Analysis of serum heat shock protein 70 (HSPA1A) concentrations for diagnosis and disease activity monitoring in patients with rheumatoid arthritis

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Abstract Heat shock proteins (HSPs) have been repeatedly implicated in the pathogenesis of rheumatoid arthritis (RA). The authors aimed to study applicability of heat shock protein 70 (HSPA1A) serum levels as a diagnostic factor and a severity indicator in patients with RA and to quantify cut-off point that predicts status of RA with highest specificity. A total of 76 patients with RA and 36 healthy adults were studied in this case-control analysis. Patients had a higher HSPA1A level than the control group (0.78 ± 0.13 vs. 0.12 ± 0.02 ng/mL, $p = 0.006$), irrespective of presence of absence of rheumatoid factor or anti-citrullinated cyclic peptide. Next, diagnostic accuracy of the HSPA1A in diagnosis of RA was evaluated (area under curve 0.71; $p < 0.05$). HSPA1A predicted status of having RA in levels above 0.42 ng/mL with more than 90 % specificity. In addition to diagnostic value, HSPA1A can distinguish between high disease activity (1.66 ± 0.75 ng/mL) and low (0.49 ± 0.1 ng/mL), moderate (0.52 ± 0.12 ng/mL), or remission phase (0.48 ± 0.11 ng/mL). Moreover, patients in remission still had a higher HSPA1A level compared to normal subject (0.48 ± 0.11 vs. 0.12 ± 0.02 ng/mL, $p < 0.05$). Our re-

sults showed that serum HSPA1A could be implemented as a specific tool to facilitate diagnosis and monitoring disease activity in patients with rheumatoid arthritis.

Keywords Rheumatoid arthritis · Heat shock protein 70 · Disease activity · Diagnostic serum marker · HSPA1A

Introduction

Rheumatoid arthritis (RA) is a chronic, systemic, and autoimmune disease caused by complex interactions between several known and unknown factors, and it is characterized by polyarticular synovitis and bony erosions (Singal et al. 1999). The activation of T cells by unidentified antigens in the susceptible host is probably the event that commences the rheumatoid process (Moodley et al. 2011). Identification of these antigens helps to better understand the pathogenesis of rheumatoid arthritis and possibly design new therapeutic regimens. Heat shock proteins (HSPs) have been repeatedly incriminated to play a part in the pathogenesis of auto-immune disease (Cwiklinska et al. 2010), including RA (Sedlackova et al. 2011; Quadri et al. 1996; Rice et al. 2008). During the course of disease, activated Synovial cells produce pro-inflammatory molecules, which lead to joint destruction (Nguyen et al. 2006). Such stress leads to the upregulation of HSP expression in synovial tissue, which protects synovial cells from apoptotic death and consequently prompts synovial cell growth and pannus formation (Hromadnikova et al. 2008).

HSPs are a family of evolutionary conserved proteins, found in all prokaryotic and eukaryotic cells (Multhoff

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2006). They play essential roles in protein-protein interactions such as folding and assisting for establishing proper protein conformation and in prevention of unwanted protein aggregation (Johnson and Fleshner 2006). HSPs are categorized according to their molecular mass, location within cell and function. Several papers addressed guidelines for the nomenclature of the human HSP families, (i) HSPB (small HSP), (ii) DNAJ (HSP40), (iii) HSPH (HSP110), (iv) HSPA (HSP70), (v) HSPC (HSP90), and (vi) as well as for the human chaperonin families HDPD/E (HSP60/HSP10) and CCT (TRiC) (Multhoff 2006; RK et al. 2012; Kampinga et al. 2009).

The 70-kDa family of heat shock proteins includes the constitutive HSC70 and stress-inducible HSP70. The stress-inducible Hsp70, HSPA1A, is an intracellular chaperone, promoting protein folding (Kiang and Tsokos 1998). HSPA1A is released from damaged cells after stress and has been found in the bloodstream of both healthy individuals and patients suffering from autoimmune diseases or other inflammatory conditions (Schick et al. 2004). It is thought that stress-inducible HSPA1A could be a warning signal to the innate immune system and in this way might be relevant to establishment of autoimmune diseases (Luo et al. 2008a). Extracellular HSPA1A could alter immune cells activity through changes in secretion of inflammatory cytokines such as TNF-alpha and IL-10, hence protecting joint from progressive destruction (Luo et al. 2008a, b).

