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The Upregulation of HLA-G1 and miRNA-34a in Lens Epithelial Cells of Diabetic Retinopathy Patients

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ABSTRACT

Background: Retinopathy of diabetes is a chronic diabetes mellitus complication affecting retinal vessels, and some ocular complications' molecular mechanisms remain obscure.

Objective: To evaluate the expression of HLA-G1, HLA-G5, miRNA-181a, and miRNA-34a in the lens epithelial cells of patients with retinopathy of diabetes.

Methods: In a case-control study, 30 diabetic patients with retinopathy, 30 diabetic patients without retinopathy, and 30 cataract patients without diabetes mellitus as the control group were enrolled after a full description with details about the study methods and objectives. The expression of HLA G1, HLA-G5, miRNA-181a, and miRNA-34a in lens epithelial cells was assessed by quantitative RT PCR. Moreover, the levels of HLA-G protein in aqueous humor were evaluated by the ELISA method.

Results: HLA-G1 expression was significantly upregulated in the retinopathy group (P=0.003). The aqueous humor of diabetic retinopathy patients contained significantly higher levels of HLA-G protein compared with the non-diabetic patients (P=0.001). miRNA-181a was significantly downregulated in the diabetic retinopathy group compared with the patients without diabetes (P=0.001). In addition, miRNA-34a was upregulated in the retinopathy group (P=0.009).

Conclusion: Taken together, the present results showed that HLA-G1 and miRNA-34a can be valuable markers for diabetic retinopathy. Our data offers new perspectives for improving the control of inflammation in the lens epithelial cells by considering HLA-G and miRNA.

Keywords: Diabetic Retinopathy; HLA-G; Lens Epithelial Cells; miRNA-181a; miRNA34a

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INTRODUCTION

Diabetes mellitus (DM) is a systemic disease associated with microvascular and macrovascular complications (1). Diabetic retinopathy (DR) is a vision-threatening chronic condition (2), and vascular detrition, neuronal, and inflammatory mechanisms are involved in its pathogenesis. A cascade of metabolic alterations such as retinal perfusion rate, the loss of pericytes, and the hypertrophy of the basement retinal membrane occur in ocular tissues during DR. The inflammation of the retina has an essential role in the pathogenesis of DR (3).

MicroRNAs (miRNAs) are short, endogenous, noncoding RNAs with a length of ~22 nucleotides that regulate gene expression through mRNA degradation and translational repression via complementarity with 3′-UTR mRNA sequences (4). Recent research has demonstrated that eye vitreous miRNAs have a key role in cellular functions such as proliferation, differentiation, apoptosis, immunity, and angiogenesis. Moreover, miRNAs may contribute to pathologic conditions such as cancer, DM, and autoimmunity (4, 5). Several studies have confirmed the important function of miRNAs in the pathogenesis and development of DR. Numerous studies have evaluated the miRNA alterations in different cell types and organs during DM development. The miRNA-34 family was demonstrated to be upregulated in diabetic rats. Therefore, in some animals, the retinal expression of miRNA-34 has demonstrated prognostic values. Moreover, the function of miRNA-34a in the proliferation and migration of retinal pigment epithelial cells in vitreoretinopathy was documented. On the other hand, the miRNA-181a was demonstrated to decrease in human posterior capsule opacification (PCO) (4-6).

HLA-G is a human leukocyte antigen class-Ib characterized by a special promoter, limited polymorphism, and restricted tissue distribution (5, 6). HLA-G can be considered one of the liable molecules for the immune-privileged status of the eye's posterior part (7). HLA-G1; the membrane-bound isoform of HLA-G; and HLA-G5 (soluble HLA-G1) can interact with receptors expressed on different cell types, such as endothelial cells, that can inhibit their proliferation and angiogenesis (6). The soluble HLA-G1 inhibits fibroblast growth factor-2 (FGF2) and corneal angiogenesis and triggers apoptosis in endothelial cells. HLA-G also plays a prominent role in peripheral immune tolerance. The effector functions of several immune cells could be mitigated upon HLA-G's engagement with inhibitory receptors expressed on these immune cells (8). HLA-G expression is regulated in various states, including epigenetics, transcriptional and post-transcriptional levels (9). The HLA-G mRNA represents a 3'-UTR polymorphism that may be engaged by different miRNAs (10). miRNA-148a, miRNA-148b, and miRNA-152 are supposed to target the HLA-G 3'-UTR (11).

