Research Article

Evaluation of HLA-G 14 base-pair Insertion/Deletion Polymorphism and Soluble HLA-G Level in Patients with Rheumatoid Arthritis in Mosul City: A Case-Control Study

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INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune illness, affecting nearly 0.5–1% of adults globally. It is distinguished by immune cell entrapment in the synovium, which promotes progressive joint damage, functional impairment, and early death. The disease is 2–3 times more likely to affect women than men [1]. RA has a considerable genetic basis [2]. The HLA system contributes to the emergence of RA, particularly HLA-DRB1 alleles. However, these alleles are only found in about 60–70% of arthritis cases [3]. Further inherited influences, including the non-classic HLA genes HLA-E, DOA, and G, were also examined for their association with RA; they were found on the sixth chromosome and play a role in immunological regulation [4]. The HLA-G locus, positioned on the 6p21.31 chromosome, is a non-typical Ig-like particle [5]. Since HLA-G is a tolerance-inducing particle, its disruption in autoimmune illnesses has harmful effects. HLA-G was categorized by the production of seven distinct forms through alternate splicing: HLA-G 5 to -7 proteins, which are soluble, while the HLA-G1-4 proteins are cell-linked [6]. Mostly through post-transcriptional mechanisms, the 3’UTR appears essential for controlling HLA-G transcription. HLA-G protein concentration is influenced by mutations in the 3’UTR, such as the 14 base-pair insertion/deletion, 3142G>C, and 3187G>A variants [7,8]. We are unaware of any research on the link between RA and the HLA-G gene variations in Mosul, Iraq. The current study is therefore focused on examining the relationship between sHLA-G levels and the 14 base-pair insertion/deletion (rs66554220) HLA-G 3’UTR variants and its associations with RA disease probability, susceptibility and activity in a cohort of Iraqi RA cases.

METHODS

Study design and patient selection

In this case-control study, 380 individuals participated in total, 190 of whom were RA cases diagnosed in accordance with the 2010 ACIR/EULAR criteria, in addition to the 190 controls. Patients were referred to the rheumatology department at Al-Salam Hospital, located in Mosul, Iraq. The ESR was determined utilizing standard methodologies. The measurement of CRP and RF was conducted utilizing a commercial latex fixation test reagent (Plasmatec, UK).

Genotyping of HLA-G gene's polymorphic 14 base-pair insertion/deletion (rs66554220) variant

Five milliliters of whole blood were drawn from each individual. For the genotype of the HLA-G 14 base-pair insertion-deletion variants, two milliliters of blood were evacuated into EDTA tubes and maintained at -70°C until testing. For the ELISA study, the remaining 3 ml of blood were collected into gel tubes and centrifuged at 2000 g for 15 min. The serum was then separated and kept at 20°C until the assay. Whole blood samples were used to obtain genomic DNA, gDNA Miniprep ReliaPrep® Kit (Catalog number: A5081, Promega, USA), obeying the guidelines of the manufacturer. Utilizing polymerase chain reaction (PCR), genotyping of the rs66554220 HLA-G (14 base pair insertion-deletion) variant's carried out corresponding to procedure outlined by Hashemi and colleges [9], the pertinent area of the HLA-G gene was augmented by the use of sequence-particular primers: forward (5’-TCACCCCTCAGTGACTGATA-3’) primer and reverse (5’-GCACAAAAGGAGGTCAGGGTT-3’) primer (Macrogen, Korea) via thermal device (BioRad, USA). PCR products were then examined under UV light after being electrophoresed on an agarose gel. DNA size in test tracks was calculated using the DNA ladder (100 bp) in the first track as a reference. The PCR products with sizes of 127 bp were for the deletion allele, and those of 141 bp were for the insertion allele. Products with sizes of 141/127 bp were for the insertion-deletion of HLA-G exon 8 (Figure 1).

sHLA-G concentration measurement

The enzyme-linked immunosorbent test (ELISA) (Shanghai YL Biont, China, Catalog No. YLA1602HU) was used to measure soluble HLA-G according to the manufacturer's instructions. Using an ELISA reader, the intensity was measured at 450 nm wavelength. A calibration curve was generated to determine the ELISA detection limits; the sensibility of the ELISA sHLA-G was determined by this process to be 0.05 U/ml.

