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The Association of The High -Mobility Group Box 1 Gene and Its Product with Rheumatoid Arthritis in Basra Province - Iraq

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***Abstract*— Background: Rheumatoid arthritis (RA) is an autoimmune disease. High mobility group box 1 (HMGB1) is a nuclear protein which considered to be a biomarker in several active systemic autoimmune diseases such as RA. The study aimed to investigate HMGB1 gene and its product in RA patients in Basra.**

Methods: The study included 26 patients and 10 healthy controls aged 21-53 yrs.*HMGB1* gene expression was detected using qRT-PCR and the serum levels of HMGB1 and Rheumatoid factors (RF)including (IgG and IgM) of study subjects were estimated by ELISA.

Results: The results showed that the mRNA expression levels of *HMGB1*was significantly higher in RA patients in compare with healthy control(4.14± 3.89 and 1, respectively). In contrast, the results showed no significant differences (*p* =0.72) between the mean of HMGB1 serum level in RA patients (3.55 ± 2.90 ng/ml) and healthy controls (3.79 ± 1.30 ng/ml) and*HMGB1* expression showed weak positive correlation to its serum levels. The level of IgG and IgM were significantly higher in RA patients in compare with healthy controls with a strong positive correlation. Moreover, there was weakly positive correlation between serum HMGB1 level and the RF-IgG(0.11) and no correlation between serum HMGB1 levels and RF-IgM(0.009) in RA patients.

Conclusion: Rheumatoid arthritis patients have elevated levels of *HMGB1* gene expression but their protein levels were normal due to the course of treatment. Furthermore, the high *HMGB1* gene expression level correlates positively with IgG levels.

Keywords*— *Rheumatoid arthritis, HMGB1, gene expression, qRT-PCR

I. Introduction

Autoimmune diseases(AIDs) occur when the immune system attacks the body causing damage in different tissues and organs. AIDs includes many diseases but the most common one are rheumatic diseases like rheumatoid arthritis (RA)[1]. Rheumatoid arthritis can be defined as a chronic inflammatory disease characterize by swelling and tenderness of the joints in addition to the destruction of cartilages and bones. The cause of this destruction is the production of autoantibodies including rheumatoid factor (RF) (IgG and IgM) and anti-citrullinated autoantibody (anti-CCP)[2].

The high -mobility group box 1 (HMGB1) was first identified by Goodwin and Colleagues in 1973 in Calf thymus [3]. It belongs to the high –mobility group (HMG) protein family which gain its name from its high electrophoretic mobility during electrophoresis in polyacrylamide gels [4]. It actsas a cytokine and it is released into the extracellular milieu. This protein is released passively in case of cellular necrosis and nearly all the nucleated cells can release it and by doing so they alert other cells to the damage happening [5]. HMGB1 has different functions including inflammation trigger, attracting cells, stem cell recruiting and activating dendritic cells as it sustains the proliferation of antigen specific T cell and help them to develop to Th1 [4].In acute and chronic inflammatory disease HMGB1 binds to soluble molecules and receptors such as RAGE, TLR2 and TLR4 leading to the activation of the cell carrying these receptors[6].

The aim of our study is to evaluate the *HMGB1* gene expression and the level of HMGB1 protein in serum of RA patients in Basra and investigate its correlations between each other and with RF.

II. Materials and Methods

A. Study subjects

Adult Rheumatoid arthritis patients(n=26) from both sexes, age 21-53 yrs. are eligible for our study. All patients were visitors of Rheumatoid arthritis center located in AL-Basra Teaching hospital. The collection of samples starts in February till August of 2018. In addition, samples (n=10) were also collected from age matched healthy control (HC).

B. Samples collection and processing

Five ml of venous blood were withdrawal from each studysubjects,1 ml were placed in EDTA tube for further molecular analysis while the remaining 4 ml were placed in a serum separation gel tube. Serum samples were separated

by centrifugation at 3000 rpm for 20 min. then 3 aliquots for each sample were made and kept at -20 °C until further analysis.

