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REVIEW ARTICLE

Association of soluble HLA-G expressions and their roles on the Risk of Recurrent Pregnancy Loss in Iraqi Populations.

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Abstract:

Background: Human Leukocyte Antigen –G HLA-G is a non-classical HLA class molecule that productions a central part in immune tolerance at the motherly fetal interface. Aim of study: The current study was designed to examine the relationship of HLA-G gene expression among recurrent pregnancy loss in Iraq. Methods: An entire of 40 females with idiopathic RPL were comprised in the training who stayed known to infertility, gynecological and obstructs department in Baghdad Hospital City from the period between February /2022–October/ 2022, and further groups comprise of 40 actually healthy persons. A three ml of blood samples were collected for polymerase chain reaction for Real Time polymerase chain reaction expression analysis quantification of HLA-G and housekeeping gene (GAPDH) in blood patients and normal samples by utilizing of Real-Time PCR process. Results: The present outcomes suggest that decreased mean of Human leukocyte antigen-G (HLA-G) gene appearance in sick females with RPL in compared with healthy controls. Conclusion: The current results identified an relationship HLA-G gene appearance and RPL development.

Keyword: Recurrent pregnancy loss, HLA-G and GAPDH

Introduction

HLA-G, a non-classical molecule within the humanoid leukocyte antigen (HLA) class, is crucial for motherly reception of the semi-allogenic fetus. Situated on chromosome 6p21.3, the HLA-G gene comprises seven introns and eight exons. Through alternate splicing of HLA-G mRNA, seven isoforms of HLA-G are produced, encompassing four membrane variants (G1-G4) and three soluble forms (G5-G7) (1). Furthermore, soluble HLA-G (sHLA-G) can arise through the detaching or proteolytic of membrane-bound HLA-G cleavage by enzymes like milieu metallo-proteinases (MMPs), as exemplified by shelter HLAG1(2). The HLA-G gene exhibits minimal allelic polymorphism and limited tissue appearance paralleled to the greatly polymorphic traditional HLA Ia genes (HLA-A, B, C). Its appearance primarily occurs at the maternal-fetal line and within immune tissues. In spite of this limited tissue distribution, HLA-G can be identified in bodily liquids as concealed soluble particles (3). Critical parts of HLA-G at the fetal motherly line involve suppressing natural killer (NK) cell-mediated cytolysis, increasing regulatory T (Treg) cell presence, and facilitating a transition from a T-helper (Th)1 to a Th2 cytokine sketch (4).

Recurrent pregnancy loss (RPL) is a multifactorial condition that may be due to genetic, anatomic, endocrine, anti-phospholipid antibody syndrome, immunologic, and environmental factors, almost 50% of cases are idiopathic(5). Several studies all over the world investigate the prevalence and etiological causes of RPL, in a study conducted by the Iraqi Minstery of Health found that the rate of recurrent spontaneous abortion increased from 11% to 22% in the last recent years and this is a serious indicator, globally 97% of recurrent spontaneous miscarriage cases occur in Africa, Asia and Latin America(6). Throughout pregnancy, preserving immunological tolerance towards the semi-allogeneic fetus is paramount. (HLA-G) stands out as a key factor associated with immune intolerance during pregnancy, exerting a central role at the maternal-fetal line. Connections between soluble HLA-G (sHLA-G) and uterine lymphocytes are pivotal in inducing maternal immune intolerance toward entering extra-villous trophoblasts, a crucial determinant for implantation of embryo. Hence, soluble HLA-G is indispensable for successful implantation of embryo. Notably, the presence of embryo-secreted sHLA-G in the culture medium emerges as an encouraging indicator for predicting an effective pregnancy

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(7). Yet, the extent of paternal soluble HLA-G appearance earlier pregnancy remains relatively underexplored. Circulating blood ranks of soluble HLA-G (sHLA-G) are notably elevated in pregnant females paralleled to non-pregnant complements. Moreover, the ranks of sHLA-G exhibit dynamic fluctuations throughout pregnancy (8).

