*Original Article*

**Microbial Biosystems 6(2) (2021) 2021.1046**
**10.21608/mb.2021.106010.1046**

Contents lists available at Egyptian Knowledge Bank
Microbial Biosystems
Journal homepage: http://mb.journals.ekb.eg/

MICROBIAL BIOSYSTEMS
INTERNATIONAL SCIENTIFIC JOURNAL OF MICROBIAL BIOLOGY

# Impact of human papilloma virus HPV on immunosenescent CD57$^{+}$ T-lymphocytes in cervical cancer patients

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INDEXED IN EMERGING SOURCES CITATION INDEX

**ARTICLE INFO**

***Article history***
Received 14 November 2021
Received revised 5 December 2021
Accepted 20 December 2021
Available online 25 December 2021


Corresponding Editor:
Elkelish AA.
Abdel-Wareth MT.

***Keywords***
CD57
cervical cancer
HLA-G
HPV

**ABSTRACT**

Human papilloma virus (HPV) related cervical cancer (CC) remains a significant cause of mortality, especially in developing nations. Practical, cheap, and responsive biomarkers are required to diagnose and prevent large-scale CCs and other cancers associated with HPV. This research aimed to investigate HPV-Human leukocyte Antigen-G (HLA-G) interaction with the cervical cancer immune modulation: Hypothesizing HPV-specific HLA markers might be accurate and cost-effective preventive biomarkers. In addition to assessing the host immune response by immunophenotyping of CD57 natural killer T-cells. Forty-five DNA and serum samples of the patients were divided into two groups; twenty-three cases with HPV infection associated with cervical cancer and twenty-two controls with HPV infection without cervical cancer. Real-Time Quantitative polymerase chain reaction (RT-PCR) was used for HLA-G messenger RNA expression. Immunophenotyping by flow cytometry was carried out using specific monoclonal antibodies against CD57 expressed on CD8+ T-cells. The results indicated that 69.6% of the cases showed HLA-G expression compared to only 22.7% of the controls, and the difference was statistically significant at $p<0.01$. Cases have eight times the risk of expression among controls since odd ratio (OR) =7.8. Also, 65.2% of the cases showed CD57$^{+}$ expression compared to 36.4% of the controls, and the difference was marginally significantly different at $p= 0.053$. Cases have nearly three times the risk of expression among controls since OR=3.3. Although the lower limit of the 95% confidence interval is <1, indicating that in the population, this increase in risk may not be present. HPV-specific HLA-G marker test might be an accurate and cost-effective preventive biomarker with potential application for cervical cancer.

**Published by Arab Society for Fungal Conservation**

## Introduction

Cervical cancer is the third most diagnosed cancer globally and the fourth-largest cause of cancer death among women in the world (Bray et al., 2018). Persistent human oncogene Papillomavirus (HPV) infection alone does not induce cervical cancer. Plenty of Known risk factors may take part in tumorigenesis initiation, such as excessive alcohol consumption, prolonged smoking, and HPV invasion (Boda et al. 2018; Ishiji 2000).

Although HPV infection is often ephemeral since the host immune system can regulate and control the virus invasion causing the lesions to regress, 10% of HPV infections may induce high grade squamous intraepithelial lesions (HSIL), and less than 1% of HPV infections induce cervical cancer (Burd, 2003). Speculating that convoluted virus-host interactions ascertain HPV- induced cancer risk and lesions advance to cancer.

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Tumor cells have many strategies to escape monitoring and regulation of the immune system. One of its tactics is that the human leucocyte antigen-G (HLA-G) molecule of non-class I is aberrantly expressed (Lin Aifen and Yan 2015). The T-cells and natural killer cells (NK) effectors inhibit HLA-G, tumor-mediated ectopic expression, which contributes to immunosuppressive T-subset cell expansion. Spliced alternative HLA-G Primary Transcript produces seven distinct isoforms, four membrane-like (HLA-G1, HLA-G2, HLA-G3, and HLA-G4). Three (HLA-G5, HLA-G6, and HLA-G7) are soluble. Most polymorphic sites are located in HLA-G at 5′URR, regulating HLA-G gene expression and 3′UTR, which affects the mRNA's production and stability (Kasakovski et al. 2018; Lin Aifen and Yan 2015; Medeiros et al. 2018).

