed the importance of osteocytes in maintaining bone homeostasis by regulating the activity of osteoclast and osteoblast cells through their gap junctions present at the dendrites[31, 32]. Furthermore, osteocytes are also responsible for regulating perilacunar/canalicular remodeling to maintain their lacuno-canalicular networks, such as osteocyte communication, mechanosensation, nourishment and systemic mineral homeostasis[29, 32, 33, 34, 35]. TGFβ signaling in osteocyte regulates perilacunar/canalicular remodeling enzyme genes such as matrix metalloproteinases (MMPs) and Cathepsin K (Ctsk) that are expressed by the osteocyte cells[33, 36]. Therefore, increase osteocyte apoptosis observed in the mild osteoarthritis affect osteocytes perilacunar/canalicular remodeling that result in reduced quality of the bone[32]. Consistent with prior reports, elevated osteocyte density was observed in the trabecular bone with increased severity of osteoarthritis[37]. This suggests that an increase in TGFβ1 activity in the severe osteoarthritis enhance osteoblast differentiation into osteocytes to increase regulation of perilacunar/canalicular remodelling and restore bone quality[38].

TGFβ1 signalling *via* SMAD3 pathway was shown to be involved in the progression of osteoarthritis. Since TGFβ1 regulates the expressions of SMAD3, TRAP, RANKL, OPG, DMP1, ALP, OCN and COL1, it is expected that TGFβ1, SMAD3 and the bone marker gene expressions are increased in the severe group[18, 19, 32, 39,, 40]. However, molecular analysis revealed that there were no differences in the gene expressions observed in this study. These could be due to genes are being expressed by cells other than osteoclast, osteoblast and osteocyte cells such as stromal cells[41]. Furthermore, factors such as RANKL and OPG has been shown to be involved in influencing bone remodelling process[20]. Although previous study demonstrated that RANKL and OPG are expressed by osteoblast cells in the osteoarthritic subchondral bone[42], the ratio of RANKL/OPG in the mild and severe osteoarthritic subchondral bone remains to be determined. Interestingly, an increase in the expression of RANKL/OPG ratio was observed in the severe osteoarthritis. This is in line with studies in human temporomandibular joint and knee osteoarthritis which demonstrated that the serum ratio RANKL/OPG in the synovial fluid were increased with the severity of disease to induce osteoclastogenesis in the osteoarthritic joints[43]. Therefore, elevated RANKL/OPG ratio enhance more bone resorption activity occurred in the subchondral bone rather than bone formation, representing an imbalance bone remodelling in the human subchondral bone.

The interrelationships between the expression of bone marker genes in the mild and severe groups were also investigated in this study. The positive correlation between both TGFβ1 and SMAD3 genes associated with TRAP, RANKL, OPG, DMP1, ALP and COL1 in mild and severe osteoarthritis indicates that the process of bone remodelling was regulated by TGFβ1/SMAD3 dependent pathway[38, 44]. This revealed that the role of TGFβ1/SMAD3 signalling in the subchondral bone was altered from being a reparative to a damaging factor as disease progressed. Furthermore, the association of osteocyte marker, DMP1, with TGFβ1,

SMAD3, TRAP, RANKL, OPG, ALP and COL1 in both groups may suggests that TGFβ1 signalling *via* SMAD3 in osteocyte also regulate for bone remodelling process[32]. However, the expression of OCN was not associated with TGFβ1 and SMAD3 in the severe group, and with DMP1 in both groups. This could be due to the expression of OCN was regulated by the other cell subpopulations in the bone[41].

A proteolytic enzyme encoded by TRAP is highly expressed in osteoclast cells required for the degradation of organic bone matrix[18]. TRAP expression is associated with ALP in severe osteoarthritis, which was not observed in mild osteoarthritis. Both TRAP and ALP are critical for dephosphorylation of OPN[45, 46]. The role of OPN is to stimulate migration and attachment of osteoclast cells to the bone[46]. When OPN is dephosphorylated, osteoclast detached from the bone, migrate through the bone surface and allow bone formation at the resorption site. The correlation between TRAP and ALP suggest that these genes co-regulate suppression of inhibitory properties of OPN in severe osteoarthritis for subsequent bone formation[47]. Moreover, TRAP, RANKL and OPG are gene expressions associated with osteoclast differention[42, 48]. The correlation between these genes indicate that RANKL and OPG influences TRAP expression to regulate osteoclastic activity in both severity of osteoarthritis [47, 49].

The expression of COL1, ALP and OCN is associated with osteoblast differentiation. The positive correlation between these genes indicates that osteoprogenitor cells in mild osteoarthritis have the potential for cell differentiation into mature osteoblasts. However, the correlation between these genes was not significant in the severe osteoarthritis, showing that these genes might also be expressed by the stromal cells[41]. These could affect the activity of osteoprogenitor cells differentiation at the bone resorption site in the severe osteoarthritis,

hence, affecting the coupled bone remodelling process. These findings revealed the complex interplay between the genes in the mild and severe osteoarthritis and illustrate a novel molecular mechanism by which the genes co-regulate in bone resorption and formation activity at the different severity of osteoarthritis.

The strength of the current study is the use of human osteoarthritic osteochondral samples to investigate the cellular and molecular mechanisms of osteoarthritis progression rather than the use of animal models or cultured cells. However, this study presents some limitations, including the inability to obtain a healthy non-osteoarthritic sample to compare the observations due to ethical reasons. Presence of samples with extensively severe osteoarthritis where the cartilage in the samples was almost completely gone. Therefore, tissues obtained from mild regions in that sample were usually considered as a severe stage of osteoarthritis. Furthermore, the use of an entire bone cell populations from the tissue samples could also affect the outcomes of the study, especially in genetic analysis. This is because cells such as stromal cells found in the subchondral bone are also known to expressed TGFβ1 and the other bone marker genes. In the future studies, further analysis in tissue localisation of proteins such as pSMAD3, osterix^{+} and nestin^{+} MSCs, and CD31^{+} endothelial progenitor cells are required to determine the level of SMAD3 phosphorylation status, activity of osteoprogenitor osteoblast and endothelial cells differentiation. This would be valuable to evaluate the cellular mechanism of TGFβ1 through SMAD3 signalling as well as to identify its effect on osteogenesis and angiogenesis at the tissue level.

In conclusion, the results of this study partially support the hypothesis where increased active TGFβ1 was observed with increased severity of osteoarthritis. The increase in TGFβ1 activity was associated with elevated osteoclastic activity, osteoblast differentiation and angiogenesis

at the tissue and cellular level. The study also demonstrated that the expression of RANKL/OPG ratio was elevated at the molecular level. These changes observed between the different severity of osteoarthritis may provide an insight that inhibition of TGFβ1 activity at any stage of osteoarthritis can be effective in preventing or reducing the progression of osteoarthritis.

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