

The Expression of $\alpha\beta3$ and Osteopontin in Articular Cartilage of Patients with Knee Osteoarthritis and Its Correlation with Disease Severity and Chondrosenescence

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Abstract

Background: Osteoarthritis(OA) is the most common joint pathological disease associated with aging,previous observations have revealed that both osteopontin (OPN) and $\alpha\beta3$ integrin are expressed in chondrocytes and both could as the biomarker for the progression of knee OA .The research aimed to comprehend the expressions of OPN and $\alpha\beta3$ integrin and the chondrocyte senescence levels in OA.

Methods: 48 cartilage tissues from normal and knee OA patients were divided into four groups of normal,minor,moderate, severe lesions based on the Mankin score. Immunohistochemistry and western blot were used to determine the expresssions of $\alpha\beta3$,OPN and SA β -gal in articular cartilage,Then Spearman's correlation was used to analyze the correlations between Mankin Scores and $\alpha\beta3$, OPN and SA β -gal. The pearson correlation analysis was used to analyze the correlation among the $\alpha\beta3$, OPN and SA β -gal.

Results: The expression of OPN and $\alpha\beta3$ and SA β -gal in articular cartilage were explored. $\alpha\beta3$, OPN and SA β -gal proteins all elvated in OA cartilage, the correlation coefficient between the Mankin score and the average optical density value of $\alpha\beta3$, OPN, SA β -gal were $r=0.60,r=0.75,r=0.87$,respectively, all $p\leq 0.001$,the correlation between the average optical density value of $\alpha\beta3$ and OPN was $r=0.3191$, $p<0.05$, the correlation between $\alpha\beta3$ and SA β -gal was $r=0.4955$, $p<0.001$, the correlation between OPN and SA β -gal was $r=0.7821$, $p<0.001$.

Conclusion: the correlation between $\alpha\beta3$, OPN and SA β -gal expression in articular cartilage might be important to the OA progression and pathogenesis. Nonetheless, more researches are needed to elucidate the exact contribution of $\alpha\beta3$, OPN and SA β -gal in the degenerative process of OA.

Introduction

Osteoarthritis(OA) is the main reason for disability and a source of societal cost in elder people. With an ageing and increasingly obese population, OA is becoming even more prevalent than several previous decades[1]. The progressive loss of articular cartilage is the most characteristic of OA that leads to chronic pain and functional restrictions in affected joints. The clinical features of OA include pain, stiffness, hypokinesia, swelling, cramps and deformities. Although clinical research on OA has been extensively studied, the etiology of the disease is still poorly understood. Several biochemical and biomechanical factors including ageing, obesity and trauma are considered in the pathogenesis[2].

OA is the most common joint pathological disease associated with aging,the aging process leads to changes in joint tissues and ultimately contributes to the development of OA[3]. Chondrocyte senescence can contribute to a decline in chondrocyte numbers due to increased cell death[4], Senescent cells exhibit increased levels of the lysosome enzyme acidic β -galactosidase called senescent-associated- β -galactosidase (SA β -gal), SA β -gal is the most commonly used senescence marker[5]. our previous study comfirmed that SA β -gal expression in articular cartilage is associated with progressive knee OA joint damage and is a potential indictor of disease severity[6].

Osteopontin (OPN) is a 44–75 KD multifunctional phosphoprotein secreted by many cell types such as synovial cells, macrophages, and chondrocytes and is present in the extracellular matrix of mineralized tissues and in extra cellular fluids, at sites of inflammation[7, 8]. OPN mRNA isolated from human OA cartilage showed enhanced expression as compared with that in normal cartilage, and human OA chondrocytes exhibited upregulated levels of OPN[9]. OPN gene locus is associated with the risk of knee osteoarthritis in Chinese Han population[10]. Elevated OPN levels in plasma, synovial fluid and articular cartilage are associated with progressive joint damage in patients with knee OA[11, 12]. OPN could be serving as a biochemical marker for the determination of disease severity and predict the progression of knee OA[11, 12].

Integrin is one of receptor of OPN[13]. There are 24 known integrin heterodimers can be classified as arginine–glycine–aspartate (RGD)-binding, the $\alpha 4$ family, leukocyte adhesion integrins, laminin-binding, and I-domain collagen-binding[13]. Previous studies confirmed that the $\alpha v\beta 3$ integrin could be expressed in chondrocytes[14]. Integrin played the function of the adhesion molecules, highlight the key role of matrix interactions in the pathogenesis of the anatomic changes in OA cartilages[13, 14]. The expression of integrins on chondrocytes is correlated with the degree of cartilage damage in human OA and could regulate cells proliferation and differentiation [14].

