ORIGINAL ARTICLE

CONTENTS

Circulating T-cell immunoglobulin and mucin domain-3(TIM-3) levels and (TIM-3) gene polymorphism in systemic lupus erythematous patients

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ABSTRACT

Aim: To determine the soluble of TIM-3 levels and how a variation in the -574 locus gene in the TIM-3 gene's promotor region contributes to SLE.

Materials and Methods: 180 participants participated in the current study: 90 SLE patients and 90 clearly healthy controls (HCs) from October 2024 to February 2025. These subjects selected from Medical City, Baghdad, Iraq. Participant's genomic DNA was collected from blood sample. Every sample was diagnosed with SLE. The enzyme-linked immunosorbent assay (ELISA) was utilized to determine the quantity of soluble TIM-3 (sTIM-3) in the serum of SLE and HC patients. The TIM-3 gene's rs10515746 gene fragment, which corresponds to the -574 locus, was amplified and genotyped using the allele-specific polymerase chain reaction (AS-PCR).

Results: Patients had significantly higher TIM-3 levels (median = 396.5 ng/ml, range = 198-698 ng/ml), than controls (median of 1618 ng/ml and a range of 1306-1999 ng/ml). The GT-TT genotypes were more prevalent in patients than controls (98.89% vs. 93.33%), with a significant difference (OR= 6.36, 95%CI= 0.75-53.92). At the allelic level, compared to controls, patients' frequencies of the mutant allele (T) were noticeably higher (90.56% *vs.* 79.44%) (OR=2.15, 95%CI= 1.15-4.03, p=0.016).

Conclusions: Individuals with SLE have significantly higher levels of soluble TIM-3, which suggests that the protein has a role in the pathophysiology of the condition and that the T allele of rs10515746 is a risk factor for SLE.

KEY WORDS: systemic lupus erythematous, T-cell immunoglobulin and mucin domain-3, Enzyme-Linked–Immunosorbent Assay, polymorphism

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INTRODUCTION

Immunocheckpoint receptors for co-stimulatory or co-inhibitory chemicals are crucial components of the immune system. It appears that the ligand galectin-9 (Gal-9) and T-cell immunoglobulin and mucin-domain-containing molecule 3 (TIM-3) are implicated in the pathophysiology of autoimmune disorders as negative checkpoint receptors [1]. A single transmembrane domain, an intracellular domain, and an N-terminal variable immunoglobulin (IgV)-like and mucin-like domain in the extracellular region are all present in TIM-family proteins. Transmembrane glycoprotein TIM-3 is primarily generated by Th1 and Th17 cells but not Th2. TIM-3 is vital for immunological tolerance since the TIM-3/Gal-9 pathway promotes apoptosis, which governs Th1 immunity, and preventing this connection makes autoimmunity worse [2, 3]. Additionally, immune cells including dendritic cells, macrophages, and natural killer cells express TIM-3 on their surface as well, when these cells overexpress TIM-3, it impairs the immunological function of the aforementioned immunocytes [4]. There have been reports linking TIM-3 polymorphism and malfunction to a number of diseases, including cancer, autoimmune illness, infections, and allergies [5]. The autoimmune condition known as systemic lupus erythematosus (SLE) is typified by multisystem inflammation and antibodies to nuclear and cytoplasmic antigens. The prevalence ranges from 20 to 50 per 100,000 women in the US. Numerous organs impacted by the illness, and the musculoskeletal,

