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Association of NF-κB1/IKKε Gene Expression with Disease Activity Indices in Patients with Early Rheumatoid Arthritis

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ABSTRACT

Background: Pain, swelling, soreness from touching, stiffness in the joints, particularly in the morning, and symmetrical polyarthritis are all symptoms of rheumatoid arthritis (RA), a prevalent form of autoimmune inflammatory arthritis.

Objective: This work was aimed to investigate whether there is a correlation between the expression of the nuclear factor kappa beta1/IKK epsilon (NF-κB1/IKKε) gene and disease activity indices in individuals who have early rheumatoid arthritis.

Patients and Methods: This study involved 50 patients with newly diagnosed RA and 30 subjects serving as controls. Patients were subjected to detailed medical history, clinical examination, and underwent evaluation of illness severity using disease severity scores (DAS-28). Laboratory investigations, and real time PCR was done for NF-B1/IKK. Examination using power Doppler ultrasonography (PDUS) was carried out on 22 of the joints that were affected by RA.

Results: NF-κB1 and IKKε gene expression levels were significantly increased in cases with positive RF and Anti-CCP compared to patients without ($p < 0.001$ for both). Also, according to disease activity; the higher the activity the more NF-κB1 and IKKε gene expression levels with significant differences ($p <0.001$). Higher erythrocyte sedimentation rate (ESR), PDUS, NF-κB1 and IKKε gene expression levels were associated with risk of higher DAS grades in univariable analysis (p=0.007, 0.023, 0.011, 0.002 respectively). However, in multivariable analysis, only higher NF-κB1 and IKKε gene expression levels were considered independent predictors of higher DAS grades (p=0.019, 0.044 respectively).

Conclusion: Our findings suggest that NF-κB1 and IKKε play an important role in RA pathogenesis and could be considered as an activity indicator.

Keywords: Rheumatoid Arthritis, NF-κB1, IKKε, Disease Activity.

INTRODUCTION

Pain, swelling, soreness from touching, stiffness in the joints, particularly in the morning, and symmetrical polyarthritis are all symptoms of rheumatoid arthritis (RA), a prevalent form of autoimmune inflammatory arthritis [(1)]. In rheumatoid arthritis (RA), the synovial membrane of the joints is affected by the immune system, leading to inflammation and damage to the joints. It affects smaller joints, such as finger joints, more than bigger joints [(2)].

The human leukocyte antigen (HLA) and major histocompatibility (MHC) genes are the ones that are considered to be the most significant contributors to an elevated risk of deteriorated rheumatoid arthritis (RA). Minor genes, such as cytokine promoters and T cell signaling genes, among others, have been linked to susceptibility and severity [(3)]. Increased expression of genes involved in inflammation may be caused by epigenetic alterations such as faulty DNA, dysregulated histone marks, or the production of microRNAs [(4)].

There is a small family of inducible transcription factors known as Nuclear Factor kappa-light-chain-enhancer of activated B cells. These factors play an essential part in the function of all mammalian cells [(5)]. It is able to govern the immunological response to infection because it affects DNA transcription, the synthesis of cytokines, cell survival, and other activities that occur inside cells [(6)].

RelA/p65, c-Rel, RelB, NF-B1 (p50), and NF-B2 (p52) are the five members of the NF-B transcription factor family that may form homo- and heterodimers. These transcription factors are involved in the regulation of gene expression. In the absence of certain triggers, these dimers remain linked to IB (inhibitors of NF-B) and are thus prevented from engaging in transcriptional activity [(7)].

The NF-kB signaling system reacts to a variety of stimuli, including cytokine and growth factor signaling to identify pathogen products, as well as DNA damage and carcinogenic stress. The activation of the NF-kappa B "canonical pathway" stimulates the formation of the IKK (IB kinase) protein complex, which contains the IKK and IKK kinases as well as the regulatory subunit IKK/O. This protein complex phosphorylates IB (Inhibitor of nuclear factor kappa-B), which in turn induces its detachment from NF-kappa B and its [(8)].

