ISSN: 2230-9926

Available online at http://www.journalijdr.com

IJDR

International Journal of Development Research
Vol. 14, Issue, 02, pp. 65054-65058, February, 2024
https://doi.org/10.37118/ijdr.27511.02.2024

RESEARCH ARTICLE OPEN ACCESS

PLACENTAL HLA-G EXPRESSION AMONG WOMEN LIVING OR NOT WITH HIV-1

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ARTICLE INFO

Article History:
Received 11th January, 2024
Received in revised form
27th January, 2024
Accepted 02nd February, 2024
Published online 28th February, 2024

Key Words:

HLA-G, HIV, Placenta,
Expression, Transmission.

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ABSTRACT

Introduction: The immune checkpoint HLA-G molecule is highly expressed in the placenta's extravillous trophoblast cells and has a pivotal role in immune tolerance during pregnancy. Since HIV-1 can infect trophoblast cells and may modify HLA-G expression to subvert host immune defenses, in this study we evaluated the HLA-G expression in third-trimester placental tissue of women living or not with HIV-1. ***Methods:*** Immunohistochemistry assay to evaluate HLA-G staining of 183 fragments of paraffin-embedded placental tissue, of which 90 from HIV-1-positive women(HIV+) and 93 from non-positive women(HIV-), were performed. ***Results:*** HLA-G staining was observed in extravillous trophoblast(EVT) cells and endothelial and Hofbauer cells, but not in syncytiotrophoblast for both. According to the magnitude of HLA-G staining, HIV-1-non-positive placenta exhibited increased HLA-G staining [negative(6.70%) and 3+(60.00%)], when compared to infected placenta, [negative(30.10%) and 3+(45.16%)] ($P<0.01$). Overall, decreases in HLA-G expression were significantly associated with pregnancy HIV-1 infection [$P<0.01$; odds ratio: 2.47(95% CI: 1.47–4.14)]. Other biomarkers of HIV-1 infection like viral load, CD4+ T-cell count, and antiretroviral therapy used during pregnancy showed no association with HLA-G. ***Conclusion:*** Our findings suggest that HIV-1 infection can modulate HLA-G expression in EVT cells, which may contribute to an immunological environment affecting the outcome of infection.

Citation*: *Mariana R. Santiago; Juliana Martinez; Talita M. Fernandes; Diego A. Souza; Gyl E. Silva; Fernando Ramalho, Silvana M. Quintana; Policardo G. da Silva; Larissa A. Cepas; Lariane A. Cepas; Eduardo A. Donadi and Ana Paula M. Fernandes, 2024. *"Placental hla-g expression among women living or not with HIV-1". International Journal of Development Research*, 14, (02), 65054-65058.

INTRODUCTION

HLA-G is a non-classical human leukocyte antigen (HLA), initially identified in the placenta's extravillous trophoblast (EVT) cells, playing a crucial function in maternal-fetal tolerance[1,2,3], avoiding immune rejection by maternal leukocytes[4]. HLA-G–expressing cells can increase the secretion of cytokines such as interleukin-6 (IL-6) and IL-8[5], induce regulatory T cells (Treg)[6], modulate antigen-presenting cells by binding to HLA-G receptors (leukocyte Ig-like receptor subfamily B member 1 LILBR1, also known as ILT2)[7,8], and limit NK cytotoxicity[9].

In addition, HLA-G may also exert its inhibitory functions in pathological conditions, including tumors[10], autoimmune diseases[11], organ transplantation[12], and viral infections[13]. In pregnant women living with human immunodeficiency virus-1 (HIV-1), studies indicate that the virus can also infect the EVT cells[14]. Soluble HLA-G (sHLA-G) levels are significantly higher in patients living with HIV-1, a finding that has been attributed to an increased HLA-G secretion from monocyte and dendritic cell intracellular stores. Indeed, HIV disease progression is also associated with increased sHLA-G levels[15] and increased surface expression of HLA-G on monocytes and T lymphocytes[16]. This suggests that the virus may control the expression of HLA-G and take advantage of its immunomodulatory properties, subverting host immune surveillance and immune

responses. Considering that HIV-1 can infect EVT cells and that the viral infection can modulate HLA-G expression. It is relevant to analyze whether EVT cells of women living with HIV-1 exhibit altered expression of HLA-G when compared to those of women not living with HIV-1. Therefore, this study aimed to evaluate the expression of HLA-G in the EVT cells of women living or not with HIV-1.