cutaneous, renal, central nervous, hematologic, cardiac, and gastrointestinal systems are among the clinical manifestations [6]. Systemic lupus erythematosus (SLE), it is thought that autoreactive T-cells are triggered by impaired self-tolerance, and that autoreactive B-cells are then encouraged to create auto-antibodies The TIM-3 genes' polymorphism may affect the immunological response [7]. Recent achievement in cancer treatment using immune checkpoint antagonists has shown that the importance of immune co-inhibitory or co-inhibitory systems in anti-tumor immune responses; however, Similar to autoimmune illnesses, immune-intercede adverse events (irAEs) are commonly caused by inhibiting inhibitory immunological checkpoint receptors [8]. Autoimmune diseases, nevertheless, restricting immune responses by either blocking co-stimulatory signals or enhancing co-inhibitory signals is a hopeful implementation. paradigm. [9]. Creation of immunological complexes due to the generation of antinuclear antibodies (ANA) [10] encourage plasmacytoid dendritic cells to express interferon alpha (IFN-α) [11]. Type I interferon (IFN) system activation, this emerges as IFN-α or IFN-inducible genes (IFN signatures) in the bloodstream, and SLE-associated autoantibodies are linked to the etiology of SLE and disease activity in SLE patients [11]. The TIM-3 ligand Gal-9 undergoes elevation in SLE patients, which coincides with the proliferation of interferon-signature genes. These data suggest that Th1 and Th17 immune responses significantly influenced by TIM-3/Gal-9 pathway, and that autoimmune disorders worsen when TIM-3/Gal-9 connection is blocked [12-14]. A previous study looked at the connection between the expression of TIM-3 on SLE peripheral blood mononuclear cells (PBMCs) patients and the activity of SLE illness [15]. A-disintegrin-like and metalloproteinase with thrombospondin type 1 motifs (ADAM) 10 or ADAM17 separates the TIM-3 stalk region, TIM-3 can be released from the cell surface, resulting in a soluble form of TIM-3 (sTIM-3), which is higher in autoimmune disease patients' serum [16-18]. Human TIM members are encoded by three genes (HAVCR1, HAVCR2, and TIMD4, which respectively encode TIM1, TIM3, and TIM4). The TIM-3 polymorphism simulates the process that gives rise to specific immunological diseases through modifications to the interconnection between TIM-3 and its ligand [19]. Human HAVCR2 variants in both the coding and non-coding parts have been interrelation with autoimmune and allergy disorders. The genome harbors several variations, notably +4259T/G (rs1036199) in the coding area and −1516G/T (rs10053538) and −574G/T (rs10515746) in the promoter site correlated with gastrointestinal cancer and rhinitis [20-21], pancreatic cancer and renal cell carcinoma. TIM-3 protein expression was detected by immunohistochemistry in women with breast cancer, according to another study. It eventually emerged that rs10053538 had a considerably greater likelihood of breast cancer than the wild-type genotype [22]. For another immune checkpoint such as Programmed cell death-1(PCD-1), a specific type of SNP in the PD-1 promoter region that corresponds to rs38084323 has been linked to the *Hepatitis* B virus (HBV), according to one investigation [23]. In the present study, we assessed the levels of circulating sTIM-3 in both healthy control volunteers and SLE patients. Also looked into how a single nucleotide variation in the -574-locus gene in the promotor region of the TIM-3 gene affects SLE and controls.

AIM

In the present study, we assessed the levels of circulating sTIM-3 in both healthy control volunteers and SLE patients. We also looked into how a single nucleotide variation in the -574 locus gene in the promotor region of the TIM-3 gene affects SLE and controls.

MATERIALS AND METHODS

THE STUDY POPULATION

Hundred and eighty subjects enrolled in this case-control study, including 90 SLE patients and 90 healthy controls (HCs). The study carried out at Autoimmune Disease Center in Medical City-Baghdad-Iraq during period from October 2024 to February 2025. All groups gave their documented consent prior to participating in the study. Systemic lupus erythematous was diagnosed clinically and confirm by Indirect immunofluorescence on Hep-2 cells remain the method of choice for detection of anti-nuclear antibodies. The ANA test has a very high (almost 100%) sensitivity for SLE but a low specificity because ANA can be seen in a variety of clinical disorders and even in healthy individuals. Anti-dsDNA antibodies seen in 40–80% of SLE patients and are very specific for the disease. The Farr assay is the preferred technique for anti-ds DNA; however, its usefulness is limited due to the requirement of employing radioactive material. The research's ethical permission was obtained by the University of Diyala's College of Medicine's scientific and ethical committees.