C. RNA Extraction

RNA was extracted from the study subjects blood samples using GENE ZOL™ Tri RNA pure kit(Korea). The quality and concentration of the extracted RNA samples were measured by Nanodrop spectrophotometer (Quawell, USA).

D. Revers transcription of RNA

cDNA was synthesized from RNA samples, 100 ng of total RNA from each sample was used to synthesized cDNA using Bioneer two step Accupower® RT-PCR pre Mix (Korea) and according to the manufacturer protocols. **Note:** different volumes of total RNA were used depending on the concertation of the extracted RNA (high concentrations required small volumes or diluted volumes while low concentrations required RNA concentrating method using vacuumed centrifugation)

E. Measuring the expression of HMGB1

The *HMGB1* expression was measured using Quantitative Real time- polymerase chain reaction (qRT-PCR),20 ng of cDNA from each samples were used to detect the expression of *HMGB1*and *GAPDH* (reference gene). The forward and the reverse primers used to show in Table 1. TaqMan was used to detect the gene expression and BYRT green was also used for the samples failed to be detected by TaqMan. The thermal cycler program used is shown in Table 2.The relative expression of the real time-PCR data was calculated according to the ΔΔCt method [7].

F. Serological study

The sera of all study subjects were tested for the level of Human High -mobility group box 1(HMGB1) protein ELISA Kit (Shanghai yehua Biological Technology Co., China).RF-IgG and IgM levels using Immunoenzymetric Assay by Rheumatoid Factor IgG and IgM kits (Demeditec /German). All kits were performed according to the manufacturer protocols.

G. Statistical analysis

The data underwent statistical analysis using a normality test at beginning followed by parametric (two sample T-test and confidence interval) and non-parametric test (Mann-Whitney U test)in MINTAB program. In addition, the Pearson correlation used to investigate the correlation between the study factors.

Table 1 Primers sequences of *HMGB1* and *GAPDH* genes

Gene	Primers sequences (5'-3')	qRT-PCR chemistry	References
HMGB1	**F:** GCTGTGCAAAGGTTGAGAGC **R:** CTCGGGTACACAGGACACAC **Probe:**HEXGATCGTCCCATCACAGTGTTG-BHQ-1	TaqMan	http://bioinfo.ut.ee/primer30.4.0
GAPDH	**F:** CAACGAATTTGGCTACAGCA **R:** AGGGGTCTACATGGCAACTG **Probe:** FAM-CCACCAGCCCCAGCAAGAGC-BHQ-1	TaqMan	http://bioinfo.ut.ee/primer30.4.0
HMGB1	**F:** TAACTGAATAGGGGCGTGGTCT **R:** GAAAATGTGCT GGCTGTAGTGG	BYRT green	[8]
GAPDH	**F:** CACCCACTCCTCCACCTTTG **R:** CCACCACCCTGTTGCTGTAG	BYRT green	[9]

Table 2*q RT- PCR Program for amplification HMGB1 and GAPDH*

Step	Cycle	Temperature	Time
GoTaq® Hot Start Polymerase activation	1	95ºC	5 min.
Denaturation	45	95ºC	15 Sec.
Annealing and extension		60ºC	1 min.

III. Results

A. Expression of HMGB1 in RA

qRT- PCR were used to measure *HMGB1* gene expression for both RA patients and HC using *GAPDH* as a reference gene. The current results showed that the mRNA expression level of *HMGB1* was significantly increased in RA patients in comparison with healthy controls (4.14± 3.89 and 1, respectively) as shown in Figure 1. For the BYRT green chemistry, the dye binding was specific to our target gene represented by one peak in the melting curve data as shown in Figure 2.

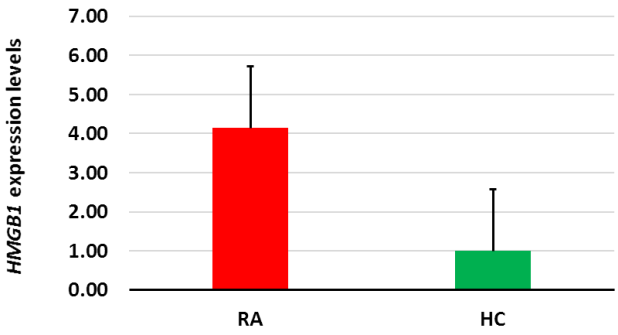

Figure 1 Relative Expression of HMGB1 Gene in RA patients and HC.

