Clinical Value of Serum β-Catenin Protein in Monitoring of Progress in Patients with Osteonecrosis of the Femoral Head

Junyuan Huang
First Affiliated Hospital of Guangzhou University of Chinese Medicine, Guangzhou
Yingchun Zhou
First Affiliated Hospital of Guangzhou University of Chinese Medicine, Guangzhou
Wei Xiao
Department of Pathogen Biology, Shenzhen University School of Medicine, Shenzhen
Peng Deng
First Affiliated Hospital of Guangzhou University of Chinese Medicine, Guangzhou
Weiguo Lu (3897988@qq.com)
First Affiliated Hospital of Guangzhou University of Chinese Medicine, Guangzhou

Research Article

Keywords: Serum β-Catenin Protein , Osteonecrosis , Femoral Head

Posted Date: May 26th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-530304/v1

Abstract

Background

Wnt/β-catenin signaling pathway is closely related to the pathogenesis Osteonecrosis of the femoral head (ONFH). β-catenin, as a major component of Wnt signaling pathway, plays a vital role in the proliferation of osteoblasts. But the effect of altering β-catenin level on the early diagnosis and progress of ONFH have not been studied. Our purpose was to investigate the role of β-catenin level in the progress of ONFH.

Method

One hundred and one patients with three stages of ONFH and fifty healthy controls were recruited between May 2016 and November 2016. We divided the patients into 32 cases of stage II, 41 cases of stage and 28 cases of stage IV according to the Association Research Circulation Osseous (ARCO) classification. We evaluated the clinical bone histomorphology, expression position and level of β-Catenin as well as the plasma β-catenin level.

Results

We found that serum β-catenin level was significantly higher in the ONFH group than those in healthy control group (P<0.001). We also found that the area under the curve (AUC) calculated by ROC curve analysis to determine the values for β-catenin levels in ONFH compared with those in the control group was 0.9358(P<0.001), where the sensitivity was 77.23% and specificity was 98.00%.

Conclusion

Our results indicated that the increased β-catenin may play a vital role in the progress of ONFH. The cutoff concentration may be used as one of the sensitive signs to assess the disease process of ONFH.

1. Introduction

Osteonecrosis of the femoral head (ONFH) is a common orthopedic disease with high disability rate[1], and this devasting disease is gradually becoming a global heathy problem. The etiology of ONFH includes traumatic and non-traumatic causes. It is an accepted acknowledged that non-traumatic ONFH may associate with several risk factors including long-term steroid treatment, excess alcohol consumption, dysbarism, genetic mutation, smoking and autoimmune disease[2–6]. Although the exact mechanisms of ONFH are now remain in dispute, but the osteocyte apoptosis is recognized highly associated with osteonecrosis.

Wnt pathways are critical in regulating cell proliferation, apoptosis and differentiation, which are contribute to the pathologies of many diseases[7]. The canonical Wnt/β-catenin pathway is involved in regulating the process of bone formation and remodeling. β-catenin, as a major component of Wnt

signaling pathway, plays a vital role in the proliferation of osteoblasts[8]. The expression of β-catenin was inhibited in ONFH. Suppressing β-catenin and Wnt signal-related molecular activities in osteoblasts and angiogenesis obviously reduces bone mass[9, 10]. And femoral head collapse always occurs after bone mass begin changed. But the effect of altering β-catenin level on progress of ONFH has not been studied. This study aimed at investigating the level of β-catenin in patients with ONFH, comparing the difference in various stages, revealing the potential relationship with disease progression.

2. Materials And Methods

2.1 Study population and sample collection

This prospective cross-sectional case-control study was conducted in 101 ONFH patients from the First Affiliated Hospital of Guangzhou University of Chinese Medicine who were diagnosed via history, Xray examinations and MRI between May 2016 and November 2016. Patients with possible confounding factors, including congenital diseases, smoking, renal dysfunction, HIV infection, diabetes mellitus, cancer and other factors were excluded. According to the ARCO staging system[11], patients were divided into 32 cases of stage II, 41 cases of stage Ⅲ and 28 cases of stage IV. Plasma was collected from patients before the arthroplasty. 50 control plasmas were collected from healthy volunteers who get medical examination during the same period. The general background of the ONFH patients and healthy controls were shown in Table 1. ONFH bone sections (n=18 totals) were obtained after THA, while bone samples in the control group with femoral neck fracture (n=6 totals) were collected. The weight-bearing zone in cartilage tissue was chosen for analysis. Bone samples were collected from healthy tissue or subchondral necrotic zone at 1-4mm below the cartilage[12-14].