DATA AND SAMPLE COLLECTION

All participants' demographic information, such as age, sex, and body mass index (BMI), residency was gath-

ered through in-person interviews using a pre-made questionnaire. Extracted two halves of five milliliters of venous blood. Serum was separated in a gel tube, with the first part (3 ml) used for the Enzyme-Linked Immunosorbent Assay to detect s TIM-3. Two milliliters of the second ingredient were put in an EDTA tube and stored at -80°C until the DNA extraction process for-574 locus of promoter area of TIM-3 gene amplification was required.

Serum preparation and storage: To ensure full coagulation, for ten minutes, the tubes centrifuged at 1900-x g at 4°C after kept for 20 minutes at ambient temperature (15–25°C), finally, the supernatant was carefully transferred to a fresh tube in order to identify sTIM-3.

T-CELL IMMUNOGLOBULIN AND MUCIN DOMAIN-3 (TIM-3) DETERMINATION USING ELISA

Sandwich enzyme-linked immune-sorbent assay technology served as the foundation for this kit. The 96-well plate was pre-coated with an anti-HAVCR2 antibody. The detecting antibody was the biotin-conjugated anti-HAVCR2 antibody. The wells were then filled with the pilot samples and standards. Wash buffer was applied. To remove the unbound conjugates subordinate incubation. After that, a biotinylated detection antibody was added to engage with the coated antibody's HAVCR2 conjugate. HRP-Streptavidin was gathered after unbound conjugates cleaned off. TMB substrates introduced to observe the HRP enzymatic response subordinate a third washing. HRP stimulated the production of a blue product from TMB, which became yellow when a stop solution was added. Measure the O.D. absorbance at 450 nm with a microplate reader. A standard curve was created to determine the sample's HAVCR2 content. The OD450 value is proportionate to the target substance's concentration.

ISOLATION DNA AND ALLELE-SPECIFIC - POLYMERASE CHAIN REACTION (AR-PCR)

A commercially available Gsync DNA extraction kit (Cat. No. GS100, Geneaid, Taiwan) was used to extract DNA from the genome. It adheres to the procedure that was recorded at the time of its development. At 260 nm/280 nm (A260/A280), the amount of DNA extracted from the samples was determined using a Biospec Nano spectrophotometer. Using allele-specific PCR (AS-PCR), the -574 locus (rs10515746) was investigated. Three distinct primers were employed to accomplish this.

Forward 1 (F1)
5′-GGCTTATGCTGGGAGTTGCT-3′
Forward 2 (F2)
5′-GGCTTATGCTGGGAGTTGCG-3′
Reverse for the F1 and F2 (R) series.
5′-GGT GTCTGATTGCCAGTGATTC-3′ [24].

The T alleles amplified using the F1 and R primers, and the G alleles amplified using the F2 and R primers. All of the expatiate fragments have a total length of 539 bp, and the samples were generally not up to par for AS-PCR using F1/R and F2/R. Overall, the segments that were magnified were 539 bp in length, each sample had to be optimized for AS-PCR by following several steps. Consequently, the 25 µl final volume produced the following nucleotide mixture on reactivity expansion: 12.5 µl of master mix (Promega, USA), 2 µl of primers (1.0 µl from the forward side and 1.0 from the reverse side), and 1.0 µl of DNA template which were all accomplished using water that has been double distilled. The target sequence amplified using the touchdown PCR method, which involved applying heat. A thermal cycler was utilized for this purpose under the following circumstances: 5 minutes at 94°C, 30 seconds at 94°C, 30 seconds at 60°C for annealing, 30 seconds at 72°C for extension, 30 seconds at 72°C for cycling, and 7 minutes at 72°C for final extension. In order to identify the amplified product for gel electrophoresis, it subjected to 1.5 g agarose in 100 ml 10x Tris-borate-EDTA (TBE) (Promega, USA) and developed with ethidium bromide.

STATISTICAL ANALYSIS

The analyses were complete utilizing the statistical program SPSS 25.0 (SPSS, Chicago). The Student t-test was used for analysis, and the continuous data's mean and standard deviation were shown. The Chi-square test applied to examine categorical variables, which reported as numbers and percentages. Using binary logistic regression, the association between SLE and rs10515746 in the promoter region of the TIM-3 gene was evaluated. Based on this test, the odds ratio (OR) and related 95% CI were calculated. A p-value of less than 0.05 indicated that a change was considered statistically significant.

