RESEARCH ARTICLE

High Serum Alpha-2-Macroglobulin Level in Patients with Osteonecrosis of the Femoral Head

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Abstract

Background: Diagnosis of osteonecrosis of the femoral head (ONFH) is complicated due to the lack of reliable serum biomarkers. Up-regulation of alpha-2-macroglobulin (A2M) gene has been reported in glucocorticoid-induced ANFH rat model. This study aimed to investigate whether the serum level of alpha-2-macroglobulin (A2M) can be used for ONFH diagnosis.

Methods: Serum protein capillary electrophoresis was performed on the sera of 36 ONFH patients. Also, human enzyme-linked immunosorbent assay was performed to evaluate the serum levels of A2M.

Results: Alpha-2 subunit level, composed of alpha-2-macroglobulin, ceruloplasmin and 2-2 haptoglobin phenotype, was increased significantly as compared to healthy subjects ($P=0.0001$). Moreover, ELISA assay confirmed significant elevation in the A2M ($P=0.037$).

Conclusion: Our findings suggest that avascular necrotic femur head presumably directly or indirectly elevates A2M in the bloodstream. Thus, serum level of A2M might be used as a reliable diagnostic tool in clinical practice.

Level of evidence: II

Keywords: Alpha-2-macroglobulin, Avascular necrosis of femoral head, Femur, Osteonecrosis

Introduction

Osteonecrosis of the femoral head (ONFH) has been considered as a devastating disease and a common cause of musculoskeletal disability (1, 2). Avascular necrosis (AVN), aseptic necrosis of bone, osteochondritis dissecans, and ischemic necrosis are different names contributing to this condition and indicating that the etiology of the disease is not entirely known (3, 4). Most ONFH patients are initially asymptomatic with no changes in their plain radiography; however, the necrosis progresses to total destruction of the hip joint in a relatively short time, usually before the fifth decade of life (1). In most patients with a lesion less than 30% of the femoral head, ONFH remains asymptomatic for more than 5 years. However, a poor prognosis with a rate of femoral head collapse greater than 85% at 2 years has been reported in symptomatic painful ONFH (5). Approximately 5% to 18% of the 500,000 total hip replacement (THR) procedures performed annually in the United States are related to ONFH (6)). Although the actual prevalence of the disease remains undetermined,

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an estimated of 10,000 to 20,000 new cases were reported each year in the United States and 300,000 to 600,000 people suffer from this disease in total there (7). Noticeably, this disease is more common among the Asian population than other people. In Taiwan, 46.3% of all THR surgeries are because of ONFH. Several statistical reports show a higher prevalence of the disease in Japan and Korea compared to Caucasian populations (8). In Japan 2500 to 3300 new ONFH cases are reported annually (1). A case series of 647 THR in Hong Kong revealed that ONFH accounted for 45.6% of all hip replacements. These data indicate that the disease is more prevalent in Asia and hence has been studied probably more thoroughly by Asian scientists and surgeons (9). Almost 75% of patients with ONFH are between 30 to 60 years old (6). Since most patients with the disease are at the peak of their productive years, there is a harmful effect on the workforce with direct economic outcome on their families. Basically, this condition has a severe socioeconomic impact on all populations in the world especially poor/middle socioeconomic classes among whom the high cost of hip replacement procedures are far beyond the reach (9). Several previous studies have reported high failure rates in total hip replacements in short-term and mid-term follow ups and the sufferance of patients from additional operations although new modern designs and surgeon awareness in THR have relatively improved the outcomes (10, 11). Core decompression is the most common available femoral head preserving technique, especially recommended in early stages (I and II) of the disease. It is usually employed before the collapse and fracture of the femoral head and/or neck to delay or avoid reconstructive surgery of femur. Most patients ultimately require total hip arthroplasty (stage III or higher) and no treatment method has proved to be completely effective in arresting the disease process before subchondral collapse or slowing the progression of femoral head destruction and osteoarthritis after subchondral collapse (9). Based on these conditions, a quick and thorough investigation can lead to the formulation of a correct diagnosis in time for the application of therapeutic methods, less debilitating for the patients (1). Hip-preserving therapies especially core decompression are most effective in the earliest stages of ONFH. Thus, the key to successful treatment lies in identifying the individuals at risk and quantifying the risk in terms of clinical and pathophysiological characteristics so that early diagnosis can be made before femoral head collapse. One of the reliable methods providing a dynamic and powerful approach to understand the spectrum of a disease with applications in screening, diagnosis, and prognosis is biomarker studies (12). A diagnostic biomarker for ONFH specifically in non-traumatic cases could lead to an early diagnosis and eventually an appropriate treatment.

