Diagnostic role of lncRNA GAS5 and its genetic polymorphisms rs2067079, rs6790 and rs17359906 in rheumatoid arthritis

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Abstract. The aim of the present study was to detect the serum levels of long non-coding RNA (lncRNA) growth arrest-specific 5 (GAS5) in patients with rheumatoid arthritis (RA) and healthy controls, and determine the association between the rs2067079, rs6790, and rs17359906 single-nucleotide polymorphisms (SNPs) of lncRNA GAS5 gene with RA risk in the Egyptian population. Reverse transcription-quantitative PCR and real-time PCR were used to measure the serum levels of lncRNA GAS5 and genotype the two distinct alleles at the SNP sites of lncRNA GAS5 gene in 200 patients with RA and 150 controls. The mean serum levels of lncRNA GAS5 were significantly lower in the patients with RA compared with the controls (P<0.0001), and the serum levels of lncRNA GAS5 were significantly negatively associated with erythrocyte sedimentation rate, C-reactive protein levels and anti-cyclic citrullinated peptide levels in the patients with RA. The TT genotype of rs2067079 SNP was significantly associated with a decreased risk of RA [TT vs. CC: Odds ratio (OR)=2.358; 95% confidence interval (CI), 1.114-5.131; P=0.045) and the risk of rs2067079 SNP reduced with a recessive pattern (TT vs. TC + CC: OR=2.374; 95% CI, 1.091-5.123; P=0.037). rs6790 SNP was associated with RA risk in the recessive model (AA vs. GA + GG: OR=2.55; 95% CI=1.39-5.32; P=0.02). No significant associations were noted between the rs17359906 SNP and RA risk (P>0.05) or between the lncRNA GAS5 levels and their respective genotypes at the three SNPs in patients with RA (all P>0.05). Based on the results of the present study, lncRNA GAS5 may serve as a biomarker for the early detection of RA. The TT genotype of rs2067079 SNP was significantly associated with a decreased risk of RA, and a reduced risk of rs2067079 SNP was observed with a recessive pattern. rs6790 SNP was associated with RA risk in the recessive model.

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Introduction

Rheumatoid arthritis (RA) is a common systemic autoimmune disorder that influences ~1% of the general population. Advanced RA can be critically morbid with a decisively fatal rate that reduces survival by 10 years (1). RA is an irreversible inflammatory disorder that is identified based on increased inflammation, chronic synovitis, advanced joint devastation and general extra-articular manifestations, such as pericarditis, neuropathy, rheumatoid nodules and vasculitis. Despite several years of research, the pathophysiology of RA remains obscure to a great extent, although it has been determined that a range of inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) participate in RA pathogenesis (2,3). Several biological processes, such as the growth and function of immune cells are regulated by long noncoding RNAs (lncRNAs). More recently, several lncRNAs have bene identified to be involved in the pathophysiology of autoimmune inflammatory disorders (4), such as RA (5), autoimmune thyroid disease and systemic lupus erythematosus (SLE) (6).

Growth arrest-specific 5 (GAS5), a type of lncRNA, is a 650-nt broad-spectrum growth repressor. lncRNA GAS5 is associated with an increased risk of SLE in mice (7). Furthermore, genetic analysis has revealed that 1q25, the chromosomal site of GAS5, is associated with the pathogenesis of SLE in humans (8).

LncRNA GAS5 is associated with RA, and it functions as an effective inhibitor of the glucocorticoid receptor (GR) through its RNA glucocorticoid response element (9). Several studies have detected lncRNA GAS5 in patients with autoimmune diseases and reported that GAS5 levels decrease markedly in these patients when compared with healthy individuals (10). Recently, genetic studies have reported several single-nucleotide polymorphisms (SNPs) that are associated with susceptibility to RA (11). Certain SNPs are present within exons; however, several other SNPs are present within introns, involving lncRNAs (12). Subsequently, lncRNA genetic polymorphisms may be associated with the molecular pathophysiology of RA, and investigating these variants may be

valuable to reveal genetic aspects linked with the development of RA.