The association of HLA-G expression with the HPV infection was explored in several research studies. The oropharyngeal cancer associations in a genome-wide association analysis (GWAS) were confined to the human leukocyte antigen (HLA) region. This was more prominent in HPV-positive tumors, implying that HLA expression may be linked to virus-related malignancies (Lesseur et al., 2016). Also, Sarmah et al. (2019) identified HLA-G re-expression as a viral escape mechanism from immune surveillance.

Recently, Syrjänen (2018) reported that HLA-G molecules were involved in estimating the risk of newborn births diagnosed with oral HPV conceiving the role of both HLA-G and HPV, based on its pronounced immunoinhibitory properties, in tumor growth and progression.

This research aimed to investigate HPV-HLA-G interaction with the cervical cancer immune modulation: Hypothesizing the potential for early-cancer detection by HPV-specific HLA markers, which might be accurate and cost-effective preventive biomarkers. As well as to assess the host immune response by immunophenotyping of CD57 natural killer T-cells.

## Materials and Methods

### *Patients and Samples*

The study participants were recruited from the Department of Clinical Pathology at Helwan University Hospitals, Cairo, Egypt. They were Forty-Five patients divided into two groups; twenty-three cases with HPV infection associated with cervical cancer and twenty-two controls with HPV infection without cervical cancer.

All patients were asked to give informed consent according to the Helsinki declaration (serial: 14-2019). None of the patients received any chemotherapy or radiotherapy. According to the FIGO (Federation International of Gynecology and Obstetrics) classification system, the clinical pathological findings for cervical cancer were determined.

### *RNA Isolation and Real-Time Quantitative polymerase chain reaction (RT-PCR)*

Total RNA was extracted using an RNA extraction kit (Promega, Madison, WI) according to the manufacturer's instructions. cDNA was prepared from 1 mg of total RNA, and the synthesized cDNA was then stored at -20°C until further use. Quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) with SYBR Green I (SGI) green was used to quantify HLA-G mRNA expression using the MiniOpticon system (Bio-Rad Laboratories). The following set of specific primers for HLA-G (88 base pair [bp]) was used: Forward primer (F): 5-CTGGAGAACGGGAAGGAGAT-30, reverse primer (R):50-GGGTGGCCTCATAGTCAAAG-3 ( Jung et al., 2009). The polymerase chain reaction was performed in 20 mL, containing 2 mL complementary DNA (cDNA), 15 mmol/L primers, and 10 mL IQ SYBR green supermix (Bio-Rad Laboratories). The melting temperature peak was 84°C for HLA-G. The value of mRNA expression was defined as 2–DDCt, using the comparative cycle threshold (Ct) quantitation method (Ct-Threshold Cycles). We used the JEG3 cell line as a HLA-G positive control (Li et al., 2012).

### *Blood cell preparation*

Fresh peripheral venous blood samples collected in EDTA tubes were incubated, for six h, in lysis buffer (NH4Cl:154.0 mM, EDTA: 0.1 mM, and KHCO3:10.0 mM) to remove red blood cells and centrifuged at 2800 rpm for 4 min to obtain the peripheral blood leukocytes (PBL), which were used for cell surface and intracellular markers analysis. For cell sorting, peripheral blood mononuclear cells (PBMC) were isolated from fresh blood samples by density-dependent centrifugation using lymphoprepTM gradient (Axis-Shield, Oslo, Norway) according to the manufacturer's instruction (Ohkawa et al. 2001).

### *Flow cytometry analysis*

All fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)- and PC5-conjugated monoclonal antibodies (mAbs) were purchased from Immunotech (Marseille, France). The human peripheral blood mononuclear cell (PBMC) separated by Lymphocyte Separation Medium (ICN Biochemicals Inc., Aurora, OH) were stained with PE-anti-ab TCR mAb, FITC-anti-CD57 mAb, and PC5-anti-CD57 mAb. The stained PBMC were analyzed by a flow cytometric analyzer (FACSCalibur, Becton Dickinson, Cockeysville, MD) with Cell Quest software (Becton Dickinson) (Takayama et al. 2003).