Previous observations have revealed that both OPN and $\alpha v\beta 3$ integrin are expressed in chondrocytes and both could as the biomarker for the progression of knee OA .We hypothesized that, in articular cartilage, OPN and $\alpha v\beta 3$ integrin might be related to the severity of disease in patients with knee OA and might be related with the chondrocyte senescence levels. So as to study the assumption, the expression levels of OPN and $\alpha v\beta 3$ integrin in various levels of damaged samples of cartilage from human-beings with OA could be determined and the correlation between OPN and $\alpha v\beta 3$ integrin can be analyzed. The current research aimed to offer a more overall comprehension of OPN and $\alpha v\beta 3$ integrin and chondrocyte senescence levels in OA.

Materials And Methods

Patients and preparation of samples This research has been verified by the Ethics Committee of Xiangya Hospital, Central South University, and all the patients offered informed consent. This study contained 8 normal healthy individuals(6 male,2 female, age: 17–55 years) and 40 patients(12 male at the age of 62–81years, 28 female at the age of 55–76 tears) with major knee OA according to the criteria of the American College of Rheumatology[15]. The samples of osteoarthritic cartilage were gathered from the 40 patients with main OA with knee arthroplasty. The normal samples could be gained from the knees of 8 age matched postmortem donors, without any history of joint pain. Informed consent of ethics could be gained from all the families and donors. After eosin and hematoxylin (HE) staining, a light microscopy was used to evaluate the histological changes of the sections,osteoarthritic changes were classified histomorphologically, using the Mankin score[16]: normal (Mankin score: 0–1), mild lesions (Mankin score: 2–5), moderate lesions (Mankin score: 6–9) and severe lesions (Mankin score: 10–14).

Immunohistochemistry. Cartilage tissues were snap-frozen in liquid nitrogen at -70°C before analysis. In analysis, cartilage were cut to $10\ \mu\text{m}$ thick slice. The slices put into the oven at 60°C for $3\sim 6\ \text{h}$, then the slices soak in xylene for 10 min twice. And soak in 100%, 95%, 85% and 75% ethanol in sequence, subsequently, add urea ($n \geq 8\text{mol/L}$) incubate at 37°C for 25min and add pancreatin dropwise for antigen retrieval, after this, 3% H_2O_2 was added for about 10 min to inactivate endogenous enzymes. Next, the slices were then incubated with diluted $\alpha\text{v}\beta 3$ antibody (1:50, BA0321, 18309-1-AP, Proteintech), OPN (1:100, Ab8448, Abcam), SA β -gal (1:50, 15518-1-AP, Proteintech) for 1 h at 37°C , respectively., overnight at 4°C , and a second antibody reaction was carried out for 20 min using biotinylated anti-rabbit IgG antibody (Proteintech), after wash, horseradish peroxidase/Fab polymer conjugate (PicTure™-Plus kit, Zymed, South San Francisco, CA) was applied to the slices for 30 min. A negative control was prepared simultaneously by neglecting the major antibody. Lastly, the staining was developed by incubation with DAB Chromogen for 20 min. To evaluate the expression of $\alpha\text{v}\beta 3$, OPN and SA β -gal, the sections were examined under a microscope at 40x magnification. The relative $\alpha\text{v}\beta 3$, OPN and SA β -gal distribution of cartilage tissue can be visualized and quantified as the Average optical density with Image-pro Plus 6.0. All densities were normalized to PBS. The final data, which were applied in all analysis, consisted therefore of a mean of three independent measurements representing the average levels of $\alpha\text{v}\beta 3$, OPN and SA β -gal in articular cartilage. The coefficient of variation (CV) of $\alpha\text{v}\beta 3$, OPN and SA β -gal expression in articular cartilage was 2 %.

Western blot. The histones were extracted from cartilage tissue in liquid nitrogen immediately after the cartilage tissue was washed and ground into small sections. Chondrocytes were lysed using 100ul/well SDS with protease inhibitor (100:1), and the protein concentration of the lysate was measured using the BCA protein assay kit (Thermo Fisher Scientific, Boston, MA, USA). 40ug protein was used in the next experiment. Proteins were separated by 12% SDS-polyacrylamide gelelectrophoresis (SDS-PAGE) (genescript, piscataway, USA) at 80v, 30min and then 120v, 60min and transferred to polyvinylidenedifluoride (PVDF) membranes with 300mA, 75min. The membranes were blocked in nonfat dry milk solution 1h, and incubated overnight at 4°C with primary antibody $\alpha\text{v}\beta 3$ antibody (1: 500, Sc-7985-R, SantaCruz), OPN (1:500, SC-21742, SantaCruz), SA β -gal (1:1000, 15518-1-AP, Proteintech) dilution buffer and then incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (1:2000) for 1 h at room temperature. β -actin was used as housekeeping protein. After that, the membranes were developed using the enhanced chemiluminescence (ECL) (NCM biotech, China) and exposed and qualified by Bio-Rad chemiDoc-XRS with image lab software (BioRad, Richmond, CA, USA).