The aim of the present study was to detect the serum levels of lncRNA GAS5 in patients with RA and healthy controls and establish the association between rs2067079, rs6790 and rs17359906 SNPs of the lncRNA GAS5 gene with RA risk in the Egyptian population.

Patients and methods

Patients. In the present study, 350 Egyptian participants were recruited between January 2019 and June 2019 from the Rheumatology and Rehabilitation Department, Fayoum University Hospital, Fayoum University, Egypt. The participants were divided into two groups: 200 patients with RA (152 females and 48 males; mean age, 42.45±0.89 years) and 150 age- and sex-matched healthy controls (112 females and 38 males; mean age, 40.85±0.99 years).

The patients were diagnosed via clinical examination, radiological outcomes and laboratory screenings, and in accordance with the 2010 Rheumatoid Arthritis Classification Criteria (13). Exclusion criteria included other autoimmune diseases and other rheumatic, inflammatory or cardiovascular diseases. Written informed consent was obtained from all participants, and this study was approved by the Ethics Committee of the Faculty of Medicine, Fayoum University. The study procedures were performed in accordance with the ethical code of the World Medical Association Declaration of Helsinki for human experiments (14).

Sample collection. Fresh venous blood (5 ml) from the antecubital vein of all participants was collected by venipuncture into EDTA-coated plain tubes to evaluate the erythrocyte sedimentation rate (ESR) using the Wintrobe method (15) and to extract the DNA for reverse transcription-quantitative (RT-q)PCR. In addition, 3-ml venous blood was collected and incubated at 37°C for 15 min and centrifuged at 3,000 x g for 10 min at room temperature to separate the serum, which was then stored in aliquots at -80°C.

Biochemical investigations. Serum was used to determine the levels of C-reactive protein (CRP), anti-cyclic citrullinated peptide (anti-CCP) antibodies and lncRNA GAS5 levels. CRP and anti-CCP levels were determined using a commercially available Human C-Reactive Protein ELISA kit (cat. no. RAB0096; Sigma-Aldrich; Merck KGaA) and an anti-CCP ELISA kit (Orgentec Diagnostika GmbH).

DNA extraction. Genomic DNA was segregated from whole peripheral blood using a QIAamp DNA blood mini-kit extraction kit (Qiagen GmbH) according to the manufacturer's protocol. Purified samples of DNA were stored at 4°C until required for PCR (16).

Real-time PCR. This phase included DNA amplification and identification of rs2067079 (C>T), rs6790 (G>A) and rs17359906(G>A) SNPs (Table I), (selected from the lncRNASNP2 database (bioinfo.life.hust.edu. cn/lncRNASNP#!/) and situated in the chromosome locus transcribed into lncRNA GAS5 utilizing Real MOD™

RT-PCR MasterMix (cat. no. 44248; iNtRON Biotechnology). The quantity of DNA template used for SNP genotyping was 20 ng/μl in the reaction mix. DNA concentration was detected by estimating the absorption at 260 nm on a Nanodrop ND-1000 spectrophotometer. The sites of genetic information for the studied SNPs are substantiated by the National Center for Biotechnology Information database (ncbi.nlm.nih.gov).

RNA extraction and RT-qPCR. Total RNA (including small RNAs) were extracted from serum using an miRNeasy mini kit (Invitrogen; Thermo Fisher Scientific, Inc.), and RNA concentration was estimated on a NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific, Inc.). Total RNA was reverse-transcribed into cDNA using a miScript II RT kit (Qiagen GmbH). Serum expression levels of lncRNA GAS5 were assessed using GAPDH as the internal control. The sequences of the primers were: GAS5 forward, 5'-AGGTRG GAGYGGGAGCATTCGG-3' and reverse, 5'-CCTGAACTG GAGCCACCAGCAGG-3'; and GAPDH forward, 5'-TAATRG GAGCCCGAGCGGGATA-3' and reverse 5'-TTTGCCCTG GAGGGACCAGCAAA-3'. All primers were part of the RT^2 qPCR Primer assay kit (Qiagen GmbH). A Maxima SYBR Green PCR kit (Thermo Fisher Scientific, Inc.) was used for amplification according to the manufacturer's protocol, using 0.4 μl of each primer (forward and reverse). The thermocycling conditions were follows: 95°C for 1 min; followed by 42 cycles at 95°C for 10 sec, 60°C for 30 sec and 72°C for 1 min. The $2^{-\Delta\Delta Cq}$ method was used normalized to the internal control to calculate the relative expression levels of lncRNA GAS5 (17).