Ethical approval

The investigation was conducted in accordance with the ethical principles delineated in the Helsinki Declaration. The local Ethics Committee of the College of Medicine, Mosul University, assessed and

Figure 1: Outcomes of polymerase chain reaction-based 14 base-pair HLA-G insertion-deletion variant. L: DNA Ladder; Lines 1,2,6,9: ins/del; Lines 3, 5, 7 and 8: del /del; Line 4: ins/ins. del, deletion/ ins, insertion.
grant approval for the research protocol and subject consent form (reference number 20 dated December 27, 2021).

**Statistical analysis**

Categorical data were represented as percent (%), and continuous data were described in terms of mean±SD. sHLA-G levels for each group are shown as medians. The chi-square test is utilized for estimating genotype and allele rates. The receiver operation characteristic was employed to compute the area underneath the curve. Utilizing the MedCalc® Statistical Software, the data were examined (version 19.1). A p-value less than 0.05 was considered a significant difference.

**RESULTS**

Table 1 summarizes the demographic facts related to both RA and healthy individuals. In this study, 190 RA cases, 165 females and 25 males (mean age 42.8±10.9 years) and 190 controls (mean age 40.5±12.1 years), were recruited.

<table>
<thead>
<tr>
<th>Variables</th>
<th>RA patients (n=190)</th>
<th>Control (n=190)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age groups n(%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 30 years</td>
<td>48(25.3)</td>
<td>52(27.3)</td>
<td>0.135</td>
</tr>
<tr>
<td>31-49 years</td>
<td>120(63.2)</td>
<td>122(64.2)</td>
<td></td>
</tr>
<tr>
<td>≥ 50 years</td>
<td>22(11.5)</td>
<td>16 (8.5)</td>
<td></td>
</tr>
<tr>
<td>DAS-28 Mean±SD = (7.42±1.13)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High activity</td>
<td>106(55.8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Moderate activity</td>
<td>41(21.6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low activity</td>
<td>34(17.9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Remission</td>
<td>9(4.7)</td>
<td></td>
<td></td>
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<tr>
<td>ESR of RA patients (Mean±SD) = 40±20.31 mm/hr.</td>
<td></td>
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<tr>
<td>CRP of RA patients n(%)</td>
<td>Positive: 159 (83.7)</td>
<td>Negative: 31 (16.3)</td>
<td></td>
</tr>
<tr>
<td>Rheumatoid factor (RF) n(%)</td>
<td>Positive: 168 (88.4)</td>
<td>Negative: 22 (11.6)</td>
<td></td>
</tr>
</tbody>
</table>

RA, Rheumatoid Arthritis; SD, standard deviation.

The 14 base-pair variations analysis shows that there are no variances in the allele frequencies and genotypes between RA cases and healthy persons. The allele occurrence for the wild allele type “del” in RA and control subjects was 78.4% (n=149) and 68.4% (n=130) (p=0.326), respectively; the mutant “ins” type allele frequency was 76.3% (n=145) and 69.5% (n=132) in RA and control subjects, respectively (p=0.411) (Table 2).

**Table 2: HLA-G 14 base-pair insertion/deletion alleles in the studied groups**

<table>
<thead>
<tr>
<th>HLA-G Allele frequency</th>
<th>RA patients (n=190)</th>
<th>Control (n=190)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA-G insertion allele</td>
<td>145(76.3)</td>
<td>132(69.5)</td>
<td>0.411</td>
</tr>
<tr>
<td>HLA-G deletion allele</td>
<td>149(78.4)</td>
<td>130(68.4)</td>
<td>0.326</td>
</tr>
</tbody>
</table>

The occurrence of the homozygous mutant genotype (insertion/insertion) of the HLA-G 14 base-pair insertion/deletion variants was 33 (17.4%) versus 29 (15.3%) in RA and healthy individuals (p>0.05), the prevalence of the heterozygosity insertion-deletion genotype was found to be 121 (63.7%) versus 111 (58.4%) (p>0.05) in RA and healthy subjects, while the deletion-deletion genotype that is wild-type was observed as 36 (18.9%) versus 50 (26.3%) in RA and healthy subjects, correspondingly (p>0.05) as demonstrated in Table 3. Soluble HLA-G levels in RA cases were considerably lower than those of healthy individuals (median=1.548 U/mL versus median=7.391 U/mL), correspondingly (p< 0.05), according to Figure 2.