Inhibitor of nuclear factor kappa-B kinase subunit epsilon, also known as I-kappa-B kinase epsilon or IKK-epsilon (IKK), is an enzyme that in humans is encoded by the I-kappa-B kinase

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epsilon gene Serine/threonine kinase that plays a significant role in the regulation [9].

The aim of this study was to investigate whether there is a correlation between the expression of the NF-κB1/IKKε gene and disease activity indices in individuals who have early rheumatoid arthritis.

MATERIALS AND METHODS

This case control study included a total of 50 patients with recently diagnosed RA and 30 volunteers of age and gender matched healthy controls, attending at Outpatient and Inpatient clinics, Department of Rehabilitation and Physical Medicine, Faculty of Medicine, Benha University Hospitals, Benha. Department of Clinical Pathology, Benha University was the location for the practical component of the research. This study was conducted between September 2020 and April 2021.

Participants in this research satisfied the categorization criteria established by the American College of Rheumatology and the European League Against Rheumatism (ACR/EULAR) [10].

Patients were subjected to detailed medical history, clinical examination, and underwent evaluation of illness severity using disease severity scores (DAS-28).

Exclusion Criteria:

Patients who were less than 16 years old, who has disease as inflammatory or autoimmune condition, or who were affected with infections were not allowed to participate in the research.

Sample collection: 6 milliliters of venous blood were withdrawn and divided between 3 tubes; EDTA tube for CBC and real-time PCR to detect NF-B1/IKK, plain tube for separation of serum (rheumatoid factor (RF), anti-cyclic citrullinated peptide (anti-CCP), and CRP) and sodium citrate tube for ESR.

Molecular Detection of NF-κB1/IKKε by quantitative real time Polymerase chain reaction:

The following procedures were followed to genotype the NF-B1/IKK gene expression in all participants using quantitative real-time PCR (polymerase chain reaction method) and SYBR Green q PCR analysis using commercially available primers: Blood EDTA was used to extract genomic RNA. By using reverse transcriptase, the recovered RNA was transformed into C DNA. Using sets of primers intended to identify the target polymorphism, the extracted DNA was amplified. Direct detection of PCR product by observing the rise in fluorescence of a DNA probe colored with SYBR Green. Whole blood genomic RNA extraction: GENEzol™ TriRNA Pure Kit was used according to the manufacturer's instruction for RNA extraction (Geneaid, UKAS, Cat.TRPD050/100/200) using Veriti Real Time PCR instrument (S/N 2990226743) (Applied Biosystems, Singapore).

The isolated RNA was converted to c DNA by reverse transcriptase: TOPscript™ RT DryMIX (dT18/dN6 plus). RT220 **Cat. No.** RT220 kits was used according to the manufacturer's instruction for isolated RNA conversion to c DNA by reverse transcriptase.

The copy DNA was amplified using sets of primers by real time PCR:

Gene expression analysis was performed using Willowfort HERAPLUS SYBR® Green qPCR Kit Cat. using the SYBR Green qPCR assay. The PCR amplification was done using Stepone Real Time PCR instrument (S/N 271003648) (Applied Biosystems, Singapore).

In a sterile micro centrifuge tube reagents were pipetted as follows:

10µl Master Mix (2x), (200nM) 1µl Forward primers 20x, (200nM) Reverse primers 20x, 20µl Nuclease free water, (up to 250ng) Volume varies DNA template. PCR was performed in thermal cycler according to the following thermal conditions: pre-PCR (1 cycle) 95°C for 10 minutes, following by 40 cycles of denaturation at 95°C for 15 seconds, and annealing and extention at 60°C for 1 minute.

Ultrasonography assessment:

22 joints underwent Power Doppler ultrasonography (PDUS) examinations, with longitudinal and transverse scanning (8–13 MHz) of the dorsal aspects of the chosen joints performed for each RA patient to assess inflammation in accordance with European League against Rheumatism (EULAR) recommendations.

***Ethical Consideration*:**

This study was ethically approved by Faculty of Medicine, Benha University Research Ethics Committee. Written informed consent of all the participants was obtained after ensuring that the goal of the study was conveyed to them. The study protocol conformed to the Helsinki Declaration, the ethical norm of the World Medical Association for human testing.