Statistical analysis. Data were analyzed using SPSS version 25 (IBM, Corp.). Plain descriptive analysis as numbers and percentages was performed for qualitative data, using standard deviations as a measurement of variation of quantitative parametric data and an inferential statistical tests. A Student's t-test was used to compare the differences between two groups. Pearson's correlation analysis was used to assess the correlation between quantitative variables; the value of the Pearson correlation coefficient (r) describes the strength and direction of a correlation ranging from -1 to +1. Receiver operating curve analysis were plotted to determine the optimal cutoff levels of serum lncRNA GAS5 to differentiate between cases and controls. The area under the curve (AUC) was calculated to evaluate the accuracy of the diagnostic tests. LncRNA GAS5 rs2067079, rs6790 and rs17359906 SNPs in the patient and control groups were in Hardy-Weinberg equilibrium. P<0.05 was considered to indicate a statistically significant difference.

Results

The present study included 350 participants: 200 patients with RA and 150 healthy controls. The mean age did not differ significantly between the two groups (P=0.245). The number of females was significantly higher than that of males in each group; however, there was no significant difference between the two groups in terms of sex (P=0.774; Table II).

The mean levels of ESR, CRP and anti-CCP antibodies were significantly higher in the patients with RA (54.02±2.31, 2.56±0.10 and 62.40±26.51, respectively) than the controls (8.05±1.04, 0.59±0.06 and 7.11±4.40, respectively) (P<0.0001).

Table I. Characteristics of the studied SNPs.

SNP	Chromosome	Position	Regulatory feature	Localization
rs2067079	1	173866073	Promoter/Enhancer	Intron
rs6790	1	173865494	Promoter/Enhancer	Exon
rs17359906	1	173867056	Promoter/Enhancer	Intron

SNP, single nucleotide polymorphism.

Table II. Characteristics of the study groups.

Characteristics	Controls, n=150	Patients, n=200	P-value
Age[a]	40.85±0.99	42.45±0.89	0.245
Sex, n (%)			0.774
Female	112 (74.6%)	152 (76%)	
Male	38 (25.4%)	48 (24%)	

[a]Mean ± standard deviation.

Table III. Laboratory parameters and lncRNA GAS5 levels in the study groups.

Factor	Controls, n=150	Patients, n=200	P-value
Erythrocyte sedimentation rate, mm/h	8.05±1.04	54.02±2.31	<0.0001[a]
C-reactive protein, mg/dl	0.59±0.06	2.56±0.10	<0.0001[a]
Anti-cyclic citrullinated peptide antibodies, U/ml	7.11±4.40	62.40±26.51	<0.0001[a]
lncRNA GAS5	1.16±0.03	0.60±0.031	<0.0001[a]

[a]$P<0.0001$. lncRNA GAS5, long non-coding RNA growth arrest specific 5.

Table IV. Multivariate regression analysis of laboratory parameters for prediction of rheumatoid arthritis.

Variable	Odd ratio (95% confidence interval)	P-value
Erythrocyte sedimentation rate, mm/h	2.89 (1.09-7.64)	0.03[a]
C-reactive protein, mg/dl	4.09 (1.35-12.38)	0.01[a]
Anti-cyclic citrullinated peptide antibodies, U/ml	1.41 (0.65-2.91)	0.04[a]
Long non-coding RNA growth arrest specific 5	0.49 (0.18-1.37)	0.17

[a]$P<0.05$.

The mean levels of lncRNA GAS5 were significantly lower in the patients with RA (0.60±0.031) than in the controls (1.16±0.03) (P<0.0001; Table III).

