DJS Vol. 46(2) (2023) –pp.96-105 ISSN: 1012-5965

Delta Journal of Science
Available online at
https://djs.journals.ekb.eg/

Research Article

CHEMISTRY

Biochemical study on serum expression of long non-coding RNA metastasis associated lung adenocarcinoma transcript 1 (MALAT1) in Behçet's disease

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Received: 27/3/2023 **Accepted:** 2 /4 /2023

KEY WORDS

Behçet's disease, MALAT1

ABSTRACT

Behçet's disease (BD) is a long-term, relapsing, and inflammatory condition that affects various body systems and is characterized by recurring oral and vaginal ulcerations, ocular abnormalities, and other symptoms.

The aim of the study was to assess the expression level of metastasis associated lung adenocarcinoma transcript 1 (MALAT1) in BD as an autoimmune disease.

The study comprised 35 BD patients (24 males and 11 females), as well as 30 healthy controls (22 males and 8 females). Blood samples were split into sera, which were then kept at -80 °C until analysis. These sera were employed in the long noncoding extraction and real-time PCR fold change detection of the MALAT1 gene.

There was a significant increase in MALAT1 expression level in cases compared with controls ($p = 0.022$). ROC curve exhibited an AUC of 0.657 (95% confidence range (CI): 0.500-0.814; sensitivity = 65.7, specificity = 100%), which indicated that MALAT1 is useful in distinguishing BD from healthy controls. MALAT1 in Behçet's disease cases may serve as diagnostic biomarkers for Behçet's disease.

Introduction

Behçet's disease (BD) is a chronic, relapsing, multisystem inflammatory disease of unknown etiology that is characterized by recurrent oral and vaginal aphthous ulcerations, vaginal ulcerations, ocular signs (such as uveitis and conjunctivitis), and, depending on the severity of the disease, additional systemic involvement. An alternative term for Behçet's illness is malignant aphthosis **(Bettiol *et al*., 2020).** Behçet's sickness can be brought on by an autoimmune process, an infectious agent, an environmental factor, and or a genetically predisposed person. The interaction of a particular genetic background with viral or environmental factors may contribute to the immune dysregulation that to some extent ripens this disease. By increasing the frequency of clinical symptoms, the vitamin D receptor (VDR) polymorphism may also contribute to the development of disease **(Dal *et al*., 2019).** Autoimmunity is no longer a sufficient explanation for the disorder; instead, it is now believed to fall midway between auto inflammatory and autoimmune. Today, it is known that Behçet's disease is an auto-inflammatory condition that affects genetically susceptible individuals and is brought on by external factors **(Kulaber *et al*., 2007).** RNA molecules longer than 200 nucleotides are called long non-coding RNAs (LncRNAs*)* **(Tano and Akimitsu, 2012).** LncRNAs are RNAs that can only encode a restricted subset of proteins. At several stages of gene expression, including transcription, post-transcriptional, translation, post-translation, and epigenetic modification, LncRNAs interact with RNA, DNA, and proteins **(Hassan *et al*., 2015).** The nuclear enriched abundant transcript 2 (NEAT2) is another name for the long intergenic non-coding RNA (LncRNAs) MALAT1 on chromosome 11q13 **(Ji *et al*., 2003).** The goal of this study was to evaluate MALAT1 (NR 002819.2) expression levels in Behçet's sickness to see whether it may be used as new biomarker for diagnosis and to link its level with disease features.

Material and Methods

A total of 65 participants were included in this study which were two groups:

Group I: 35 Behçet's patients [11 females (31.4%) and 24 males (68.6 %)] attended the Fayoum University Hospital's, Rheumatology Department as outpatients for evaluation and assessment or as inpatients to be investigated within the department, they were diagnosed as Behçet's according to

the International Study Group criteria for Behçet's disease **(Davatchi *et al*., 2014).** Patients under the age of 18 and those with other autoimmune or immune deficiency illnesses, diabetes, renal failure, liver failure, heart failure, or cancer were excluded from the study.