Table 1

General background

	ONFH	Controls	*P* value
Age(mean±SD)	42.77±14.646	38.24±11.191	>0.05
Gender			
Male	52	28	>0.05
Female	49	22	

2.2 Bone morphology observed by H&E staining

Necrotic bone samples collected from necrotic area and healthy control femoral head were fixed in 4% formaldehyde for 24h at room temperature. After being decalcified by subsequently using 10% EDTA over 2 weeks, the samples were embedded in paraffin wax. Specimens cut longitudinally into 5-μm sections

were stained with hematoxylin-eosin(H&E). The stained sections were observed through the microscope (BX53, Olympus Corp., Japan)[12-14].

2.3 Immunohistochemistry for β-catenin

Immunohistochemistry was used to further analyze the expression of protein β-catenin. The femoral head tissue sections obtained from previous sections/2.2 were processed by avidin-biotin-peroxidase complex (ABC) to detect the presence of activated β-catenin. After being deparaffinized in xylene and rehydrated by a graded series of alcohols to water, and then incubated in 0.3 % H2O2 for 1h to quench the endogenous peroxidase activity the sections were incubated with primary rabbit anti-human β-catenin antibody (1:200; Santa Cruz Biotechnology, USA) in a solution consisting of 1% bovine serum albumin and 0.05% sodium azide in 0.1M PBS for 24h at 4°C. After three washes with PBS, the specimens were exposed to biotinylated goat anti-rabbit IgG diluted 1:200 in PBS for 4 h at room temperature. Next, the peroxidase reaction was developed for 10 min in 0.05M Tris buffer (pH 7.6). Slides were covered with DEPEX mounting medium, and observed under a microscope (BX53, Olympus)[12-14].

2.4 Western blotting for β-catenin

The bone samples were washed by 0.9% NaCl and PBS and lysed by NET-Triton lysis buffer. Aliquots of lysates were electrophoresed on SDS-PAGE with Tris-glycine running buffer and then the proteins were transferred to poly membranes (BioRad). Nonspecific binding of the antibodies to the membrane was blocked by a one hour incubation with TBS/Tween20 (0.05 mM Tris, 0.15 mM NaCl, pH 7.6; 1% Tween 20) containing 5% w/v non-fat dried milk for 1h and were then incubated in TBS/Tween20 with 5% w/v non-fat dried milk supplemented with specific rabbit anti-human β-catenin antibody (1:1000; Santa Cruz Biotechnology, USA) overnight at 4°C. Horseradish peroxidase-conjugated anti-β-actin (Santa Cruz Biotechnology, USA) was used as loading control. Signals were detected using a diluted (1:5000) secondary polyclonal antibody (goat anti-rabbit conjugated with peroxidase) and the membranes were immersed in ECL detection solution (Santa Cruz, USA). The protein bands were quantified using an Epson GT-8000 laser scanner[12-14].

2.5 β-catenin level measurements by Enzyme-linked immunosorbent assay

Levels of β-catenin in plasma were analyzed by a commercial sandwich enzyme-linked immunosorbent assay (ELISA) (IBL, Germany). The concentration of β-catenin in the samples was determined by comparing the O.D. of the samples to the standard curve. ROC curves were used to draw data from the results obtained in this study, and the cut-off value was set to provide optimal diagnostic accuracy and likelihood ratios for the level of β-catenin[12-14].

2.6 Statistical analysis

Statistical analysis was performed using SPSS 23.0 and GraphPad Prism v7.0. Nonpaired t-tests with the significance level of $p<0.05$ were used for statistical analysis.

2.7 Ethical approval

All patients and healthy volunteers signed informed Consent. This study was carried out with the approval of the responsibility by the Ethics Committee of the First Affiliated Hospital of Guangzhou University of Chinese Medicine.

3. Results

3.1 Radiography and pathology evaluation of ONFH patients and control subjects

Figure 1A-D show the X-ray results from normal control group and ONFH patients. Figure 1A shows a normal joint space in the femoral head, which also has a regular shape and uniform spherical density. Figure 1B shows the nonuniform density, disappearance of local bone trabeculae, and mildly narrowed joint space, which was corresponding to ARCO stage II. Figure 1C shows the collapse of the articular surface, osteosclerosis and the preservation of joint space, which was a sign of stage III. Figure 1D shows acetabulum changes, subchondral collapse and degenerative arthritis. Figure 1E-H show the general appearance of femoral head sections. Figure 1E shows the homologous trabecular bone of the control subject without any evident destruction. Figure 1F shows the disorganized bone trabeculae and rough surface cartilage in the necrotic region. Figure 1G and 1H show the distinct collapse of the femoral head. Furthermore, the deteriorated and severely destroyed, even obvious stripped in the cartilage structure has shown in Figure 1H. Images from HE staining are shown in Figure 1I-L. Figure 1I shows a healthy and complete trabecular bone structure with lots of osteocytes embedded in the control bone samples. Figure 1J-K show that with the increase in the stages, the bone trabeculae displayed increasing empty lacunae resulting from the loss of osteocytes. Figure 1M shows that the ratio of empty lacunae in the control group was significantly lower than that in ONFH groups of each stage ($p<0.001$). Although no differences were found between stage III and stage IV ($p>0.05$), the ratio of empty lacunae in both stages III and IV were higher than that in stage II ($p<0.001$).