The serum levels of lncRNA GAS5 were significantly negatively correlated with ESR (r=-0.64, P<0.001), CRP (r=-0.38, P<0.001), and anti-CCP antibodies (r=-0.58, P<0.001) in RA patients (Fig. 1).

ROC curve analysis revealed high diagnostic value for lncRNA GAS5 with respect to the diagnosis of RA, with 89% sensitivity, 70% specificity, 0.75 area under the curve and 76% accuracy (Fig. 2). Multivariate regression analysis was used on ESR, CRP, anti-CCP and lncRNA GAS5 levels to determine which parameters independently predicted RA. The results showed that ESR [odds ratio (OR)=2.89, P=0.03], CRP (OR=4.09, P=0.01) and anti-CCP antibodies (OR=1.41, P=0.04) were independent predictors for RA (Table IV).

Table V shows the genotype and allele frequencies of rs2067079, rs6790 and rs17359906 SNPs of the lncRNA GAS5 gene in patients with RA and normal controls. The TT genotype of rs2067079 SNP was significantly related to a decreased susceptibility of RA [TT vs. C: OR=2.358; 95% confidence interval (CI), 1.114-5.131; P=0.045]. Moreover, there was a
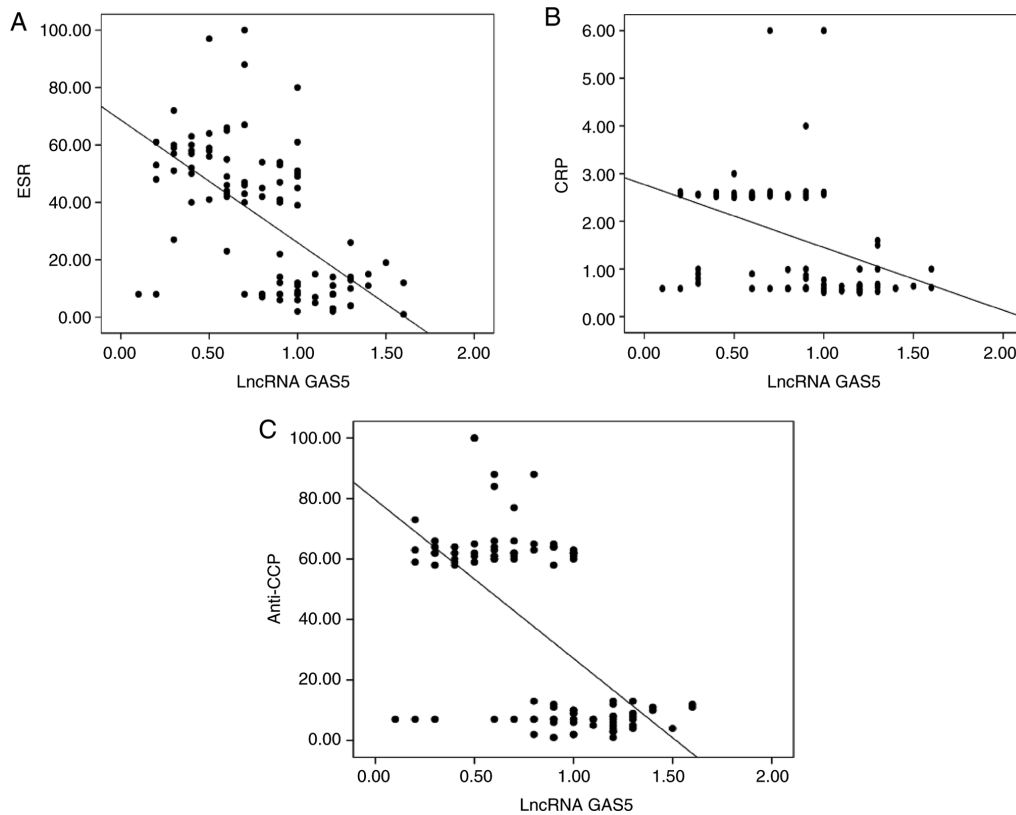
Figure 1. Correlations between lncRNA GAS5 and (A) ESR, (B) CRP and (C) anti-CCP in patients with RA. lncRNA GAS5, long non-coding RNA growth arrest-specific 5; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; anti-CCP, anti-cyclic citrullinated peptide; RA, rheumatoid arthritis.
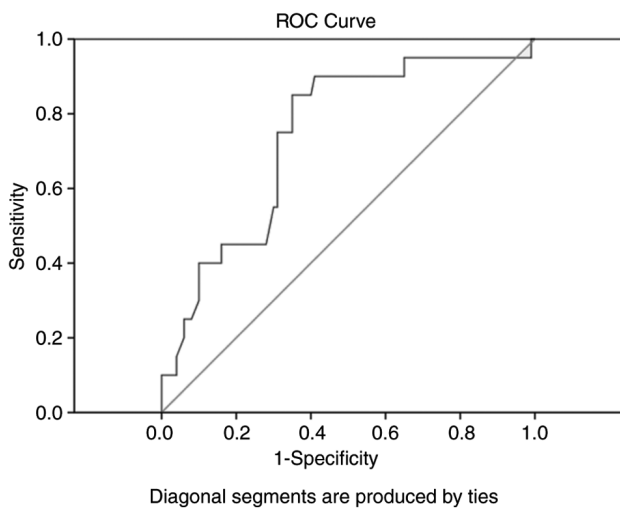