Group II: A control group of 30 healthy people [8 females (26.7%) and 22 males (73.3 %)] were also included in the study. This study, which was carried out in accordance with the Helsinki Declaration received permission from the ethics committee of the Fayoum University Faculty of Medicine. Its reference number is R 320 (2009). Legal guardians of the participants verbally agreed to take part in the study after being made aware of its goal.

Detailed history taking and full clinical examination was performed. Behçet's Disease Current Activity Form (BDCAF) score was calculated. In addition to complete laboratory investigations MALAT1 expression level (NR_002819.2) in serum was done.

Blood sample collection and storage

Each subject had 10 mL of blood drawn aseptically, divided into four parts, and the first component was placed in a simple vacutainer tube without anticoagulant and kept at 37°C for half of an hour before being centrifuged for 10 minutes at 3000 rpm. The determination of the liver function tests (AST, ALT), kidney function tests (urea, creatinine), and CRP and ESR were done using serum samples.

After allowing the remaining material to coagulate for 15 minutes, it was collected in serum separator tubes and centrifuged at 4000 Xg for 10 minutes. Sera were separated and stored at -80 °C before analysis. Real-time PCR fold change analysis and lengthy noncoding extraction of the MALAT1 gene were performed using those sera (NR 002819.2).

Long non coding RNAs extraction

Detection of MALAT1 (NR_002819.2) gene expression levels in serum was done using the iRNeasy mini kit and procedure for purification of serum total RNA, including long noncoding RNA (Qiagen, Valencia, CA, USA).

The NanoDrop® (ND)-1000 spectrophotometer was used to assess and quantify the purity of RNA samples (NanoDrop Technologies, Inc. Wilmington, USA).

Conversion of RNA into complementary DNA (cDNA) by reverse transcription (RT)

Reverse transcription was carried out on total RNA using the miScript II RT kit (Qiagen, Valencia, CA, USA) in a final volume of 20 uL RT reactions.

Quantitative Realtime PCR (qPCR) for detection of long-non coding RNAs

This step was completed using the RT2 SYBR Green ROX q PCR Master Mix kit and method for quantifying long non-coding RNA (Qiagen, Valencia, CA, and USA).

The table below used as the programming guide for the real-time cycler (DNA-technology thermo cycler, DT light 4S1, Russian).

Table (1): Real-time cycler programming

Cycles	Duration	Temperature (°C)
1	**10 min.**	**95**
40	**15 s**	**95**
40	**min**	**60**

The serum expression levels of the examined LncRNAs MALAT1 were measured using pre-made primers, with GAPDH serving as an internal control. Cycle threshold (Ct) in real-time PCR is the measure of how many cycles are required for the fluorescent signal to cross the threshold. Gene expression was assessed in comparison to an internal control (2Ct). For relative fold change, 2Ct was used.

Melting curve tests were done after the PCR cycles were completed to confirm the particular production of the expected PCR result. To standardize the expression pattern and to quantify the target Long noncoding RNA compared to other genes, GAPDH was used.

The number of qPCR cycles required for the fluorescence signal to exceed a particular threshold is known as the cycle threshold (Ct) value. By subtracting the GAPDH Ct values from the target Ct values we could obtain Ct. Fold change in long noncoding RNAs Malat1 expression was computed using the equation:By subtracting the ΔCt of the control samples from the ΔCt of the disease samples we can calculate ΔΔCt.

The long non-coding RNA is up-regulated when the FC is positive; it is down-regulated when the FC is negative. Because -Ct for control participants equals zero and 20 equals one, the control value was taken to equal 1.

Statistical analysis of data

We used version 18 of SPSS software statistics computer package to arrange, tabulate, and statistically analyze the data collected (SPSS Inc, USA). The mean, median, standard deviation (SD), standard error, and range of quantitative data were determined. The Kolmogorov-Smirnov (KS) test was employed as a normality check. For comparison of any two or three groups, the Mann-Whitney-U test or the Kruskal Wallis test were utilized. The Independent-t test was employed in all other cases. Numbers and percentages were utilized to present

qualitative data, and the chi-square (2) test was performed to determine significance. A Spearman correlation was used (SPSS, IBM Corporation, New York).

