Clinical significance of high mobility group box 1/toll-like receptor 4 in obese diabetic patients

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Abstract. High mobility group box 1 (HMGB1) is an alarmin that may link to obesity and type 2 diabetes mellitus (T2DM). The present study analyzed the correlation between HMGB1/ Toll-like receptor 4 (TLR4) and certain biochemical parameters in obese (OB) diabetic patients. 40 normal glucose tolerant subjects (NGT) and 40 patients with newly diagnosed T2DM were enrolled. All patients were further divided into non-obese NGT (NGT-NOB), obese NGT (NGT-OB), non-obese T2DM (T2DM-NOB) and obese T2DM (T2DM-OB) groups according to body mass index (BMI). The levels of HMGB1 in serum were quantified using ELISA, whereas the mRNA expression levels of TLR4 in peripheral blood mononuclear cells were assessed using reverse transcription-quantitative PCR. The results suggested that the levels of HMGB1 and TLR4 were higher in NGT-OB and T2DM-NOB groups compared with those in NGT-NOB group. Similarly, the levels of these two markers were higher in T2DM-OB group compared with those in NGT-OB group. Correlation analysis indicated that the levels of HMGB1 and TLR4 were positively correlated with triglyceride (TG), fasting plasma glucose (FPG) levels and BMI, whereas a negative correlation between HMGB1 and high density lipoprotein (HDL) was noted. Linear regression analysis suggested that HMGB1 was associated with FPG and TG levels, whereas TLR4 was strongly associated with TG levels and BMI. The results demonstrated that the expression levels of HMGB1 and TLR4 in patients with T2DM or obesity were increased, which were associated with glycolipid metabolism disorders. Therefore, the HMGB1/TLR4 may serve a role in inflammatory process associated with obesity and T2DM.

Key words: High mobility group box 1, Toll-like receptor 4, Type 2 diabetes mellitus, Obesity, Regression analysis

OBESITY is a disorder, associated with a state of chronic low-grade inflammation. This condition has become a major public health concern. A adiposity is an independent risk factor, which plays a specific role in the development of T2DM [1, 2]. A number of studies have reported that >90% of patients with T2DM are overweight or OB [3]. Individuals who are overweight or OB exhibit local and systemic chronic low-grade inflammation or para-inflammation. These disorders are central links between T2DM and obesity based on slightly elevated leukocyte counts and levels of C-reactive protein and inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) [4, 5]. TNF-α is an important proinflammatory cytokine that is strongly associated with the development of insulin resistance in T2DM [6].

HMGB1 is a non-histone chromatin-associated protein that migrates rapidly during electrophoretic separation and was named according to this property, it binds to TLR4 causing the release of proinflammatory cytokines via the NF-κB signaling pathway [7]. Recently, HMGB1 has received increasing attention [8, 9]. Under normal conditions, nuclear HMGB1 can bind to and bend DNA to aid with chromosome architecture organization and gene transcription regulation. However, in the extracellular space, HMGB1 functions as a cytokine to activate the immune system and mediate a variety of physiological and pathological responses [10, 11]. Aseptic injury to cells increases the levels of HMGB1 in the serum and
tissues [12]. Therefore, HMGB1 is associated with low-grade inflammatory diseases, such as obesity and T2DM [13].

Toll-like receptors (TLRs) are a major class of pattern-recognition receptors (PRRs). TLR4 is closely associated with and significantly contribute to the pathogenesis of T2DM among all TLRs [14]. HMGB1 is an important endogenous activator of TLR4 and the interaction between HMGB1 and TLR4 causes the release of pro-inflammatory cytokines via the NF-κB signaling pathway [8]. The aim of the present study was to analyze the association between HMGB1/TLR4 levels and glycolipid metabolism in obese patients with newly diagnosed T2DM.