A limited number of studies reported elevated levels of HSPA1A and HSP binding proteins in RA synovial tissue (Schett et al. 1998), but there is no comprehensive data regarding HSPA1A in serum of patients with RA.

In the current study, first, we evaluated whether serum levels of HSPA1A could be utilized as a diagnostic factor in diagnosis of RA. We considered RA as an outcome to determine the percentile values for HSPA1A to derive a possible cut-off point for HSPA1A in which it is associated with RA. Second, we intended to investigate whether there is correlation between HSPA1A levels and severity of rheumatoid arthritis. Furthermore, we investigated serum HSPA1A levels in patients with RA to see if HSPA1A could differentiate RA from other states associated with increased HSPA1A levels, such as type 2 diabetes mellitus (Nakhjavani et al. 2010). Finally, correlation of other well-established rheumatoid arthritis markers and hsp70 levels were examined to see if HSPA1A acts as a dependent or independent serum marker in prediction of RA status.

Methods

We performed a case-control study to discover the importance of HSPA1A as a serum maker in diagnosis and predicting the prognosis of rheumatoid arthritis disease. Seventy-six patients along with 36 healthy adults were recruited from

rheumatology clinic of Vali-Asr Hospital affiliated with the Tehran University of Medical Sciences. In addition, 73 sex- and BMI-matched type 2 diabetic patients were selected from diabetes clinic of Vali-Asr Hospital (36 patients being newly diagnosed and 37 with a 5-year history of having type 2 diabetes). Rheumatoid arthritis was diagnosed according to American College of Rheumatology criteria (Aletaha et al. 2010). Briefly patients were defined based on their score in four categories of joint involvement, serology, acute phase reactants, and duration of symptoms.

Exclusion criteria were acute or chronic renal failure, glomerulonephritis, congestive heart failure, acute infections, pregnancy, thyroid disorders, and hospital admission in last 3 months. None of the studied women were on hormone replacement therapy. There were no comorbid disease such as asthma, diabetes, and other autoimmune diseases present.

Patients were stratified into four groups, including those in remission, those with low disease activity, moderate disease activity, and high disease activity based on DAS 28 (disease activity score) score (van der Heijde et al. 1990). DAS 28 is a severity score, comprised of various variables, scaling from 2 to 10. A score more than 5.1 shows high disease activity, less than 3.2 indicates low disease activity, and below 2.6 is considered remission.

Research was performed according to the Declaration of Helsinki principles and was approved by the ethics committee of the Tehran University of Medical Sciences. All participants gave written informed consent before enrollment.

Anthropometric measurements and physical examination of patients Demographic and anthropometric data including age, sex, height, weight, and duration of disease were recorded. BMI (kg/m^2) was calculated according to the Quetelet formula. Blood pressure was measured in sitting position and was remeasured after 5 min and averaged. Total number of swollen joints, joints with tenderness and extra-articular involvement were recorded.

Biochemical analysis HSPA1A was measured using a quantitative sandwich enzyme-linked immunosorbent assay (ELISA) (EKS-715, Stressgen). The intra- and inter-assay coefficient of variation (CV) ranged between 4.5 and 7 %.

Creatinine was measured using calibrated Jaffe method (Parsazmoon, Karaj, Iran). High sensitivity C-reactive protein (CRP) was assessed by a two-site ELISA (CAN-CRP-4360, Diagnostic Biochem). The sensitivity of the assay was 10 ng/L. Intra- and inter-assay CV were 8 and 10 %, respectively.

Anti-citrullinated cyclic peptide (CCP) levels were determined by ELISA kit (EA 1505-9601, Euroimmun), which measures human IgG antibody against CCP. Rheumatoid factor (RF) was assessed by the latex agglutination method.

Statistical analysis Variables are presented as mean $\pm$ standard error of mean (SEM) or number (percent). Independent samples and ANOVA analysis were employed for group comparisons as indicated. Linear regression was used for data modeling. Two-tailed p value <0.05 was considered as statistically significant. The statistical package SPSS 17 for Windows was used for analysis.