In summary, decreased ranks of HLA-G may contribute to a heightened inflammatory immune sketch, as evidenced in females experiencing recurrent pregnancy loss (RPL). The HLA-G antigen serves to shield fetal tissues from (NK) cells and cytotoxic lymphocytes by impeding their action. Diminished appearance of HLA-G has stayed noted in circumstances pretention risks to pregnancy, including pre-eclampsia and recurring spontaneous abortion, affirming its protecting role in pregnancy (9). Thus, in the existent training, we aimed to estimate the gene expression levels of HLA-G in blood testers of recurrent pregnancy loss sick and other fit pregnant females (control) via real-time PCR.

Materials and methods

Study Design and Population:

A case-control work was directed on the succeeding training groups throughout the dated from the first of 2022 to the last of 2022. Issues that stood registered in this training were characterized into two groups. The first group consist of forty women were analyzed as idiopathic RPL sick with at minimum two repeated pregnancy fatalities prior to the 20th week (aged (20- 44) years old); were subdivided into two sub-groups, two baby loss and three baby loss. They were regularly attending to the infertility and gynecological and obstructs department in Baghdad hospital City during the period of (February /2022– October/ 2022). The other groups included forty Age-matched apparently healthy pregnant females were kindly recruited as a controlling group in this study.

We excepted sick with smoking or alcohol usage. RPL sick with structural, hormonal, chromosomal (patients or their partners), infectious, auto-immune, diabetic mellitus, thyrodism, hypertension or thrombotic reasons stood also excepted from this training.

Sampling Criteria

Three milliliters of blood testers were aseptically composed via vein puncture by means of throwaway syringes. The samples were then transported into EDTA tubes and promptly frozen at -20°C to prevent repetitive defrosting and freezing. This was done in preparation for Real-Time PCR examination to quantity the appearance of HLA-G and the housekeeping gene (GAPDH) in both patient and normal blood samples. This study adhered to the ethical guidelines of the infertility, gynecological, and obstetrics department at Baghdad Hospital City, and verbal knowledgeable consensus was got from all contributors.

Methodology

Total RNA extraction

Entire RNA was extricated from blood testers by means of the TRIzol® reagent kit (easy-BLUE™ Total RNA) ensuing the producer's directions.

DNase I Management

The extricated RNA underwent treatment with DNase I enzyme to eliminate any residual genomic DNA. This process was carried out using the DNase I enzyme kit rendering to the protocol provided via Promega establishment, USA:

Table (1): DNase I Management groundwork for every response:

Mix	Volume
Total RNA 100ng/ µl	10 µl
DNase I enzyme	1 µl
10X buffer	4 µl
DEPC water	5 µl
Total	20 µl

Subsequently, the mix stayed keep warm for 30 minutes at 37°C. Following this, 1µl of stop reaction stayed additional, and the mixture was keep warm at 65°C for 10 minutes to de-activate the enzyme of DNase.

cDNA synthesis

DNase-I preserved RNA testers stood likewise used in cDNA production phase for via by means kit of M-MLV Reverse Transcriptase and completed rendering to establishment directions as next tables::

Table (2): cDNA master combination

RT master mix	Volume
Total RNA 100 ng/ µl	8µl
miRNA RT primer 20 pmol	1 µl
DEPC Water	1 µl
Total	10 µl

Following that, the RNA and primer stayed subjected to denaturation at 65°C for 10 minutes, surveyed via immediate freezing on ice.

Table (3): Glyceraldehyde 3-phosphate dehydrogenase GAPDH gene RT master mix:

RT master mix	Volume
RT master mix	10ul
M-MLV RTase (200u)	1ul
5X M-MLV RTase reaction buffer	4ul
100mM DTT	2ul
Dntp	2ul
RNase inhibitor	1ul
Total	20ul

Next, the tubes stood located in a twister and concisely spun downward. The RNA was then transformed into cDNA in a thermos-cycler below the specified circumstances:

Table (4): GADPH gene thermocycler circumstances

Step	Temperature	Time
cDNA synthesis (RT step)	42 °C	1 hour
Heat inactivation	95 °C	5 minutes

qPCR Thermo-cycler conditions

Following this, the qPCR platter stayed laden, and the thermos-cycler practice described in Table 5 was implemented.