**Materials and Methods**

**Patients**

A total of 80 participants (male \( n = 40 \), female \( n = 40 \)) aged 21–60 years (mean age, 44 ± 9 years) were enrolled from the Third Affiliated Hospital of Xinxiang Medical University. The patients (male \( n = 20 \), female \( n = 20 \)) with newly diagnosed T2DM aged 21–60 years (mean age, 44 ± 10 years) were recruited from the inpatients department, whilst control subjects (male \( n = 20 \), female \( n = 20 \)) aged 27–60 years (mean age, 45 ± 9 years) were recruited from the health management center of the same hospital. The study protocol was approved by the Ethics Committee of the Third Affiliated Hospital of Xinxiang Medical University (Xinxiang, China; approval no.: k2017-038-01). The glucose status of all participants was confirmed by performing the 75 g oral glucose tolerance test, and individuals were diagnosed with T2DM or as NGT subjects based on the World Health Organization diagnostic criteria (1999) [15]. In the morning, following an 8-h fast, a fasting glucose blood test was performed in all participants. Following the initial venipuncture, the participants were asked to drink 75 g glucose and were subjected to a second venipuncture at 2 h post glucose consumption. The NGT group consisted of 40 subjects and the T2DM group consisted of 40 patients with T2DM. According to the obesity criteria of Diabetes Branch of the Chinese Medical Association (2004) and the American Heart Association/American College of Cardiology/The Obesity Society Guidelines for the Management of Overweight and Obesity in Adults (2013), individuals with a BMI ≥28 kg/m² were diagnosed with obesity [16, 17]. All participants were divided into the following four groups: i) T2DM-non-obese (NOB, \( n = 20 \)); ii) T2DM-obese (OB, \( n = 20 \)); iii) NGT-NOB (\( n = 20 \)); and iv) NGT-OB (\( n = 20 \)). In addition, the exclusion criteria were as follows: Cardiovascular disease, infectious, hepatic or renal disease, autoimmune diseases, tumor, pregnancy, excessive alcohol consumption [defined as an average daily consumption of alcohol >20 g/day (140 g/week) in men and >10 g/day (70 g/week) in women], acute and chronic complications of diabetes, and stage 2 hypertension (resting blood pressure ≥160/100 mmHg).

**Collection of blood samples and biochemical analysis**

The blood samples were collected via venipuncture in the morning following an overnight fast of ≥8 h. The serum samples were obtained by centrifugation at 4°C at 1,200 × g for 5 min and immediately used for the measurement of FPG, TG, HDL, total cholesterol (TC) and low density lipoprotein (LDL) levels. The concentration levels of all these biochemical indicators were determined using a fully automated biochemical analyzer. The aliquots for the HMGB1 assay were stored at −80°C until analysis via an ELISA (cat. no. SEA399Hu; Cloud-clone Corp.) according to the manufacturer’s instructions.

**Reverse transcription-quantitative PCR (RT-qPCR)**

Peripheral blood mononuclear cells (PBMCs) were isolated from the whole blood samples via Ficoll-Paque density gradient (Beijing Solarbio Science & Technology Co., Ltd.) centrifugation at 4°C at 1,200 × g for 5 min. Total RNA was extracted from PBMCs using RNAiso Plus (Takara Biotechnology Co., Ltd.) and reverse transcribed into cDNA following an initial incubation at 42°C for 60 min and 95°C for 5 min using PrimeScript™ 1st Strand cDNA Synthesis Kit (cat. no. 6110A; Takara Biotechnology Co., Ltd.) and an ABI 7500 Fast Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The RNA samples were stored at −80°C. The sequences of primers used for quantitative PCR (qPCR) were as follows: GAPDH forward, 5’-AGG TGG GTG TAAGCGATTG-3’ and reverse, 5’-TGTA GACCATGTAGTTGAGGTC-3’; and TLR4 forward, 5’-CGAGGAAGAGAAGACACCAGT-3’ and reverse, 5’-C ATACCTCACTGTTCTGTTG-3’. qPCR was performed using SYBR® Select Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) and an ABI 7500 Fast Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The following thermocycling conditions were used for qPCR: 50°C for 2 min; 95°C for 10 min; followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min; 95°C for 30 sec; and 60°C for 15 sec. The mRNA expression levels were quantified using the 2^(-ΔΔCq) method and normalized to the internal reference gene GAPDH [18]. RT-qPCR was performed in triplicate.

**ELISA**

The expression levels of HMGB1 in the peripheral blood samples were detected using the aforementioned
ELISA kit. In brief, the samples (100 μL/well) were added to microplates and incubated for 1 h at 37°C. Following washing with a wash buffer, a HMGB1 peroxidase-conjugated monoclonal antibody was added and incubated with the samples for 90 min at 37°C. After washing with a wash buffer, 100 μL chromogenic substrate was added to each well and developed at room temperature in the dark for 30 min. Finally, the stop solution was added to each well. The optical density value of each well was measured at a wavelength of 450 nm. The HMGB1 concentration in the testing samples was calculated using the standard curve. All samples were analyzed in duplicate.