Glucocorticoid (GC) administration is considered as the most common risk factor for non-traumatic ONFH and so far, several candidate genes had been introduced to become up-regulated in GC induced ONFH in animal models (13). Upregulation of a number of genes such as

A2M (alpha-2-macroglobulin), collagen type II alpha 1 (Col2A1) and melanoma inhibitory activity (MIA) was previously reported in ONFH animal models. Amongst the candidate genes, A2M is considered as a highly upregulated molecule in animal models of ONFH. Since A2M could be measured in the blood, it has the potency to become a serum biomarker. Taking into account the colorful roles of A2M in hemostasis, chondrogenesis and osteogenesis, in the present study, A2M is measured at the serum level in ONFH patients to assess its diagnostic biomarker applicability (14).

Materials and Methods
Population
The study was approved by the local ethical committee (IR.MUMS.fm.REC.1394.393) and a signed informed consent was obtained from each study participant prior to blood collection. Patients with longstanding history of steroid consumption and established ONFH were recruited at two regional hospitals. The diagnosis of non-traumatic ONFH was established on the basis of the clinical history and a combination of radiology, CT scan, or MRI. Patients who were admitted for surgical treatment were classified into different stages based on Ficat and Arlet classification. Patients in stage 2 and 3 received surgical core decompression of the hip and patients with late stages and painful hip underwent total hip replacement. The inclusion criteria were ONFH and glucocorticoid intake. Patients with a history of hip injury, rheumatoid arthritis and sickle cell anemia were excluded from the study.

Blood Sample collection
Blood samples of 36 ONFH patients with glucocorticoids use in two regional hospitals were collected from February 2014 to December 2015. The venous blood samples were centrifuged at 1000 x g for 15 minutes; the sera were aliquoted in labeled tubes and stored at –80°C before use.

Capillary Electrophoresis of Serum Proteins
An automated multi-capillary zone electrophoresis (CE) instrument (Capillarys®, 4.51 software version; Sebia) was used for human serum protein analysis. Briefly, using the Capillarys β1-β2+® reagent set (Sebia), the proteins were separated at 7 kV for 4 min in 15.5 cm × 25 μm fused-silica capillaries (n=8) at 35.5 °C in a pH=10 buffer with online detection at 200 nm.

Enzyme-linked Immunosorbent Assay
Enzyme-linked immunosorbent assay (ELISA) was performed for A2M protein using Abcam® human alpha 2 macroglobulin in vitro competitive ELISA kit (ab108888). The experiment was carried out according to the manufacturer's instructions. Briefly, the reagents (1X dilute M, 1X wash buffer, 1X biotinylated alpha 2 macroglobulin, and 1X conjugated SP) were initially prepared and the standards were made according to the Abcam standard dilution preparation table. Following the ELISA plate preparation, all experiments were performed in triplicates. A PerkinElmer microplate-reader (victor x5) was used to measure the absorbance

at 450 nm. Finally, data calculation was accomplished based on the standard curve.

Statistical Analysis

The mean A2M protein concentration in patients was compared with the normal mean values using one-tailed t-test. The correlation between A2M and alpha 2 was investigated with Pearson's Product-Moment Correlation test. The statistical evaluation of the results was performed using SPSS version 22.0 software (Chicago, IL, USA). All values were expressed as mean±SD. A *P≤0.05* was considered as statistically significant. The normality of distribution was checked by the Kolmogorov-Smirnov test.