RESULTS

DEMOGRAPHIC FEATURES OF THE PATIENTS

The relationship between demographic characteristics and SLE is shown in Table 1. The mean age of 51.12±10.59 years for the patients was considerably lower than the mean age of 55.26±15.85 years for the controls. While controls had a more balanced male-to-female ratio (58.89% male, 41.11% female), SLE patients

Table 1. Features of the research population's demographics

Variables	Patients (n=50)	Controls (n=50)	p-value
Age, years Mean±SD Range	51.12±10.59 16.0-82	55.26±15.85 9.0-85	**0.041**
Gender Male Female	16(17.78%) 74(82.22%)	37(41.11%) 53(58.89%)	0.001
BMI, kg/m^2 Mean±SD Range	25.03±3.81 18.78-34.37	25.13±3.76 20.34-31.46	0.819
Residence Rural Urban	27(30%) 61(67.78%)	29(32.22%) 63(70%)	0.747

SD: standard deviation, BMI: body mass index
Source: Own materials

Table 2. Median level of sPD-1 in patients with SLE and controls

Variables	Patients (n=90)	Controls (n=90)	p-value
sPD-1, ng/ml **Mean ± SD** **Median** **Range**	1630.5±175.07 1618.0 1306-1999	396.5±96.05 396.5 198-698	<0.001

Source: Own materials

Table 3. The prevalence of multiple genotypes and allele of rs10515746 polymorphism in SLE patients and control

Rs10515746 Polymorphism	Patients (n=90)	Controls (n=90)	P-value	OR(95%CI)
Genotypes GG GT TT HWE	1(1.11%) 15(16.67%) 74(82.22%) 0.808	6(6.67%) 25(27.78%) 59(65.56%) 0.654	0.256 0.065 0.032	1.0 7.19(0.88-64.25) 3.6(0.39-32.87)
Dominant model GG+GT TT	31(34.44%) 59(65.56%)	16(17.78%) 74(82.22%)	0.012	2.43(1.22-4.86)
Recessive model GT+TT GG	89(98.89%) 1(1.11%)	84(93.33) 6(6.67%)	0.09	6.36(0.75-53.92) 1.0
Alleles T G	163(90.56%) 37(20.56%)	143(79.44%) 17(9.44%)	0.016	1.0 2.15(1.15-4.04)

Source: Own materials

are overwhelmingly male 82.22%. At p = 0.001, this difference is extremely significant. There is no discernible difference between the mean BMI of SLE patients (25.03±3.81 kg/m^2) and controls (25.13±3.76 kg/m^2) (p = 0.819).

STIM-3 SERUM LEVEL IN SLE PATIENTS

It was discovered that the data pertaining to the blood levels of s TIM-3 was not regularly distributed. As a result, the median and range of these data were used, and the non-parametric Mann Whitney U test was used for analysis. In patients with SLE, Patients' TIM-3 was significantly greater than controls' (median = 396.5 ng/ml, range = 198-698 ng/ml) *versus* patients (median = 1618 ng/ml, range = 1306-1999 ng/ml) (Table 2).

MOLECULAR ASSAY

This study examined the relationship between SLE infection and the SNP rs10515746 in the TIM-3 gene's promoter region. PCR is used for allele-specific genotyping. Figure 1 displays the results of the PCR. GG, GT, and TT were the three genotypes in which the SNP was