3.2 Immunohistochemistry for β-catenin

Both necrotic cartilage region and healthy region of the femoral head positive for β-catenin are evaluated by immunohistochemical staining (Figure 2). Intact articular cartilage and low-level presence of β-catenin were observed in healthy samples (Fig 2.A). The level of β-catenin was increased among with ARCO stage

progress and was accompanied by the rise of empty bone lacuna (Figure 2B-D). Furthermore, with the progression of disease, the superficial layer of articular cartilage had become rough, disordered and even structurally disappeared. group and ONFH group with different stages.

3.3 Western blot quantitative analysis of ONFH patients and control subjects

The expression of β-catenin was determined by Western blotting (Figure 3). The level of β-catenin in cartilage samples of ONFH patients was significantly higher than those in control group (P<0.001). Among the three ARCO stages, the level of β-catenin in ONFH patients increased following the severity of X-ray findings increased.

3.4 Plasma β-catenin level quantity of ONFH patients and control subjects

The result of plasma β-catenin level measured by ELISA and potential relation between other clinical data was shown in Table 2. The plasma β-catenin level was significantly higher in ONFH patients compared with control subjects (Figure 4A). The level of β-catenin was significantly different among patients with ONFH at various ARCO stages (p<0.001). According to multiple comparisons, difference of plasma β-catenin level was found between stage II and stage III (p<0.01), and between stage II and stage IV (p<0.001) (Figure 4B). In addition, β-catenin level in post-collapse patients was higher than that in pre-collapse patients (Figure 4C). The area under the curve (AUC) which was calculated by ROC curve analysis to determine the values for β-catenin levels in ONFH compared with those in the control group was 0.9358 (p<0.0001), where the sensitivity was 77.23% and specificity was 98.00% (cutoff, 45.99 pg/ml) (Figure 4D).

Table 2

Plasma β-catenin levels in ONFH patients and control subject and potential relation between other clinical data. Data presented as mean ± SD.

Groups	Cases	β-catenin level (pg/mL)	Comparison	*P* value
Control	50	20.14 ± 1.715	Control vs ONFH	< 0.001
ONFH	101	66.99 ± 3.032		
Pre-collapse	32	49.30 ± 4.649	Pre-collapse vs Post-collapse	< 0.001
Post-collapse	69	75.20 ± 3.478		
ARCO stages				< 0.001
Stage II	32	49.30 ± 4.649	II vs III	< 0.01
Stage III	41	72.54 ± 4.864	III vs IV	>0.05
Stage IV	28	79.10 ± 4.773	II vs IV	< 0.001
Etiology				0.0821
Alcohol-induced (a)	41	71.61 ± 4.397		
Steroid-induced (b)	32	55.57 ± 5.988		
Idiopathic (c)	12	72.85 ± 6.443		
Traumatic (d)	16	73.61 ± 7.618		

4. Discussion

In the present study, we investigated the relationship between β-catenin levels and disease process in patients with ONFH. We found that in both protein level and plasma level, β-catenin was significantly higher in the ONFH group, especially in the post-collapse patients than that in control group, and it was positively associated with ARCO stages, but not with etiology. To the best of our knowledge, this is the first study effectively explain the correlation between β-catenin level and the disease severity of ONFH. Our finding suggests that β-catenin could possibly be used as a biomarker to assess the progress of ONFH.

Wnt/β-catenin signaling pathway which regulate the differentiation of bone marrow mesenchymal stem cells is closely related to the regulation of bone balance [15]. β-catenin mainly exists in the cytoplasm, but few of them also exists in the cell membrane and the nucleus. And as the core targe and important regulator, it plays a crucial role in Wnt/β-catenin pathway. In the normal Wnt/β-catenin signaling pathway, β-catenin will pass from the cytoplasm into the nucleus for promoting the proliferation and differentiation