Figure 2. ROC curve analysis of long non-coding RNA growth arrest specific 5 as diagnostic marker of rheumatoid arthritis. ROC, receiver operating characteristic.

reduced risk of rs2067079 SNP with a recessive pattern (TT vs. TC + CC: OR=2.374; 95% CI, 1.091-5.123; P=0.037). The A allele of the rs6790 SNP was present at a higher frequency in the patients compared with the controls, but the difference was not statistically significant (P=0.06). The rs6790 SNP was associated with the risk of RA in the recessive model (AA vs. GA + GG: OR=2.55; 95% CI, 1.39–5.32; P=0.02). Regarding rs17359906 SNP, no significant difference was noted between patients and controls with regard to genotype or allele.

The association between lncRNA GAS5 levels and their corresponding genotype frequencies in the three SNPs were next analyzed. No significant differences were noted in the levels of lncRNA GAS5 amongst the different genotypes in patients with RA (Table VI).

Discussion

RA is an aggressive eradicative disorder characterized by joint destruction. The pathophysiology of RA is complicated and includes individual (genetic predispositions) and environmental (bacterial or viral infections) factors (18).

Recent studies have reported that lncRNAs are associated with numerous biological processes, particularly with immune cell function and the genetic pathogenesis of immune-related disorders (4,19).

Furthermore, lncRNAs serve diverse crucial roles, which involve suppressing the transcriptional machinery, influencing mRNA stabilization, regulating DNA modulation, promoting RNA degradation and inducing immune cell apoptosis, in several physiological processes (20). A previous study has reported that the expression of ~85 lncRNAs in CD14^{+} monocytes in patients with RA is significantly regulated by TNF-α and IL-6 receptor inhibitors. These results suggest that lncRNAs are involved in RA pathophysiology (21).

LncRNA GAS5, located at chromosome 1q25, which was initially segregated from mice NIH 3T3 cells, is a small nucleolar RNA host gene (22,23). LncRNA GAS5 levels are decreased in CD4^{+} T and B cells in patients with RA and SLE compared with control healthy individuals (24). The expression of lncRNA

Table V. Genotype and allele frequencies of SNPs of lncRNA GAS5 in the different study groups.