Statistical analysis SPSS software for Windows (version 22; IBM SPSS, Armonk, NY, USA) was utilized for data analysis and management. One-way analysis of variance (ANOVA) was applied to study the difference between multiple groups in the mean values between multiple groups. Then Spearman's correlation and linear regression analysis were used to analyze the correlation between the average optical density of $\alpha\text{v}\beta 3$, OPN and SA β -gal in the articular cartilage with the degree of degeneration. The pearson correlation analysis was used to analyze the correlation among the average optical density of $\alpha\text{v}\beta 3$, OPN and SA β -gal. $P < 0.05$ indicated statistical significance.

Results

Histology and Immunohistochemistry. The microscopic images of osteoarthritic changes in HE staining, $\alpha\beta 3$, OPN and SA β -gal immunohistochemical staining were presented in Fig. 1.

Each Group's Mankin Grading System Based on the grading system of Mankin, 48 biopsies in total gained from 8 normal people and 40 patients were ascribed severally to the severe, moderate, minor, and normal groups. 8 normal samples with a Mankin score of 0–1, 12 samples with a Mankin score of 2–5, 15 samples with a Mankin score of 6–9, and 13 samples with a Mankin score 10–14 were included in this study. Table 1 illustrated the Mankin scores of each group.

Table 1
The mankin score of each group

group	samples	Mankin score
normal	8	0.8750 \pm 0.3536
Minor OA	12	3.2000 \pm 1.2293
Moderate OA	15	7.8667 \pm 1.1255
Severe OA	13	12.3077 \pm 1.3156

Validation of $\alpha\beta 3$, OPN and SA β -gal expression by western blot. The western blot results showed that the $\alpha\beta 3$, OPN and SA β -gal protein levels in OA cartilage tissues were higher than normal tissues (Fig. 2), which was consistent with immunohistochemistry results.

Improved Expression of $\alpha\beta 3$ Protein in Cartilage Tissues of OA. The expression levels of $\alpha\beta 3$ in the different degrees of damaged cartilage tissues were showed in Table 2. $\alpha\beta 3$ located in the cell membrane, was demonstrated to be expressed in the OA and normal groups. In comparison to normal, minor, and moderate groups, a higher expression degree of $\alpha\beta 3$ was shown in the severe group (Table 2).

Table 2
The average optical density and apoptosis rate of each group

group	OPN	$\alpha\beta 3$	SA β -gal
normal	0.0996 \pm 0.0111	0.0208 \pm 0.0688	0.0214 \pm 0.0040
Minor OA	0.1518 \pm 0.0332*#	0.0347 \pm 0.0102	0.0520 \pm 0.0088
Moderate OA	0.1953 \pm 0.0515*#	0.0569 \pm 0.0151*	0.0930 \pm 0.0165*
Severe OA	0.1263 \pm 0.0360*#	0.0587 \pm 0.0109*●	0.2471 \pm 0.0737*▲

For OPN, the difference of average optical density in each group showed statistical significance,*p < 0.05 each OA group vs normal,# P < 0.05 comparison among each OA group; for $\alpha\beta 3$, *p < 0.05 means Moderate and Severe OA group vs normal,respectively,●p < 0.05 Moderate vs Severe OA group;for SA β -gal,*p < 0.05 means Moderate and Severe OA group vs normal,respectively,●p < 0.05 Moderate vs Severe OA group.

Enhanced OPN Protein Expression in Cartilage Tissues of OA. The expression levels of OPN in the different degrees of damaged cartilage tissues were illustrated in Table 2. OPN located in the extracellular matrix, was demonstrated to be expressed in the OA and normal groups. In comparison to normal, minor, and moderate groups, a higher expression degree of OPN was shown in the severe group (Table 2).

Elevated SA β -gal expression in Cartilage Tissues of OA. The expression levels of SA β -gal in the different degrees of damaged cartilage tissues were presented in Table 2. SA β -gal located in the Intracellular and extracellular matrix, was demonstrated to be expressed in the OA and normal groups. In comparison to normal, minor, and moderate groups, a higher expression degree of SA β -gal was shown in the severe group (Table 2).

Correlations Between Mankin Scores And $\alpha\beta$ 3, Opn And Sa β -gal

According to the Spearman rank correlation analysis, the correlation coefficient between the average optical density value of $\alpha\beta$ 3 and the Mankin score was $r = 0.60$, $p < 0.001$ as Fig. 3A showed.