of osteoblast, and keeping bone balance. On the contrary, when Wnt signaling pathways was suppressed, β-catenin will be degraded in the cytoplasm, thus weakening the proliferation and differentiation of osteoblast, causing a decline in bone mass[16]. However, many studies in recent years have shown that Wnt/β-catenin signaling pathway has different effects on osteoblasts of different differentiation stages, and it needs precise regulation to maintain bone balance[17, 18]. At present, numerous studies indicated that ONFH is related to osteocyte apoptosis and bone remodeling, with increased osteocyte apoptosis and bone resorption in the necrotic area of the femoral head, but increased osteoblast activity in the sclerotic area around the necrotic area[19-21], these results were consisted with the phenomenon we found in the present study. According to the results of immunohistochemistry and plasma, our research also showed that the expression of β-catenin in ONFH patients was higher than that in normal control group, and it was positively associated with ARCO stages. At the early stage of ONFH, corresponding to ARCO □, bone cell apoptosis was the main manifestation of femoral head lesions. Therefore, the expression of β-catenin might be decreased. Due to most patients at the early stage of ONFH were asymptomatic and very rare in clinical, they were not enrolled in our research., we will further study this problem. However, sclerosis rim can be found in X-ray film at the stage of ARCO II, which mean the osteoblast activity was enhanced. Thus, in theory, as the disease progresses, the osteoblast activity increases and so does the expression level of β-catenin. Although some animal experiments have shown that the expression of β-catenin in rats with femoral head necrosis was decreased[22-24], but this was not contradictory, because these animal experiments were only reflect the situation of early stage of ONFH.

In addition, the results from X-ray film, view of femoral head section and HE staining together showed that the pathological features of ONFH are apoptosis of osteocytes and rising number of lacunae. The ratio of lacunae was increased as the disease proceeded, which confirmed that the samples collected were trustworthy and can prove the pathological alterations in different stages. Although the exact mechanisms of ONFH are now still in dispute, but the femoral head cartilage damage caused by osteocyte apoptosis is seen highly associated with osteonecrosis[25].

There are certain limitations in this study. First, we have not evaluated the levels of β-catenin in stage I, which makes it difficult to calculate the early stage of ONFH. Second, a relatively small sample has limited the accuracy of the research. Even with these limitations, our study first demonstrated that β-catenin could possibly be used as a signal of disease progression in ONFH.

Declarations

Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and national research committee and all methods were carried out in accordance with relevant guidelines and regulations.

Consent for publication

Not applicable.

Availability of data and materials

The datasets and materials are available from the corresponding author on reasonable request.

Competing interests

The authors have no conflict of interest to declare.

Funding

This study was partially supported by project from Guangdong Science and Technology Department [NO.KTP2020346].

Authors' contributions

Weiguo Lu conceived the study design. Junyuan Huang and Yingchun Zhou designed methods and experiments, carried out the laboratory experiments, analyzed the data, interpreted the results and wrote the paper. Wei Xiao made the statistical analysis of the databases. Weiguo Lu revised the manuscript critically for important intellectual content. All authors read and approved the final manuscript.

Acknowledgements

The authors are grateful to their laboratory colleagues for the specimens they provided.

References

1. ZHANG Y, LIU Y, ZHOU G, et al. Fibular Allograft for Osteonecrosis Prevention in the Management of Femoral Neck Fractures: Clinical Outcome and Biomechanical Evaluation [J]. Journal of Biomaterials and Tissue Engineering, 2015,
2. MICROSURGERY DEPARTMENT OF THE ORTHOPEDICS BRANCH OF THE CHINESE MEDICAL DOCTOR A, GROUP FROM THE O, BONE DEFECT BRANCH OF THE CHINESE ASSOCIATION OF R, et al. Chinese Guideline for the Diagnosis and Treatment of Osteonecrosis of the Femoral Head in Adults [J]. Orthopaedic surgery, 2017, 9(1): 3–12.