Statistical analysis

Using Statistical program for Social Science (IBM Corporation Released 2017), the data that was acquired was cleaned up, coded, tabulated, and then uploaded to a personal computer. Armonk, New York: IBM Corporation, 2005. IBM SPSS Statistics for Windows, Version 25.0. The Kolmogorov-Smerinov test was carried out in order to examine the degree to which the data followed a normal distribution. The mean, as well as the standard deviation (SD), for numerically parametric data. Non-numerical data broken down by frequency and proportion.

The Student T Test, the Mann Whitney Test (also known as the U test), The Kruskal-Wallis test, the Chi-Square test, and Fisher's exact test were all used in the process of doing analytical statistics. In order to evaluate how strongly two quantitative factors are associated with one another, a correlation study was carried out. The receiver operating characteristic curve, more often known as the ROC Curve, is used to assess the sensitivity and specificity of quantitative diagnostic tests that classify patients into one of two categories.

The area under the curve (AUC) suggests that a test has high accuracy if it has a value more than 0.9, that the range 0.7–0.9 indicates moderate accuracy, that the range 0.5–0.7 indicates poor accuracy, and that 0.5 implies a chance result. In order to make accurate predictions about risk variables, logistic and ordinal regression analyses were employed in conjunction with generalized linear models. The odds ratio as well as the confidence interval up to 95 percent were computed. If a p value is less than 0.05 at a confidence interval of 95 percent, then it is deemed significant.

RESULTS

This study showed that the mean age of the 50 patients was 49.3±9.7 years: 42 (84%) females and 8 (16%) males. The mean disease duration of the RA cases was 6±2 months.

Table 1 shows the demographic data and characteristics of subjects participated in the study. The family history of RA was positive in 15 (30%) cases. Low activity was detected in 5 (10%) cases, moderate in 7(14%) cases and high activity in 38 (76%). Regarding laboratory parameters, RF was positive in 47 (94%) cases, anti CCP was positive in 44 (88%) cases. Regarding drugs received by RA patients, 40 cases (80%) were on regular steroid, 42 (84%) cases treated with hydroquin, 43 (86%) cases received MTX, Imuran was used in 2 cases, leflunomide was taken in 12 cases, cyclophosphamide in 1 case, salazopyrin was taken by 4 cases, no patients were on biologic therapy.

Table (1): Characteristics of the studied subjects

Variable	RA (n=50)	Control (n=30)	P value
Age (years)	49.3±9.7	47.7±5.8	0.231
Sex (female: male)	42:8	22:8	0.248
TLC ($X10^9$/L)	7.5±1.8	7.7±1.8	0.117
Hemoglobin (g/dL)	10.6±1.1	13.4±0.5	<0.001
Platelet ($X10^9$/L)	292±71.1	244.2±60.3	0.007
ESR (mm/h)	67.6±15.4	8.9±1.4	<0.001
BMI (Kg/m^2)	23.7±3.1		
Disease duration (months)	6±2		
Morning stiffness (minutes)	104.3±95.8		
Number of tender joints	3.5 ±2.1		
Number of swollen joints	2.1±1.8		
DAS 28	5.5±1.2		
PDUS	11.86 ± 2.91		
Positive RF titer (U/mL)	81.1±19.12		
Positive Anti CCP (U/mL)	52± 12.10		
NF-κB1	199171.7± 47792.75	3527.1±875.32	**<0.001**
IKKε	17458.5± 4164.5	4820.4±1105.11	**<0.001**

Table 2 shows that NF-κB1 and IKKε in patients who had positive RF and Anti-CCP tests had gene expression levels considerably greater than patients who did not have positive tests ($p < 0.001$ for both). Also, regarding disease activity; the greater active form the higher NF-κB1 and IKKε gene expression levels with significant differences ($p <0.001$).

Table (2): Comparisons between NF-κB1 and IKKε gene expression levels regarding RF, Anti CCP, and disease activity status.