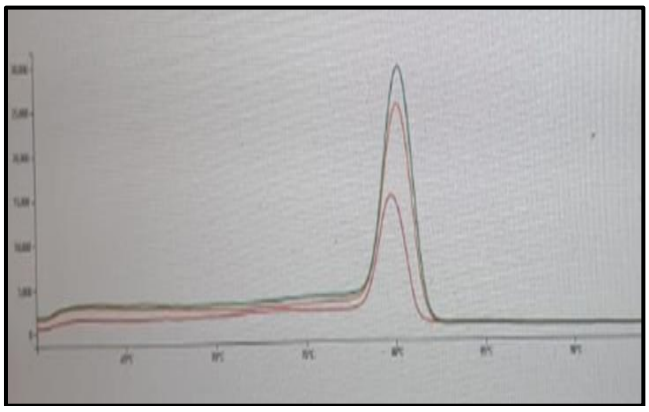

Figure 2 Melting curve for HMGB1 gene for three different samples showing the one Peak curve using BYRT green chemistry in quantitative RT-PCR

B. *Serum Human High mobility group B1 protein (HMGB-1) levels in RA*

The results showed no significant differences (*p* =0.36) between the mean of serum Human HMGB1 levels in RA patients (3.55 ± 2.90 ng/ml) and healthy controls (3.79± 1.30 ng/ml) (Figure 3).

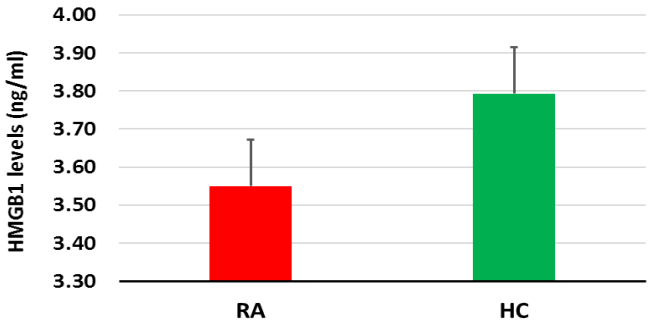

Figure 3 HMGB1 levels in RA patients and HC. No significant differences between RA and HC groups. The HMGB1 levels were measured by ELISA

C. *Correlation between HMGB1 expression and serum HMGB1 levels*

The results of *HMGB1* expression showed weak positive correlation to its serum levels as the gene expression results showed high levels of expression in compare with the serum levels of HMGB1 measured by ELISA. The correlation coefficient was 0.14 and it was non-significant under $p < 0.05$ as shown in Figure 4.

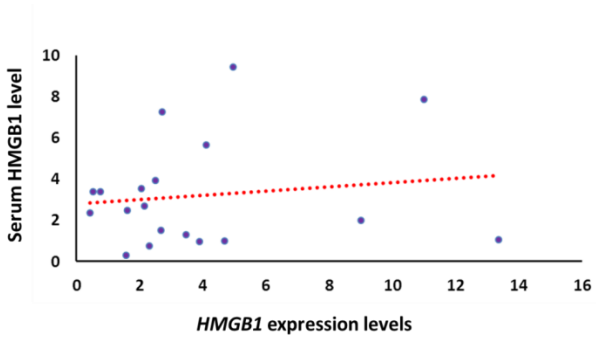

Figure 4 The correlation of HMGB1 expression with its serum levels The correlation coefficient was 0.14 and non-significant under $p < 0.05$.