Statistical analysis
The data are presented as the mean ± standard deviation. Statistical analyses were performed using GraphPad Prism software (version 5.0; GraphPad Software, Inc.). The differences among the groups were analyzed using two-way ANOVA and multiple comparisons using Bonferroni’s test. Pearson’s correlation analysis was performed to evaluate the correlations between variables. Specifically, |r| < 0.3, 0.3 ≤ |r| ≤ 0.8 and |r| > 0.8 were considered to indicate weak, significant and strong correlations, respectively. Multiple linear regression analysis was performed to further explore the associations between the variables and the expression levels of HMGB1 and TLR4. The regression models consisted of variables that correlated linearly with HMGB1 and TLR4. All statistical tests were two-tailed and p < 0.05 was considered to indicate a statistically significant difference.

Table 1 Clinical and laboratory characteristics of the study population

| Parameter     | NGT                  | T2DM                  | F-value | p-value |
|---------------|----------------------|-----------------------|---------|---------|
|               | NOB (n = 20)         | OB (n = 20)           |         |         |
|               |                      |                       |         |         |
| Age (years)   | 40.10 ± 7.47         | 42.30 ± 7.39          | 42.50 ± 7.57 | 41.70 ± 10.17 | 0.125 | 0.945 |
| BMI (kg/m²)   | 22.13 ± 2.25         | 29.03 ± 0.69b         | 24.22 ± 2.68ad | 32.24 ± 2.05bde | 101.229 | <0.001 |
| FPG (mmol/L)  | 4.79 ± 0.34          | 4.85 ± 0.49           | 11.72 ± 3.48bd | 10.96 ± 2.96bd | 39.403 | <0.001 |
| TC (mmol/L)   | 4.27 ± 0.47          | 4.45 ± 0.48           | 4.69 ± 0.48b | 4.98 ± 0.74bc | 7.058 | <0.001 |
| TG (mmol/L)   | 0.92 ± 0.36          | 1.09 ± 0.39           | 1.91 ± 0.67bd | 3.57 ± 0.76bde | 91.409 | <0.001 |
| HDL (mmol/L)  | 1.62 ± 0.44          | 1.54 ± 0.44           | 1.29 ± 0.22 | 1.25 ± 0.28bc | 2.804 | 0.045 |
| LDL (mmol/L)  | 2.60 ± 0.33          | 2.67 ± 0.29           | 2.91 ± 0.47c | 3.00 ± 0.64bc | 3.561 | 0.018 |

Results
Characteristics of patients
The main clinical characteristics and laboratory data of the study participants are presented in Table 1. The study group included 40 female and 40 male patients. No significant differences were noted among the four subgroups with regard to age (F = 0.125; p > 0.05; Table 1) and sex (χ² = 0.000; p > 0.05; data not shown). BMI and the FPG, TC, TG and LDL levels were significantly increased in the T2DM-NOB and T2DM-OB groups compared with those noted in the NGT-NOB group (p < 0.05 or 0.001). Higher BMI and higher levels of FPG, TC, TG and LDL were observed in the T2DM-OB group compared with those of the NGT-OB group (p < 0.05 or 0.001), whereas no significant changes were noted in the levels of TC and LDL between the T2DM-NOB and NGT-OB groups (p > 0.05). Moreover, it was a remarkable fact that the levels of TG were significantly higher in the T2DM-OB group compared with those of the T2DM-NOB group (p < 0.001). However, the T2DM-OB group exhibited lower levels of HDL compared with those of the NGT-NOB and NGT-OB groups (p < 0.05). The aforementioned comparisons indicated that both obesity and diabetes affected the levels of metabolism-associated biomarkers (Table 1).

Serum HMGB1 levels
Higher serum levels of HMGB1 were observed in the NGT-OB groups compared with those of the NGT-NOB group (p < 0.01), whereas no significant differences were noted between the T2DM-NOB and T2DM-OB groups. Increased HMGB1 expression levels were observed in the T2DM-OB group compared with those in the NGT-OB group (p < 0.01). Moreover, HMGB1 levels were significantly higher in the T2DM-NOB group compared with those in the NGT-NOB group (p < 0.01).
with those in the NGT-NOB group (p < 0.001; Fig. 1).

**Assessment of TLR4 gene expression levels in PBMCs**

The expression levels of TLR4 were measured via RT-qPCR analysis in human PBMCs and the results indicated that they were significantly increased in the NGT-OB group compared with those noted in the NGT-NOB group (p < 0.01). Increased TLR4 gene expression levels were observed in the T2DM-NOB and NGT-OB groups (p < 0.01). Moreover, the TLR4 gene expression levels were significantly higher in the T2DM-NOB group compared with those in the NGT-NOB group (p < 0.001) (Fig. 2).