A, rs2067079 C>T

Genotype	Controls, n=150 (%)	Patients, n=200, (%)	P-value	OR (95% CI)
TT	6 (4)	3 (1.5)	0.045[a]	2.358 (1.114-5.131)
CT	36 (24)	50 (25)	0.777	0.951 (0.732-1.261)
CC	108 (72)	147 (73.5)	Reference	
C allele	252 (84)	345 (86.2)	0.396	0.942 (0.763-1.148)
T allele	48 (16)	55 (13.8)	Reference	
Dominant model				1.042 (0.804-1.343)
CC	108 (72)	147 (73.5)	0.8	
CT + TT	42 (28)	53 (26.5)	Reference	
Recessive model				2.374 (1.091-5.123)
TT	6 (4)	3 (1.5)	0.037[a]	
CT + CC	144 (96)	197 (98.5)	Reference	

B, rs6790 G>A

Genotype	Controls, n=150 (%)	Patients, n=200, (%)	P-value	OR (95% CI)
AA	12 (8)	40 (20)	0.09	2.78 (1.265.88)
GA	66 (44)	80 (40)	0.56	1.06 (0.64-1.78)
GG	72 (48)	80 (40)	Reference	
G allele	210 (70)	240 (60)	0.06	1.49 (1.04-2.12)
A allele	90 (30)	160 (40)	Reference	
Dominant model				1.33 (0.82-2.15)
GG	72 (48)	80 (40)	0.49	
GA + AA	78 (52)	120 (60)	Reference	
Recessive model				2.55 (1.39-5.32)
AA	12 (8)	40 (20)	0.02[a]	
GA + GG	138 (92)	160 (80)	Reference	

C, rs17359906 G>A

Genotype	Controls, n=150 (%)	Patients, n=200, (%)	P-value	OR (95% CI)
AA	16 (10.6)	18 (9)	0.61	1.098 (0.761-1.574)
GA	70 (46.7)	89 (44.5)	0.2	1.269 (0.864-1.839)
GG	64 (42.7)	93 (46.5)	Reference	
G allele	198 (66)	275 (68.8)	0.07	1.149 (0.979-1.349)
A allele	102 (34)	125 (31.2)	Reference	
Dominant model				0.839 (0.679-1.044)
GG	64 (42.7)	93 (46.5)	0.13	
GA + AA	86 (57.3)	107 (53.5)	Reference	
Recessive model				0.848 (0.588-1.209)
AA	16 (10.6)	18 (9)	0.36	
GA + GG	134 (89.4)	182 (91)	Reference	

[a]P<0.05. OR, odds ratio; CI, confidence interval.

GAS5 increases during growth arrest induced by serum starvation or suppression of mammalian target of rapamycin (24).

Furthermore, GAS5 can stimulate cell cycle arrest at the G0/G1 phase by suppressing the transcription of genes, such as glucocorticoid receptors and androgen, indicating that GAS5 participates in the pathophysiology of RA and SLE by inducing cell cycle arrest. GAS5 also serves as a microRNA (miR)sponge, such as for miR-21. As miR-21 is generally

Table VI. Association of lncRNA GAS5 levels with their respective genotype in the patients with rheumatoid arthritis.

Variable	Genotype	n	lncRNA GAS5	P-value
rs2067079 C>T	CC	147	0.65	0.405
	CT	50	0.58	
	TT	3	0.90	
rs6790 G>A	GG	80	0.89	0.551
	GA	80	0.56	
	AA	40	0.61	
rs17359906 G>A	GG	93	0.68	0.519
	GA	89	0.81	
	AA	18	0.51	

lncRNA GAS5, long non-coding RNA growth arrest specific 5.

implicated in enhancing Th17 cell differentiation (25,26) and participates in the pathogenesis of several autoimmune disorders, the decreased expression of GAS5 may be involved in the pathophysiology RA and SLE via uncontrolled miR-21 function in Th17 cells (27). In the present study, the serum levels of lncRNA GAS5 were investigated in patients with RA and healthy controls. Considering the criteria for RA diagnosis, ESR, CRP levels, and anti-CCP antibody levels were assessed and the results showed that the mean values of these parameters significantly increased in the patients compared with the controls (P<0.0001). The mean levels of lncRNA GAS5 significantly decreased in the patients with RA compared with the controls (P<0.0001).