As for the Spearman rank correlation analysis, the correlation coefficient between the average optical density value of OPN and the Mankin pathology score was $r = 0.75$, $p < 0.001$ as Fig. 3B showed, after the Spearman rank correlation analysis, the correlation coefficient between the average optical density value of SA β -gal and the Mankin score was $r = 0.87$, $p < 0.001$ as Fig. 3C showed.

Correlations Among $\alpha\beta$ 3, Opn And Sa β -gal In Cartilage Tissue

According to the Pearson correlation analysis, the correlation between the average optical density value of $\alpha\beta$ 3 and OPN was $r = 0.3191$, $p = 0.0306$, $p < 0.05$, as the Fig. 4A showed. the correlation between the average optical density value of $\alpha\beta$ 3 and SA β -gal was $r = 0.4955$, $p < 0.001$, as the Fig. 4B showed, the correlation between the average optical density value of OPN and SA β -gal was $r = 0.7821$, $p < 0.001$, as the Fig. 4C showed.

Discussion

OA is an age-related degenerative joint disease characterized by progressive degradation in articular cartilage, ageing contributes to OA related to the following factors: age-related inflammation, cellular senescence, mitochondrial dysfunction and oxidative stress[17]; therefore, cellular senescence is one of the hallmarks of ageing, and chondrocytes have many feature that are characteristic of senescent cells during ageing and during OA[18]. Senescent chondrocytes accumulate with age and are present at higher

numbers in human OA cartilage compared with age-matched healthy cartilage[19]. The profile of inflammatory and catabolic mediators present during the pathogenesis of OA is strikingly similar to the secretory profile observed in classical senescent cells. During OA, chondrocytes exhibit increased levels of various senescence markers, such as SA β -gal activity, senescence of cells within joint tissues may play a pathological role in the causation of OA due to shifting of the balance between extracellular matrix (ECM) synthesis and degradation, through changes influence the expression of catabolic factors resulting in increased production of matrix metalloproteinases (MMPs) and cytokines, reduced levels of collagen type II and aggrecan synthesis, and increased production of reactive oxygen species (ROS) [18, 20].

Previous studies confirmed that human OA chondrocytes exhibited upregulated levels of OPN[9]. Elevated OPN could be serving as a biochemical marker for the determination of disease severity and predict the progression of knee OA[11, 12]. Regarded as a proinflammatory cytokine and a multifunctional protein, OPN has altered expression involved in several pathological conditions, for example, sepsis, cancer metastasis, rheumatoid arthritis, osteoporosis, cardiovascular diseases, and OPN also has beneficial roles in wound healing, bone homeostasis, and extracellular matrix (ECM) function and some other stress forms[21, 22]. Our previous study indicated that in chondrocytes, OPN promoted the production of MMP13 and activation of NF-kappaB pathway by increasing the abundance of p65 and phosphorylated p65 and translocation of p65 protein from cytoplasm to nucleus, notably, inhibition of NF-kappaB pathway by inhibitor suppressed the production of MMP13 induced by OPN treatment[23], and OPN could up-regulate expression of IL-6 and IL-8 cytokines in human OA chondrocytes[24]. In line with this findings, our results showed that OPN expression level in the severe OA group were higher than the normal, minor, and moderate groups. Furthermore, the expression of OPN was proved to relate to the level of severity in the degeneration of cartilage according to the Mankin score. Therefore, we deduce that OPN may play as a destructive factor in OA progression and pathology.

Our data revealed a marked increase of α v β 3 integrin levels in articular cartilage of patients with knee OA compared with controls. The chondrocytes express several integrins including α v β 3, and α v β 3 integrin is a receptor of OPN, integrins could mediate cells adhesion, regulate cells proliferation and differentiation, affect cartilage homeostasis[13]. Cartilage oligomeric matrix protein (COMP) promotes cell attachment via two independent mechanisms involving cell surface CD47 and α v β 3 integrin and that a consequence of cell attachment to COMP is the specific induction of fascin-stabilized actin microspikes[25]. Dysregulated α v β 3 and CD47 signaling in OA pathogenesis and suggest that activation of α v β 3 and CD47 signaling in many articular cell types contributes to inflammation and joint destruction in OA[26]. Although the previous studies confirmed the important role of α v β 3 integrin in the progression of OA, however, it was the first time to determined that the expression of α v β 3 integrin in OA cartilage was elevated.