D. *Rheumatoid factor (IgG level and IgM level) in RA*

The level of IgG was significantly higher in RA patients in compare with healthy controls (p=0.028), the mean of IgG level was 88.33 ± 171.84 ng/ml while the mean level of IgG in healthy controls was 1.55 ± 4.37 ng/ml (Figure 5 A & B). Regarding the positive and negative cases of RA patients in terms of IgG levels, the results showed that 31.81 % of RA patients were positive for RF IgG leaving 68.18% of RA patients as negative cases, for healthy controls 100% were negative. The current study showed that level of IgM in RA patients in compare to healthy controls (p=0.10)and the means were 30.55 ± 77.24 ng/ml and 2.12 ±3.32 ng/ml, respectively. Regarding the positive and negative cases of RA patients in terms of IgM levels. The results showed that 27.27% of RA patients were positive for IgM leaving 72.72% of RA patients as negative cases. For healthy controls 100% were IgM negative.

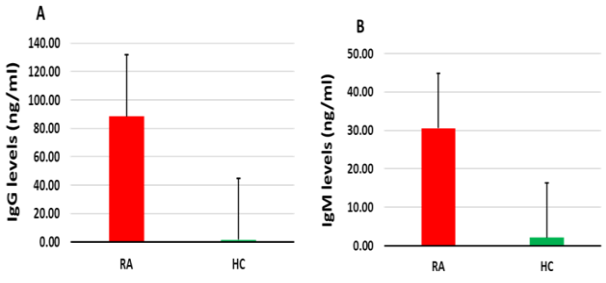

Figure 5 RF (IgG, IgM) levels in RA patients and HC. **A.** IgG levels in RA patients and HC. **B.** IgM levels in RA patients and HC. The RF levels were measured by ELISA

From plotting the serum IgG values with the serum IgM levels in RA patients, the results showed strong positive correlation between the two factors. The R value was 0.65 (Figure 6)

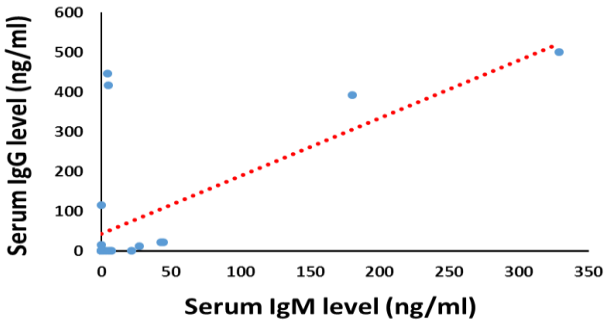

Figure 6 The correlation between IgG and IgM in RA. The R value was 0.65

E. The correlation of serum levels of HMGB1 with RF in RA

The correlation between the serum levels of HMGB1 and RF (IgG and IgM) can be shown as follow:
From plotting the HMGB1level values with the IgG levels in RA patients, the results showed a weak positive correlation between the two factors. The R value was 0.11(Figure 7 A).while plotting the HMGB1 level values with the IgM levels in RA patients showed no correlation between the two factors. The R value was 0.01 (Figure 7B).

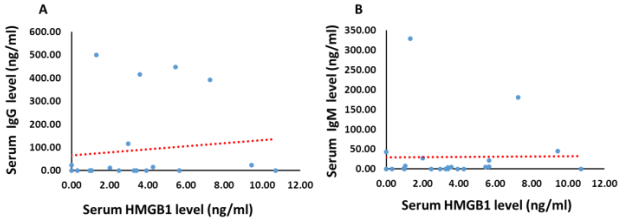

Figure 7 The correlation between HMGB1 and other study parameters. **A.** The correlation between HMGB1 and RF-IgG levels in RA. The results showed a weak positive correlation. **B**. The correlation between HMGB1 and serum RF-IgM levels in RA. The results showed no correlation.

IV. Discussion

Normally RA patients have high levels of HMGB1 in serum and in joints specially in the pannus regions. HMGB1 have functions involved in promoting proteolytic enzymes activity and the maturation of osteoclasts[10, 11]. Our results of *HMGB1* expression in RA showed a significant elevation in the expression of the gene in compare to healthy controls as reported previously in many studies[12, 13].Technically the gene expression data was reliable as the BYRT green dye showed specificity to the target genes as the one peak of melting curve showed. This elevation can be explained by the function of HMGB1 as a pro-inflammatory cytokine that is released from macrophages[14-16]. HMGB1 also can be released from neutrophils, monocytes and endothelial cells which are stimulated by inflammation. It can activate macrophage to produce TNF-α, IL-1 and other pro-inflammatory cytokines and it promotes the recruitment of inflammatory cells during RA which is a chronic inflammatory disease[17]. Moreover, the implication of HMGB1 in the pathogenicity of arthritis is documented[18, 19]. It may contribute to the disease progress, as it is also promotes cell proliferation, modulation, migration and adhesive features. In addition, the extracellular HMGB1prolonged pro-inflammatory process [20, 21].