Results

Patient characteristics are summarized in Table 1. Patients were more likely to have a higher average HSPA1A level than the control group (Fig. 1; 0.78 ± 0.13 vs. 0.12 ± 0.02 ng/mL, $p=0.006$).

Receiver operating characteristic (ROC) curve analysis was performed to evaluate diagnostic accuracy of HSPA1A in discriminating patients with RA from control subjects. The area under curve for HSPA1A was 0.71 (Fig 2; $p<0.05$). HSPA1A tended to be a more specific test in diagnosing RA than being a both specific and sensitive test. Next, we investigated cut-offs in which HSPA1A predicts state of having RA. Table 2 summarizes sensitivity and specificity for selected values of HSPA1A as the test variable and state of having RA as the state variable. We presume the 0.42 ng/mL value as the optimum cut-off point, maximizing both specificity (91 %) and sensitivity (49 %).

In order to verify application of HSPA1A in prediction of severity of rheumatoid arthritis, patients were stratified into four groups based on a severity score, DAS28, computed based on number of swollen and tender joints, serum ESR or CRP levels, and patient's global health: patients in remission and patients in low, moderate, or severe status. We compared mean levels of HSPA1A within these four groups using one-

way ANOVA and post hoc analysis. HSPA1A level were significantly higher in patients with high activity of RA compared to other groups (1.66 ± 0.75 ng/mL, $p<0.001$; Fig. 3). Interestingly, HSPA1A levels in patients within remission phase were still higher than control subjects (0.48 ± 0.11 vs. 0.12 ± 0.02 ng/mL, $p<0.05$). However, there was not any significant difference in HSPA1A levels between patients in remission or patients with low (0.49 ± 0.1 ng/mL) or intermediate (0.52 ± 0.12 ng/mL) disease activity. Since HSPA1A level in the studied population was not distributed normally, we have verified our results after transforming HSPA1A values to natural logarithms. After transformation, data were normally distributed (Fig. 1S). One-way ANOVA revealed significant difference of mean HSPA1A levels in patients with high disease activity compared to other patients (geometric mean $\pm$ SEM, 1.52 ± 0.45 ng/mL, $p=0.001$). Patients in remission had a significantly higher transformed HSPA1A values compared to normal subjects (geometric mean $\pm$ SEM, 0.41 ± 0.16 vs. 0.24 ± 0.06 ng/mL, $p=0.003$).

Given these results supporting the relative specificity of HSPA1A in distinguishing high disease activity from other severity grades and patients in remission from healthy subjects, we then examined other inflammatory markers to further address advantages and disadvantages of application of HSPA1A in patients with RA. Statistical comparison of HSPA1A, ESR, and CRP between patients with various disease severities revealed highly significant difference in ESR levels between patients in remission (15.4 ± 5.95) or patients with low (18.2 ± 8.01 ng/mL) or intermediate (24.88 ± 10.65 ng/mL) disease activity with patients in high disease activity (44.57 ± 18.22 ng/mL). Unlike HSPA1A, neither ESR and nor CRP were specific enough in distinguishing patients in remission (ESR 15.4 ± 5.95 ng/mL, CRP

Table 1 Baseline characteristics of patients with rheumatoid arthritis

		Male ($n=13$)	Female ($n=63$)	All ($n=76$)
	Age (years)	49.3 ± 13.5	47.0 ± 11.5	47.4 ± 11.8
	Disease duration (years)	7.8 ± 1.36	9.0 ± 0.75	8.8 ± 0.66
	Cr (mg/dL)	1.0 ± 0.02	0.8 ± 0.05	0.8 ± 0.02
	ESR (mm/h)	20.2 ± 6.54	24.0 ± 1.87	23.3 ± 1.88
	CRP (mg/L)	49.5 ± 9.97	55.3 ± 5	54.4 ± 4.47
	HSPA1A (ng/mL)	0.769 ± 0.27	0.791 ± 0.15	0.78 ± 0.13
Data are expressed as mean $\pm$ SEM	RF	+	75 % ($n=9$)	78.7 % ($n=59$)
		–	25 % ($n=3$)	21.3 % ($n=16$)
<i>Cr</i> creatinine, <i>ESR</i> erythrocyte sedimentation rate, <i>CRP</i> C-reactive protein, <i>HSPA1A</i> heat shock protein A1A, <i>RF</i> rheumatoid factor, <i>CCP</i> anti-citrullinated cyclic peptide	Anti-CCP	+	58.3 % ($n=7$)	60.3 % ($n=38$)
		–	41.7 % ($n=5$)	40 % ($n=30$)
	Extra-articular involvement	+	16.7 % ($n=2$)	5.3 % ($n=4$)
		–	83.3 % ($n=10$)	94.7 % ($n=71$)

