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Altered HLA Class I and HLA-G Expression Is Associated with IL-10 Expression in Patients with Cervical Cancer

Josefa Antonia Rodríguez[a] Liliana Galeano[a] Diana María Palacios[b, c]
Constanza Gómez[d] Martha Lucía Serrano[a] María Mercedes Bravo[a]
Alba Lucía Combita[a, c]

[a]Grupo de Investigación en Biología del Cáncer, Instituto Nacional de Cancerología, [b]Departamento de Patología, Fundación Santa Fe de Bogotá, [c]Facultad de Medicina, Universidad Nacional de Colombia, y [d]Grupo de Patología, Instituto Nacional de Cancerología, Bogotá, Colombia

Key Words
Histocompatibility antigen class I · HLA-G · Interleukin-10 · Cervical cancer

Abstract
Although high-risk human papillomaviruses (HPVs) are an important risk factor in the etiopathogenesis of cervical cancer, increasing evidence suggests that the ability to avoid immune surveillance seems to be linked to the transforming potential of HPV and a rapid progression to cancer. In other cancer models, IL-10 contributes to impair anti-tumor immune response either by downregulating human leukocyte antigen Class I (HLA-I) expression or by increasing HLA-G expression. To comprehend how these alterations could contribute to evasion of immune surveillance in cervical cancer, we analyzed HLA-I, HLA-G and IL-10 expressions by immunohistochemistry in 63 biopsies from patients with cervical intraepithelial neoplasia III (CIN-III) and cervical cancer. Immunohistochemistry showed absent or weak HLA-I expression in 50/59 cases. In these cases, a high percentage had loss of heterozygosis. IL-10 and HLA-G expression were observed in 46.6 and 27.6% of cases, respectively. Concurrent upregulation of IL-10 was found in 87.5% of HLA-G positive cases ($p = 0.000$). Similarly, a significant association between IL-10 expression and HLA-I downregulation was found ($p = 0.028$). Finally, we observed higher HLA-G expression in patients with HLA-I downregulation than in those with normal HLA-I expression ($p = 0.004$). Our results suggest that, in cervical cancer, the IL-10 expression may induce an immunosuppressive environment by upregulating HLA-G expression and downregulating HLA class I expression.

Introduction

Although infection with high-risk human papillomavirus (HPV) is an important risk factor in cervical cancer etiopathogenesis, there is increasing evidence supporting that the immune system plays a pivotal role in determining the outcome of HPV infections [1]. Evidence suggests that immunosuppressed individuals are more susceptible to developing persistent HPV infections and cervical intraepithelial HPV-associated neoplastic lesions [2–4]. This suggests that the ability to avoid immune surveil-

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Alba Lucía Cómbita Rojas
Av. 1 No. 9-85
Bogotá (Colombia)
Tel. +57 1 334 0959
E-Mail acombita@cancer.gov.co

lance might be linked to the transforming potential of HPV and to the rapid progression of cervical cancer [1].

There are several mechanisms that could explain the complex cancer-host immune interactions and immune escape of cancer cells into the surrounding environment. One mechanism frequently linked to tumor escape from host immune surveillance is an alteration of human leukocyte antigen (HLA) expression on tumor cells [5, 6]. Thus, a complete loss of human HLA class I molecules results in resistance to cytotoxic T lymphocyte (CTL)-mediated lysis, but it in turn renders tumor cells susceptible to natural killer (NK) cell-mediated killing. Total or partial loss of HLA class I molecules expression has been reported in different human tumors [7–9]. In cervical cancer, loss of HLA class I occurs frequently and is mainly caused by genetic aberrations in the 6p21.3 region [10–13]. It has been reported that at least 50% of multiple HLA allele loss is caused by loss of heterozygosis (LOH) [14, 15].

Among other strategies that have been developed by tumor cells to avoid recognition by different immune effectors, the non-classical HLA-G seems to be one of the most powerful molecules for the suppression of the innate and/or adaptive immune response by multiple pathways of the immune system [16]. In fact, it has been reported that HLA-G inhibits cytotoxicity of CD8+ T cells and also NK cells, as well as T-cell alloproliferation [17, 18]. Additionally, HLA-G affects dendritic cell maturation, migration, trafficking, antigen presentation and their cross-talk with T and NK cells [19]. Finally, it could also modulate and shift the release of cytokine productions from T-helper Th1 to Th2 profile [20, 21]. Numerous studies have indicated that HLA-G expressed in cancer patients contributes to tumor progression and could be a significant prognostic factor [22].