**Fig. 1** The mean levels of HMGB1 in serum of each group. HMGB1, high mobility group box 1; NS, no statistical significance; NGT, normal glucose tolerant; NOB, non-obese; OB, obese; T2DM, type 2 diabetes mellitus.

**Fig. 2** Gene expression levels of TLR4 in PBMCs of each group. NGT, normal glucose tolerant; NOB, non-obese; OB, obese; PBMCs, peripheral blood mononuclear cells; T2DM, type 2 diabetes mellitus; TLR4, toll-like receptor 4.

**Pearson’s correlation and multiple linear regression analyses of the expression levels of HMGB1 and TLR4 in all participants**

The correlation between HMGB1 levels and clinical parameters was analyzed. The serum levels of HMGB1 were positively correlated with BMI (r = 0.39; p < 0.001), and TG (r = 0.62; p < 0.0001) and FPG (r = 0.54; p < 0.0001) levels, whereas negatively correlated with HDL (r = −0.25; p < 0.05) levels. The gene expression levels of TLR4 were positively correlated with HMGB1 (r = 0.55; p < 0.001) levels, BMI (r = 0.60; p < 0.0001), as well as TG (r = 0.68; p < 0.0001) and FPG (r = 0.47; p < 0.0001) levels. The data are summarized in Figs. 3 and 4.

The variables that were identified to be significantly correlated using Pearson’s correlation analysis were entered in a multivariate model using HMGB1 and TLR4 as the dependent variables. The expression levels of HMGB1 were still positively correlated with the levels of FPG and TG (p < 0.05), whereas the expression levels of TLR4 was positively correlated with BMI and TG levels (p < 0.05). In contrast to these findings, the expression levels of HMGB1 were no longer correlated with BMI and HDL levels, whereas those of TLR4 were no longer correlated with FPG levels. These data are summarized in Table 2.

**Discussion**

HMGB1 functions as damage-associated molecular pattern molecule produced by necrotic or activated immune cells such as monocytes and macrophages. However, it cannot be secreted by the Golgi apparatus or the endoplasmic reticulum pathway owing to the deficiency of the necessary signal peptide structure. It is actively secreted from cells via an atypical vesicle-mediated pathway, which is stimulated by lysophosphatidylcholine (LPC). A number of studies have established that the protein expression levels of HMGB1 are increased in the adipose tissues of OB patients as well as in OB mice [19-21]. Giacobbe et al. [22] demonstrated that the expression levels of HMGB1 in OB women were higher in serum compared with those noted in NOB subjects and a significant positive correlation was reported between serum HMGB1 levels and BMI. Arrigo et al. [23] reported that HMGB1 served an important role in the inflammatory process associated with childhood obesity. Our previous study further demonstrated increased expression of HMGB1 in the visceral adipose tissues of high-fat diet mice [24].

TLR4 is a protein that belongs to the PRR family and is considered to be an important molecule for the development of chronic inflammation [25]. Previous studies...
Fig. 3  Pearson’s correlation between the serum levels of HMGB1 and clinical parameters. The levels of serum HMGB1 were positively correlated with (A) BMI, (B) TG and (C) FPG, and (D) negatively correlated with HDL. BMI, body mass index; FPG, fasting plasma glucose; HDL, high density lipoprotein; HMGB1, high mobility group box 1; TG, triglyceride.

Fig. 4  Pearson’s correlation analysis of TLR4 gene expression levels with HMGB1 and clinical parameters. The gene expression levels of TLR4 were positively correlated with (A) BMI, (B) TG, (C) FPG and (D) HMGB1. BMI, body mass index; FPG, fasting plasma glucose; HDL, high density lipoprotein; HMGB1, high mobility group box 1; TG, triglyceride; TLR4, toll-like receptor 4.
levels in the T2DM-NOB group were significantly higher in the T2DM-OB group compared with those of the T2DM-NOB group, suggesting that the effects of blood glucose affected the development of glycolipid metabolism disorders more severely compared with the effects of obesity on HMGB1 expression. However, TLR4 expression levels were significantly higher in the T2DM-OB group compared with those of the T2DM-NOB group, suggesting that both hyperglycemia and obesity could affect the expression levels of TLR4.