Our results also indicated a marked increase of SA β -gal expression in knee OA articular cartilage compared with controls. SA β -gal is a widely used marker for cellular senescence, SA β -gal provides in situ evidence that senescent cells may exist and accumulate with age in vivo[27]. In addition, the cause of the age-related loss of chondrocyte function may be progressive senescence of articular cartilage

chondrocytes marked by a decline in mitotic activity, increased expression of SA β -gal and erosion of telomere length. The persistent SA β -gal expression in articular cartilage raised the speculation that SA β -gal might also influence degenerative cartilage diseases such as OA[28]. Our study showed the presence of a correlation between the pathological features of OA articular cartilage and the increase of SA β -gal, and a correlation between SA β -gal and OPN, a correlation between SA β -gal and α v β 3 integrin also presences.

Our results indicated that there was a correlation between α v β 3 and OPN, and a correlation between α v β 3 and SA β -gal, and a correlation between OPN and SA β -gal also presence. These were in line with the former studies [29–31].Due to the ligand-receptor relationship between OPN and α v β 3, we infer that elevated OPN through the α v β 3 integrin affect the SA β -gal expression and influence the cellular senescence.

Conclusion the correlation between α v β 3, OPN and SA β -gal expression in articular cartilage might be important to the OA progression and pathogenesis. Nonetheless, more researches are needed to elucidate the exact contribution of α v β 3, OPN and SA β -gal in the degenerative process of OA.

Declarations

Acknowledgements None.

Authors' Contributions Chao Cheng, Hua Yang and Fangjie Zhang designed the experiment. Chao Cheng, Jian Tian, Shuguang Gao, and Hua Yang performed the experiment. Zhihong Zhou, Ruiqi Yang, and Kai Xiao analyzed the data. Wei Guo and Li Liu helped in experiment . Chao Cheng, Hua Yang, Jian Tian, Shuguang Gao and Fangjie Zhang wrote and revised the paper. All authors read and approved the final manuscript .

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Availability of data and materials The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate This study was approved by our Institutional Ethics Committee (201503192), and each participant or the legally authorized representative of the participant was aware of and agreed to the study.

Consent for publication

All the authors agreed to publish this study in the "J Orthop Surg Res".

Competing interests The authors declare that there are no conflicts of interest.