Table 3: Genotypes occurrences of 14 bp HLA-G insertion-deletion (rs66554220) variants in cases with RA and healthy controls

<table>
<thead>
<tr>
<th>14-base-pair insertion/deletion alleles</th>
<th>Patients n(%)</th>
<th>Control n(%)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>ins/ins</td>
<td>33(17.4)</td>
<td>29(15.3)</td>
<td>0.378</td>
</tr>
<tr>
<td>ins/del</td>
<td>121(63.7)</td>
<td>111(58.4)</td>
<td>0.154</td>
</tr>
<tr>
<td>del/del</td>
<td>36(18.9)</td>
<td>50(26.3)</td>
<td>0.420</td>
</tr>
</tbody>
</table>

Figure 2: Value of sHLA-G in patients with RA (median=1.548) and healthy individuals (median=7.391) (p=0.042).

The validity properties of sHLA-G when used as a biomarker to distinguish RA patients from controls and predict the disease diagnosis are shown in Table 4.
ROC curve analysis of the sHLA-G showed that the specificity and sensitivity were 75.6% and 64.4%, respectively, at a threshold of < 1.871 U/mL, where sHLA-G was used to distinguish between RA and healthy individuals. The AUC was 0.643, and the p-value was 0.0193, as shown in Figure 3A, while Figure 3B demonstrates the ROC curve when using the sHLA-G in differentiating between active and inactive RA.

Correspondingly, the study shows a substantial adverse link between sHLA-G concentration and ESR (r=0.245, p = 0.012; Figure 4A). As well, there is a statistical difference (p<0.05) between the sHLA-G level and the DAS-28 activity score. The sHLA-G level is lower in patients who have high disease activity (median=4.1 U/mL), while it is higher in patients who are in remission (median=11.8 U/mL), as shown in Figure 4B. Figure 4C shows a statistically significant relationship between sHLA-G level and RF factor (p>0.05).

**DISCUSSION**

HLA-G is an immunomodulatory particle; it keeps the adaptive and innate responses of cells like macrophages and T and B lymphocytes under control [10]. Variants in the HLA-coding and noncoding regions may have an impact on the molecule's biological properties; variants in the promoter area and 3'UTR affect the HLA-G gene expression degree, besides how much sHLA-G is present [11]. The HLA-G genome variations in 3'UTR in addition to 5'UTR control its expression and are related to an augmented hazard of autoimmune disorders [12]. The mRNA stabilization and protein production rates are influenced by 14 base-pair insertion-deletion in the 3'UTR, which may be a hazard factor for RA development [13,14]. In the current research, we evaluated the genotype and allele occurrences of the 14 base-pair variants and the likelihood of RA development in a cohort of Iraqi inhabitants. However, we found no statistically substantial discrepancy in the allele or genotype frequency (p>0.05) between the RA and control groups. The 14 base-pair insertion allele was the principal allele in our RA cases, in contrast to controls. Previous studies suggested that the insertion allele was linked to decreased levels of bound and sHLA-G [15].