The received operating characteristic (ROC) curve was utilized to assess the discrimination value of MALAT1 for case differentiation, as well as to select optimal sensitivity and specificity cut-points. P 0.05 was used to interpret the findings of the significance tests.

Results

Thirty-five patients with BD (mean age, 36.1 years, SD= 10.8), consisting of 24 men and 11 females with percentages of 68.6% and 31.4%, respectively, and 30 healthy controls (consisting of 22 males and 8 females with percentages of 73.3% and 26.7%, respectively). In the current study, participants had mean ages of 39.1 years and SDs of 5, and there were no statically significant differences in age or sex between the two groups (p= 0.146 and 0.674, respectively). Table (2, 3) summarizes the demographic and biochemical characteristics of BD patients and controls.

Table (2): distribution of age and sex of patients and controls

Variable	**Cases (N =35)**	**Control (N =30)**	**P. value**
Age (years) mean± SD	36.1 ± 10.8	39.1 ± 5	0.146*
Sex N (%)			0.674**
Female	11 (31.4%)	8 (26.7%)	
Male	24 (68.6%)	22 (73.3%)	

*Independent-t test **Chi-squared test

Table (3): Biochemical parameters of BD cases

Laboratory investigations	
ESR (mm)	47.57± 29.3
CRP (mg/dl)	10.6 ± 9.84
ALT (µL)	16.33 ± 6.71
AST (µL)	33.4± 15.9
Urea (mg/dl)	26.9 ± 8.59
Creatinine (mg/dl)	0.4 to 1.3

ALT: alanine transaminase
AST: aspartate transaminase
ESR: Erythrocyte Sedimentation Rate
CRP: C- reactive protein

At comparison between MALAT1 expression level in cases and controls there was statistically significant increase in cases (N=35, and with median =5.03, and the range was from 0.02 to 27.01) versus controls (N=30, with median = 1, and the range was from 1 to 1) (p-value =0.022) Table (4).

Table (4): Serum expression level of MALAT1 in cases and control

	Cases (N=35)			Control (N=30)			P-value#
	Median	Range		Median	Range		
MALAT1	5.03	0.02	27.01	1	1	1	**0.022***

#Mann-Whitney U test *Significant

There was no correlation between MALAT1expression level and the characteristics of BD patients Table (5).

Table (5): Relation between MALAT1 expression and characteristics of BD patients

		MALAT1			P-value
		Median	Range		
Sex	Female	5.70	0.19	24.61	0.186#
	Male	2.07	0.02	27.01	
Family H/O	Negative	4.64	0.02	27.01	0.920#
	Positive	5.11	0.08	21.42	
Genital ulcer	Absent	3.05	0.08	24.51	0.873#
	Present	5.07	0.02	27.01	
Cutaneous Lesions	Absent	5.11	0.15	27.01	0.333#
	Erythema nodosum	2.67	0.02	24.51	
Activity	No	3.15	0.18	24.51	0.930#
	Yes	5.03	0.02	27.01	
BDCAI	0	3.15	0.18	24.51	0.194##
	1	2.07	0.02	21.42	
	2	8.13	6.54	24.42	
	3	4.25	0.08	27.01	
	4	0.18	0.18	0.18	
	7	24.61	24.61	24.61	
Fundus Ex	Normal	5.22	0.02	24.42	0.832#
	Uveitis	3.65	0.15	27.01	
Vascular	Positive	7.01	0.55	27.01	0.059#
	Negative	3.65	0.02	24.61	
Pathergy test	Negative	5.03	0.08	27.01	0.561#
	Positive	3.1	0.02	24.61	
Joint manifestations	Positive	5.93	0.02	27.01	0.139#
	Negative	1.08	0.15	24.51	
CNS	Headache	5.17	1.08	24.42	0.608##
	CVS	5.03	0.19	24.51	
	NAD	1.08	0.02	27.01	

BDCAF: Behçet's Disease Current Activity Form #Mann-Whitney U test ## Kruskal-Wallis test *Significant

There was not any correlation between MALAT1 and other biochemical parameters as shown in Table (6).

