Comparison of Serum Levels of 14-3-3 ETA Proteins between Rheumatoid Arthritis, Osteoarthritis and Normal Controls

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14-3-3 ETA protein, a joint-derived biomarker that up-regulates inflammatory cytokines which enhances local and systemic inflammation and may lead to destructive changes in joints, is thought to be a good diagnostic marker for early RA. To assess the usefulness of serum levels of 14-3-3 ETA in the diagnosis of RA. This is a case-control study which involved 3 groups: group 1 included 30 RA patients, group 2 included 30 primary osteoarthritis patients and group 3 included 30 healthy controls. All study subjects were assessed using laboratory investigations as CBC, ESR, CRP, RF, ACPA as well as serum levels of 14-3-3 ETA protein which were measured through ELISA technique. Mean ± SD levels of 14-3-3 ETA were significantly higher among RA compared to OA and control groups (0.7(0.5), 0.2 (0.1) and 0.3(0.1) ng/ml, respectively) with a sensitivity of 79.3%, specificity of 81.7%, positive predicted value of 86% and negative predicted value of 81%. 14-3-3 ETA also had high diagnostic OR (1478.04). A statistically significant correlation (r= 0.259) was found between serum levels of 14-3-3 ETA and ESR. In conclusion, 14-3-3 ETA is a novel marker for RA that should be used in conjunction with RF and ACPA for diagnosis of the disease.

14-3-3 ETA is a protein marker that has been reported to be significantly higher in Rheumatoid Arthritis (RA) patients' synovial fluid compared to healthy individuals and other rheumatic diseases such as osteoarthritis (OA), ankylosing spondylitis (AS) and gout [1].

The current RA diagnosis is based on the presence of arthritis, autoantibodies including rheumatoid factor (RF) and anti-citrullinated peptides antibodies (ACPA) in addition to acute phase reactants as erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP). However, sometimes it can be extremely hard to diagnose this disease in its early stage [2].

RA autoantibodies are of great diagnostic and prognostic values. Specificity of ACPA for RA is excellent: More than 90% of patients with ACPA have or will soon develop RA. However, its sensitivity has been variable with values ranging from 56–80% in established RA [3]. One of the most useful roles of ACPA testing is the diagnosis of early RA. In early RA (less than two years), ACPA was found in 41–81% [4].

Sensitivity of RF in RA ranges between 60% to 90% and its specificity ranges between 50-85%. RFs are immunoglobulins directed against Fc portion of IgG. IgM is the most commonly tested isotype of RFs due to its large size and hence being easier to detect. Testing for RF is tricky because it can be present in many rheumatic and non-rheumatic diseases such as infections. For instance, RF can be present in up to 76% of patients with HCV infection [5].

Furthermore, it can be found in patients with other autoimmune diseases such as sjogren syndrome, cryoglobulineamia, SLE, MCTD and others. Being non-specific, this made researchers and clinicians in need for

new disease markers. Accordingly, the search for newer more sensitive and specific markers for RA is still ongoing [6].

14-3-3 regulatory proteins are of seven isoforms: α/β, γ, δ/ζ, ε, η, θ/τ and σ. Such proteins are present inside cells and act as adapters that can combine together to form a cuplike structure named the amphipathic groove. They are capable of interacting with many other intracellular proteins to stimulate or inhibit their activities. These interactions may include protein trafficking and cellular signaling. Interestingly, 14-3-3 ETA is thought to be unique among them as its externalization in RA can release heat shock proteins (HSPs) from human peripheral blood mononuclear cells (PBMCs) and B lymphocytes under heat stress [4,7].

14-3-3 ETA is thought to have a direct ability to induce factors linked to inflammation and radiographic damage. 14-3-3 ETA has been shown to induce inflammatory factors such as IL-1 and IL-6 and is linked to the process of joint damage as it also induces factors such as RANKL and MMPs [8].

This study aimed to assess the usefulness of serum levels of 14-3-3 ETA protein in diagnosis of RA in comparison with OA patients and healthy controls; furthermore, to correlate its serum levels with the laboratory parameters of disease activity in RA patients.

Patients and Methods

This study was conducted as a case control comparative study that took place in Clinical Pathology and Rheumatology Departments, Suez Canal University Hospital, Ismailia, Egypt. The study was approved by the institutional review board and ethics committee of Suez Canal University. Subjects fulfilling eligibility criteria were invited to join the study. All participants signed an informed consent upon their approval.