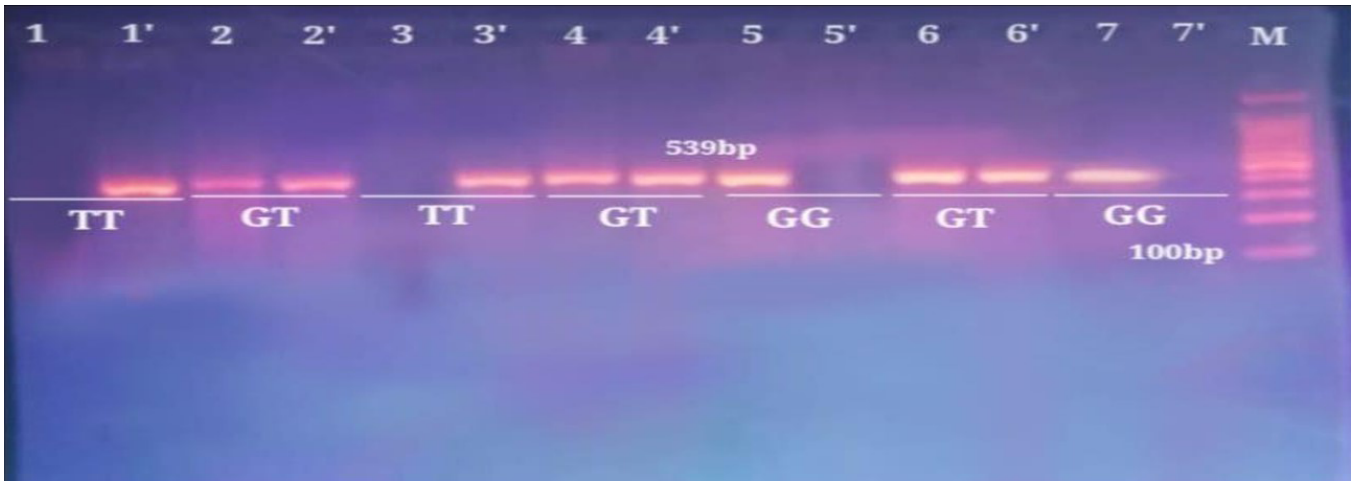

Fig. 1. Genotype patterns of rs10515746 in the promoter region of TIM-3 gene allele-PCR conceived under UV transluminator. M: DNA marker, lanes 1, 3: TT genotype, lanes 2 and 6: GT genotype, lanes 5 and 7; GG genotype
Source: Own materials

found. These genotypes' distribution in patients and controls was in good agreement with Hardy-Weinberg equilibrium.

ASSOCIATION OF RS10515746 WITH SLE INFECTION

The homozygous genotype (TT) was more frequent in patients with SLE (82.22%) than controls (65.56%) with a significant difference (p=0.032). Although the heterozygous genotype (GT) was less common in patients than controls (16.767% *vs*. 27.78%), the difference was not significant. The GT-TT genotypes were more prevalent in patients than controls (98.89% *vs*. 93.33%), with a significant difference (OR= 6.36, 95%CI= 0.75-53.92), suggesting that this polymorphism has recessive inheritance. At the allelic level, patients had a significantly higher frequency of the mutant allele (T) than controls (90.56% *vs*. 79.4%) (OR=2.15, 95%CI= 1.15-4.03, p=0.016) (Table 3).