MATERIALS AND METHODS

Participant recruitment: This case-control study was carried out at the University Hospital of the Ribeirão Preto Medical School, University of São Paulo, Brazil. We analyzed 183 fragments of paraffin-embedded placental tissue, of which 93 samples were from women living with HIV-1 (HIV+) and 90 from women not living with HIV-1 (HIV-). All the pregnant women samples in this study are HIV-1 non-transmitting mother-infant, and the placentas were obtained from successful third-trimester (37 weeks or older) pregnancies, collected between 2008 and 2012. The patients with other comorbidities were excluded. The study was approved by the Ethics Committee of the College of Nursing of Ribeirão Preto, University of São Paulo, Brazil (protocol # 1330/2011), and all participants signed the Free and Informed Consent Form. This study was carried out by The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans.

Clinical data: Clinical and laboratory data of the HIV+ group, such as viral load, circulating CD4+ lymphocyte count (CD4+ T-cells), and type of antiretroviral therapy (ART) used during pregnancy were retrieved. Data from patients' medical records available in the electronic files of the University Hospital of the Ribeirão Preto Medical School were consulted.

Immunohistochemical analysis of HLA-G: Four-micrometer-thick sections of paraffin embedded tissue blocks were cut and mounted on polylysine-coated slides. Tissue sections were dewaxed in xylene and rehydrated in graded series of ethanol. Antigen retrieval treatment was performed at 96 °C for 40 min in 10 mmol/L sodium citrate buffer, pH 6.0 (Synth, Diadema, Brazil). The endogenous peroxidase activity was blocked by treating the slides with two consecutive baths of 3% hydrogen peroxide in methanol. To block nonspecific reactions, tissue sections were washed three times (5 min each) in phosphate-buffered saline (PBS) 0.01M (pH 7.2) and incubated in PBS enriched with 1% nonfat milk. Samples were incubated overnight with the primary monoclonal antibody 5A6G7 anti-HLA-G antibody (EXBIO Antibodies, Vestec, Czech Republic). The 4H84mAb recognizes the free heavy chain of all the HLA-G isoforms. After incubation with the primary antibody, the slides were treated with streptavidin complex Ultra–Biotin–Peroxidase (EP-USA/500; Signet, Belmont, CA) for 40 min at 37 °C. Finally, the sections were revealed with diaminobenzidine solution (Sigma, Saint Louis, MO) at 37 °C for 10 min, and counterstained with hematoxylin for the 60s, washed with water, dehydrated, and mounted. A cytotrophoblast from the first-trimester human placenta was used as an HLA-G–positive control. Negative control was performed in placenta tissue, omitting the primary antibody. One experienced pathologist blindly analyzed, under a light microscope, all specimens encompassing the entire placenta sample at different. According to the percentage of stained placenta cells, HLA-G expression was stratified into five categories adopting the score used by Martinez et al[17]. Negative staining was defined when less than 5% of the EVT cells expressed HLA-G (–); positive staining when 6-25% of the EVT cells expressed HLA-G (1+); 2+ (26-50%); 3+ (51-75%) and 4+ (>75%).

Statistical analysis: The chi-square test was applied with a significance set at 5% and a logistic model was applied. For this analysis, the Odds Ratio (OR) was estimated with a 95% confidence interval (95% CI), using the software SAS® 9[18].

RESULTS

The clinical and pathological findings of our series of patients are shown in Table 1. The average ages of the HIV+ and HIV patients were 28 years (SD = ±6.25) and 24 years (SD ±5.54), respectively. In both groups, the majority of the participants were Caucasian and had completed high school, were unemployed, and had up to nine years of schooling. Most of the women living with HIV-1 were single mothers, while women not living with HIV-1 were married.

Table 1. Demographic characteristics of women living or not with HIV-1

Demographic parameters	HIV+ (%)	HIV- (%)
Marital status		
Single	51	14
Married	15	19
Living in common law	19	54
Separated	3	0
Divorced	4	3
Widowed	1	0
Ancestry		
Caucasian	64	40
Admixture	19	38
African American	10	12
Years of schooling		
Up to nine years	57	32
From 9 to 12 years	28	58
More than 12 years	6	0
Undeclared	2	0
Employment		
Yes	32	26
No	61	64