These findings are consistent with those of Mayama *et al* (10), who investigated the expression of lncRNA GAS5 in various autoimmune diseases. They revealed that GAS5 expression significantly decreased in the CD4$^+$ T and B cells of patients with RA compared with healthy controls.

Wu *et al* (28) also reported that GAS5 expression significantly decreased in the plasma of patients with SLE compared with healthy controls. Furthermore, Li *et al* (29) examined three lncRNAs (Linc0597, lnc-DC and GAS5) in patients with SLE and found that the levels of these lncRNAs was significantly decreased in patients with SLE compared with healthy controls.

Several studies have proposed that numerous lncRNA-related SNPs are associated with the pathophysiology of autoimmune disorders. The GAS5 gene exists in the disease susceptibility region in the mice BXSB strain and causes glomerulonephritis with SLE. A locus on chromosome 9p21, including lncRNA ANRIL, is significantly associated with predisposition to type II diabetes. Furthermore, several related SNPs alter the transcription and processing of ANRIL transcripts in the pathogenesis of human diseases (30,31).

In the present study, the association between rs2067079, rs6790 and rs17359906 SNPs of the lncRNA GAS5 gene with RA risk in the Egyptian population was assessed. The TT genotype of rs2067079 SNP was significantly associated with a decreased risk of RA. Moreover, there was a reduced risk of RA with the rs2067079 SNP with a recessive pattern.

Li *et al* (29) performed a study to elucidate the possible association between the rs2067079 C>T SNP of lncRNA GAS5 and SLE risk, but found no significant association. Eftekharian *et al* (32) reported an association between rs2067079 SNP of lncRNA GAS5 and multiple sclerosis risk. They found a significantly higher percentage of the T allele of rs2067079 SNP in patients with MS than in healthy controls, and showed that this variant was associated with MS risk in a co-dominant and recessive patterns (32).

Several studies have revealed that certain SNPs in the promoter region of GAS5 can regulate its expression and are associated with the susceptibility to carcinomas. For example, rs6790 SNP is a biomarker for adverse effects resulting from chemoradiotherapy in patients with nasopharyngeal carcinoma (33). CHIP-seq data revealed that rs6790 SNP is located in an active promoter region or enhancer region, and *silico* analysis detected that this variant has a considerable feature of expression quantitative trait locus. Both CHIP-seq data and *silico* analysis revealed the involvement of rs6790 SNP in the variations of GAS5 expression. In addition, rs6790 was revealed to exert repressor effects of GAS5 on the transcriptional activity of glucocorticoid receptor in autoimmune disorders (32).

The A allele of rs6790 SNP existed at a higher frequency in the patients compared with the controls, but it was not statistically significant. rs6790 was associated with the risk of RA in the recessive model. Wu *et al* (34) reported that the genotype frequencies of the rs6790 SNP of lncRNA GAS5 gene was significantly associated with the risk of RA, but they did not reveal the relation of this SNP with RA risk with dominant and recessive patterns. Thus, further studies are required to explain the specific role of lncRNA GAS5 and its polymorphisms in RA development.

In conclusion serum lncRNA GAS5 levels were significantly decreased in patients with RA, which indicates that it is a candidate biomarker for the early diagnosis of RA, highlighting its potential for use in the early detection of RA for improved treatment outcomes. The TT genotype of rs2067079 SNP of lncRNA GAS5 was significantly associated with a decreased risk of RA. Furthermore, there was a reduced risk of rs2067079 SNP with a recessive pattern, whereas rs6790 SNP was associated with RA risk in the recessive model.

Availability of data and material

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

AME, HSES, NKA and SNG performed the experiments and participated in data analysis. SS recruited the participants and participated in data analysis. All authors participated

in designing the study, performing statistical analyses and writing the manuscript. All authors have read and approved the final manuscript. AME, HSES, NKA, SNG and SS confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Written informed consent was obtained from all participants, and this study was approved by the Ethics Committee of the Faculty of Medicine, Fayoum University. The study procedures were performed in accordance with the ethical code of the World Medical Association Declaration of Helsinki for human experiments.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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