3. MONT M A, JONES L C, HUNGERFORD D S. Nontraumatic osteonecrosis of the femoral head: ten years later [J]. Journal of Bone & Joint Surgery American Volume, 2006, 88(5): 1117–32.
4. ZHAO D W, YU M, HU K, et al. Prevalence of Nontraumatic Osteonecrosis of the Femoral Head and its Associated Risk Factors in the Chinese Population: Results from a Nationally Representative Survey [J]. Chinese Medical Journal, 2015, 128(21): 2843–50.
5. GANGJI V, ROOZE M, MAERTELAER V D, et al. Inefficacy of the cementation of femoral head collapse in glucocorticoid-induced osteonecrosis [J]. International Orthopaedics, 2009, 33(3): 639–42.
6. WESSEL J H, DODSON T B, ZAVRAS A I. Zoledronate, smoking, and obesity are strong risk factors for osteonecrosis of the jaw: a case-control study [J]. J Oral Maxillofac Surg, 2008, 66(4): 625–31.
7. MOHAMMED M K, SHAO C, JING W, et al. Wnt/β-catenin signaling plays an ever-expanding role in stem cell self-renewal, tumorigenesis and cancer chemoresistance [J]. Genes & Diseases, 2016, 3(1): 11–40.
8. PEI J, FAN L, NAN K, et al. Excessive Activation of TLR4/NF-κB Interactively Suppresses the Canonical Wnt/β-catenin Pathway and Induces SANFH in SD Rats [J]. Rep, 2017, 7(1): 11928.
9. YU Z, FAN L, LI J, et al. Lithium prevents rat steroid-related osteonecrosis of the femoral head by β-catenin activation [J]. Endocrine, 2016, 52(2): 380–90.
10. FUWEN, ZHANG, KUN, et al. miR-29a promotes osteoblast proliferation by downregulating DKK-1 expression and activating Wnt/β-catenin signaling pathway [J]. Advances in clinical and experimental medicine: official organ Wroclaw Medical University, 2019, 28(10): 1293–300.
11. GARDENIERS J W M. The Arco Perspective for Reaching One Uniform Staging System of Osteonecrosis [J]. Bone Circulation & Vascularization in Normal & Pathological Conditions, 1993,
12. GONG S-D, CHEN X-J, CHEN Z-Q, et al. Elevated plasma cartilage oligomeric matrix protein (COMP) level are associated with the progression of non-traumatic osteonecrosis of femoral head [J]. Clinica Chimica Acta, 2019, 490(214 – 21.
13. CHEN X J, YANG F, CHEN Z Q, et al. Association of reduced sclerostin expression with collapse process in patients with osteonecrosis of the femoral head [J]. Int Orthop, 2018, 42(7): 1675–82.
14. HE M, GONG S D, CHEN X J, et al. Plasma C-terminal cross-linking telopeptide of type II collagen as a biomarker in advanced stages of femoral head osteonecrosis [J]. Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie, 2019, 111(1213–20.
15. MAJIDINIA M, AGHAZADEH J, JAHANBAN-ESFAHLANI R, et al. The roles of Wnt/beta-catenin pathway in tissue development and regenerative medicine [J]. Journal of cellular physiology, 2018, 233(8): 5598–612.
16. REGARD J B, ZHONG Z, WILLIAMS B O, et al. Wnt signaling in bone development and disease: making stronger bone with Wnts [J]. Cold Spring Harbor perspectives in biology, 2012, 4(12):
17. GLASS D A, BIALEK P, AHN J D, et al. Canonical Wnt Signaling in Differentiated Osteoblasts Controls Osteoclast Differentiation [J]. Developmental Cell, 2005, 8(5): 751–64.

18. EIJKEN M, MEIJER I M, WESTBROEK I, et al. Wnt signaling acts and is regulated in a human osteoblast differentiation dependent manner [J]. Journal of cellular biochemistry, 2008, 104(2): 568–79.
19. MUTIJIMA E, DE MAERTELAER V, DEPREZ M, et al. The apoptosis of osteoblasts and osteocytes in femoral head osteonecrosis: its specificity and its distribution [J]. Clinical rheumatology, 2014, 33(12): 1791–5.
20. RONY L, PERROT R, HUBERT L, et al. Osteocyte staining with rhodamine in osteonecrosis and osteoarthritis of the femoral head [J]. Microscopy research and technique, 2019, 82(12): 2072–8.
21. WANG C, WANG X, XU X L, et al. Bone Microstructure and Regional Distribution of Osteoblast and Osteoclast Activity in the Osteonecrotic Femoral Head [J]. Plos One, 2014, 9(5): e96361-.
22. ZHANG C, ZOU Y-L, MA J, et al. Apoptosis associated with Wnt/β-catenin pathway leads to steroid-induced avascular necrosis of femoral head [J]. BMC musculoskeletal disorders, 2015, 16(1):
23. PEI J, FAN L, NAN K, et al. Excessive Activation of TLR4/NF-kappaB Interactively Suppresses the Canonical Wnt/beta-catenin Pathway and Induces SANFH in SD Rats [J]. Scientific reports, 2017, 7(1): 11928.
24. LIU G, LUO G, BO Z, et al. Impaired osteogenic differentiation associated with connexin43/microRNA-206 in steroid-induced avascular necrosis of the femoral head [J]. Experimental and molecular pathology, 2016, 101(1): 89–99.
25. MAGNUSSEN R A, GUILAK F, VAIL T P. Cartilage degeneration in post-collapse cases of osteonecrosis of the human femoral head: altered mechanical properties in tension, compression, and shear [J]. Journal of Orthopaedic Research, 2010, 23(3): 576–83.