### *Statistical Analysis*

Statistical analysis was carried out using the SPSS software program. To compare the means of HLA-G immunoreactive scores, we used the Mann-Whitney test. The association of HLA-G expression with HPV infection and host immune response was calculated using the Pearson Chi-square Test, where P <0.05 was considered statistically significant.

## Results and discussion

The current study targeted 45 HPV- exposed women. Twenty-three with cervical cancer act as a case group, and 22 non-cancer patients act as a control group. The following tables and graphs show the differences between the cases and control. The mean age among cases (39.39 ± 5.37) was slightly higher than that of the patients (38.64 ± 5.97), but the difference is statistically non-significant since p>0.05 (Table 1).

There is no significant difference between cases and controls regarding HIV history since p>0.05. Although the history of HIV appears to be a risk factor since OR>1, but as the lower limit of the 95% confidence interval is below one, this means that in the population, the increase in the risk of cervical cancer with positive HIV history may not be present (Table 2).

Also, there is no significant difference between cases and controls regarding hormonal contraceptives since p>0.05. An odd ratio of 1.1 indicated that hormonal contraceptive use is unrelated to cervical cancer risk. There was a significant difference between cases and controls regarding smoking history since p<0.05. Current smoking is associated with an about 5-fold increase in the risk of cervical cancer (OR=4.87) (Table 2).

Regarding HLA-G expression, 69.6% of the cases showed expression compared to only 22.7% of the controls, and the difference was statistically significant as p<0.01. Cases have eight times the risk of expression among controls since OR=7.8 (Table3, Figure1).

For CD57 expression, 65.2% of the cases showed expression compared to 36.4% of the controls, and the difference showed a marginally significant difference as p= 0.053. Cases have nearly three times the risk of expression among controls since OR=3.3, although the lower limit of the 95% confidence interval is <1. This increase in the risk may not be present (Table 4, Figure 2).

In the control group where women were infected with HPV without cervical cancer incidence, there was an association between HLA-G expression and CD57 expression (p=0.009). On the other hand, in the cases group, no association was found (Table 5).

Table (6) shows a strong association in the cases group, where a strong association was found between CD 57 expression and hormonal contraceptive usage (p=0.010). No other association was found.

Logistic regression was performed to ascertain the effects of HLA-G expression, CD57 expression, and smoking history on the likelihood that participants have cervical cancer. The logistic regression model was statistically significant, $\chi 2(4) = 17.80$, $p < 0.005$. The model explained 41.0% (Nagelkerke R2) of the variance in cervical cancer disease (Table 7) and correctly classified 73.0% of cases (Table 8). The only significant predictor of cancer was HLA-G expression. The odds of having cervical cancer is 0.25 times less with a one-unit decrease in HLA-G expression when CD57 and smoking are kept constant (Table 9). Also, decreasing the CD57 expression and smoking were associated with decreasing the likelihood of exhibiting cervical cancer.

HPV-related CC remains a significant cause of morbidity and death, especially in developing nations. Practical, cheap, and responsive biomarkers are required to diagnose and prevent large-scale CCs and other cancers associated with HPV (Schiffman and Wentzensen, 2013). Based on its pronounced immuno-inhibitory properties, HLA-G has been suggested as a probably pronounced biomarker and a therapeutic target for a wide range of cancers and viral infections underexplored in cervical cancer to date (Gimenes et al.2014).

Given the potential impact of HLA-G on the clinical course of HPV, cervical lesions, and cancer development, a better understanding of HLA-G involvement in cervical cancer leads to two fundamental aspects (Fahim et al. 2018): Characterization of a new biomarker for the diagnosis and monitoring of cervical cancer, necessary for the screening of patients. Identification of lesion and progression mechanisms for HLA-G-driven immune systems will contribute to the development of HLA-G modulation strategies for treatment (Rouas-Freiss et al., 2014).

Therefore, the present study was designed pertinently to investigate HPV-HLA-G interaction in the context of immune modulation in cervical cancer, exploring the possible influence of HLA-G on the clinical course of HPV infection. As well as to assess the host immune response by immunophenotyping of natural killer T-cells.