		NF-κB1 gene expression	P value	IKKε gene expression	P value
RF u/ml	**Negative**	8155.9±2036.1	0.019	9794.9±1248.5	0.01
	Positive	211364.2±52841.0		17947.7±4483.32	
Anti CCP u/ml	**Negative**	18180.7±4542.3	0.021	12185.9±2023.5	0.05
	Positive	223852.3±55923.4		15005.5±3750.25	
Low disease activity		5390.9±2784.3	**P1=0.639**	7232.8±1551.4	P1=0.122
Moderate disease activity		6927.0±1000.5	**P2<0.001**	8437.5±862.1	P2<0.001
High disease activity		260082.7±640518.3	P3<0.001	20465.8±13135.0	P3<0.001

P1= low versus moderate, P2= low versus high, P3= moderate versus high

Figure (1A&B): ROC curve shows sensitivity analysis for the diagnosis ability of NF-κB1 and IKKε gene expression for RA and disease activity; figure 1A: the curve shows that NF-κb1 and IKKε gene expression can significantly diagnose RA with high accuracy AUC (AUC 0.95, 0.95 respectively), sensitivity 88%, and 92%, specificity 90% and 90% respectively. Figure (1B) shows that NF-κB1 and IKKε gene expression can significantly detect active RA with high accuracy regarding NF-κB1 (AUC 0.985) and perfect AUC regarding IKKε gene expression, sensitivity 97.4%, and 100%, specificity 100% and 100% respectively.

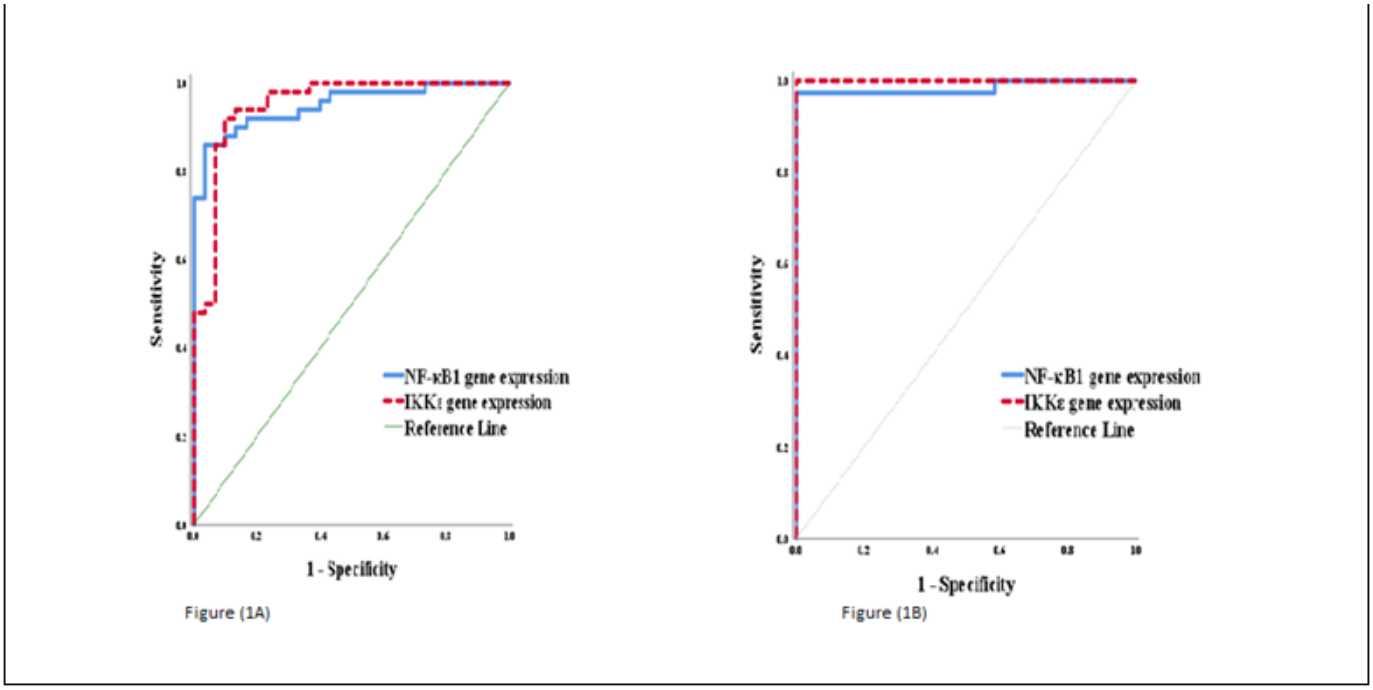

Figure (1 A): ROC curve showing the predictability of NF-κB1 and IKKε gene expression for diagnosis of RA, (1B): ROC curve showing the predictability of NF-κB1 and IKKε gene expression for RA disease activity