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Figures

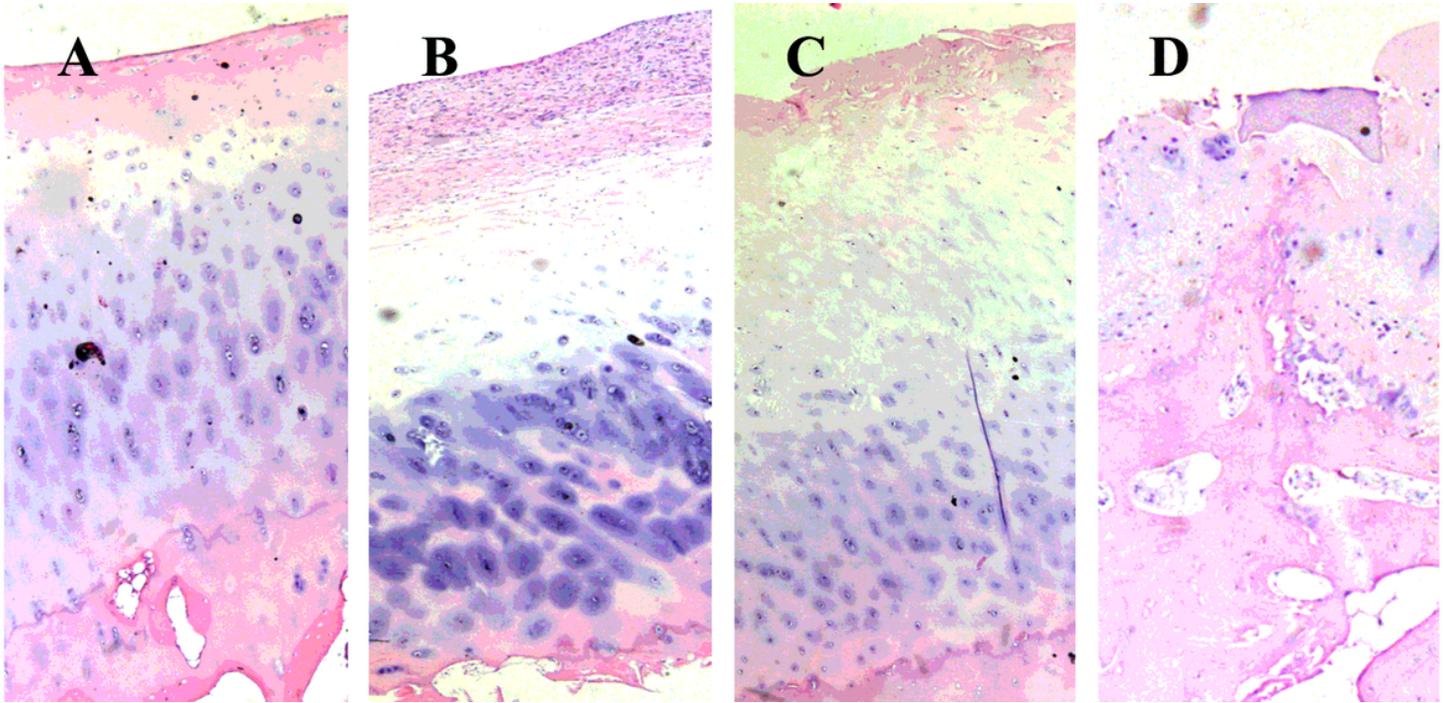


Figure 1

A HE staining $\times 40$ The normal cartilage under the microscope, the surface layer was slightly uneven, no cracks were formed, a few cells proliferated, the arrangement was not regular, the tide line was intact, and there was no pannus formation. The Mankin score was 0-1. The cartilage surface layer of the OA group has erosion and crack formation, local full-thickness loss and surface fibrosis, cell proliferation disorder, clustering or reduction of loss, tidal line destruction and capillary passage or disappearance, Mankin score was 2-14. B immunohistochemistry analysis $\times 40$ showed the $\alpha v \beta 3$ in normal cartilage(A), in minor lesions cartilage(B), in moderate lesions cartilage(C), in severe lesions cartilage(D). C immunohistochemistry analysis $\times 40$ showed the OPN in normal cartilage(A), in minor lesions cartilage(B), in moderate lesions cartilage(C), in severe lesions cartilage(D). D immunohistochemistry analysis $\times 40$ showed the SA β -gal in normal cartilage(A), in minor lesions cartilage(B), in moderate lesions cartilage(C), in severe lesions cartilage(D).

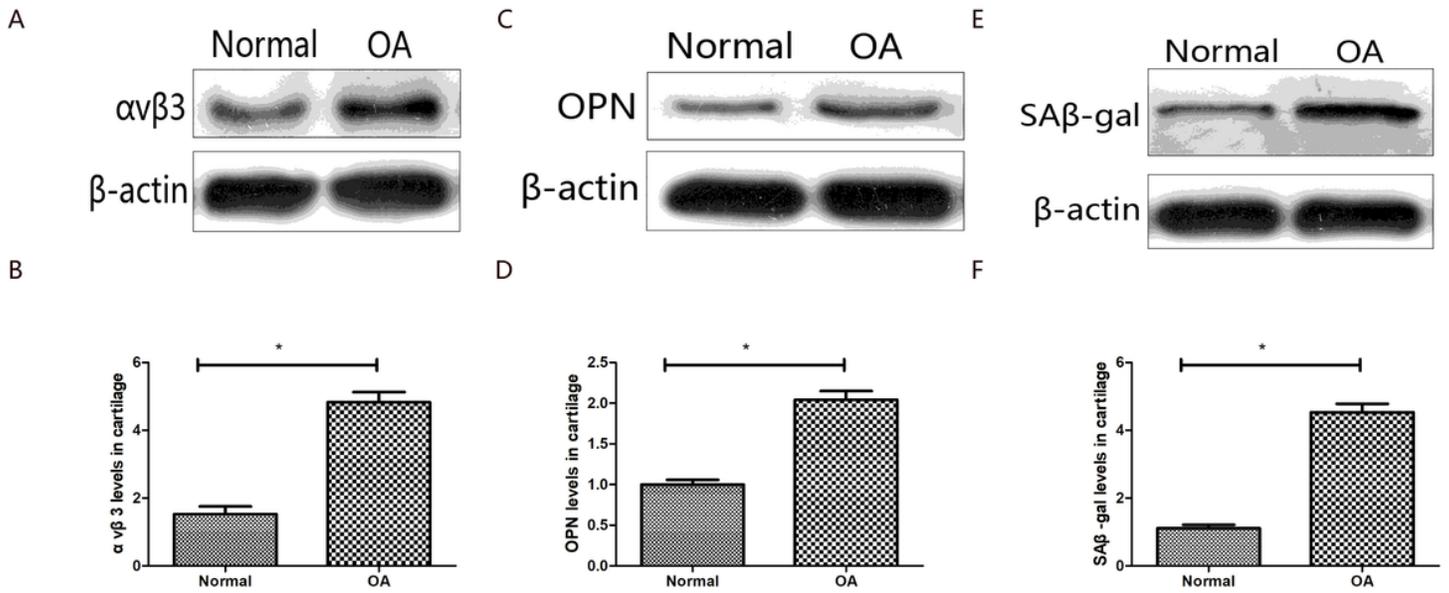


Fig-2

Figure 2

A The $\alpha v\beta 3$ protein expression in normal and OA cartilage. B The $\alpha v\beta 3$ protein expression in normal and OA cartilage was significant difference . (* means $P < 0.05$, versus normal group). C The OPN protein expression in normal and OA cartilage. D The OPN protein expression in normal and OA cartilage was significant difference . (* means $P < 0.05$, versus normal group). E The SA β -gal protein expression in normal and OA cartilage. F The SA β -gal protein expression in normal and OA cartilage was significant difference . (* means $P < 0.05$, versus normal group).