In cervical cancer it is well-known that progression is associated with a shift from Th1 to Th2 cytokine production. This shift toward a Th2 cytokine profile, characterized by IL-10 secretion, is associated with progression of premalignant lesions to cancer [23, 24]. It has been reported that IL-10 can contribute to an impaired anti-tumor immune response either by downregulating HLA class I expression allowing tumor escape from lysis mediated by CTLs [25], or by increasing HLA-G expression allowing HLA class I-deficient cells to escape from NK-mediated lysis through interaction with killer inhibitory receptors on NK cells [26–29]. In lung cancer, IL-10 secretion can contribute to the progression by downregulating HLA class I expression and selective HLA-G induction on tumor cells [30–32]. In cervical cancer, the HLA-G and IL-10 expression has been shown to be higher in tumor cells than in normal cervix, and may be involved in early carcinogenesis. However, there is no clear association between HLA-G and IL-10 expression in cervical cancer [33]. To evaluate the contribution of these mechanisms in cervical cancer, we analyzed the expression of HLA class I, HLA-G and IL-10 at the tumor cell surface in biopsies of patients with CIN III and invasive cervical cancer.

Materials and Methods

Study Population

Sixty-three patients with CIN III and invasive cervical cancer stage IBI–IVB according to the International Federation of Gynecologists and Obstetrics (FIGO), attending the outpatient gynecology clinic at the Instituto Nacional de Cancerología (INC), in Bogotá, Colombia, from October 2004 to October 2005, were enrolled in this study. Patients were not included if they had undergone any treatment before, if they showed prior or concurrent second malignancies or if they had been pregnant. All individuals were subjected to a short interview concerning medical, gynecologic and sexual history. Written informed consent was obtained from all patients according to the guidelines of the institutional review board. This study was reviewed and approved by the INC Medical Ethics Committee.

Tissue and Blood Samples

During the first gynecological examination, cervical scrapes were collected from each patient using a spatula and a brush and placed in tubes containing 5 ml phosphate-buffered saline (1× PBS) and 0.05% thimerosal to determine the presence of HPV DNA. Cervical biopsies were obtained and cryopreserved in Tissue Freezing Medium (Triangle Biomedical Sciences, Durham, N.C., USA) for further LOH analyses and immunostaining procedures. Blood samples were also collected and peripheral blood mononuclear cells (PBMCs) were isolated from fresh heparinized blood by Ficoll-Hypaque density gradient centrifugation (Sigma) and used as normal control for LOH analysis.

Microdissection and DNA Extraction

To minimize the possibility of contaminating the tumor samples with normal cells, the following protocol for tumor microdissection was used. Fixed cryopreserved tissue sections of 4 μm, stained with hematoxylin and eosin, were microdissected by a proficient pathologist according to a procedure based on the previously described by Ramal et al. [34], but with some modifications. An insulin needle was used to microdissect tumor cells (approx. 500 cells) which were then incubated in 100 μl of digestion buffer containing 1× standard Taq reaction buffer, 0.1 mg/ml proteinase K and 1% Tween-20 for 2 h at 56°C. Proteinase K was heat-inactivated by incubating at 95°C for 10 min. After centrifugation for 10 min at 4°C to remove debris, the digestion product was aliquoted and frozen at –20°C until its use. Normal DNA from PBMCs was purified by using a DNA easy blood isolation kit (MO BIO Laboratories, Inc., Carlsbad, Calif., USA) according to the manufacturer's instructions.