In addition, a significant positive correlation was noted between HMGB1 levels or TLR4 expression and TG and FPG levels, suggesting that glycolipid metabolism may affect the production of HMGB1. Yin et al. [33] assessed the protein expression levels of HMGB1 and TLR4 following liver injury in diabetic mice and revealed that umbelliferone inhibited diabetic inflammation by downregulating the expression levels of HMGB1 and TLR4. Liu et al. [34] further demonstrated that apolipoprotein E−/− mice exhibited significantly increased levels of aortic inflammation and atherosclerosis, as well as enhanced expression levels of HMGB1 and increased concentration levels of TC and TG. In addition, administration of simvastatin to apolipoprotein E−/− mice markedly attenuated vascular inflammation and the atherosclerotic lesion area and decreased the aortic expression of HMGB1 [34].

Table 2: Multiple linear regression analysis of HMGB1 and TLR4

| Parameter | Unstandardized regression coefficient | Standardized regression coefficient | t | p-value |
|-----------|--------------------------------------|-----------------------------------|---|--------|
| TLR4      | 55.980 (±32.855)                     | 0.222 (±1.704)                    | 0.093 |
| Age (years) | 4.549 (±8.582)                      | 0.049 (±1.150)                    | 0.254 |
| BMI (kg/m²) | 9.056 (±23.130)                     | 0.045 (±0.018)                    | 0.986 |
| FPG (mmol/L) | 68.778 (±29.008)                   | 0.277 (±2.452)                    | 0.017 |
| TC (mmol/L)    | −67.827 (±159.813)                  | −0.049 (±0.086)                   | 0.932 |
| TG (mmol/L)    | 345.576 (±106.562)                  | 0.473 (±2.118)                    | 0.038 |
| HDL (mmol/L)    | −121.659 (±231.198)                 | −0.052 (±0.248)                   | 0.805 |
| LDL (mmol/L)    | −50.511 (±184.363)                  | −0.029 (±0.248)                   | 0.805 |

HMGB1, high mobility group box-1 Protein; TLR4, toll like receptor 4; BMI, body mass index; FPG, fasting plasma glucose; TC, total cholesterol; TG, triglyceride; HDL, high density lipoprotein; LDL, low density lipoprotein; B, unstandardized regression coefficient; β, standardized regression coefficient; t, observation value of t-test statistics.

have indicated that saturated fatty acids can induce inflammation by activating the TLR4 signaling pathway [26, 27]. The activation of TLR4 by saturated fatty acids is an important mechanism potentially underlying the initiating effect of obesity on chronic low-grade inflammation. Moreover, certain actions of HMGB1 are mediated via TLRs [28-30]. TLR4 is the receptor of HMGB1, which induces NF-κB activation and increases the production and release of cytokines, when HMGB1 combines with TLR4 on the surface of the immune cell membrane [31].

In the present study, the expression levels of the HMGB1/TLR4 were associated with BMI and specific blood biochemical indices. The results indicated that the expression levels of the HMGB1 and TLR4 were significantly higher in the OB groups compared with those of the NOB groups with the exception of the expression of HMGB1 between the T2DM-OB and T2DM-NOB groups. In the present study, a significant positive correlation was identified between HMGB1 expression and BMI in all participants, which was consistent with previous studies [23, 32]. Moreover, the results indicated that the serum HMGB1 levels and the TLR4 expression levels in the T2DM-NOB group were significantly increased compared with those of the NGT-NOB group, which suggested that dysglycemia may also increase HMGB1 levels in addition to obesity. The results revealed no significant differences in serum HMGB1 levels between the T2DM-NOB and T2DM-OB groups, which suggested that the effects of blood glucose affected the development of glycolipid metabolism disorders more severely compared with the effects of obesity on HMGB1 expression. However, TLR4 expression levels were significantly higher in the T2DM-OB group compared with those of the T2DM-NOB group, suggesting that both hyperglycemia and obesity could affect the expression levels of TLR4.
In the present study, additional analysis was performed using linear regression and the results suggested that serum HMGB1 expression levels were strongly associated with FPG and TG levels and that TLR4 expression levels were associated with BMI and TG levels, indicating that HMGB1/TLR4 affected the development of impaired glucose tolerance in the body.

In conclusion, the present study indicated that HMGB1 and TLR4 were highly expressed in the serum and PBMCs, respectively, of patients with obesity and T2DM compared with the corresponding expression levels noted in the control groups. The expression levels of HMGB1 and TLR4 were positively correlated with specific parameters associated with obesity and glycolipid metabolism. Therefore, HMGB1/TLR4 may serve as a marker of obesity and obesity-associated metabolic disease. Due to the small sample size of the present study, a larger study population should be recruited in a future study to verify the results obtained in the current analysis. Furthermore, the expression levels of HMGB1 and the levels of inflammation in adipose tissue require additional evaluation in the future studies.

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Disclosure

The authors declare that they have no competing interests.

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