Figures

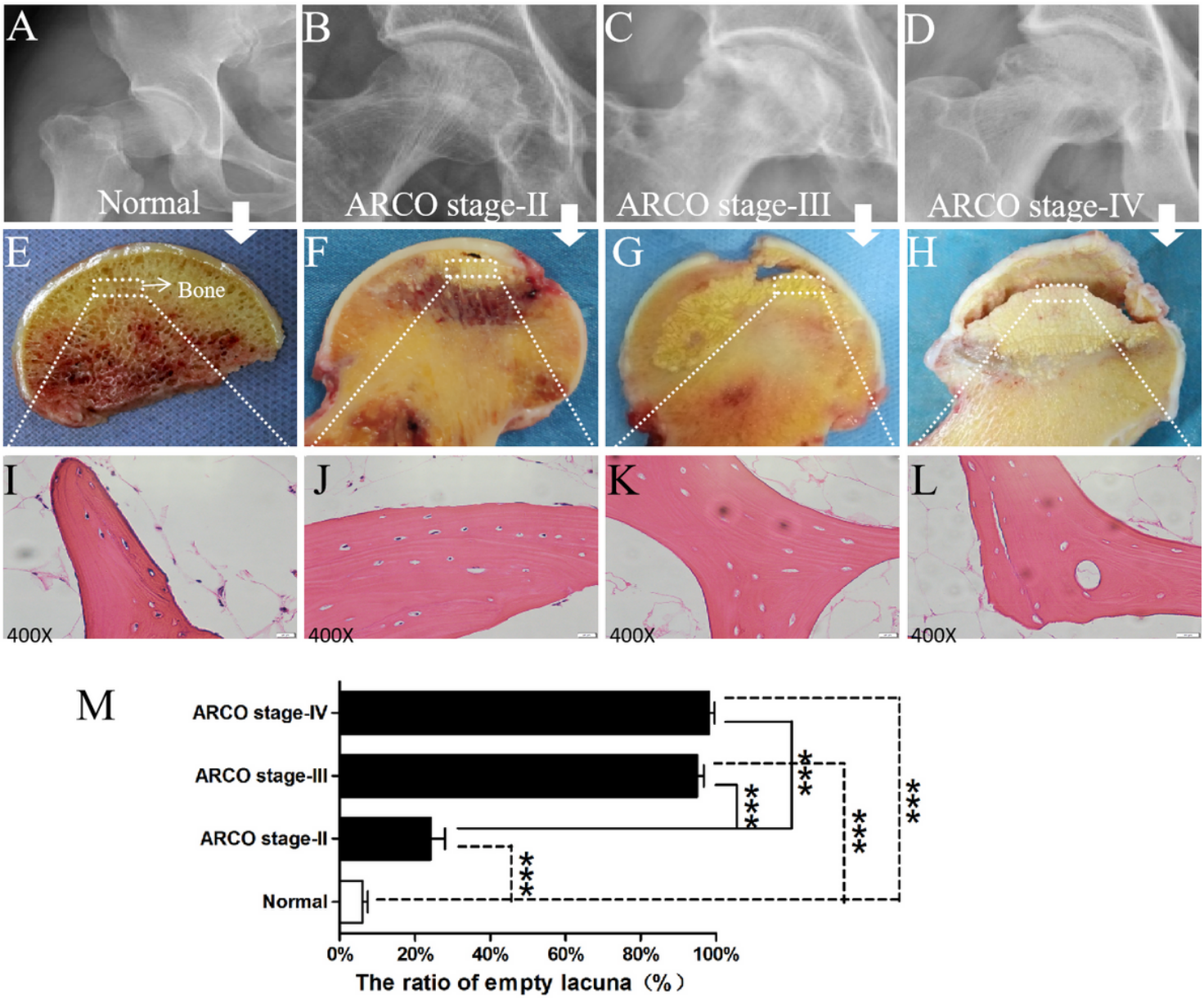


Figure 1

A-D. X-ray images of control subject and ONFH patients with different ARCO stage. Figure 1E-H. General appearance in the bone and cartilage samples of control subject and ONFH patients with different ARCO stage. The white dashed boxes indicated the regions collected for further analysis. Figure 1I-L. Histopathological features of control and ONFH bone. *** represents p<0.001.