HLA-G status recognition will contribute to an improved range of cancer patients who may benefit from personalized immune treatment or neoadjuvant full biological therapy. Several research studies investigated HLA-G expression and plasma sHLA-G levels in cancer and their association with clinical parameters. The high frequencies of HLA-G expression in tumor cells have successively been observed in various solid tumor types and hematological malignancies as it was initially

**Table 1** The difference in the mean age between cases and controls

| | Cases (n=23) | Controls (n=22) | t | P -value |
|---|---|---|---|---|
| | Mean ± SD | Mean ± SD | 0.45 | 0.66 |
| **Age/years** | 39.39 ± 5.37 | 38.64 ± 5.97 | | |

**Table 2** Cross-tabulation between the incidence of cervical cancer (CC), HIV history, hormonal contraceptive usage, and smoking

| | Cases | Controls | OR ($CI_{95\%}$) | $X^2$ | P-value |
|---|---|---|---|---|---|
| HIV history | N (%) | N (%) | 2.0 (0.17-23.78) | 0.31 | 0.58 |
| +ve | 2 (8.7) | 1(4.5) | | | |
| -ve | 21 (91.3) | 21 (95.5) | | | |
| | Cases | Controls | OR ($CI_{95\%}$) | $X^2$ | P-value |
| Hormonal contraceptive use | N (%) | N (%) | 1.1 (0.34-3.76) | 0.037 | 0.848 |
| +ve | 9 (39.1) | 8 (36.4) | | | |
| -ve | 14 (60.9) | 14 (63.6) | | | |
| | Cases | Controls | OR ($CI_{95\%}$) | $X^2$ | P-value |
| Smoking | N (%) | N (%) | 4.87 (1.12-21.20) | 4.87 | 0.027 |
| +ve | 10 (43.5) | 3 (13.6) | | | |
| -ve | 13 (56.5) | 19 (86.4) | | | |

*Odd Ratio (OR), Confidence interval (CI)

**Table 3** The difference in human leucocyte antigen (HLA-G) expression between cases and controls

| | Cases | Controls | **OR ($CI_{95\%}$)** | **$X^2$** | **P-value** |
|---|---|---|---|---|---|
| HLA-G expression | N (%) | N (%) | | | |
| +ve | 16 (69.6) | 5 (22.7) | 7.8 (2.04-29.5) | 9.91 | 0.002 |
| -ve | 7 (30.4) | 17 (77.3) | | | |

*Odd Ratio (OR), Confidence interval (CI)

**Table 4** The difference in the CD57 expression between cases and controls

| | Cases | Controls | OR ($CI_{95\%}$) | $X^2$ | P-value |
|---|---|---|---|---|---|
| CD57 expression | N (%) | N (%) | | | |
| +ve | 15 (65.2) | 8 (36.4) | 3.3 (0.97-11.13) | 3.75 | 0.053 |
| -ve | 8 (34.8) | 14 (63.6) | | | |

described in melanoma (Paul et al. 1998), in addition to breast cancer (Gimenes et al. 2014; He et al. 2010; Jeong et al. 2014; Provatopoulou et al. 2012), Lung cancer (Cao et al., 2011; Schütt et al. 2010; Yie et al. 2007) hepatocellular carcinoma (Gorantla & Kirkwood, 2014; A. Lin et al. 2010; Lin Aifen et al. 2011; Park et al. 2012; Rouas-Freiss et al. 2014), colorectal cancer (Guo et al. 2015; Zeestraten et al. 2014; Zhang et al.2018), gastric cancer(Tuncel et al. 2013), esophageal carcinoma (Lin Aifen et al.2011; Zheng et al. 2014) nasopharyngeal carcinoma (Cai et al. 2012), laryngeal lesions (Cai et al. 2012), renal cell carcinoma(Ibrahim et al. 2003; B. L. Li et al. 2009) and thyroid carcinoma (De Figueiredo Feitosa et al. 2014).