Considering the importance of the above-mentioned pathways in the development of DR, the purpose of the present study was to evaluate HLA-G1, HLA G5, miRNA-181a, and miRNA-34a expressions in lens epithelial cells (LECs), and HLA-G protein in aqueous humor, comparing them among patients with DM, DR, and non-diabetic cataract patients.

MATERIALS AND METHODS

Subjects

In a case-control study, 30 retinopathy of diabetes patients, 30 diabetic patients without retinopathy, and 30 cataract patients without diabetes mellitus as the control group were enrolled after a full description with details about the study methods and objectives. The diagnosis of diabetes was in accordance with the American Diabetes Association (ADA) Guidelines. This study was conducted with the ethical approval of the local Ethical Committee according to the Helsinki Declaration. After a full explanation

of the surgical procedures and complications, written informed consent was derived from all contributors. Cataract surgery was carried out using extracapsular extraction or phacoemulsification techniques between May 2016 and April 2017 at Bou Ali Hospital, Sari, Iran. The exclusion criteria were any history of inflammatory or autoimmune diseases, history of ocular diseases and ocular surgeries, and the use of NSAIDs or steroids in the past three months. The same surgeon performed cataract surgeries. The patients were divided into the following three groups: cataract patients without DM (no-DM), patients with DM, and patients with DR. Table 1 demonstrates brief demographic information of the study participants.

Collection of Samples

During the cataract surgery, an anterior capsule sample was taken and immediately transferred into RNAlater solution for 24-72 h before the RNA extraction. Also, 0.1-0.2 mL of aqueous humor was obtained via paracentesis at the mid-lateral aspect using a 26 G needle. The undiluted aqueous samples were stored in a −70 °C freezer before the Enzyme-Linked Immunosorbent Assay (ELISA) analysis.

RNA Extraction from Tissue Specimens

The total RNA was extracted from epithelial cells using Accuzol™ reagent (Bioneer, South Korea), based on the manufacturer's instructions. The RNA content of each sample was quantified using a PicoDrop instrument (PicoDrop, UK). DNase I (Thermo Scientific, #EN0521, Auckland, New Zealand) treatment was carried out to remove any contamination with DNA prior to the first strand complementary DNA (cDNA) synthesis.

Specific cDNA Synthesis of miRNAs

Stem-loop primers were used for specific cDNA synthesis of miRNAs. The total RNA was reversely transcribed using the Revert Aid First Strand cDNA synthesis kit (Thermo Scientific, #K1622, Auckland, New Zealand) following the manufacturer's instructions. Real-time quantitative polymerase chain reaction (RT-qPCR) was performed using SYBR Premix Ex Taq II (Takara, Japan) with specific primers represented in Table 2 for HLA-G1, HLA-G5, and GAPDH as the internal control. Similarly, snU6 was used for the normalization of miRNA-181a and miRNA-34a expressions. All RT-qPCR reactions were performed by the Bio-Rad iQ5 Real-Time PCR instrument (Bio-Rad Laboratories, Hercules, CA, USA). The utterance levels of target genes were determined $2^{-\Delta\Delta CT}$ way and our results were definedas fold changes in gene expression.

HLA-G Protein Assay by ELISA Method

HLA-G protein levels in aqueous humor and the other groups were measured by an ELISA kit (East Biopharm Co Hangzhou, China) following the manufacturer's procedures. Briefly, for the test wells, aqueous humor was added, and then both the HLA-G antibody and streptavidin-HRP were added to the reaction. Then, the plate was incubated for 60 min at 37 °C. After washing, the chromogen solutions were added to each well. The plate was incubated for a further 10 min at 37 °C in the dark. Then, the stop

Table 1. Demographic information of the study participants

Parameter	Mean±SD			P value
	no-DM	DM	DR	
Age	68.61±9.57	68.13±7.58	63.84±8.96	0.066
Sex	16 M/14 F	13 M/17 F	12 M/18 F	0.558
HbA1c (%)	5.2±0.48	7.9±0.88	9±0.79	<0.0001
Duration of DM (years)	-	11.16±3.35	14.85±3.68	0.0004