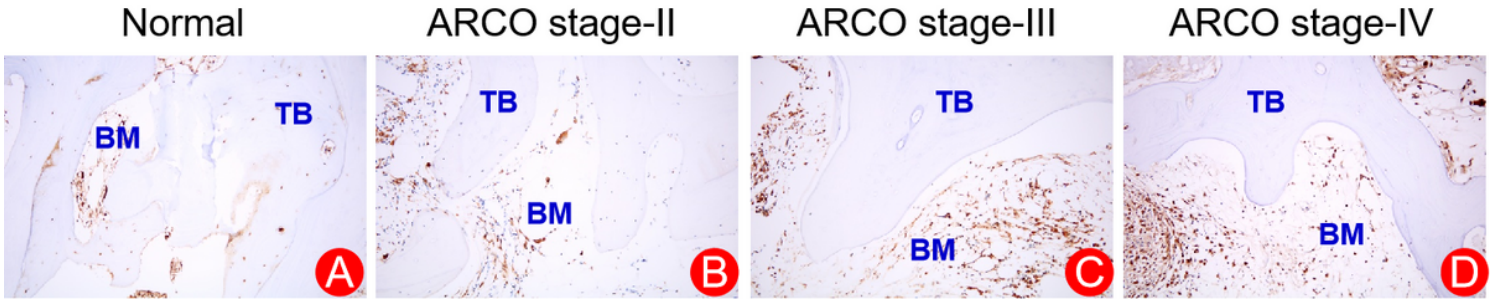
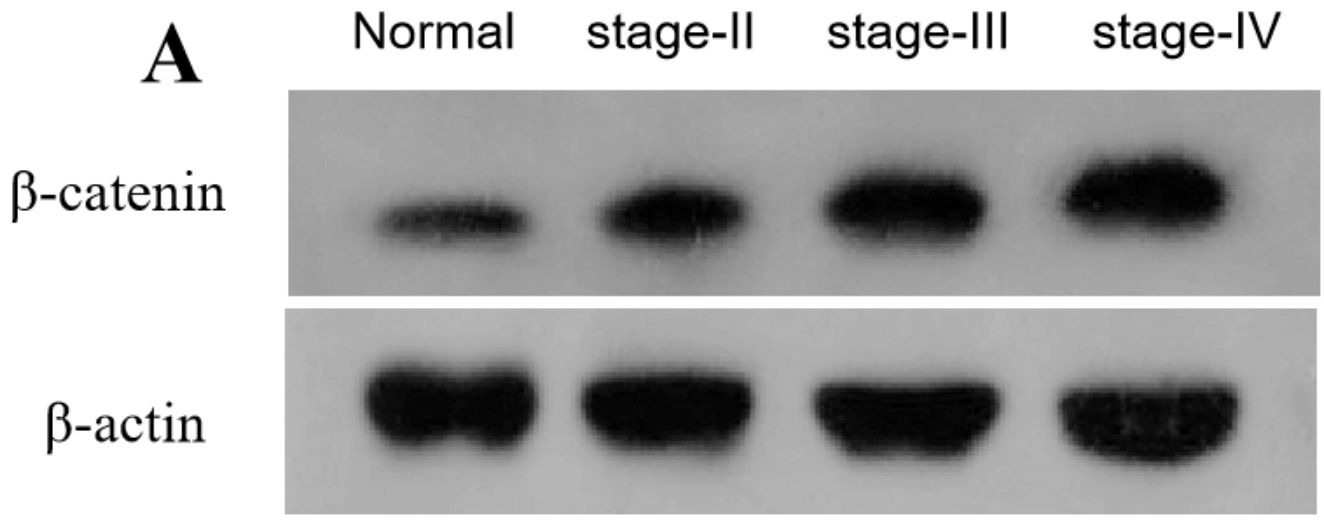


Figure 2

Immunohistochemistry results for β-catenin of cartilage samples in control group and ONFH group with different stages.



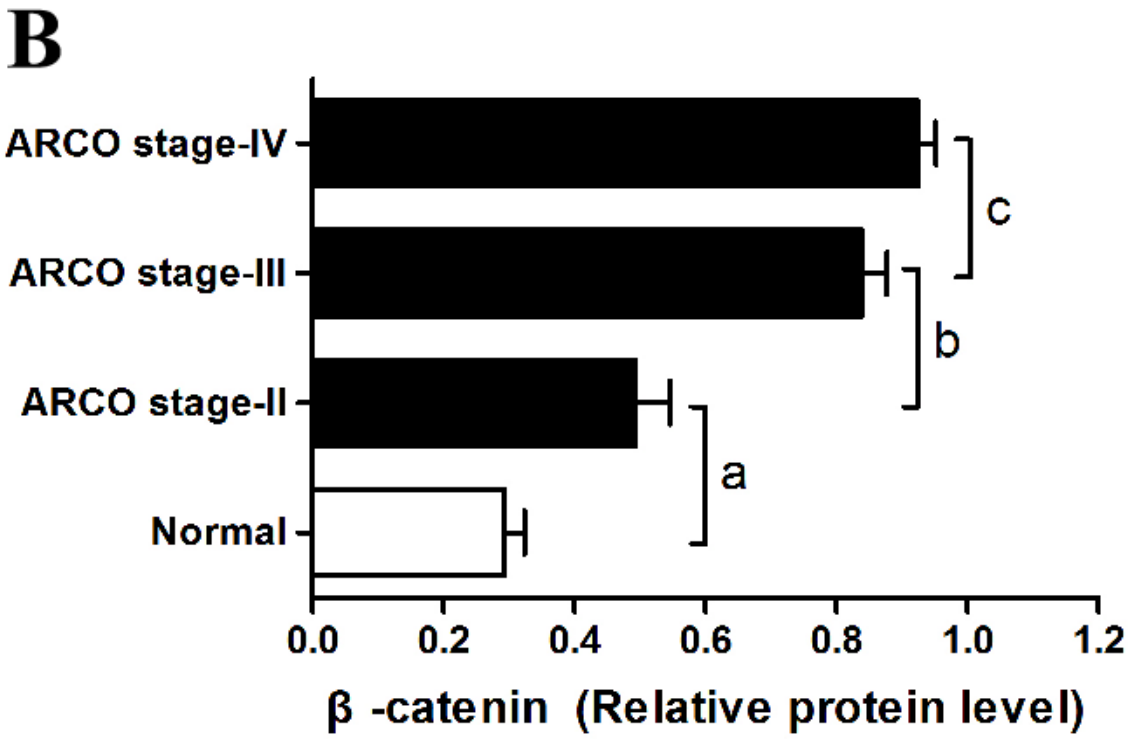


Figure 3

Western blot analysis of cartilage samples for the β-catenin. Level of β-catenin is increased with the ARCO stage (A). The histogram represents western blotting analysis (B). Values are the means ± SEM. ap<0.001 vs the control group; bp<0.001 vs the stage II group; cp<0.001 vs the stage III group.