Nevertheless, there is still a discrepancy between the increased expression of HLA G and cancer patients' clinical parameters. In contrast, the degree of increased HLA-G expression and other clinical parameters has been significantly affected, including advanced disease, low histologic stage, higher tumor grades, metastasis, limited survival, increased tumor size, recurrence of tumors, or tumor invasion in several tumor types (Yan 2018a).

**Table 5** The association between HLA-G expression and other parameters in the two groups

| | | Control HLA-G expression | | P-value | Cases HLA-G expression | | P-value |
|---|---|---|---|---|---|---|---|
| | | negative | positive | | negative | positive | |
| CD57 Expression | negative | 14 | 1 | 0.009 | 2 | 6 | 0.679 |
| | positive | 3 | 4 | | 5 | 10 | |
| HIV | negative | 16 | 5 | 0.579 | 6 | 15 | 0.529 |
| | positive | 1 | 0 | | 1 | 1 | |
| Hormonal contraceptive | negative | 11 | 3 | 0.848 | 5 | 9 | 0.493 |
| | positive | 6 | 2 | | 2 | 7 | |
| Smoking | negative | 14 | 5 | 0.312 | 6 | 7 | 0.620 |
| | positive | 3 | 0 | | 1 | 9 | |

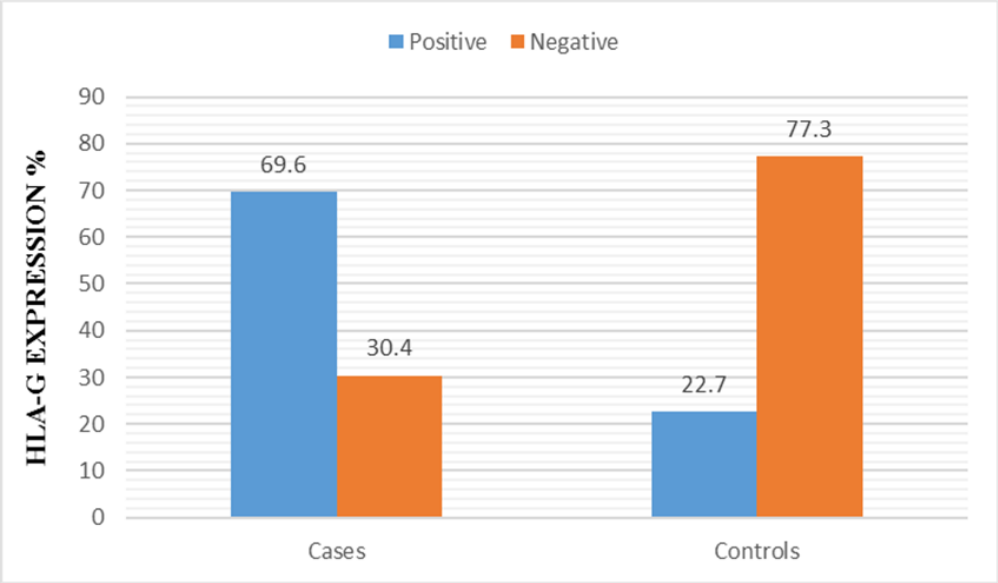


**Fig 1.** The difference in the HLA-G expression between cases and controls.

**Table 6** The association between CD57 expression and other parameters in the two groups

| | | Control CD57 Expression | | P-value | Cases CD57 Expression | | P-value |
|---|---|---|---|---|---|---|---|
| | | negative | positive | | negative | positive | |
| HLA-G expression | negative | 14 | 3 | 0.009 | 2 | 5 | 0.679 |
| | positive | 1 | 4 | | 6 | 10 | |
| HIV | negative | 14 | 7 | 0.484 | 7 | 14 | 0.636 |
| | positive | 1 | 0 | | 1 | 1 | |
| Hormonal contraceptive | negative | 9 | 5 | 0.604 | 2 | 12 | 0.010 |
| | positive | 6 | 2 | | 6 | 3 | |
| Smoking | negative | 12 | 7 | 0.203 | 4 | 9 | 0.645 |
| | positive | 3 | 0 | | 4 | 6 | |