Fig. 1 HSPA1A levels in the sera of control subjects and patients with rheumatoid arthritis. Serum HSPA1A levels in patients were significantly higher than that of control subjects ($p < 0.05$). Each bar represents mean level $\pm$ SEM of HSPA1A for control subjects ($n = 36$) and patients with rheumatoid arthritis ($n = 76$)

42.02 $\pm$ 22.78 ng/mL) from control subjects (ESR 12.8 $\pm$ 5.12 ng/mL, CRP 37.8 $\pm$ 12.86 ng/mL). Interestingly, there was not any significant correlation among HSPA1A, ESR, and CRP.

Mean HSPA1A serum levels for patients with positive rheumatoid factor were not significantly different from those who were negative for rheumatoid factor. Similar results were obtained for patients with positive anti-CCP serum marker, and those with negative anti-CCP (Table 3).

Since there are other states associated with elevated HSPA1A, such as diabetes, we wondered if HSPA1A is specific in diagnosing severe states of rheumatoid arthritis. Comparison of mean serum HSPA1A levels of patients with diabetes with patients with RA revealed higher serum HSPA1A levels in patients with RA compared to newly diagnosed diabetic patients (0.52 $\pm$ 0.23 ng/mL, $p < 0.05$). Although mean levels of HSPA1A were not significantly higher than that of patients with five years duration of diabetes (0.87 $\pm$ 0.31 ng/mL), but RA patients with high disease activity still had a significantly higher levels of HSPA1A compared to both groups of patients with type 2 diabetes.

Fig. 2 Receiver operating characteristic curve statistics for serum HSPA1A levels in diagnosis of rheumatoid arthritis. The area under the curve of 0.71 implies a significant diagnostic accuracy of HSPA1A in patients with rheumatoid arthritis. In addition, based on the curve, HSPA1A serum concentration of 0.42 ng/mL could serve as an optimum cut-off point with maximum sensitivity and specificity for predicting state of rheumatoid arthritis

Discussion

Heat shock proteins play essential roles maintaining the overall cellular protein hemostasis (Morteza et al. 2013). Their dysfunction is implicated in life-threatening disorders, including Alzheimer's, Parkinson's, and cardiovascular disease. Due to their inducibility, strong immunogenicity, and high inter-species homologies, HSPs are among major factors considered to participate in autoimmune disease. Substantial evidence points to a role of immunity to HSPA1A in rheumatoid arthritis (Moodley et al. 2011). Schett and colleagues demonstrated upregulation of HSPA1A expression in synovial tissue

Table 2 ROC curve statistics for serum HSPA1A levels in predicting RA status

HSPA1A	Sensitivity	Specificity
0.185	0.907	0.08
0.21	0.733	0.444
0.23	0.73	0.5
0.26	0.73	0.55
0.31	0.53	0.86
0.38	0.49	0.88
0.42 ^a	0.49	0.91
0.45	0.467	0.91
0.47	0.427	0.94
0.55	0.32	0.79

^a Best overall cut-off having least distance on curve

Fig. 3 Serum HSPA1A level in various disease activity levels. HSPA1A level were significantly higher in patients with high activity of RA compared to other groups (1.66 ± 0.75 , $p < 0.001$) and control subjects had a significantly lower HSPA1A level compared to patients within the remission phase (0.48 ± 0.11). Each point represents mean level $\pm$ SEM of HSPA1A for control subjects ($n=36$), patients in remission ($n=36$), and patient with low ($n=12$), moderate ($n=28$), and high ($n=11$) disease activity

of patients with RA, but not in patients with osteoarthritis, indicating effective cellular stress mechanisms ongoing in RA synovial tissue (Schett et al. 1998). Moreover, expression of HSPA1A is increased by inflammatory cytokines, such as TNF- α (Kang et al. 2009). The resistance of synovial cells to stress is associated with the expression of HSPs in synovial tissues, which can protect cells from apoptotic death (Hromadnikova et al. 2008; Nguyen et al. 2006; Schett et al. 1998). Although the anti-apoptotic role of HSPA1A has been demonstrated in a number of studies (Borges et al. 2012), several other studies have shown that HSPA1A is expressed in RA synovial tissue (Sedlackova et al. 2009) and knock-down of HSPA1A protects RA synoviocytes from stress-induced apoptosis via Akt signaling pathway (Kang et al.