Results

A total of 36 non-traumatic ONFH patients (17 males and 19 females, 39±10 years old ranging 25-60 years) were enrolled in this study. According to the clinical history and a combination of radiology, computed tomography or magnetic resonance imaging, the femur head was 50% bilaterally and 50% unilaterally involved in ONFH patients. Majority of the patients (69%) were at stage 3 of Ficat and Arlet classification. All patients had received at least one sort of GC. The clinical and biological characteristics of the patients are summarized in Table 1.
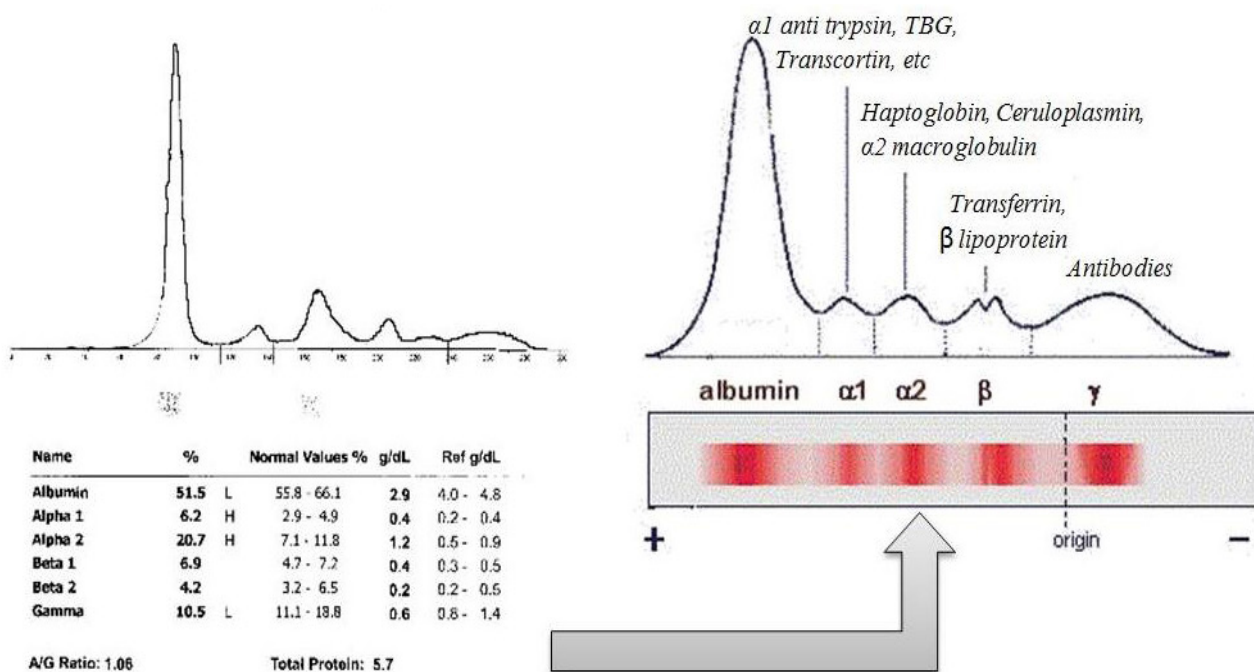
Capillary Electrophoresis of Serum Proteins