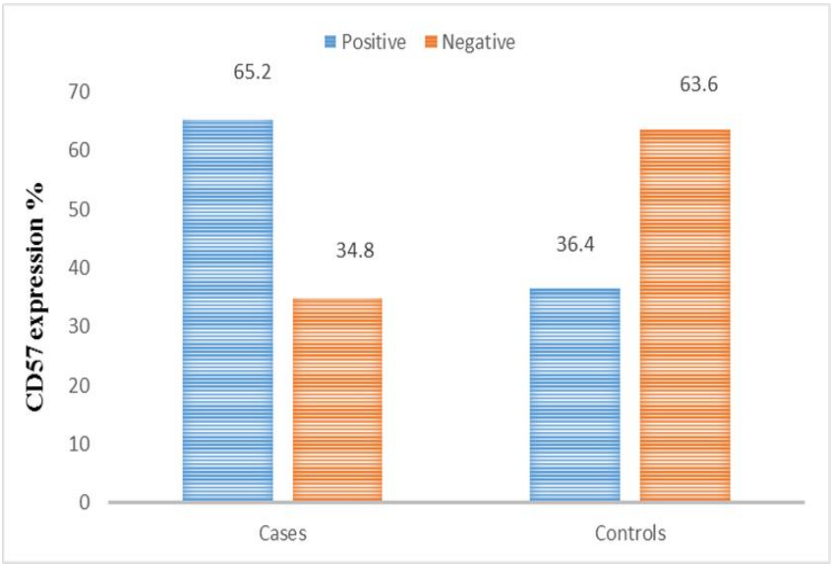


**Fig 2.** The difference in the CD57 expression between cases and controls.

**Table 7** Values of the log likelihood function, Cox and Snell's R-square (R2), Nagekerke's R-square (R2) for the logistic regression

| | -2 Log likelihood | Cox & Snell R Square | Nagelkerke R Square |
|---|---|---|---|
| Step 1 | 45.758[a] | 0.309 | 0.411 |

a. Estimation terminated at iteration number 5 because parameter estimates changed by less than 0.001. Step 1: is the full model specified in the logistic regression.

**Table 8** Classification table to test the accuracy of predicting the cervical cancer incidence in HPV infected patients

| | | | Predicted | | |
|---|---|---|---|---|---|
| | | | Cervical cancer | | Percentage Correct |
| | | | Yes | No | |
| Step 1[a] | Cervical cancer | Yes | 16 | 7 | 69.6 |
| | | No | 5 | 17 | 77.3 |
| | Overall Percentage | | | | 73.3 |

a. The cut value is 0.500

**Table 9** The estimate results for the logistic regression analysis

| | | Estimated regression Coefficient | Standard error around coefficient | Wald $\chi^2$ | df | P-value. | OR (relative risk) | 95% C.I. for OR | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Lower | Upper |
| Step 1[a] | HLA_G (1) | -1.587 | .731 | 4.709 | 1 | .030 | .205 | .049 | .858 |
| | CD57(1) | -1.123 | .754 | 2.219 | 1 | .136 | .325 | .074 | 1.426 |
| | Smoking (1) | -1.514 | .874 | 2.998 | 1 | .083 | .220 | .040 | 1.221 |
| | Constant | 1.657 | .650 | 6.494 | 1 | .011 | 5.241 | | |

a. Variable(s) entered on step 1: HLA-G, CD57 and smoking. Df: degree of freedom, OR: Odd ratio.

Other tumors such as bladder TCC (Gan et al., 2010) and acute myeloid leukemia (Gros et al., 2006) have not been found to be associated with HLA G expression. Although several studies have shown the aggressive behavior in the case of cancer, histologic form, metastases of tumor node or shorter survival time of patients with chest cancer, papillary thyroid carcinoma, and lung carcinoma, among others, to be correlated with high serum sHLA-G levels, no strong association was found between plasma sHLA-G levels and clinic pathologies.

Many factors can, in particular, affect the expression of sHLA-G (Zheng et al. 2014). Upregulated plasma IL.10 was associated with elevated sHLA-G rates in primary esophageal squamous cell carcinoma (Zheng et al. 2014). Besides, sHLA-G levels in acute myeloid leukemia were associated with earlier myelodysplasia and higher leukocytosis (Gros et al. 2006).