Regarding obstetric history, women living with HIV-1 gave birth to an average of 3 children (range 1-10), 20% of them had previous miscarriages, 87% had access to prenatal care and 51% had cesarean deliveries. Women not living with HIV-1 had an average of 2 children (range 1-7) and 14% had miscarriages, 100% had access to prenatal care and 74% had vaginal delivery. Clinical and laboratory data indicated that 83.7% of women not living with HIV-1 had a viral load of <10,000 copies/mL, 44.1% had CD4+ T-cell count between 200-499 cells/mm3 and 95.1% of the patients used protease inhibitors (PI) in a combination of antiretroviral drugs. The expression of HLA-G protein in human placental tissue was visualized as a brown-stained area, primarily observed in the cytoplasm of EVT, endothelial cells, and Hofbauer cells, but syncytiotrophoblast cells were not immunoreactive for HLA-G (Figure 1). This expression pattern of HLA-G was found in the placentas of women living or not with HIV-1.

The analysis of HLA-G expression according to the HIV status revealed that HIV+ samples presented a higher frequency of negative staining (30.1%) and a lower frequency of 3+ staining (45.2%). Whereas, HIV samples exhibited a lower frequency of negative staining (6.7%) and a higher frequency of 3+ staining (60.0%). Overall, HIV+ placentas exhibited decreased HLA-G expression when compared to HIV-samples ($P<0.01$; Table 2). Regression logistic and chi-square tests were performed to verify the influence of HIV-1 infection on HLA-G expression in the placental tissue. When we compared the group according to the percentage of HLA-G staining 4+ vs 0 and HLA-G staining (1+,2+,3+) vs 0, our data showed that patients under control HIV-1 infection decreases the HLA-G expression in the placenta ($P<0.01$, odds ratio =2.47, Cl: 1.47-4.14 and $P<0.01$, odds ratio =2.45, Cl:1.51-3.98), respectively. Lastly, it was performed the comparison of viral load, abortion, CD4+ T-count cells, and type of ART used by women stratifying by HLA-G staining in the placentas, and no significant difference was found.