DISCUSSION

This study revealed SLE patients were significantly younger than the controls (51.12±10.59 years), with an average age of 55.26±15.85 years. Females make up 82.22% of SLE patients, and this difference is statistically significant p=0.001. Comparable with past report was found Men's and women's anticipated rate of incidence are 0.9 (95% CI 0.7 to 1.1) and 1.9 (95% CI 1.7 to 2.2) per 100,000 person-years, respectively. In the male population, the age-specific incidence rate of SLE peaks at 2.2 (95% CI 1.0 to 3.4) per 100,000 person-years between ages of 65 and 70. The incidence peaks at 3.6 (95% CI 2.9 to 4.3) cases per 100,000 person-years in women aged 20 to 25 [25]. Critically differentiated T-lymphocytes' surface expression of TIM-3 has related to the pathophysiology of autoimmune disorders driven by Th1 by negatively controlling T-cell response. Autoimmune complex deposition and autoantibody synthesis are hallmarks of SLE, a Th1-dependent human autoimmune illness [26-27]. In this work, we showed that SLE patients had considerably higher serum sTIM-3 levels than HCs. It has proposed that the TIM-3/Gal-9 co-inhibitory pathway is substantial autoimmune modulator [28]. SLE disease activity correlates with T-cell TIM-3 expression; furthermore, it has shown that SLE patients have a larger CD3+CD4+TIM3+ T-cell subset than HCs. Patients with SLE also showed high serum levels of Gal-9 [29]. Patients with SLE also showed elevated serum levels of Gal-9 [30-31]. It can conclude that the stimulation of the TIM-3/Gal-9 pathway functions as an anti-immune mediator in patients with SLE. By lowering the levels of anti-double stranded DNA antibodies, intraperitoneal Gal-9 treatment improved the proteinuria and arthritis in lupus-prone mice [32]. On the other hand, this route can modulated by the sTIM-3 that released from TIM-3 conveyed on the immune cell surface [33]. T-cell exhaustion may caused by the TIM-3/Gal-9 interconnection; however, sTIM-3 appears to have different effects against this feedback process. It should note that a soluble form of a receptor might not necessarily cause receptor preventing; additional research is required to identify the origin of serum sTIM-3 and its function in the pathophysiology of SLE. The current study accordance to previous study examined the soluble TIM-3 levels were considerably greater in 65 Japanese subjects with SLE. The study's clinical data and medical histories were gathered by looking through the patients' electronic medical records compared to those

in controls (1363 pg/mL; IQR, 1097–1673; p=0.0015) and SLE patients (2123 pg/mL (IQR, 229–7235) [34]. In contrast to recent study, Jin et al. revealed that there was not any interesting association between SLEDAI scores and sTIM-3 levels that elevated serum IL-17 levels linked to elevated serum sTIM-3 scales in SLE subjects, and that serum sTIM-3 scales were discernable lower in SLE patients with lupus nephritis [35]. This study found the predominance of homozygous genotype (TT) was significantly greater in SLE patients versus to controls. Patients were less likely than controls to have the heterozygous (GT) genotype, but the variation was not statistically considerable. These results indicated a relationship between Iraqi SLE-patients and the -574 locus polymorphism. Numerous investigations have shown that the aberrant behavior and loss of function of CD8+ T-cells in relation to SLE caused by the immunoglobulin-and mucin domain-containing molecule-3 (TIM-3). Similar findings from our work Cai and associates were showed the elevated expression of TIM-3 ligand (galactin-9) mRNA in SLE patients suggested that TIM proteins may have a role in the pathophysiology of SLE [36]. While, Li et al reported the systemic lupus erythematosus in a Chinese population is not linked to polymorphisms of the TIM-1 and TIM-3 genes [37]. According to Guo et al., Chinese people are susceptible to renal cell carcinoma (RCC) due to TIM-3 polymorphisms [38]. TIM-3's binding to its ligand, galectin-9, can make the Tim-3 protein more expressed when regulatory T-cells (Treg) are stimulated. This causes helper T-cells to die, and a decrease in TIM-3 linked to a rise in Th2 cells and a decrease in Th1 cells [39]. Investigations into the association of TIM3 SNPs with several autoimmune diseases, including type I diabetes and Ankylosing Spondylitis (AS), have been conducted [40]. Unfortunately, no prior research has examined the relationship between the genes of the -574 locus and SLE infection. Nonetheless, it may be inferred that the presence of thymine rather than guanine in the -574 locus of the TIM-3 gene's promoter area may enhance the transcription of this gene, possibly as a result of an increase in RNA polymerase to the promoter region. Overexpression of TIM-3 transcriptions can hinder T-cell function and increase the likelihood that SLE will cause the illness.

CONCLUSIONS

Our findings demonstrate that sTIM-3 levels correlated with SLE disease activity and that circulating levels of sTIM-3 are higher in SLE patients than in healthy persons. According to these results, the T allele of rs10515746 is thought to be a risk factor for SLE infection. The association between the GT+TT genotype and patients with this genotype may benefit from closer monitoring and more aggressive therapies to achieve better disease control. Screening for the rs10515746 polymorphism could be useful in identifying disease activity, enabling personalized treatment approaches.

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CONFLICT OF INTEREST

The Autors declare no conflict of interest

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