Our sample consisted of three groups. Each group included thirty subjects. The first group included thirty adult RA patients fulfilling the 2010 ACR–EULAR classification criteria for RA [9]. The second group included thirty primary OA patients according to the OA ACR criteria [10]. The third group included thirty healthy volunteers of our hospital workers who are not known to have any chronic illness, matching other groups for age and sex. All study subjects were selected randomly from the patients and blood donors attending the Rheumatology Department and Blood Bank of Suez Canal University Hospital respectively. We excluded patients with psoriatic arthritis and other rheumatic or autoimmune diseases. Patients with other systemic diseases or infections such as hepatic or renal diseases were also excluded.

Study subjects were assessed using detailed clinical history and examination. Biochemical investigations included CBC, which was done by automated blood cell counter (Cell-Dyn 3700, USA); serum levels of ESR were measured manually using the westergren method; CRP and RF were measured using fully automated auto-analyzer Cobas c501 (Roche diagnostics, Manheim, Germany). ACPA values were assessed through the Elecsys anti-CCP assay on the Cobas e 411 auto-analyzer (Roche Diagnostics, Mannheim, Germany) for all subjects from the three groups according to the manufacturer's recommendations which included the use of 2 calibrators. The range of calibrators concentrations was 0-200 U/ml and the measuring range of the assay was <7–1000 U/ml with a cut-off level 17 U/ml. The Elecsys anti-CCP immunoassay is a two-step IgG-capture test with streptavidin-coated microparticles and electrochemiluminescence detection. 14-3-3 ETA protein serum levels were evaluated using manual enzyme-linked immunosorbent assay (ELISA) (Catalog Number. CSB-EL026289HU) where antibody specific for YWHAH has been pre-coated onto a microplate. Standards (7 concentrations: 0.625, 1.25, 2.5, 5, 10, 20, 40ng/ml) and samples were pipetted into the wells and any YWHAH present was bound by the immobilized antibody. After removing any unbound substances, a biotin-conjugated antibody specific for YWHAH was added to the wells. After washing, avidin conjugated Horseradish Peroxidase (HRP) was added to the wells. Following a wash to remove any unbound avidin-enzyme reagent, a substrate solution was added to the wells and color developed in proportion to the amount of YWHAH bound in the initial step. The color

development was stopped and the intensity of the color was measured using a microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 570 nm. Then a log-log ELISA curve was made manually for the calibrators where absorbance readings were put on the y axis while concentrations of the 7 calibrators were put on the x axis to get the measurements of the study subjects' samples. The minimum detectable dose of human YWHAH is typically less than 0.156 ng/ml. The assay used has high sensitivity and excellent specificity for detection of human YWHAH.

Statistical Analysis

Data were tabulated using spreadsheet. Data analysis was done using Statistical Package for Social Solution (*SPSS v.23*) software to determine the statistical inference of the variables in question. Data were grouped as discrete and continuous variables: Discrete variables were presented in the form of frequency and percentage tables. Inferences were done using Yates corrected chi-square and Fisher's Exact tests were used. Continuous variables were presented in the form of means and standard deviation (SD). Non-parametric tests such as Mann-Whitney U and Kruskal-Wallis tests as well as Spearman's correlation test were used. *P*-value < 0.05 was considered significant.

Results

Demographic data were obtained from all study participants of the three groups. There was no statistical difference between the three groups in terms of age and sex. Mean (SD) age for RA group was 45.5(9.5) years, OA group was 50.3(8.8) and control group was 46.2(6.9) years old (*P*=0.1). Females represented 96.7% of RA group, 83.3% of OA group and 86.7% of control group (*P* =0.23).

RF was positive in 23(76.7%) subjects in the RA group compared to 2(6.7%) subjects only in each of the OA and control groups. RA patients had significantly higher RF titers (mean ± SD) compared to OA and control groups (123.2±10.3, 15.3±5.4, 14.4±5.2 IU/ml respectively). ACPA was positive in 29 RA patients and no subjects from the second and third groups were positive for ACPA. Serum levels of ACPA were 64.4±6.6 IU/ml in the RA group compared to 9.2±1.5 and 8.9±1.7 IU/ml in OA and control groups respectively.