Table (5): qPCR Thermo-cycler circumstances

qPCR step	Temperature	Time	Repeat cycle
Initial Denaturation	95 °C	5min	1
Denaturation	94 °C	20 sec	45
Annealing\Extension Recognition	58 °C	30 sec	

qPCR Thermo-cycler circumstances

Following this, the qPCR platter stood laden, and the thermos-cycler procedure described in Table 5 was implemented.

Fold change (target / HKG) = 2CT ΔCT

Statistical investigation

The statistical investigation stayed completed by means of IBM SPSS Statistics version 26. Incessant variables stayed reported as mean ± standard deviation (SD), while definite variables stayed existing as proportions and occurrences. Numeric information stayed assessed for ordinariness using the Kolmogorov-Smirnov examination, and decisions were made regarding the distribution (normal or non-normal) accordingly. The independent sample t-test stayed practical to parallel means between typically disseminated variables in two groups. The chi-square examination stood utilized to examine associations between definite variables. Receiver operating characteristic (ROC) curve examination stayed directed to determine the cutoff value predicting a positive finding, along with associated measures such as region below the curve (AUC), precision, specificity, sensitivity, and significance flat (P). Implication stayed fixed at a P-value of fewer than 0.05, with a greatly important flat considered at 0.01 or lower.

Results

3.1. Features of the training populace

The existing study enrolled ٤٠ females with RPL and 40 healthy controlling issues. The demographic features of sick and controlling issues are revealed in table (6). According to age, the mean age of females with RPL was 32.72 ± 4.82 years old and that of control subjects stayed 33.16 ± 4.63 years old and there stood no important variance between different groups (P = 0.701). According to BMI, patient group displayed a no important variance (p< 0.05) paralleled to controlling groups, (22.46 ±1.49) vs (22.79 ± 1.34) respectively. The frequency distribution of females with RPL rendering quantity of pregnancy defeat was shown in table (1). Patients with two pregnancy loss accounted for 22 (55.0 %), whereas, patients with three pregnancy loss accounted for 18 (45.0%), therefore, the present results show high women with recurrent pregnancy have with two pregnancy loss but in non-significant manner (P = 0.527).

Table (6): Features of women with recurrent pregnancy loss and healthy control

Characteristic	Patients (n=٤٥)	Healthy Control (n=40)	P
Age (years)	32.72 ± 4.82	33.16 ± 4.63	٠.٧٠١
BMI kg/m2	22.46 ±1.49	22.79 ± 1.34	٠.٢٨٩
Previous pregnancy loss			
Mean ± SD	2.45 ± 0.58		
Two, n (%)	22 (55.0 %)		0.527
Three, n (%)	18 (45.0 %)		
HLA-G gene expression	1.28 ± 1.08	11.37 ± 6.99	< 0.001

Amounts of HLA-G) gene appearance)

(HLA-G) gene appearance in females with RPL was highly significant decrease than healthy control subjects (1.28 ± 1.08 vs 11.37 ± 6.99], respectively, P < 0.001).

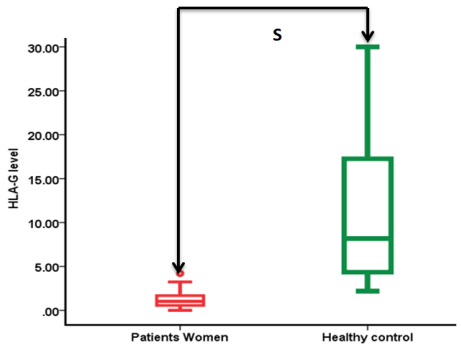

Figure 1: HLA-G gene expression in females with recurring pregnancy defeat and controlling subjects. S: statistically significant P < 0.05.