Unlikely, the serum levels of HMGB1 in RA was normal which might be explained by the effect of anti-inflammatory drugs that has been taken by the patients like methotrexate which reported to inhibit T- cells activation[22]. Similar results were reported for other AIDs such as medium vessel vasculitis (MVV), large vessel vasculitis (LVV), and Juvenile Idiopathic Arthritis(JIA) [19]in which the levels of HMGB1 gradually decreased to normal after treatment of the disease [23].In addition, there are many factors that may affect the serum level of HMGB1 in AID such as age, sex, environmental and clinical factors[19, 24].

Although the serum levels of HMGB1 in RA were normal but the correlation betweenHMGB1 gene and its protein in the serum showed a weak positive correlation which confirm the elevation of the protein but the interference of many factors (drug, age and sex) may affect the result of the protein level in the serum[23, 25].

The RF level (IgG, IgM) in RA is high in compare to healthy controls [2, 26], both antibodies showed a strong correlation in RA patients [26]. Despite this elevation in IgG and IgM some RA patients are negative to these antibodies as in many other study reported previous[27].

The positive correlation between HMGB1 and IgG can be explained by the normal presence of IgG in chronic inflammation which is the case in RA [28]while IgM normally found in acute inflammation which explain that there is no correlation between HMGB1 and IgM[29]. Moreover, IgM has a relatively low affinity for modified self-components[30].

In conclusion, HMGB1 gene expression is associated with RA patients in Basra. Despite the effector factors on its level in serum with confirmation of its correlation to IgG which is a characteristic feature of chronic inflammation such as in RA.

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References

1.Scott DL, Pugner K, Kaarela K, et al. The links between joint damage and disability in rheumatoid arthritis. Rheumatology (Oxford) 2000;39:122-32.

2.Too CL, Rönnelid J, Yusoff YM, et al. Increased IgG Rheumatoid Factor-Positivity in the Asian Rheumatoid Arthritis Patients Irrespective of Ethnicity. Ethnicity (n) 2014;47:40.2-13.3.

3.Goodwin GH, Sanders C, Johns EW. A new group of chromatin-associated proteins with a high content of acidic and basic amino acids. Eur J Biochem 1973;38:14-9.

4.Martinotti S, Patrone M, Ranzato E. Emerging roles for HMGB1 protein in immunity, inflammation, and cancer. Immunotargets Ther 2015;4:101-9.

5.Andersson U, Tracey KJ. HMGB1 is a therapeutic target for sterile inflammation and infection. Annu Rev Immunol 2011;29:139-62.

6.Wähämaa H, Schierbeck H, Hreggvidsdottir HS, et al. High mobility group box protein 1 in complex with lipopolysaccharide or IL-1 promotes an increased inflammatory phenotype in synovial fibroblasts. Arthritis Res Ther 2011;13:R136.

7.Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001;25:402-8.

8.Zhang W, An F, Xia M, et al. Increased HMGB1 expression correlates with higher expression of c-IAP2 and pERK in colorectal cancer. Medicine 2019;98:e14069-e14069.

9.Jiang Z-H, Peng J, Yang H-L, et al. Upregulation and biological function of transmembrane protein 119 in osteosarcoma. Experimental & Molecular Medicine 2017;49:e329-e329.

10. Andersson U, Harris HE. The role of HMGB1 in the pathogenesis of rheumatic disease. Biochim Biophys Acta 2010;1799:141-8.

11. Liang S, Lv ZT, Zhang JM, et al. Necrostatin-1 Attenuates Trauma-Induced Mouse Osteoarthritis and IL-1β Induced Apoptosis via HMGB1/TLR4/SDF-1 in Primary Mouse Chondrocytes. Front Pharmacol 2018;9:1378.