Amplification of Microsatellites

As was previously published [35], a panel of 11 short tandem repeat (STR) markers was selected to define HLA and β_2m LOH based upon their degree of polymorphism [36]. Eight STR markers are located in the short arm of chromosome 6. Seven hybridized in the region 6p21.3 where the genes coding for HLA are located (D6S291, D6S1618, D6S1583, D6S273, D6S265, D6S105 and D6S276). One is telomeric to the short arm of chromosome 6 at 6p25 (D6S1617). To define whether or not LOH is caused by total chromosome loss, we included a STR (D6S311) located on the long arm of chromosome 6. Two lasting STRs are located on chromosome 15 (D15S209 and D15S126), and flank the β_2m locus in the 15q21 region. The forward primers were stained with two different fluorochromes: 6-FAM in D6S311, D6S291, D6S265, D6S105, D15S126, and D15S209 markers, and TET in D6S1618, D6S273, D6S1583, D6S1617 and D6S276 markers. They were then used in STR analysis.

LOH Analysis

Polymerase chain reaction (PCR) and capillary electrophoresis for LOH analysis were performed as previously described in the 14th International Histocompatibility Workshop, 2002 [http://www.microbiol.unimelb.edu.au/14ihiws/]. Briefly, the PCR was performed in a final volume of 25 μl of reaction mixture containing 1× Taq polymerase buffer, 1–4 pmol of each primer pair, 2 mM dNTP, 1.5 mM $MgCl_2$, 0.25 U Taq polymerase, and 200 ng of normal DNA obtained from PBMCs (no tumor DNA) or DNA obtained from tumor cells. The thermal cycling profile, using a PTC-100 Cycler (MJ Research, Inc.) was as follows: an initial denaturating step at 96°C for 5 min, 33 cycles of amplification (denaturating at 96°C for 1 min, annealing at 55°C for 2 min, and elongation at 72°C for 1 min), finally, an extension step at 72°C for 6 min. Afterwards, 1 μl of the PCR product was added to a mix that contained 12 μl of formamide and 0.5 μl of internal molecular weight standard TAMRA 500. The samples were then homogenized and heat-denatured at 95°C for 3 min. Capillary electrophoresis was performed using the polymer POP4 for 30 min at 60°C using an ABI PRISM® 310 DNA sequencer (Applied Biosystems, Foster City, Calif., USA).

The analysis was performed using GeneScan® analysis software (Applied Biosystems), and the alleles were assigned with Genotyper software (Applied Biosystems). STR markers were informative when the PCR product obtained from the normal DNA of PBMCs showed two different alleles corresponding to a heterozygous individual. LOH was assigned when a signal reduction of more than 25% in one allele was observed in the tumor sample compared to the same STR marker in the control sample [34]. Haplotype loss was assigned when a tumor sample showed LOH in three or more STR markers [34, 37].

LOH was calculated as:

$$\frac{\text{height of tumor allele 2/height of tumor allele 1}}{\text{height of normal allele 2/height of normal allele 1}}$$

where allele 2 is the long allele and allele 1 is the short one.

Antibodies and Reagents

Immunohistochemistry study was performed using the following monoclonal antibodies (mAbs): W6/32 (1:100 dilution in PBS 1×) which recognizes a determinant expressed on the heavy chain of β_2m-associated HLA-A, -B, and -C (BD Pharmingen, San Diego, Calif., USA), E10 (1:100 dilution in PBS 1×), IgG2a, anti-human IL-10 mAb (Santa Cruz Biotechnology, Santa Cruz, Calif., USA), and 4H84 (1:500 dilution in PBS 1×), IgG1, anti-denatured HLA-G α1 domain of the heavy chain (BD Pharmingen). Immunocomplex detection was performed using the DakoCytomation LSAB2 System HRP kit (Dako, Carpinteria, Calif., USA).

Immunohistochemistry

Immunohistochemistry analysis was performed on 63 frozen cervical biopsies. Briefly, 4 μm-thick cryostat sections were cut and placed on glass slides. After 15 min of air-drying, the sections were fixed for 10 min in ice-cold acetone and stored at –20°C until its use. Non-specific binding sites were blocked by incubating slides with 20% AB serum/PBS for at least 15 min at room temperature. Endogenous peroxidase activity was blocked by incubation with 3% hydrogen peroxide for 15 min. The slides were then incubated for 30 min at room temperature with the corresponding dilution of anti-HLA class I mAb (W6/32), anti-IL-10 mAb (E10) or anti-HLA-G mAb (4H84). Incubation with PBS was used as a negative control. After three washes with 1× PBS, biotinylated goat anti-mouse immunoglobulins (Dako) were applied and incubated at room temperature for 30 min. The slides were then incubated with an avidin-biotin-peroxidase conjugate for 30 min. The immunocomplexes were developed with a fresh 3,3′-diaminobenzidine tetrahydrochloride solution. The slides were counterstained with hematoxylin, dehydrated in alcohol, and cleared in xylene before mounting.