The alpha 2 zone measured by capillary electrophoresis increased significantly in ONFH patients compared with the baseline (0.5-0.9 g/dL, 9.45%) of healthy individuals, (12.40±2.97) (*P=0.0001*) [Figure 1]
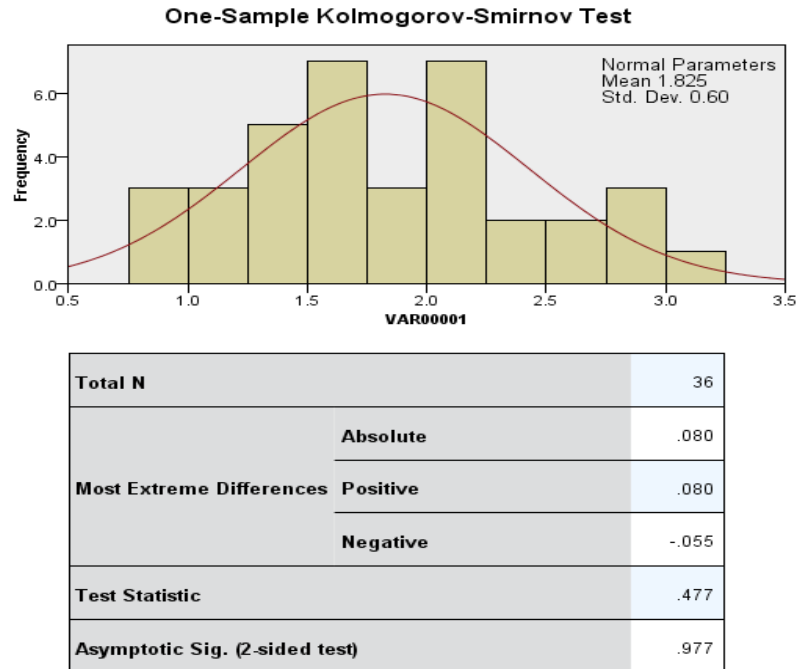
Table 1. The clinical and biological characteristics of the ONFH patients													
Number (%) and age (25-45 yr)		Consumption of GCs (%)	Femur bone involvement (%)		Drug abuse N (%)		Alcohol consumption N (%)		Chemotherapy N (%)		Renal disorders N (%)		Grade		
Male	Female		BL	UL	+		+		+		+		2	3	4
17 (47%)	19 (53%)	36 (100%)	18 (50%)	18 (50%)	16 (44%)	10 (27%)	33 (92%)	3 (8%)	32 (89%)	4 (11%)	31 (87%)	5 (13%)	5 (13%)	25 (69%)	6 (18%)

GCs: Glucocorticoids; BL: bilateranl; UL: Unilateral

Name	%		Normal Values %	g/dL	Ref g/dL
Albumin	51.5	L	55.8 - 66.1	2.9	4.0 - 4.8
Alpha 1	6.2	H	2.9 - 4.9	0.4	0.2 - 0.4
Alpha 2	20.7	H	7.1 - 11.8	1.2	0.5 - 0.9
Beta 1	6.9		4.7 - 7.2	0.4	0.3 - 0.5
Beta 2	4.2		3.2 - 6.5	0.2	0.2 - 0.5
Gamma	10.5	L	11.1 - 18.8	0.6	0.8 - 1.4

A/G Ratio: 1.06   Total Protein: 5.7

Figure 1. Capillary electrophoresis of serum patients compared with the baseline (0.5-0.9 g/dL, 9.45%) of healthy individuals (12.40±2.97) (Left); Capillary electrophoresis composition of serum (Right).

Figure 2. The statistical analysis of serum concentration of patients' A2M compared with the mean of normal A2M level.

ELISA

The Kolmogorov-Smirnov test showed no significant deviation from the normal curve for A2M data ($P=0.977$).

Serum concentration of A2M was determined using standard curve and expressed as mg/L. Patients' A2M were compared with the mean of normal A2M level indicating a range of 1.49-1.79 g/L in the ELISA kit. The results demonstrated a significant increase in the level of A2M ($P=0.037$). It was also significantly enhanced compared to the upper limit of normal A2M level ($P=0.04$). The ELISA results for antibodies against A2M correlated to alpha 2 proteins are shown in Figure 2.

Discussion

Nowadays, researchers have encountered with some limitations regarding the etiology and treatment of ONFH, including: 1) lack of understanding the interaction between the disease and the bone cellular abnormalities especially in the early stages of the disease; 2) lack of suitable animal models; and 3) lack of informative biomarker to evaluate the activity status or the prognosis of the disease (14, 15). Finding an informative biomarker that can diagnose the disease especially in its early stages has been the interest of many researchers. In addition to diagnosis, plasma biomarkers could be useful in screening, routine assessment of disease recurrence, and response to therapy without accompanying imaging studies or biopsies. In present study, we measured the level of alpha-2-macroglobulin protein in ONFH diseases serum. Alpha-2 subunit level, composed of alpha-2-macroglobulin, ceruloplasmin and 2-2 haptoglobin phenotype, was increased significantly as compared to healthy subjects.

Upregulation of mentioned protein has reported in other studies (13, 16). Carli AV. and his colleagues showed elevated plasma A2M levels during GC treatment in rodents. They suggested A2M raise could play an important role in the host reparative response to GC-associated effects (16). Ramadori G et al. showed GC could modulate A2M and apolipoprotein E gene expression by raising the steady-state levels of alpha-2-macroglobulin-specific messenger RNA and reducing apolipoprotein E-specific transcript in cultured rat liver fat-storing (Ito) cells (17). Milosavljevic TC et al. showed GCs could mediate transcriptional activation of the A2M gene (18).