The association and correlation of NF-κB1 and IKKε gene expression levels to the clinical characteristics are presented in Table 3.

Table (3): Correlation of NF-κB1 and IKKε gene expression levels with the studied parameters.

	NF-κB1 gene expression		IKKε gene expression	
	Rho	**P value**	**Rho**	**P value**
Age(years)	0.184	0.201	0.236	0.151
BMI(Kg/m^2)	-0.208	0.148	-0.226	0.114
TLC(X10^9/L)	-0.026	0.816	-0.041	0.718
Hemoglobin (g/dL)	-0.104	0.101	-0.103	0.201
Platelet (X10^9/L)	-0.123	0.147	-0.156	0.168
ESR (mm/h)	0.776	**<0.001**	0.749	**<0.001**
Creatinine (mg/dL)	0.200	0.136	0.259	0.072
ALT (U/L)	0.198	0.167	0.202	0.160
FBS (mg/dL)	-0.138	0.339	-0.116	0.420
TG (mg/dL)	-0.164	0.256	-0.174	0.228
CRP (mg/L)	-0.045	0.757	-0.057	0.694
RF(U/mL)	0.22	0.137	0.207	0.163
Anti CCP(U/mL)	0.211	0.170	0.182	0.237
Disease duration (months)	0.041	0.777	0.012	0.935
Morning stiffness(minutes)	0.981	**<0.001**	0.972	**<0.001**
DAS 28 (score)	0.979	**<0.001**	0.994	**<0.001**
Number of tender joints	0.901	**<0.001**	0.926	**<0.001**
Number of swollen joints	0.876	**<0.001**	0.902	**<0.001**
PDUS	0.782	**<0.001**	0.831	**<0.001**

NF-κB1 and IKKε gene expression levels were significantly positively correlated (r= 0.88, p value <0.001).

Regression analysis was conducted for prediction of RA and disease activity are shown in Table 4.

Table (4): Logistic regression analysis for prediction of RA development.

	Univariate			**Multivariate**		
	P value	**OR**	**95% CI**	**P value**	**OR**	**95% CI**
NF-KP1	0.001	1.003	1.001-1.010	0.008	1.002	1.001-1.012
IKKε	<0.001	1.002	1.001-1.012	0.006	1.004	1.001-1.007

OR, odds ratio; CI, confidence interval. Logistic regression analysis was used.

DISCUSSION

The nuclear factor that allows B-cells to produce kappa free light chains has many components (NF-B1) that play a significant part in the regulation of a number of different biological processes. Numerous research indicated a correlation between the overexpression of members of the NF-B1 family and autoimmune conditions such as psoriasis, rheumatoid arthritis (RA), and systemic lupus erythematosus (SLE) [(12)].

It is generally agreed that NF-B is one of the most essential regulators of the expression of proinflammatory genes. The NF-B1 signaling pathway plays a significant part in the course of the RA illness, as well as the inflammatory process and the survival of synovial cells [(12)].

Immunoreactive IKK protein may be found in high levels in primary fibroblast-like synoviocytes that have been isolated from the synovium of individuals with RA and osteoarthritis. TNF- and IL-1 boost the action of IKK, which ultimately results in the breakdown of endogenous IκBα and the translocation of NF-B into the nucleus. This ultimately resulted in the activation of collagenase-1, IL-6, and IL-8, as well as ICAM-1 [(13)].

According to the findings of this research, the levels of expression of NF-B1 and IKK were much higher among Egyptian RA patients than among healthy controls, and these differences were statistically significant (p =0.001 for each).