2009), which uphold the importance of HSPA1A role in RA pathogenesis and suggest that HSPA1A has dual functionality depending on its location, cell type, and stimulus type (Dybdahl et al. 2002; De Maio 2011). On the other hand, Schick and colleagues (2004) demonstrated that Hsc70, and not HSPA1A, was overexpressed in RA synovial tissues. However, there are several studies showing increased HSPA1A expression in synovial tissues of patients with RA (Kang et al. 2009; Sedlackova et al. 2009).

Thus far, there have been several serological markers, such as CRP and ESR, proposed to represent the disease activity of RA. However, none of these markers have definitely allowed for accurate monitoring of RA disease activity. To our knowledge, few studies to date have experimentally tested the serum levels of HSPA1A in a group of patients with rheumatoid arthritis. A study by Sedlackova et al. (2011) showed nonsignificant difference in serum HSPA1A levels in patients with RA compared to control subjects. However, the participants in their study were only rheumatoid factor-positive. Hayem and colleagues (1999) showed increased concentrations of anti-HSPA1A antibodies in patients with RA, which its concentration was correlated to RA activity.

We have used an approach involving both rheumatoid factor-positive and factor-negative patients. Specificity of ELISA kit used in this study has been tested previously (Multhoff and Hightower 2011). We observed that serum HSPA1A levels are elevated in patients with rheumatoid arthritis and could be used as a diagnostic test for rheumatoid arthritis. Increased serum levels of HSPA1A in patients with RA were independent of serum RF and anti-CCP status. ROC curve analysis revealed that HSPA1A tends to be a specific test, rather being sensitive in RA diagnosis.

We demonstrated significant increase in HSPA1A serum levels in patients with high RA activity compared to those in remission, or those with low/moderate RA activity. Interestingly, patients in remission still had a significantly higher serum HSPA1A levels compared to control subjects. The overlap of normal subjects and patients with inactive RA is large. While their mean may be different there does not appear to be a cut-off point that differentiates the two groups with this number of participants and future experiments with more participants could help in assigning this cut-off point.

RF and anti-CCP have prognostic and diagnostic value in RA. We did not observe any association between these factors and serum HSPA1A levels. In addition, the association between serum HSPA1A levels and RA activity highlights the importance of HSPA1A as an independent diagnostic and prognostic factor in rheumatoid arthritis.

Finally, in order to verify that serum HSPA1A levels could differentiate high disease activity in RA patients from other states associated with increased HSPA1A levels, we performed the same measurements on patients with newly diagnosed and chronic diabetes. It has previously been shown that

Table 3 Mean serum HSPA1A levels in RF +/- or anti-CCP +/- patients

		Mean serum HSPA1A level (ng/mL)	p value
RF	+	1.10	0.85
	-	1.42	
Anti CCP	+	0.69	0.27
	-	0.99	

p values show the significance of difference in mean values of HSPA1A between groups using the t-test

HSPA1A heat shock protein A1A, RF rheumatoid factor, CCP anti-citrullinated cyclic peptide

diabetes is associated with increased serum HSPA1A levels (Nakhjavani et al. 2010). The ANOVA revealed a significantly higher HSPA1A level in RA patients with high disease activity compared to both groups of patients with diabetes. In addition, mean HSPA1A levels were significantly higher in patients with RA compared to newly diagnosed diabetic patients, but not patients with chronic diabetes.

We are aware that population included in this study was too small to draw any definitive conclusions. However, the results of this study are compatible with suggestion of HSPA1A as danger signals during inflammation. In a clinical perspective, circulating HSPA1A as markers of rheumatoid arthritis may possibly become of diagnostic and severity index advantage.

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