12. Andersson U, Erlandsson-Harris H. HMGB1 is a potent trigger of arthritis. Journal of Internal Medicine 2004;255:344-350.

13. Jiang W, Pisetsky DS. Mechanisms of Disease: the role of high-mobility group protein 1 in the pathogenesis of inflammatory arthritis. Nat Clin Pract Rheumatol 2007;3:52-8.

14. Byun K, Yoo Y, Son M, et al. Advanced glycation end-products produced systemically and by macrophages: A common contributor to inflammation and degenerative diseases. Pharmacology & Therapeutics 2017;177:44-55.

15. Yang Z, Li L, Chen L, et al. PARP-1 Mediates LPS-Induced HMGB1 Release by Macrophages through Regulation of HMGB1 Acetylation. The Journal of Immunology 2014;193:6114-6123.

16. Wang J, Li R, Peng Z, et al. HMGB1 participates in LPS-induced acute lung injury by activating the AIM2 inflammasome in macrophages and inducing polarization of M1 macrophages via TLR2, TLR4, and RAGE/NF-κB signaling pathways. Int J Mol Med 2020;45:61-80.

17. Wang C, de Souza AW, Westra J, et al. Emerging role of high mobility group box 1 in ANCA-associated vasculitis. Autoimmun Rev 2015;14:1057-65.

18. Cecchinato V, D'Agostino G, Raeli L, et al. Redox-Mediated Mechanisms Fuel Monocyte Responses to CXCL12/HMGB1 in Active Rheumatoid Arthritis. Front Immunol 2018;9:2118.

19. Zhu B, Zhu Q, Li N, et al. Association of serum/plasma high mobility group box 1 with autoimmune diseases: A systematic review and meta-analysis. Medicine (Baltimore) 2018;97:e11531.

20. Yanai H, Ban T, Taniguchi T. High-mobility group box family of proteins: ligand and sensor for innate immunity. Trends Immunol 2012;33:633-40.

21. Harris HE, Andersson U, Pisetsky DS. HMGB1: a multifunctional alarmin driving autoimmune and inflammatory disease. Nat Rev Rheumatol 2012;8:195-202.

22. Kumar P, Banik S. Pharmacotherapy options in rheumatoid arthritis. Clin Med Insights Arthritis Musculoskelet Disord 2013;6:35-43.

23. Singh A, Feng Y, Mahato N, et al. Role of high-mobility group box 1 in patients with acute obstructive suppurative cholangitis-induced sepsis. J Inflamm Res 2015;8:71-7.

24. Wu Y-L, Chien M-H, Chou Y-E, et al. Association of EGFR mutations and HMGB1 genetic polymorphisms in lung adenocarcinoma patients. Journal of Cancer 2019;10:2907-2914.

25. Ke S, Shi H, Shi W, et al. Down-regulation of HMGB1 induces apoptosis and inhibits invasion and migration of MCF-7 breast cancer cells through targeting SMARCC1. Int J Clin Exp Pathol 2017;10:2764-2773.

26. Hussein RH, MezherAl-Rayahi IA, Taha K. Rheumatoid factor isotypes in a sample of Iraqi rheumatoid arthritis patients. Journal of Global Pharma Technology 2018;10:141-145.

27. Choi S-T, Lee K-H. Clinical management of seronegative and seropositive rheumatoid arthritis: A comparative study. PLOS ONE 2018;13:e0195550.

28. Brückner C, Lehmann C, Dudziak D, et al. Sweet SIGNs: IgG glycosylation leads the way in IVIG-mediated resolution of inflammation. Int Immunol 2017;29:499-509.

29. Kindt TJ, Goldsby RA, Osborne BA, et al. Kuby immunology. New York: W.H. Freeman, 2007.

30. Arai S, Maehara N, Iwamura Y, et al. Obesity-associated autoantibody production requires AIM to retain the immunoglobulin M immune complex on follicular dendritic cells. Cell Rep 2013;3:1187-98.