Quantitative variables presented as mean±standard deviation. No-DM: Patients without diabetes mellitus; DM: Patients with diabetes mellitus and without retinopathy; DR: Diabetic patients with retinopathy. HbA1C :Hemoglobin A1C

Table 2. The sequence of the primers for the quantitative Real-Time PCR

Primer	Sequence (5' to 3')
U6 F	GCTTCGGCAGCACATATACTAAAAT
U6 R	CGCTTCACGAATTTGCGTGTCAT
miRNA-181a F	GAACATTCAACGCTGTCGGTGAGT
miRNA-181a R (universal reverse primer)	GTGCAGGGTCCGAGGT
miRNA-34a F	CGGTATCATTTGGCAGTGTCT
miRNA-34a R (universal reverse primer)	GTGCAGGGTCCGAGGT
HLA-G1 F	CTGGTTGTCCTTGCAGCTGTAG
HLA-G1 R	CCTTCCTTACCTGAGCTCTTCTTTCT
HLA-G5 F	GAAGAGGAGACACGGAACACCA
HLA-G5 R	TCGCAGCCAATCATCCACTGGA
GAPDH F	CATGAGAAGTATGACAACAGCCT
GAPDH R	AGTCCTTCCACGATACCAAAGT

F: Forward; R: Reverse; miRNA: MicroRNA; HLA-G: Human Leukocyte Antigen -G; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

solution was put in the reactions, and the OD was measured at 450 nm.

Statistical Analysis

The data were first evaluated by the D'Agustino normality test. Then, the parametric data were analyzed by one-way ANOVA followed by Tukey's multiple comparison test, and the non-parametric data were assessed by the Kruskal-Wallis test followed by Dunn's multiple comparison test. The parametric values are evaluated as the means±SD, and non-parametric values are represented as the median±range. The P-values below 0.05 were considered statistically significant. It was performed using GraphPad Prism software (version 6; GraphPad, San Diego, USA).

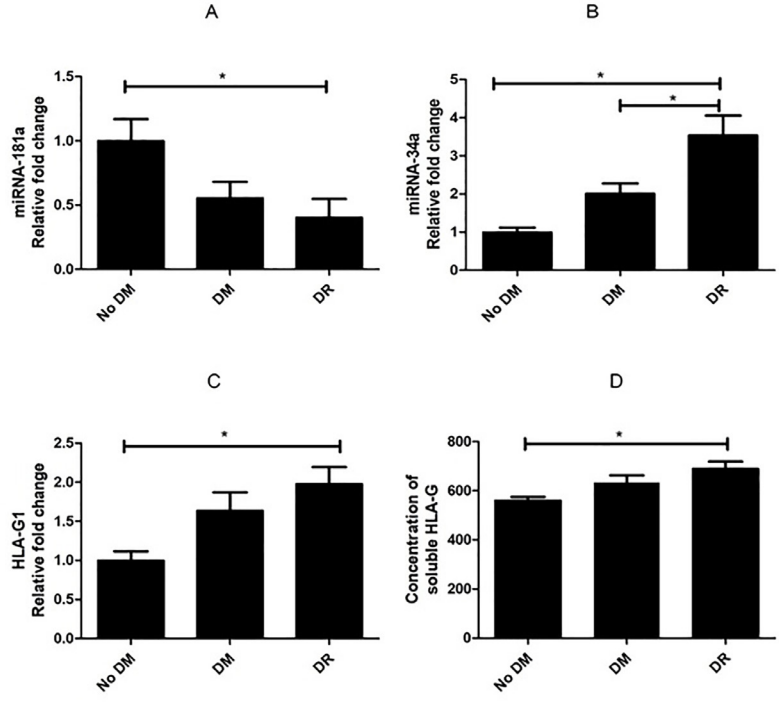

Figure 1. (A) miRNA-181a gene expression in the experimental groups by real-time PCR. The data are expressed as the median±range, n=30 in each group; (B) relative expression of miRNA-34a; (C) relative expression of HLA-G1 in experimental groups; (D) The comparison of expression of the soluble HLA-G protein in experimental groups using ELISA technique. (B, C, D) The data are expressed as the mean±standard deviation, n=30 in each group (*P<0.005). DM: Patients with diabetes mellitus and without retinopathy; DR: Diabetic patients with retinopathy