**Table 4:** Validity parameters of sHLA-G when employed as biomarker to distinguish RA patients from controls (* represent the optimal cut-off value)

<table>
<thead>
<tr>
<th>Criterion</th>
<th>Sensitivity%</th>
<th>Specificity%</th>
<th>+PV</th>
<th>-PV</th>
<th>Youden index (accuracy rate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt;1.267 (Highest sensitivity)</td>
<td>97.87</td>
<td>19.99</td>
<td>50.6</td>
<td>50.1</td>
<td>0.241 (24)</td>
</tr>
<tr>
<td>=&gt;1.871</td>
<td>64.4</td>
<td>97.8</td>
<td>95.7</td>
<td>65.7</td>
<td>0.400 (40)</td>
</tr>
<tr>
<td>&gt;31.254 (Highest specificity)</td>
<td>13.33</td>
<td>97.78</td>
<td>6.00</td>
<td>0.89</td>
<td>0.163 (16)</td>
</tr>
</tbody>
</table>

Figure 3: sHLA-G ROC curve [Sensitivity = 64.4%; Specificity = 75.6%; Cut-point value > 1.871 U/mL; AUC = 0.643; 95% CI=0.535 - 0.741, p=0.0193] (A). B. sHLA-G ROC curve for differentiating active from inactive RA [Sensitivity=70.6%; Specificity=70.0%; Cut-point value > 2.1082 U/mL; 95% CI=0.512- 0.802, AUC = 0.669, accuracy 41.4%, p=0.045] (B).

Figure 4: The association between the sHLA-G score and ESR in RA patients (r= -0.245, p=0.012) (A). The correlation between DAS 28-Score and sHLA-G concentration in RA patients (B). The association between RF factor and sHLA-G concentrations in RA patients (C).

These locations are connected to the HLA-G gene's post-transcriptional modulation; changes there have an impact on mRNA synthesis [16]. The mRNA instability is more associated with the 14 base-pair insertion allele [17]. Ablation of the 14 nucleotide bases leads to more stable messenger RNA than the addition of the 14 bases does [9]. However, no statistically significant correlation was found between the 14 base-pair polymorphism and the incidence of RA, according to a meta-analysis established by Lee and his colleges [18]. Mariaselvam et al. research on south Indian people [7], Catamo et al. study on Brazilians [19], and Hashemi et al. study on the Iranian population [9] all produced similar negative
results. The 14-base pair polymorphism has a clear consequence for protein expression levels and might be a hazardous issue for RA development. Low sHLA-G concentrations were connected with the 14 bp del/del genotype (p<0.01) in this study. Our results show that a lower sHLA-G value was shown in cases in contrast to controls (p>0.05), these lower levels may be the reason why the inflammatory state characteristic of RA disease developed. Our findings are consistent with other reports that have been published. According to Verbruggen et al., RA patients had considerably lower sHLA-G levels than controls [20]. Veit and colleagues also found the same results [21]. According to the ROC curve study, sHLA-G may act as an immunological biomarker for the development and severity of RA. According to studies, low sHLA-G values cannot repress the immune system's self-reactive cells, which results in tolerance loss, the likelihood of acquiring autoimmunity, and the emergence of autoimmune disorders that result in rheumatoid arthritis [7,17]. Additionally, the lower sHLA-G values in cases with RA suggest that HLA-G may be engaged in RA pathophysiology; decreased sHLA-G shows that T lymphocytes and natural killer cell actions really aren’t effectively inhibited by sHLA-G in RA [22]. When we examined any potential relationships between the sHLA-G level and the disease’s clinical indicators (DAS-28 and ESR, RF), there was a substantial link between the disease activity metrics and the sHLA-G level (p<0.05). Similar findings were found by Dreaj et al. in Iraq [23], besides Rizzo and colleagues [24]. This indicates that sHLA-G is linked to illness physiopathology, suggesting HLA-G as a potential indicator to evaluate disease severity in addition to the prognosis in RA patients.

Study limitations

The study is limited by the tiny sample size and the fact that it was conducted exclusively on the 3’UTR of the HLA-G gene. It is crucial that the identified associations be validated through research involving larger patient and control populations that are representative of various populations.

Conclusion

The findings revealed a strong association between sHLA-G and RA susceptibility, in addition to a correlation between sHLA-G level and disease activity and severity.

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Conflict of interests

No conflict of interest was declared by the author.

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Data sharing statement

Supplementary data can be shared with the corresponding author upon reasonable request.

REFERENCES