The staining intensity of HLA class I expression in the tumor cells was scored as described by Feenstra et al. [36]. At normal HLA class I expression, staining of the tumor cells showed the same intensity as the surrounding stromal cells, at weak HLA class I expression, staining of the tumor cells was significantly less compared to staining of the surrounding stromal cells, and when negative, no staining of the tumor cells was observed. Necrotic or keratinized tumor cells were not taken into consideration. To analyze HLA-G expression, sections of cytotrophoblast from human placenta served as an HLA-G-positive control. HLA-G expression was graded as follows: negative, 1 for focal expression or weak expression, 2 for >75% of expression.

Statistical Interpretation

Statistical analysis was performed using SPSS 18.0 software. The percentage of heterozygosity for individual STR markers was calculated as the ratio of normal peripheral blood samples that were heterozygous to the total population analyzed. The percentage of LOH was calculated by applying the formula described above only for those individuals whose peripheral blood was heterozygous. χ^2 test or, where appropriate, Fisher's exact test was used to assess differences in LOH frequency and correlations between HLA class I, HLA-G and IL-10 expression. The survival rates were calculated by the Kaplan-Meier method, and the differences between the survival curves were determined by the log-rank test. Overall survival was defined as the interval from the beginning of treatment to death or last visit date. For all tests, p values <0.05 were considered statistically significant.

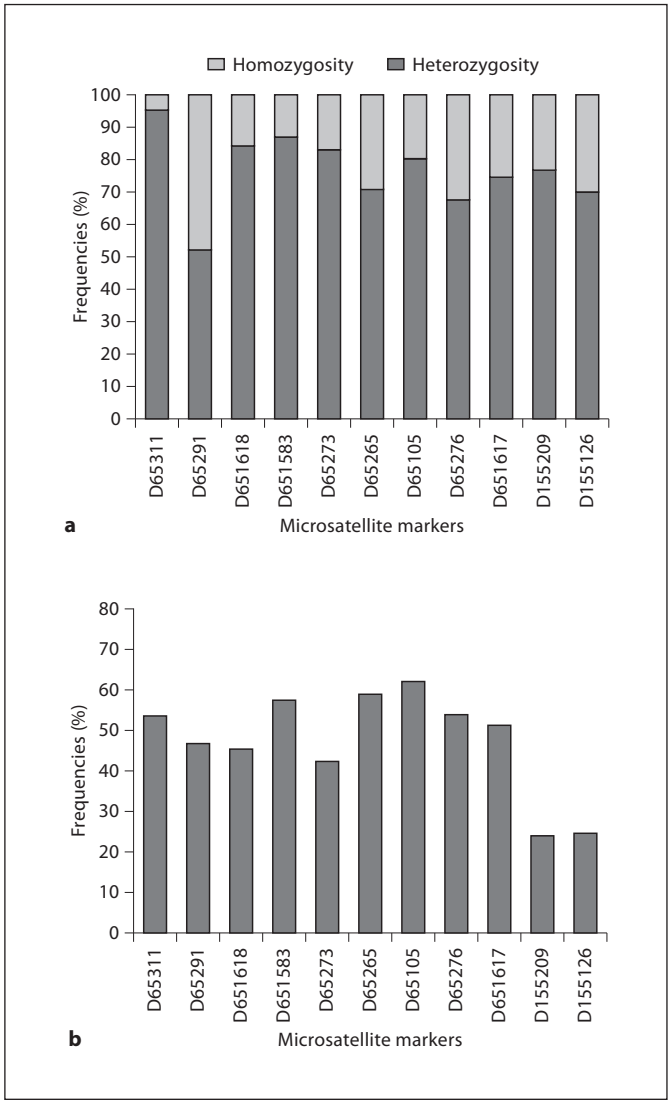

Fig. 1. Percentage of heterozygosity (**a**) and LOH frequencies (**b**) of eleven microsatellite markers in 63 microdissected tumor cells and normal cells from cervical cancer patients.