There are, among various researches, inconsistencies in the same type of tumor. For example, Kren et al. (2012) reported that HLA-G is upregulated and correlated with worse forecasts in renal cell carcinoma tissues. In contrast, Reimers et al. (2014) have shown a poor prognosis and markedly worse survival of low HLA-G expression.

Interestingly, HLA- G as a potential therapeutic target in cancer, In human hepatocellular carcinoma (HCC) cell lines, a significant increase occurs in natural killer (NK) cell-mediated lysis, which prevents tumor progression if HLA-G expression is reduced by applying vectors containing the small, interfering RNA specifically aimed at HLA-G genes (Zeng et al. 2013).

However, it is noteworthy that various therapies induce negative HLA -G tumors to express HLA-G, thereby preventing cancer. A multicenter work has shown that combined five aza 2' deoxycytidine and interferon (INF) α therapies in vitro (Wastowski et al. 2013) may induce high HLA G expression rates in glioblastoma. The serum level of sHLA-G has significantly increased in melanoma patients under INF α immunotherapy (Ugurel et al. 2001). Also, it has been reported that the radiosensitivity of human tumor cell lines could be modulated with expression of HLA-G1: the expressing cell HLA-G1 was more active in human melanoma M8 and cell lines K562 in human erythrocyte leukemia (Gallegos et al. 2014).

The different ethnic groups of the patient cohorts, varying patient selection parameters, methods of action used, and factitious surgical errors may explain these inconsistencies. A variety of pathways to clarify the immunomodulatory role of HLA-G in cancer have been suggested. The polarization that is non-effective with viral degradation (Amiot et al. 2014) enables viral maintenance and lesion development (Fahim et al. 2018), which may be caused by HLA-G Th2 (IL-4, IL-5 IL-10). Genetic modifications of invasive cervical lesions can modify HLA-G expression in cancer cells in different types of tumors (Lin Aifen and Yan, 2018b).

Other factors include genetic variations; a single HLA allele in the 6p2.3 regions may be attributed to low HLA-G expression, particularly in cervical cancer (Guimarães et al., 2010). The loss of HLA-G expression may be an initial case of cervical cancer. The loss of heterozygosis in the TAP area could trigger failures in transmitting the HLA-expression (Vermeulen et al., 2007).

We believe that HPV infection downregulates major histocompatibility complex molecules of class I, in line with other viral models. Though there is no conclusive proof that HPV affects such cancers, HPV infection is reported to occur in cervical cancer (Gameiro et al., 2017; Scott et al. 2001), and the patients in this sequence have seen HPV DNA. It remains to be established if HPV is impaired by HLA-G expression ( Lin Aifen and Yan, 2015).

However, it has been stated that high-risk HPV oncoproteins may obstruct the HLA I promoter gene heavy chain and regulate latent membrane protein-2 and antigen-processing-1 (TAP-1) transporter protein responsible for the processing and transportation of peptides in the groove (Vambutas et al. 2001).

Furthermore, by maintaining HLA heavy-chain class I in the Golgi complex (Ashrafi et al. 2005), HPV E5 Protein downregulates HLA Class I molecules. The action of HLA-G expression in HPV-associated cervical lesions is also essential (Ashrafi et al., 2005). HLA-G expression was observed in atypical, undetermined glandular cells and was slowly decreased from intraepithelial cervical neoplasia-1 (CIN1) to CIN2-3 and ICC (Dong et al. 2010).

In conclusion, why some cervical cancer patients express HLA-G while others do not is yet determined. Further studies are needed with a large number of participants to recognize HPV influence in invasive cervical malignancies with HLA-G expression. In addition, more research is needed to explain HLA-G and HPV molecular aspects, which may contribute to future applications for cervical cancer diagnosis. Furthermore, we demonstrated that the HLA-G test is an important biomarker diagnostic/prognostic to detect cervical cancer and monitor the disease stage, including evaluating the risk of cervical lesion progression.

**Declaration of competing interest**

The authors declare that they have no competing interests.

**Acknowledgments**

Non to be declared.

**Funding**

No funding was received to conduct this research.

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