RESULTS

Expression of miRNA-181a and miRNA-34a in LECs

As represented in Figure 1A, the miRNA-181a expression was significantly downregulated in the DR group in comparison with the no-DM group (P=0.001). No significant difference was found between the DM and DR groups regarding their miRNA-181a expressions (P=0.279). Compared with the no-DM and DM groups, miRNA-34a was upregulated in the DR group ($p < 0.001$ and P=0.009, respectively; Figure 1B).

Expression of HLA-G1, HLA-G5, and Soluble HLA-G

While HLA-G5 expression was consistently undetectable in all the study groups, HLA-G1 expression was significantly upregulated in the DR group (P=0.003; Figure 1C).

As demonstrated in Figure 1D, there was also an increase in soluble HLA-G levels in aqueous humor in the DR group compared with the no-DM group (P=0.001). In addition, the soluble HLA-G levels of DM patients were elevated in comparison with the no-DM group (P=0.09).

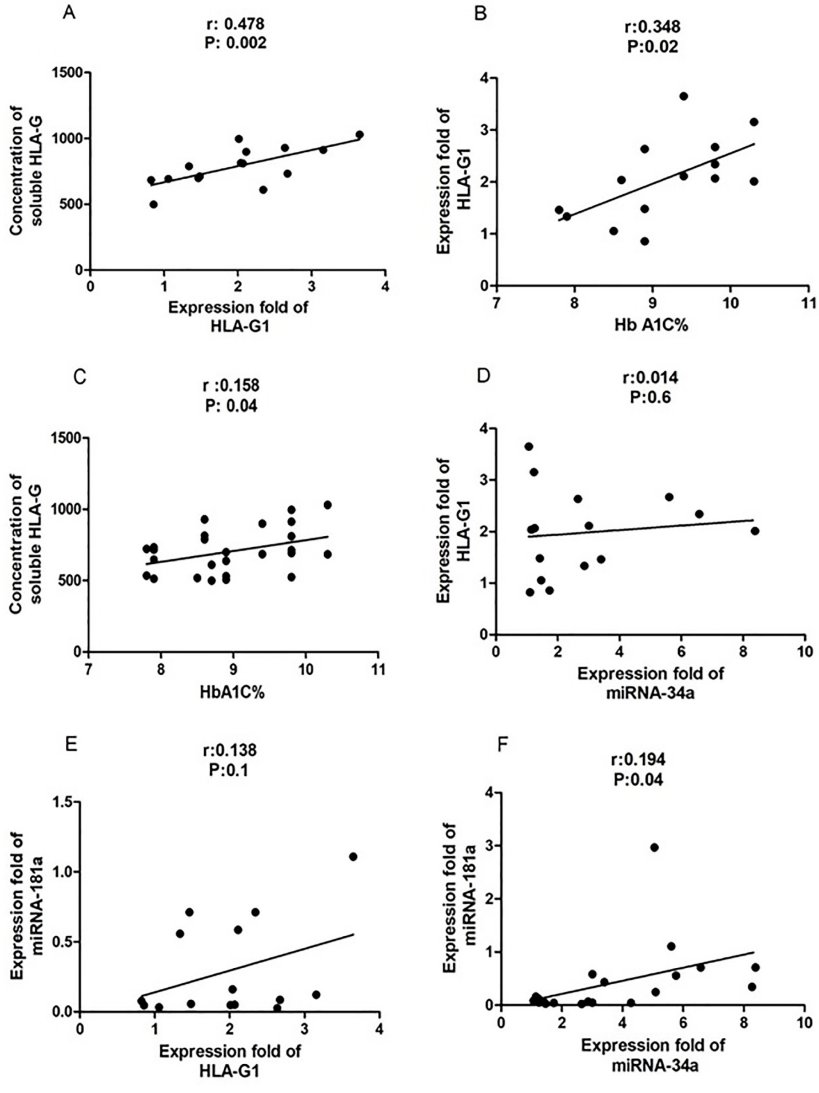

Figure 2. (A) The correlation between HLA-G1expression in LECs and HLA-G protein in aqueous humor; (B) HLA-G1 expression in LECs and HbA1C% in blood; (C) HLA-G protein in aqueous humor and HbA1C% in blood; (D) HLA-G1 and miRNA-34a expression in LECs; (E) Correlation between HLA-G1 and miRNA-181a expression in LECs and (F) miRNA-34a and miRNA-181a expression in LECs. miRNA: MicroRNA;HLA-G: Human Leukocyte Antigen-G; HbA1C :Hemoglobin A1C