Table (6): Correlation between MALAT 1 and other biochemical parameters

Group / Parameter	Behçet's disease group N = 35	
	MALAT 1	
	Correlation coefficient (r)	P. value
ESR	0.091	0.603
CRP	-0.077	0.559
Creatinine	0.066	0.706
Urea	0.292	0.089
ALT	0.105	0.547
AST	0.291	0.090

**Correlation is considered a significant correlation at the 0.01 level (2-tailed).
*Correlation is considered a significant correlation at the 0.05 level (2-tailed).
ALT: alanine transaminase,
AST: aspartate transaminase,
ESR: Erythrocyte Sedimentation Rate,
CRP: C- reactive protein.

ROC curve analyses were performed to determinate the diagnostic value of MALAT1 expression level through all the BD patients and healthy controls. Figure (1) and Table (7) provide examples of the ROC curve. It was discovered that MALAT1 was helpful in differentiating BD from healthy controls. With a 65.7 sensitivity and a 100% specificity, MALAT1expression level had an AUC of 0.657. These findings suggested that MALAT1 expression level may be a viable biomarker for BD diagnosis.

Fig. (1): ROC curve for detecting BD using MALAT1

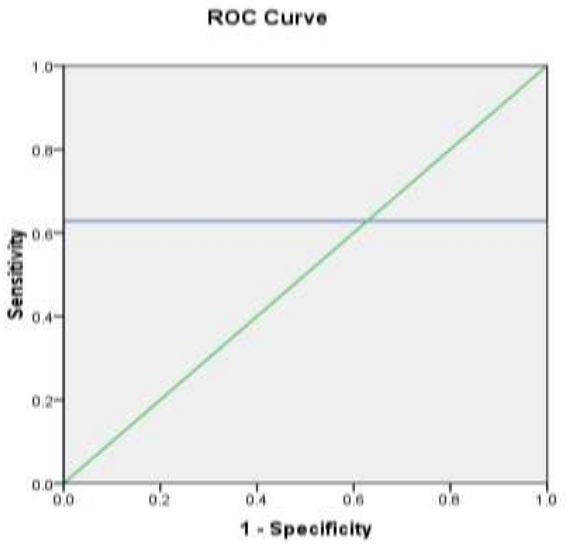

Table (7): Validity of MALAT1 expression level for differentiating between cases and control

Variable	AUC 95% CI	P-value	Cut-off point	Sensitivity	Specificity
MALAT1	0.657 (0.500-0.814)	**0.030***	1.04	65.7	100.0

Discussion

Recurrent oral aphthous ulcers, vaginal ulcerations, ocular symptoms, and other systemic involvement are hallmarks of Behçet's disease **(Bettiol *et al.*, 2020).** The clinical signs and symptoms of BD vary by nation, geography, and ethnicity. The clinical characteristics of BD have also been linked to human leukocyte antigen (HLA)-B51, onset age, and gender. The condition is frequently found in the third and fourth decades but can also be seen after the age of 50 or in childhood.

Although BD affects both sexes equally, guys typically experience a more severe course. Genital ulcers and erythema nodosum are more prevalent in women, but ocular symptoms, vascular lesions, and pustular lesions are more severe in men **(Bang *et al*., 2013).**

Molecules longer than 200 nucleotides are known as long non-coding RNAs (lncRNAs). Many biological processes, including the maturation and operation of immune cells, are actively regulated by lncRNAs.

LncRNAs have recently been shown to play a role in the etiology of rheumatic illnesses such SS, SLE, and RA **(Li *et al*., 2018).**

MALAT1, may also nomenclature as Neat2 (nuclear enriched abundant transcript 2), is a long intergenic non-coding RNA (lincRNA) with a number of nucleotides more than 8,000 nucleotide that is present on chromosome 11q13. As lncRNA sequences are highly conserved across species, this implies that MALAT1 may have crucial biological activities. MALAT1 was first proposed as a prognostic marker for stage I non-small cell lung cancer (NSCLC) in 2003 when it was discovered to be significantly linked with the metastasis of early-stage NSCLC. Studies on MALAT1 have increasingly increased since that time **(Ji *et al*., 2003).**

The goal of this study was to determinate the expression level of metastasis associated lung adenocarcinoma transcript1 (MALAT1) in Behçet's disease as an autoimmune disease, and to correlate its level with disease characteristics.