This study and several similar studies denote the effect of GC in regulation of A2M especially in its up-expression. In this regard, capillary electrophoresis was performed on the serum of patients receiving GCs. For the six serum capillary electrophoresis accepted zones, all six peaks were observed (albumin, alpha 1, alpha 2, beta 1, beta 2 and gamma). Alpha 1 peak includes a combination of α1-antitrypsin, thyroxine-binding globulin (TBG), and transcortin. Alpha 2
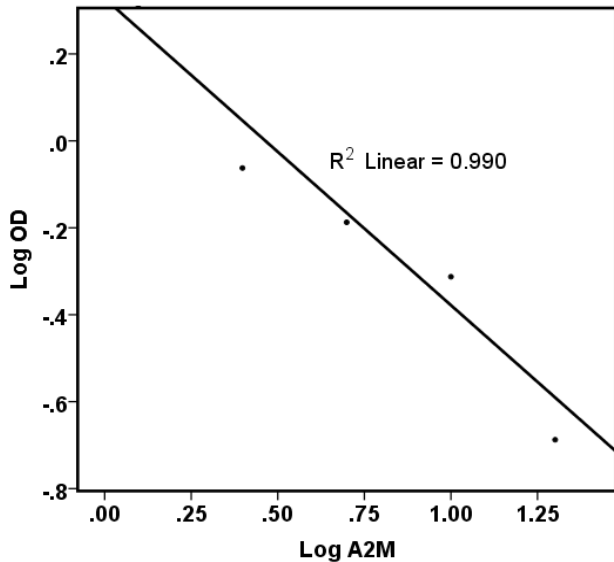
Figure 3. Linear regression between A2M concentration and its absorbance.

peak contains haptoglobin, ceruloplasmin, and A2M. Measurement of alpha 2 in ONFH patients showed an elevation in comparison to normal values. The elevation could be due to an increase in each alpha 2 component especially A2M protein. Thus, A2M in ONFH patients' sera was measured, which demonstrated a significant increase in A2M protein level. Surprisingly, Soyfoo MS et al. did not observe an elevated serum level of A2M and α1-antitrypsin in their cohort of non-traumatic ONFH patients, however, they declared cryofibrinogenemia, a fibrinolysis defect, in their patients and, more importantly in their multifocal ONFH patients (19). Amdo TD et al. elucidated there was a high serum level of α1-antitrypsin and A2M and delayed euglobulinlysis time in cryofibrinogenemia as opposed to Soyfoo's findings (20). In another study, Chen Y et al. reported that the levels of complement component 3 (C3), C4, inter-α-trypsin inhibitor heavy chain H4 and A2M in the serum of patients with ONFH have reduced, although several other studies did not confirm their results (14, 21-23). The difference amongst the results could be related to various stages of disease and dissimilar environmental conditions as well as technical issues. Thus, it is recommended to stage the disease and determine the size of necrotic lesions of the femoral head and then correlate them with A2M serum level in future studies. To minimize the co-founder effects, it is suggested to perform similar studies on different categories of ONFH patients such as glucocorticoid, alcohol, or drug induced and idiopathic ONFH patients separately. Hence, the effect of each inducer could be compared to the A2M serum level of normal individuals and studied independently.

Besides, regards to alpha fraction in electrophoresis, moving toward the negative portion of the gel (i.e., the negative electrode), the next peaks after albumin, involve the alpha1 and alpha2 components. The alpha2-protein fraction is comprised of Ceruloplasmin, alpha2-macroglobulin, and haptoglobin. Since renal failure and acute inflammation (resulting from acute-phase reactants) can increase the alpha2-protein band, the patients' information and the stage of disease could be helpful (24). Furthermore, larger sample sizes could help increase the power and ensure the correct conclusion respecting whether these protein could be a reliable diagnostic tool in clinical practice.

To sum up, the present study demonstrated that patients with nontraumatic ONFH have significantly higher serum levels of A2M than normal population.

We declare that there are no conflicts of interest.

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