Correlation between HLA-G, HbA1C%, miRNA-34a, and miRNA-181a Expressions

We analyzed the HLA-G1 mRNA expression in LECs and soluble HLA-G protein concentrations in aqueous humor for correlations and demonstrated that there is a significant positive correlation between them in the DR patients. (r=0.478, P=0.002; Figure 2A). There was also a notable correlation between HLA-G1 expression in LECs and the percentage of blood HbA1C in DR patients (r=0.348, P=0.02; Figure 2B). The concentrations of soluble HLA-G in aqueous humor have also correlated with the percentage of blood HbA1C (r=0.158, P=0.04; Figure 2C). In addition, we analyzed our data to detect any possible correlations between HLA-G1 and miRNA-34a or miRNA-181a as well as between miRNA-181a and miRNA-34a. Despite there being no significant correlations between HLA-G1 and miRNA-34a or between HLA-G1 and miRNA-181a (Figure s 2D and E), there was a weak positive correlation between miRNA-34a and miRNA-181a expressions in LECs (r=0.198, P=0.04; Figure 2F).

DISCUSSION

The current study was conducted to present one of the best immune tolerance-related anti-inflammatory markers involved in preventing adverse immune reactions. This is the first research designed for the assessment of HLA-G, miRNA-181a, and miRNA-34a expression in LECs on basis of our knowledge.

The findings of our study provided justification for targeting HLA-G and microRNAs in LECs as these molecules have immunomodulatory effects in transplantation, cancer, and immune privilege sites such as eyes and testes.

Our previous study demonstrated a low expression of the HLA-G gene in diabetes patients (12). This study can be considered an important investigation in the elucidation of the underlying mechanisms involved in DR and the discovery of reliable biomarkers and novel therapeutic strategies in this regard. Today, inflammatory pathways and destructive mechanisms related to DR are demonstrated, and the function of inflammation and angiogenesis in the pathogenesis of DR is widely confirmed (13, 14). However, there are significant gaps in detecting HLA-G in ocular tissues (15). Several studies on the detection of HLA-G protein in adult corneas have concluded that this protein may have a key role in the establishment of the corneal immune-privileged sites (15, 16). Our observations of the constitutive HLA-G expression in non-diabetic individuals verify the talent function of HLA-G in the eye immune-privileged condition. The eye immune-regulation pathways might prevent ocular cell inflammation and maintain tissue integrity (17). These mechanisms may be involved in the prevention of any detrimental inflammation. Svendsen et al. observed that retinal pigment epithelium cells stimulated with IFN-γ and TNF-α significantly upregulated the HLA-G, and concluded that HLA-G might be an important retinal epithelial element during inflammation (7). Moreover, Feng et al. demonstrated that the inflammatory cytokine quantity such as IL-1β, IL-6, IL-8, IL-17, and TNF-α in aqueous humor of DR patients are upregulated and may have a key function in the pathogenesis of the disease (18). In this research, we demonstrated that higher levels of HLA-G and its secretion might be a regulating mechanism for reducing inflammation in LECs in patients with DR in comparison with the control group. Fons et al. reported that soluble HLA-G1 molecule demonstrates anti-angiogenic properties through the inhibition of angiogenesis, induction of endothelial cell apoptosis, and the inhibition of FGF2-induced capillary-like tubule formation via the CD160 receptor pathway (6).