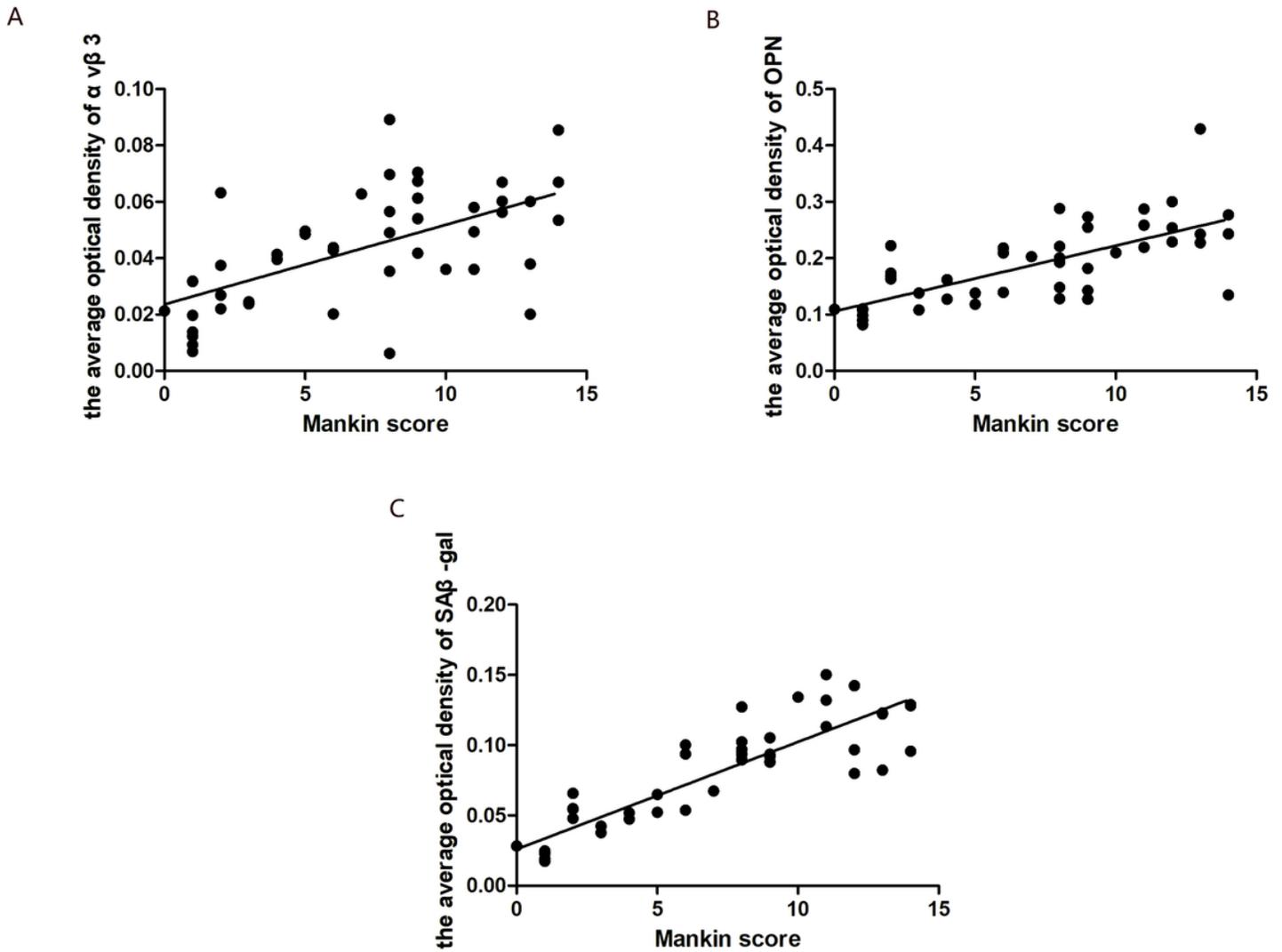


Fig-3

Figure 3

A the correlation coefficient between the average optical density value of $\alpha v\beta 3$ and the Mankin score was $r=0.60$, $p<0.001$ B the correlation coefficient between the average optical density value of OPN and the Mankin score was $r=0.75$, $p<0.001$ C the correlation coefficient between the average optical density value of SA β -gal and the Mankin score was $r=0.87$, $p<0.001$