Table 1. Clinicopathological characteristics of cervical cancer patients

Age, years		43.08 (25–63)
Tumor size, cm	Before treatment	6.09 (1–12)
	After treatment	1.47 (0–6)
FIGO stage	CIN III	9
	IBI–II	10
	IIA–B	20
	IIIB	21
	IVB	3
HPV DNA	Simple infection	
	HPV 16	57/63 (90.4%)
	HPV 33	3/63 (4.7%)
	HPV 52	1/63 (1.5%)
	Multiple Infection	
	HPV 18 and 53 or	
	HPV 16 and 18	2/63 (3.0%)

Results

Clinical Characteristics of Patients

The clinical characteristics of the study subjects are listed in table 1. A total of 63 female patients with a diagnosis of CIN III and invasive cervical cancer stage IBI–IVB were enrolled in this study. The age range was between 25 and 63 years and the mean age of the patients was 43.08 years (±10.81 SD). Tumor sizes before treatment ranged from 1.0 to 12 cm. 13 patients (20.6%) underwent surgical treatment, 5 patients (7.9%) were treated with surgery and radiotherapy, 26 (41.3%) received combined chemoradiotherapy, and 12 patients (19%) received only radiotherapy. 7 (11.1%) patients did not accept any therapy. The mean follow-up time was 38.93 months, with a time range from 5 to 74 months. All samples were positive for HPV DNA. The most prevalent HPV type was 16. Other, less frequent HPV types were 18, 53, 33, 52, and 58. Only 3% (2/63) had a multiple infection (table 1).

Analysis of LOH in 6p21.3 and 15q21 and HLA Class I Expression

LOH was analyzed in 63 cases. Four different patterns were found with STR analysis: partial LOH, total LOH, retention of heterozygosis, and homozygosis. Partial LOH was defined when one STR marker in the tumor sample had a signal intensity ≤25% of that observed in the same STR marker in the control sample. Total LOH was defined as the presence of only one STR allele in the tumor sample. Retention of heterozygosis was defined when the two alleles of STR amplified in the microdissected tumor cells showed a similar profile to that of the normal peripheral blood sample. Finally, homozygosis was defined as the presence of only one allele signal in the control samples. In this case, the marker was not informative. Homozygosis was observed in 19.6% of cases, while the percentage of heterozygosis varied from 51.8 to 86.4% depending on the marker (fig. 1a). LOH frequencies in the HLA region varied from 24.1 to 62% depending on the STR analyzed (fig. 1b). The total LOH in the 6p21.3 region was 81% (51 out of 63 cases), while in the 15q region, 31.3% (15 out of

48 cases) were found to have LOH. When we analyzed the LOH according to cervical cancer stage (fig. 2a), a high frequency of LOH was observed in more advanced cervical cancer, but only the D6S105 marker showed a significant variation between different stages (Pearson's χ^2, p = 0.044). Haplotype loss was defined in 6p21.3 when more than three consecutive microsatellite markers were lost. Haplotype loss was found in 62.7% (32 out of 51 cases). It was higher in patients with advanced cervical cancer than in those with early stages (fig. 2b).

The HLA class I expression was analyzed in 59 cases. Of these cases, 15.2% (9/59) showed normal HLA class I expression and were used as an internal control of HLA class I expression. Of the 50 remaining cases, 22% (11/50) showed a weak expression and 78% (39/50) displayed a total loss of HLA class I expression (fig. 3a–d). Although an association between decrease of HLA class I expression and LOH in the 6p21.3 and 15q regions has been observed in other cancer models, in this study no significant difference in the frequency of LOH was observed among patients who had an alteration in HLA class I expression. Table 2 shows the analysis of LOH in 6p21.3 and 15q21 regions in cervical cancer patients with normal HLA class I expression. In the 6p21.3 region, 33.3% (3/9) did not show LOH, 11.1% (1/9) presented LOH and 55.6% (5/9) had haplotype loss. Among the patients who had altered HLA class I expression (table 3), 18.0% (9/50) did not show LOH, 32.0% (16/50) presented LOH and 50% (25/50) had haplotype loss (Pearson's χ^2, p = 0.353). When the expression of HLA class I was analyzed in relation to the 15q LOH, in patients who had a normal expression of HLA class I, 44.4% (4/9) did not have LOH and 55.6% (5/9) showed LOH. Among patients who had altered HLA class I expression, 74.3% (26/35) did not have LOH and 25.7% (9/35) showed LOH (Fisher's exact test, p = 0.097).