Our results agreed with **Olfat *et al*., (2020)** and **Manal *et al*., (2021)** for the assessment of MALAT1 level expression where its expression level was significantly higher in SLE patients than healthy controls **(Olfat *et al*., 2020 and Manal *et al*.,2021).**

Our results also agreed with **Huaxia Yang *et al*., (2017),** who observed up-regulation of MALAT1 in the immune cells of patients with SLE (systemic lupus erythematous). Because it can function as a competitive endogenous RNA (ceRNA) to block the inhibitory effect of miRNAs on the expression of inflammatory factors, MALAT1's role as a key inflammatory regulator may help to explain this **(Huaxia *et al*., 2017).** Therefore, MALAT1 was proposed to be implied in BD as an autoimmune disease. Our study showed that there were highly significance in ESR and CRP ($P \leq 0.001$), that was in agreement with **Koca, (2018)**. While his study showed the reverse of our study AST, ALT results where our study showed significance in AST, ALT activity (p. value ≤ 0.001,

0.018 respectively) that might be due to the severity of disease in our study.

Ataş *et al.*, (2020) showed that there were no statistically difference in serum urea and creatinine in agreement with our results which showed the same results (p. value= 0.291, 0.814 respectively).

Mounir *et al.*, (2018) which showed that there were statistically insignificant ESR level, and highly statistically significant difference (P<0.001) regarding CRP level being higher in patients with BD than healthy control group agreed with our results.

Limitation that should be put in consideration, the relatively small sample size therefore, further studies with large samples in other ethnic groups are required particularly to study and explain the correlation between these markers and different clinical data in BD.

Conclusion

MALAT1 was differentially expressed in serum of BD patients and had a potential diagnostic value for the detection of the disease.

Conflict of interest: none
Funding: This work did not receive any specific grant.

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دراسة بيوكيميائية حول تعبير الحمض النووي الريبوزي الطويل الغيرمشفر المرتبط بسرطان الغدة الرئوية١ (MALAT1) في مرض بهجت

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مرض بهجت (BD) هو حالة التهابية طويلة الأمد ومنتكسة وتؤثر على أجهزة الجسم المختلفة وتتميز بتقرحات متكررة في الفم والمهبل وتشوهات في العين وأعراض أخرى.

ان الهدف من الدراسة هو تقييم مستوى التعبيرالخاص بالحمض النووي الريبوزي الطويل الغير مشفر المرتبط بسرطان الغدة الرئوية ١ (MALAT1) في مرض بهجت كمرض مناعي ذاتي.

اشتملت الدراسة على ٣٥ مريضًا مصابًا بمرض بهجت (٢٤ ذكرًا و ١١ إناثًا) ، بالإضافة إلى ٣٠ مريضًا سليمًا (٢٢ ذكرًا و ٨ إناث). تم تقسيم عينات الدم وفصل الامصال ، ثم حفظت في درجة حرارة -٨٠ درجة مئوية حتى التحليل. تم استخدام هذه الأمصال في الاستخراج الطويل غير المشفر واكتشاف تغيير أضعاف PCR في الوقت الحقيقي لجين MALAT1.

اوضحت النتائج ان هناك زيادة كبيرة في مستوى التعبيرالخاص بجين ال MALAT1 في الحالات مقارنة مع الضوابط (ع = ٠.٠٢٢). أظهر منحنى ROC قيمة AUC تبلغ ٠.٦٥٧ (نطاق ثقة ٩٥٪ (0.500-0.814 :CI) ؛ الحساسية = ٦٥.٧ ، النوعية = ١٠٠٪) ، مما يشير إلى أن MALAT1 مفيد في تمييز BD عن عناصر التحكم الصحية. قد يكون MALAT1 في حالات مرض بهجت بمثابة مؤشرات بيولوجية تشخيصية لمرض بهجت.