In the current research, the expression of levels was shown to be greater among patients who had high or moderate disease activity in comparison to those who had low disease activity. Additionally, its levels correspond with indicators of activity such as ESR levels, the length of morning stiffness, the number of joints that are sensitive and swollen, the DAS-28, and PDUS. **Elkhawaga *et al.*** [(14)] found a connection between the NF-B1 polymorphism and the development of RA. **Wang *et al.*** [(15)] believed that IKK was an activity indicator for RA back in

2005. Despite this, **Gomes da Silva *et al.* [16]** observed that there was no association between the NF-B1 gene polymorphism and DAS28 in a group from Brazil.

Previous clinical research stated that the NF-B/IKK signaling pathway was connected to the activity of RA [12]. Synoviocytes in RA that express IKK2 had the potential to stimulate the activation of NF-B. In addition, it has been stated that the phosphorylation of regulatory protein IB is what drives NF-B activation, which is an interesting finding. In 2017, **Salinas and coworkers [12]** found a notable difference between the NF-B1 expression levels of individuals with clinical disease activity and those in clinical remission.

NF-B1 and IKK gene expression levels were significantly higher among patients with positive RF and positive Anti-CCP results compared to patients with negative results ($p < 0.001$ for both), which agreed with the findings of **Sánchez-Maldonado *et al.* [17],** who discovered a significantly increased risk of developing RA in Anti CCP-positive patients who carried the NFKB1rs4648110A/A genotype or the NFKB2rs115748.

In the present work, ROC curve (Fig1A) showed that NF-κB1 and Ikkε gene expression can significantly diagnose RA with high accuracy AUC (AUC 0.95, 0.95 respectively), sensitivity 88%, and 92%, specificity 90% and 90% respectively. Regarding diagnosis of RA activity, ROC curve showed that NF-κB1 and IKKε gene expression can significantly detect active RA with high accuracy regarding NF-κB1 (AUC 0.985) and perfect AUC regarding IKKε gene expression, sensitivity 97.4%, and 100%, specificity 100% and 100% respectively. This agreed with **Sánchez-Maldonado and his colleagues [17]** who found that at early and late stages of joint inflammation, activated NFKB was detected in the synovium of RA patients. **Sabir *et al.* [18]** also found that, Among the NKPF category, NFKB1 found to be increased, in the RA sample, whereas NFKB2 and RELB are decreased.

Zhou *et al.* [19] showed that IKKε-/- mice with CIA [Collagen induced Rheumatoid Arthritis (CIA) mice model] had less inflammatory response than wild type mice model (WT mice). That mean, the clinical RA articular damage rates were significantly decreased in IKKε-/- mice with significance decrease in NF-κB expression. Also, inflammatory responses were remarkably decreased than those of the negative contr

ols in WT mice administrated with IKKε inhibitor amlexanox. Thus, IKKε induced the NF-κB signaling pathway by stimulation the activation of inflammatory cytokines, and inflammatory response. IKKε has been proved to be correlated with the activity and severity of RA[12].

In the present study, Regression analysis was conducted for prediction of higher DAS activity grade. Higher ESR, PDUS, NF-κB1 and IKKε gene expression levels were associated with risk of higher DAS grades in univariate analysis. However, in multivariable analysis, only higher NF-κB1 and IKKε gene expression levels were considered independent predictors of higher DAS grades among RA cases.

This result was consistent with that of **Wang *et al.* [15],** who verified the role of the NF-B p65 subunit in cytokine-induced up-regulation of the human IKK gene in many cell types by physically interacting with the functional kappa B site on the IKK promoter.

CONCLUSION

Our findings suggest that NF-κB1 and IKKε play an important role in RA pathogenesis and could be used as activity indicators of motion. Considering the limitations of existing RA treatments, modulating the expression of these transcripts may represent a promising therapeutic target despite their significant side effects—remain the primary therapy to manage inflammation and avoid potential consequences.

We are aware that a significant drawback of this study is the small sample size, and the chosen genes may be challenging to quantify in routine clinical settings. Nevertheless, they may help determine individuals who have not shown clinical benefit at a secondary or tertiary care hospital with a rheumatology department.

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