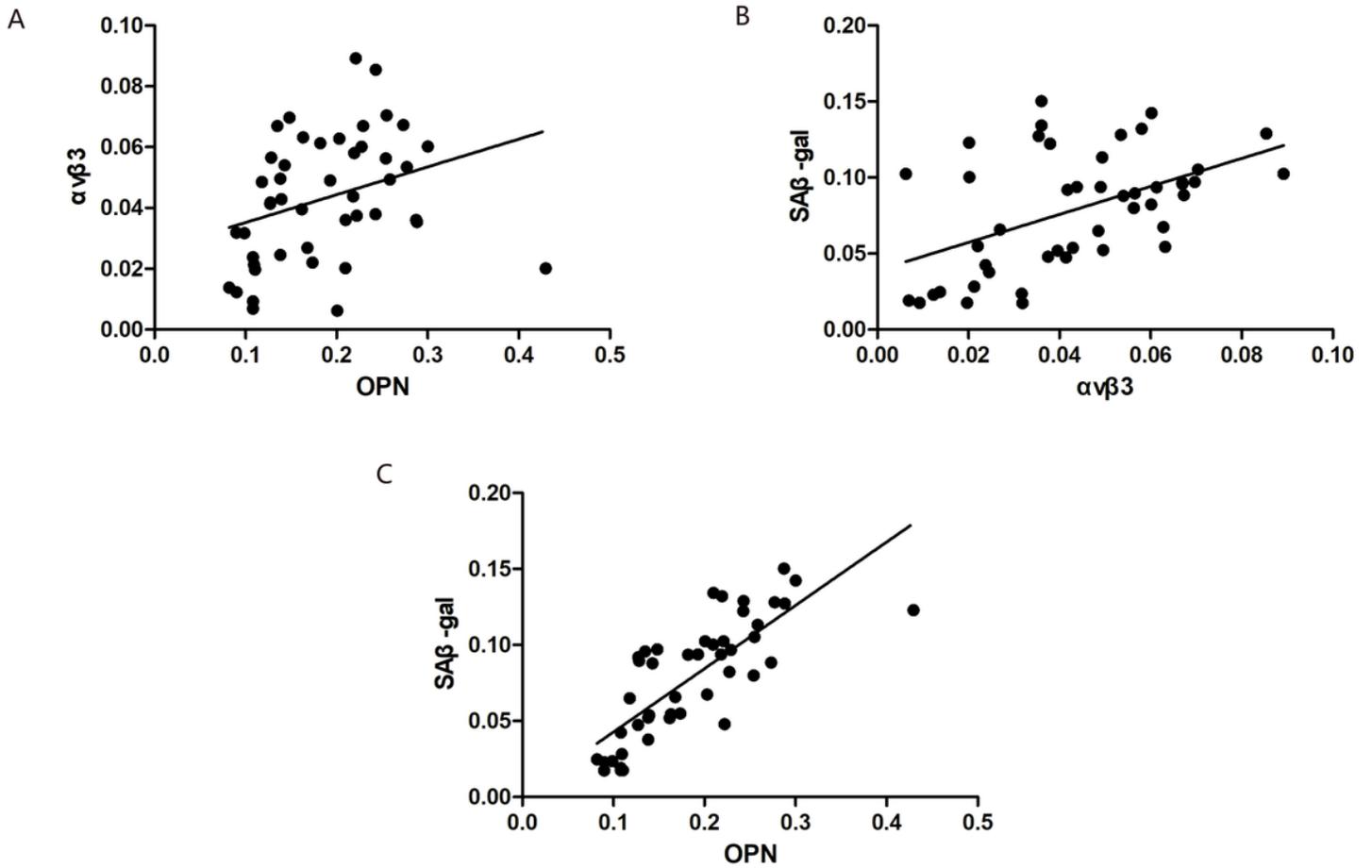


Fig-4

Figure 4

A the correlation between the average optical density value of $\alpha\beta3$ and OPN was $r=0.3191$, $p=0.0306$, $p<0.05$ B the correlation between the average optical density value of $\alpha\beta3$ and SA β -gal was $r=0.4955$, $p<0.001$ C the correlation between the average optical density value of OPN and SA β -gal was $r=0.7821$, $p<0.001$

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- [Fig1COPN.tif](#)
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- [renamed3499b.tif](#)