Markers of inflammation including ESR and CRP were significantly higher in the RA group compared to the other groups. Levels of ESR were 32.5±8.2 IU/ml in the RA group, 21.97±6.6 IU/ml in the OA group and 16.1±7.3 IU/ml in the control group. CRP levels were 17.9±5.04 for the RA group compared to 5.7±2.8 and 4.9±2.4 IU/ml in OA and control groups respectively (table 1).

Table 1. Laboratory Findings among the Studied Groups.

Variables	OA group (n=30)	RA group (n=30)	Control (n=30)	*P*-value
Positive RF	2 (6.7%)	23 (76.7%)	2 (6.7%)	<0.001^*
RF Mean±SD (IU/ml)	15.3±5.4	123.2±10.3	14.4±5.2	<0.001$*
Positive ACPA	0	29 (96.7%)	0	
ACPA (U/ml)	9.2±1.5	64.4±6.6	8.9±1.7	<0.001$*
ESR (mm/hour)	21.97±6.6	32.5±8.2	16.1±7.3	<0.001$*
CRP (mg/L)	5.7±2.8	17.9±5.04	4.9±2.4	<0.001$*
14-3-3 ETA protein	0.2±0.1	0.7±0.5	0.3±0.1	<0.001*¥

RF = rheumatoid factor, ACPA = anti-citrullinated peptides antibodies, ESR = erythrocyte sedimentation rate, CRP = C-reactive protein. *P*<0.05 is significant. Statistically significant p value is marked with *, ^ Chi-Square test, $ Mann Whitney U test, ¥ Kruskal Wallis test.

Serum levels of 14-3-3 ETA proteins were significantly higher among RA compared to OA and the control groups (Mean ± SD = (0.7(0.5), 0.2 (0.1) and 0.3(0.1) respectively) (P <0.001) (table 1). We divided our sample based on sex and RF positivity to compare the serum levels of 14-3-3 ETA proteins. However, there was no difference between males and females (0.4±0.3 vs 0.5±0.4) nor between RF positive and negative patients (0.7±0.5 vs. 0.8±0.6) (table 2).

Table 2. Comparison of serum levels of 14-3-3 ETA Protein based on Sex and RF positivity in All Study Subjects

	14-3-3 ETA protein	*P* value
Sex		
Males	0.4±0.3	NS
Females	0.5±0.4	
RF		
Positive	0.7±0.5	NS
Negative	0.8±0.6	

Mann-Whitney U test (*P*>0.05 is not significant (NS).

There was a statistically significant positive correlation between 14-3-3 ETA protein and ESR. However, there was no correlation between 14-3-3 ETA protein with age, ACPA and CRP (table 3).

ROC curve for 14-3-3 ETA protein for diagnosis of RA showed a 79.6% accuracy. A cutoff value of ≥0.25 was found to be 79.3% sensitive and 81.7% specific for RA diagnosis (Figure 1) (table4).

Logistic regression analysis of markers associated with RA showed that ESR (OR=1.24, P =0.001) and 14-3-3 ETA protein (OR 1478.04, P=0.01) are highly associated with the presence of RA (table 5).

Table 3. Correlation of 14-3-3 ETA Protein with other variables in All Study Subjects

	14-3-3 ETA protein	
	Correlation coefficient	*P* value
Age	-0.038	NS
ESR	0.259	0.047*
ACPA	0.109	NS
CRP	-0.079	NS

* *P* value >0.05 is not significant (NS).

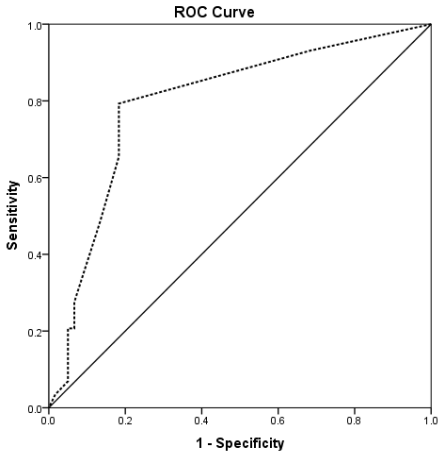

Figure 1. ROC curve for 14-3-3 ETA protein for diagnosis of RA showed a 79.6% accuracy

Table 4. Area Under the Curve of 14-3-3 ETA for diagnosis of RA

Area	Std. Error	*P* value	Asymptotic 95% Confidence Interval	
			Lower Bound	Upper Bound
0.796	0.052	<0.001	0.694	0.898

* *P* value <0.05 is significant.