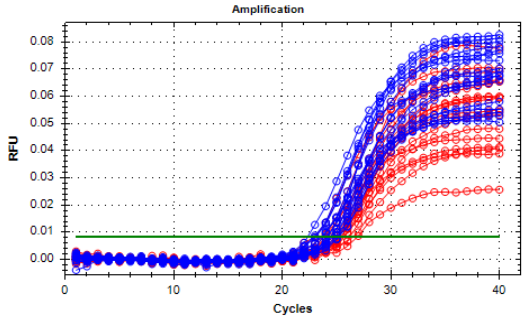

Figure (2): The Real Time PCR augmentation plots of HLA-G gene expression in patienta and heathy control samples.

Diagnostic accuracy of (HLA-G) gene

Receiver operating characteristic (ROC) examination stood achieved to show the analytic precision of using HLA-G expression to distinguish females with RPL from healthy control subjects. An optimal HLA-G gene expression cut-off value of less than 3.01 resulted in an AUC rate of 0.980 (95% [CI], 0.956- 1.000, P < 0.001), sensitivity of 92.5%, specificity of 92.5%, PPV of 92.5%, and NPV of 92.5%.

Table (7): Roc curve of HLA-G gene appearance

Characteristic	HLA-G gene expression
Cutoff value	> ٣.01
P value	< 0.001
Sensitivity %	92.5 %
Specificity %	92.5%
PPV %	92.5 %
NPV %	92.5%
AUC (95% CI)	0.980 (0.956- 1.000)

Figure (3): ROC investigation of HLA-G for the scheming of probable prognostic cutoff value.

Relationship among appearance of HLA-G and number of pregnancy loss

The comparison of HLA-G levels according to number of pregnancy loss has stood approved out and the outcomes stayed proved in table (8). Mean levels of HLA-G were 1.31 ± 0.701 and 1.19 ± 0.676 , in patients with two pregnancy defeat and sick with three pregnancy loss separately; the mean levels was lower in sick with three pregnancy defeat in comparison with in sick with two pregnancy loss, but the variance was no important (P = 0.492).

Table (8): Relationship among gene appearance of HLA-G and .pregnancy loss

	Number of pregnancy loss		
	Two n = 22	Three n = 18	P
Gene expression of HLA-G			
Mean± SD	1.31 ± 0.701	1.19 ± 0.676	0.492 † NS
Range	0.03 – 3.8	0.09- 4.20	

Discussion

Throughout embryo implantation and pregnancy, the motherly immunity system interfaces closely with foetal trophoblast cells. To prevent refusal of the semi-allogeneic fetes, apparatuses that regulate the motherly immunity system need to be activated (10). (HLA-G) productions a crucial role in successful pregnancy implantation. A developing body of scientific literature indicates that HLA-G is indispensable for foetal intolerance via preventing the cytotoxic action of T and NK cells. (11). It is crucial to comprehend the molecular and biochemical traits of HLA-G and its derivatives owing to the varied impact polymorphisms of this antigen on pregnancy outcomes. HLA-G, situated on the small armrest of chromosome 6p21.1–6p.21.3, was the initial trophoblast HLA to be identified (12). From the perspective of pregnancy progression, HLA-G polymorphism appears to be particularly significant. Initially identified on the superficial of trophoblast cells during initial pregnancy, HLA-G is thought to impact the implantation process of the fertilized egg into the uterine mucosa. Expression of HLA-G is detected in ova shortly next fertilization at the oocyte phase. Studies have indicated that serum ranks of HLA-G in pregnant females are 2-5 intervals upper than those in non-pregnant persons (13).