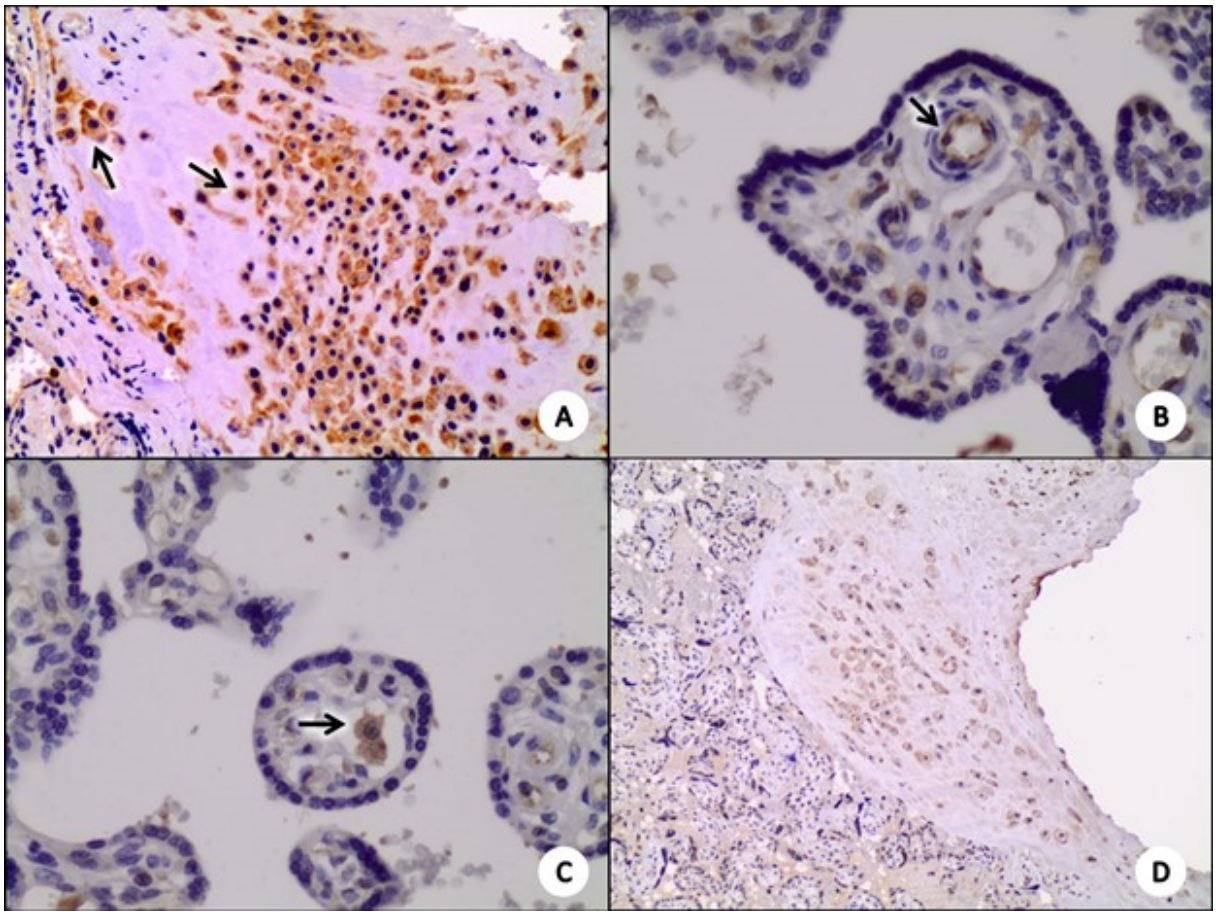

Figure 1. Representative photomicrographs of the expression of HLA-G protein in human placental tissue. Extravillous trophoblast cells (A, D), endothelial cells (B), and Hofbauer cells (C) presented cytoplasmic immunoreactivity for HLA-G (arrows), whereas syncytiotrophoblast cells were HLA-G negative (D)

Table 2. Immunohistochemistry HLA-G staining frequency (0 to 4+) of placental samples, stratified according to clinical variables observed in women living with HIV-1.

	HLA-G staining			*P-Value**
	0	1,2,3	4	
	(≤ 5%)	(6-75%)	(>75%)	
	N (%)	N (%)	N (%)	
HIV-1 Status				
Not living with HIV-1	6 (6.7)	54 (60.0)	30 (33.3)	<0,01
Living with HIV-1	28 (30.1)	42 (45.2)	Absorption	
Abortion				0,25
Non	31 (20.7)	78 (52.0)	41 (27.3)	
Yes	3 (9.1)	18 (54.5)	12 (36.4)	
Viral Load				
<10,000 copies/mL	24 (31.2)	35 (45.4)	18 (23.4)	0,92
>10,000 copies/mL	4 (28.6)	6 (42.8)	4 (28.6)	
$CD4^+$ T-cells				
≥500 cells/mm^3	6 (24.0)	11 (44.0)	8 (32.0)	0,37
200–499 cells/mm^3	2 (8.3)	13 (54.2)	9 (37.5)	
<200 cells/mm^3	0 (0)	5 (71.4)	2 (28.6)	
ART				
Without PI	2 (28.6)	3 (42.8)	2 (28.6)	0,99
With PI	20 (31.7)	26 (41.3)	17 (27.0)	
ART: antiretroviral therapy PI: protease inhibitors				

DISCUSSION

HLA-G was initially described at the maternal-fetal interface[19,20]. It exerts several immunomodulatory effects by directly binding to the inhibitory receptors KIR2DL4, ILT-2, and ILT-4 that are present on NK, T cells, and antigen-presenting cells, being beneficially implicated in embryo implantation and fetal survival. Nevertheless, it is potentially detrimental when expressed in chronic infections conversely[21,22]. In the present study, we investigated the HLA-G expression in the placenta tissue of pregnant women living with HIV-1 submitted to ART and in healthy pregnant women. A previous study analyzed the HLA-E and HLA-G expression in the first trimester placentas of women not living with HIV-1 and showed that HLA-G was expressed in the EVT, endothelial, and Hofbauer cells, but not in the perivillous trophoblast and syncytiotrophoblast[20]. Our results showed that HLA-G molecules were expressed in the