Table 5. Logistic Regression Analysis of Markers of RA

	B	S.E.	*P* value	OR	95% C.I. for OR	
					Lower	Upper
Female Sex	6.116	5.274	NS	452.92	0.015	13966915.87
ESR 1st Hour	0.220	0.067	0.001	1.24	1.092	1.42
14-3-3 ETA protein	7.298	2.816	0.010	1478.04	5.922	368917.71
Constant	-14.147	6.232	0.023	0.000		

B= Beta factor; S.E= Standard error; OR= Odds ratio; CI= Confidence interval

P value >0.05 is not significant (NS).

Discussion

Early diagnosis and treatment are now the main basis of the current treat to target strategy adopted by most of RA management guidelines. Since the current RA associated antibodies such as RF and ACPA are not very sensitive for early diagnosis of RA, there is unmet need for more sensitive and specific markers for detecting early disease. Furthermore, assessment of disease activity is sometimes very hard as many of disease activity scores rely on the presence of tender joints, patients' and physicians' global assessment. This is sometimes inaccurate especially in patients with other concomitant diseases such as fibromyalgia syndrome and secondary OA. Additionally, ESR and CRP are not specific for RA disease activity and may be deceiving in the presence of infection.

Results obtained from the present study showed that serum levels of 14-3-3 ETA proteins were significantly higher in RA patients compared to OA patients and healthy controls (P<0.001). On the other hand, there was no difference in its levels between OA and control groups. Comparing between males and females, there was no difference in serum levels of 14-3-3 ETA proteins. Furthermore, RF positivity did not have any effect on the serum levels of 14-3-3 ETA proteins.

Previous studies compared serum 14-3-3 ETA levels expression in RA, OA and other rheumatic diseases in addition to healthy controls. They showed that its levels in RA were significantly higher than in other autoimmune diseases, OA patients and healthy controls [3, 11].

Furthermore, studies found that serum 14-3-3 ETA levels were significantly higher in early RA patients with radiographic changes versus those without radiographic changes [12].

Additionally, studies suggested that serum levels of 14-3-3 ETA in arthralgia patients could predict patients who

progressed to definite RA [13]. On the other hand, whether ACPA and RF can predict arthralgia in patients who develop to RA, results were inconsistent. For instance, a study of 148 patients with arthralgia suggested that elevated 14-3-3 ETA and ACPA titers, but not RF, were associated with the development of RA [14]. Another study showed that 67% of patients were 14-3-3 ETA positive and levels of 14-3-3 ETA and RF, but not ACPA, were significantly higher in patients who progressed radiographically to RA [15].

In the present study, there was statistically significant correlation between serum levels of 14-3-3 ETA protein with ESR but not with age, CRP, RF and ACPA levels. In this prospect, similar results were found by other authors [11]. Further studies are needed to determine whether 14-3-3 ETA protein can be used for monitoring of disease activity and the development of a score using it instead of ESR and CRP can be very beneficial in the previously mentioned patients. More importantly, serum levels of 14-3-3 ETA do not vary according to age, sex, and maintenance at room temperature, several freeze-thaw cycles, or potential interfering factors in peripheral blood including RF.

During this study, it was suggested that the optimal cutoff point to diagnose RA was measured using ROC curves (≥0.25 ng/mL) with a sensitivity of 79.3%, specificity of 81.7%, and positive predicted value of 86% and negative predicted value of 81%. The AUC was 0.796, which is significantly high with very low standard error (0.052). The 14-3-3 ETA protein also had high diagnostic odds ratio (1478.04).

A similar study that compared established RA to healthy subjects demonstrated a significant AUC of 0.89 with an optimal cut-off of ≥0.19 ng/mL yielding 77.0% sensitivity, 92.6% specificity, a positive predicted value of 70.0%, a negative predicted value of 80.0% and likelihood ratio of 10.4 [11].

Another study comparing early RA with healthy controls demonstrated a significant AUC of 0.90. The ROC curve yielded a sensitivity of 73%, a specificity of 91%, and likelihood ratio of 8.0 [13].

In conclusion, 14-3-3 ETA protein is significantly elevated in patients with RA. While not considered a standard test, the 14-3-3 ETA test may be used with other lab tests in the early diagnosis of RA. The positive correlation between 14-3-3 ETA protein and ESR suggests that it might be useful for monitoring of inflammation associated with RA. However, further larger follow-up studies are required to confirm these results.

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