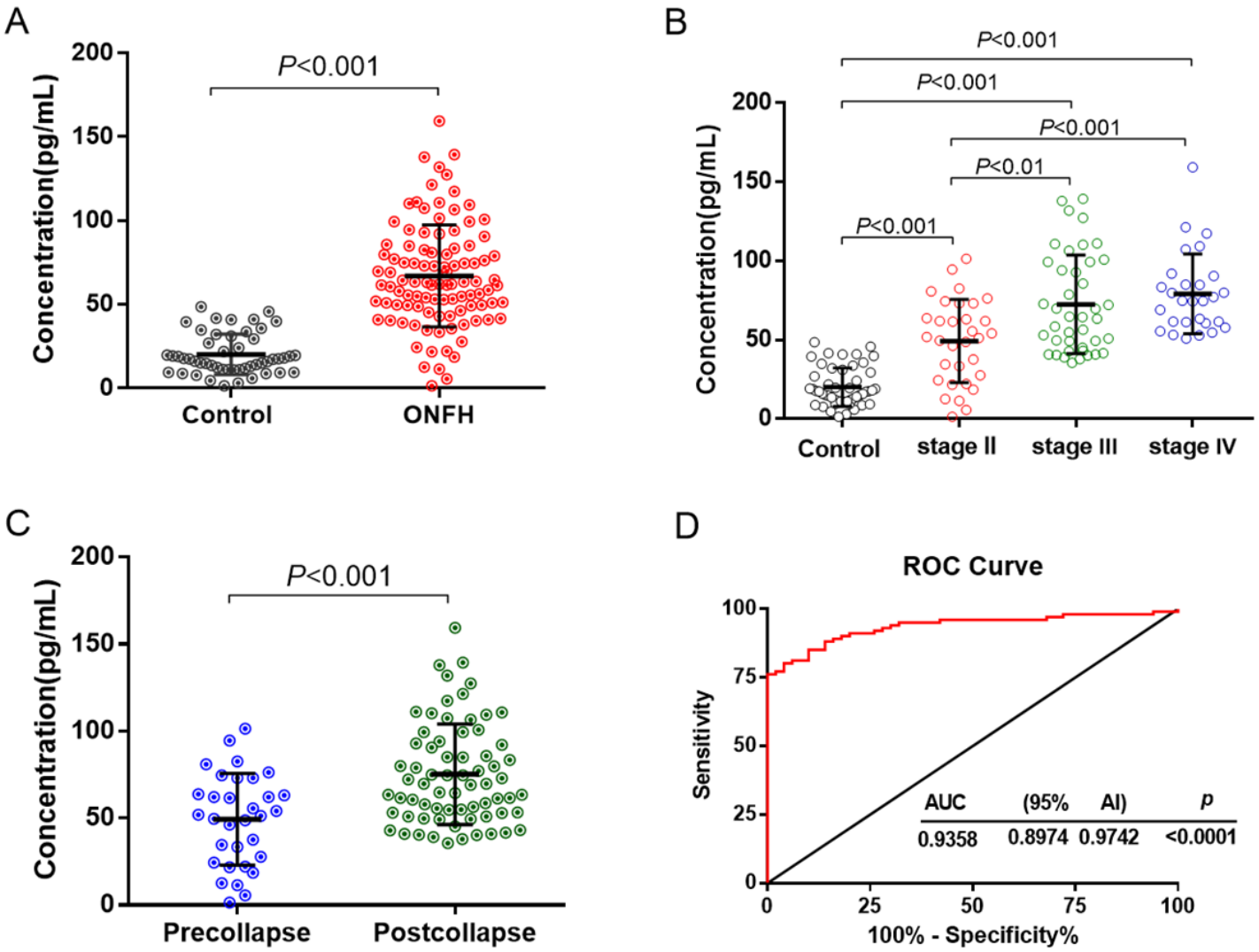


Figure 4

Overview shows plasma β-catenin level in ONFH patients and control individuals. β-catenin levels with statistical differences among different groups(A-C). Receiver operating characteristic (ROC) curve and the area under the curve (AUC) in association with the sensitivity and specificity of ONFH (D).