EVT, endothelial, and Hofbauer cells, but not in the syncytiotrophoblast. Remarkably, the presence of HIV-1 has been detected histologically during maternal infection in Hofbauer cells. The modulation of HLA-G expression may reflect immune-regulatory predominance in the placenta as important mediators of protection in the fetus-maternal interface during ongoing HIV-1 exposure[23]. In the context of HIV-1 transmission in women ART-naive during pregnancy, studies have been analyzed indicating the association between certain *HLA-G* alleles and/or 3'UTR polymorphisms. These alter HLA-G expression and consequently influence the susceptibility to transmission (placental or peripheral) of HIV-1[24,25]. Among these 3'UTR polymorphisms, the 14 bp-INS/DEL has been the most studied and associated with the magnitude of HLA-G production, being consequently associated with the risk of mother-to-child transmission (MTCT) of HIV-1[26–31]. Recently, Hong et col. investigated the role of *HLA-G* in MTCT of HIV-1 focusing on the HLA-G polymorphisms showed that certain maternal *HLA-G* alleles (*G*01:01:02*) and 3'UTR (+3187G and UTR-1) polymorphisms were associated with an increased risk during pregnancy transmission[31]. However, such associations have not been strong enough to be considered a disease marker. Few polymorphic sites along regulatory regions have been extensively evaluated regarding their function, and probably a combination of regulatory transcriptional and post-transcriptional elements may account for the final HLA-G expression.

Few studies on HLA-G expression were conducted in the placenta of women living with HIV-1 and its effect on vertical infection. Our results indicated that in the placental tissue of women living with HIV-1, HLA-G expression in the EVT cells was decreased when compared to that in the placenta of women not living with HIV-1. It has been postulated that decreased HLA-G expression would exert greater NK and CD8+T cell activation, which could result in the increased ability of these cells to target viral-infected cells and thus decrease the risk for HIV-1 transmission[29–33]. It is important to remember that all the pregnant women samples in this study are HIV-1 non-transmitting mother-infant. In agreement with our data, Moodley and Bobat[33] related increased HLA-G expression in the placenta of women living with HIV-1 associated with increased mothers' viral log load and the increased risk of viral infection for infants in a group of pregnant ART-naive. The decrease of HLA-G expression observed can be explained by the direct action of the HIV-1 that downregulates the HLA-I expression, thus avoiding the recognition of the infected cells by CD8+ T lymphocytes and NK cells[24]. Some studies aimed to observe the expression of the molecule HLA-G in different tissues and cells in the early stage of infection by HIV and its progression. Corroborating our findings, Derrien and colleagues[31] demonstrated HLA-G downregulation in cell culture infected with HIV-1, suggesting that this may be an escape mechanism that inhibits viral peptides' presentation to CD8 + T cells[3].

In contrast, it has been reported that the HLA-G expression was increased on monocytes and T lymphocytes obtained from patients living with HIV-1 treated or not with antiretroviral therapy. This hypothesizes an indirect effect of HIV-1 infection, since both cell subtypes could not be infected[16]. In addition, Donaghy and colleagues[15] observed high levels of soluble HLA-G in HIV-infected serum levels when compared with the control group, correlating that molecule to the pathogenesis of HIV by inducing tolerance. In agreement with the findings, Mudarca and colleagues[34] identified the systemic increase of soluble HLA-G in patients living with HIV-1, correlated with CD4+ T-count cells and virus response to antiretroviral treatment. Interestingly, it was observed decreased levels of soluble HLA-G in patients in which the replication of HIV-1 was suppressed during ART, while the soluble HLA-G levels remained elevated in people living with HIV-1 individuals who rapidly progressed to AIDS[28]. The interference of antiretroviral drugs on the modulation of HLA-G expression has been analyzed. Cabello et al.[35] reported an increased number of monocytes expressing HLA-G in patients using antiretroviral drugs when compared to that in untreated patients. Rivero et al.[36] evaluated the expression of HLA-G in different HIV therapeutic regimens and revealed that nucleoside analog reverse-transcriptase inhibitors increased HLA-G expression in circulating CD4+ monocytes and lymphocytes, whereas protease inhibitors did not affect lymphomononuclear cell HLA-G expression. However, the decrease of HLA-G expression in the placenta tissue of women living with HIV-1 under ART showed it could be related to the decreased progesterone levels in women living with HIV-1 receiving ART, since this hormone induces HLA-G expression in the placenta tissue[37].

CONCLUSION

In comparison with placentas of women not living with HIV-1, the expression of HLA-G in the EVT cells was decreased in women living with HIV-1 under ART. The expression level of HLA-G was not influenced by factors such as viral load, CD4+ T-cell count, and the use of PI with ART. These observations suggest that, in the context of viral infections, the expression of HLA-G is a complex process modulated by many factors such as stage of infection, drug therapy, and cytokine expression patterns, which may contribute to an immunological environment affecting the outcome of infection.

Acknowledgements: We acknowledge the financial support provided by the São Paulo Research Foundation (FAPESP) (Master's funding in Brazil, FAPESP 11/16885-6).

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