Rendering to the outcomes of real-time PCR, the appearance of HLA-G gene stayed highly significant decrease in females with RPL in appraisal with healthy control, 1.28 ± 1.08 versus 11.37 ± 6.99 respectively, (P< 0.001). HLA-G alleles comprises a 14 bp portion in exon 8 that its obliteration chiefs to important reduction in appearance of HLA-G mRNA and protein(14). Trainings exposed that HLA-G productions an significant part in the pathogenesis of recurrent pregnancy loss. Consequently, any insufficiency in HLA-G appearance is related with RPL(15). Additional research proposed that the reduction in soluble HLA-G (sHLA-G) ranks may be linked to alterations in the equilibrium among cytokines of pro- and anti inflammatory , which are related with abortion (16). Specifically, earlier studies have documented a reduction in IL-10 appearance, a key promoter of HLA-G construction during typical pregnancies. This deficiency in IL-10 secretion may partly account for the observed decline in soluble HLA-G appearance among the women in our abortion cohort (17).

The existing outcomes similar to the results of Mosaferi et al., (18), The test group displayed an important reduction in the appearance gene of the HLA-G (P < 0.001) paralleled to the controlling group. Additionally, the appearance of HLA-G stayed notably diminished in placenta testers from the examination group compared to the controlling group. These outcomes are dependable with the outcomes reported via Akhter et al. (19), who demonstrated a important reduction in the appearance of HLA-G among individuals suffering recurring miscarriage compared to those with usual pregnancies, as well as Cheng et al., (17), who noted reduced ranks of soluble HLA-G (sHLA-G) in women experiencing abortion compared to pregnant individuals. Conversely, no important disparity in sHLA-G levels stayed detected among non-pregnant females and those with abortion. Nevertheless, the findings of certain other studies do not align with the current results, as indicated by Bhalla et al., (20), Assessed the appearance of HLA-G through immune-histochemistry stain in placental tissue from patients with recurrent miscarriage, comparing it with a healthy control group. The findings revealed that there stayed no important variance in the rate of HLA-G appearance among the two sets.

Diverse trainings displayed polymorphism that insertion/deletion of HLA-G is considerably related with recurring miscarriage (21). Sipak-Szmigiel et al., (22), noted that the experimental group exhibited a higher risk of pregnancy failure correlated with the occurrence of the HLA-G allele. A parallel study stayed carried out via Abbas et al., 23, conducted an analysis of HLA-G polymorphisms involving 120 females with recurring impulsive abortion and 120 females experiencing unfussy pregnancy in India. According to the novelists' findings, the HLA-G allele stayed additional prevalent among women with recurring impulsive abortion. Specifically, the HLA-G 010108 allele stayed detected in 0.4% of females with recurring impulsive abortion, while it was entirely absent in those with uncomplicated pregnancies.

Examining findings from various studies reveals conflicting results when evaluating the impact of individual factors, like serum HLA-G levels, on recurrent miscarriage. Ultimately, it can be risked that artificial sHLA-G analogs capacity be utilized to treat sure pregnancy linked sicknesses and aid duplicate. Nevertheless, a thorough comprehension of the pathophysiology underlying these illnesses is essential previously considering such interventions (18).

The existing training validated the appearance of HLA-G in predicting recurrent pregnancy loss which is more significant for recognition the development of early analysis of recurrent pregnancy loss. The present study show 37 of 40 patients (92.5%) had HLA-G expression inferior than the cut off rate (>3.01) in paralleled to only 3 of 40 healthy issues (7.5%) had HLA-G expression lesser than the cut off rate (>3.01), and the variance stayed highly important (P> 0.001) table (7). ROC

curve investigation displays that the HLA-G expression cutoff rate stayed >3.01 with sensitivity, specificity, PPV and NPV ranks of 92.5%, 92.5%, 92.5% and 92.5% separately. The region below the curve (AUC) of the (ROC) stayed 0.980 (0.956- 1.000) for these gene, representing that these gene could calculate the illness strictness of recurrent pregnancy loss.

Conclusions

In our training designates that sHLA-G expressions in relation to Recurrent Pregnancy Loss (RPL). We anticipate that our findings will inspire additional investigation directed at defining whether a combination of multiple HLA-G genetic factors along with the sHLA-G examination of parent body fluids holds clinical significance for successful implantation.

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