Several studies have shown that miRNAs have an important role in the development of DM and related complications (19, 20). Similar to our results, Kong et al. demonstrated the serum levels of miRNA-34a, miRNA-

124a, miRNA-30, miRNA-146a, miRNA-9, miRNA-29a, and miRNA-375 increased in type 2 DM patients (21). They reported that miRNA-34a was downregulated in patients with uveal melanoma. It has been demonstrated that miRNA-34a is an important regulator of p53-mediated apoptosis and its dysregulation can lead to retinal neurodegeneration during DM (22). In the present study, we concluded that miRNA-34a elevation in DR patients might have a detrimental function on the proliferation of LECs.

The miRNA-181a-5p is another well-known miRNA regulating the vascular endothelial growth factor (VEGF)-receptor-1. High concentrations of glucose have been shown to downregulate the expression of miRNA-181a-5p in human retinal endothelial cells (23). In this research, a low expression of miRNA-181a in LECs in DR patients is following a previous study in a PCO eye. However, although several miRNAs such as miRNA-148a, miRNA-148b, and miRNA-152 have been proposed to target the 3'-UTR of HLA-G transcripts (24), we did not detect any correlation between miRNA-34a, miRNA-181a, and HLA-G1 expressions in this study.

Undoubtedly, a better understanding of the underlying molecular mechanisms of DR pathogenesis can obtain a platform for the expansion of reliable markers for early detection of the complication, and the development of therapies that can specifically target defective pathways. Based on our knowledge, this is the first report of HLA-G upregulation in the ocular tissues during diabetic retinopathy. With respect to the anti-inflammatory roles of HLA-G, this upregulation may protect ocular cells against detrimental inflammation.

Furthermore, we have shown the correlation between HLA-G1 mRNA expression in LECs and soluble HLA-G protein concentrations in aqueous humor (r=0.478, P=0.002; Figure 2A). Also, it is a notable correlation between HLA-G1 expression in LECs and the percentage of blood HbA1C in DR patients (r=0.348, P=0.02; Figure 2B). The concentrations of soluble HLA-G in aqueous humor also correlated with the percentage of blood HbA1C (r=0.158, P=0.04; Figure 2C). In addition, we analyzed our data to detect any possible correlations between HLA-G1 and miRNA-34a or miRNA-181a as well as between miRNA-181a and miRNA-34a. Despite there being no significant correlations between HLA-G1 and miRNA-34a or between HLA-G1 and miRNA-181a (Figure s 2D and E), there was a positive correlation between miRNA-34a and miRNA-181a expressions in LECs (r=0.198, P=0.04; Figure 2F). In addition, aligned with this study, miRNA-34a positively correlated with metabolic disorders such as hypertension and diabetes (25)

In this study, dysregulation of miRNA-181a and miRNA-34a in LECs of DR patients may be related to a wide range of metabolic changes during DM. Although the affected miRNAs are in upstream or downstream of several signaling modulators, miRNA replacement strategies may be effective in ameliorating DR complications. The in silico analysis has shown the relationship between the diseases with miRNA. Kumar and et al. (2003) reported an association of epithelial ovarian cancer downregulation and upregulation of miRNA-205 and miRNA-34a (26).

The limitations of this study were the sample size and obtaining cytokines kits. Furthermore, there are some inconsistent studies on the miRNA roles. Thus, inflammatory evaluation of eye epithelial cells in different diseases may be useful in future research

CONCLUSION

Since these genes are involved in the notch signaling pathway, as direct target genes of miR-34a (4), they could be an important molecule in the repression of inflammations. The correlations between HLA-G1 and miRNA-34a or miRNA-181a have shown a regulatory role for miRNAs in target suppression and therapeutic function in

inflammation. Taken together, the present results showed that HLA-G1 and miRNA-34a can be valuable markers in diabetic retinopathy. This specific research offers new perspectives for improving the control of inflammation in lens epithelial cells by considering the HLA–G and miRNA.

ETHICAL APPROVAL

This study was conducted based on the local Ethical Committee's ethical codes according to the Helsinki Declaration. After an explanation of the surgical function and complications, written informed consent was obtained from all the participants.

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AUTHORS' CONTRIBUTION

Saeid Abediankenari carried out the study conception and design. Elnaz Taghvaei and Narjes Jafari performed the experiments and data acquisition. Fatemeh Abedian and Ahmad Ahmadzadeh analyzed the data and reviewed the manuscript. All the authors read and approved the final manuscript

Conflict of Interest: None declared.

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