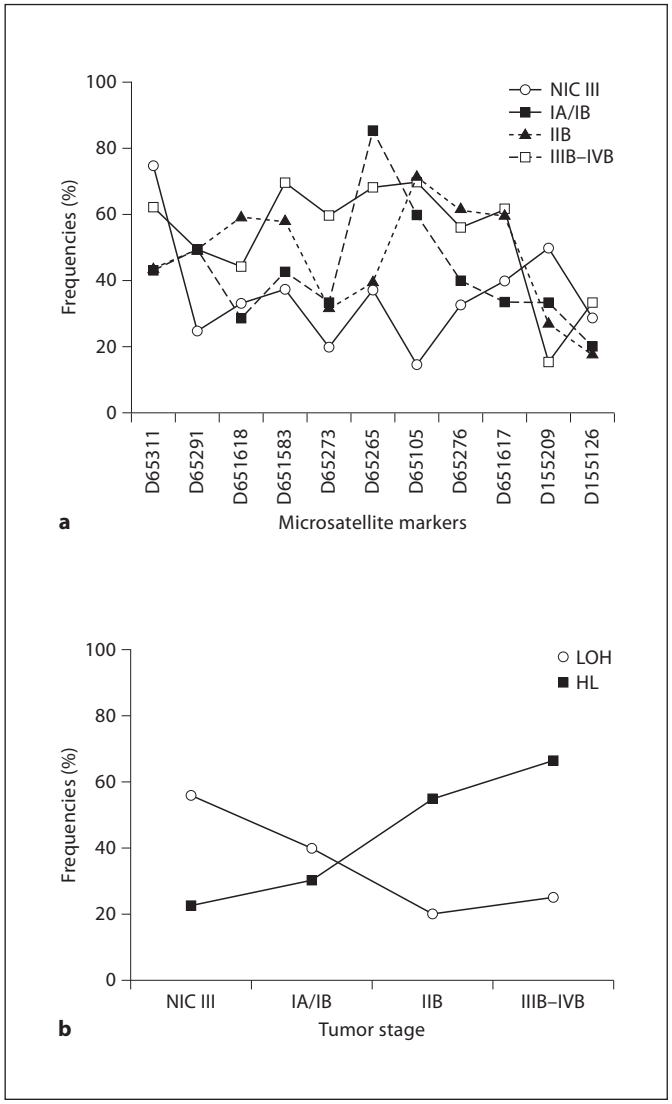

Fig. 2. LOH frequencies according to tumor stage of cervical cancer patients (**a**) and LOH and LH frequencies (**b**) of eleven microsatellite markers in 63 microdissected samples.

Association of IL-10 Expression and Downregulation of HLA Class I Expression

It has been suggested that IL-10 may negatively regulate HLA class I expression at the tumor cell surface, thus allowing the escape from tumor cell lysis mediated by CTLs. In this study, a significant association was found between IL-10 expression and decrease of HLA class I expression (Pearson's χ^2, p = 0.028). Among the patients who expressed IL-10 cytokine, 48.1% (13/27) had a total loss of HLA class I expression, 25.9% (7/27) had weak, and 25.9% (7/27) had normal HLA class I expression (table 4). However, among the patients who did not have IL-10 expression, a high percentage (80.6%, 25/31) was also HLA class I negative.

IL-10 Expression Is Associated with HLA-G Expression in Cervical Cancer Samples

Overall, IL-10 was expressed in 46.6% (27/58) of cases, while HLA-G protein expression was observed in 27.6% (16/58) of cases. A significant association was found between IL-10 and HLA-G expression (Fisher's exact test, p = 0.000). In most of the HLA-G-positive cases, 14/16 (87.5%) exhibited IL-10 expression. Similarly, a high percentage (69%, 29/42) of the HLA-G-negative cases did not express IL-10 (table 4). It has been reported that HLA-G expression status is associated with tumor histologic

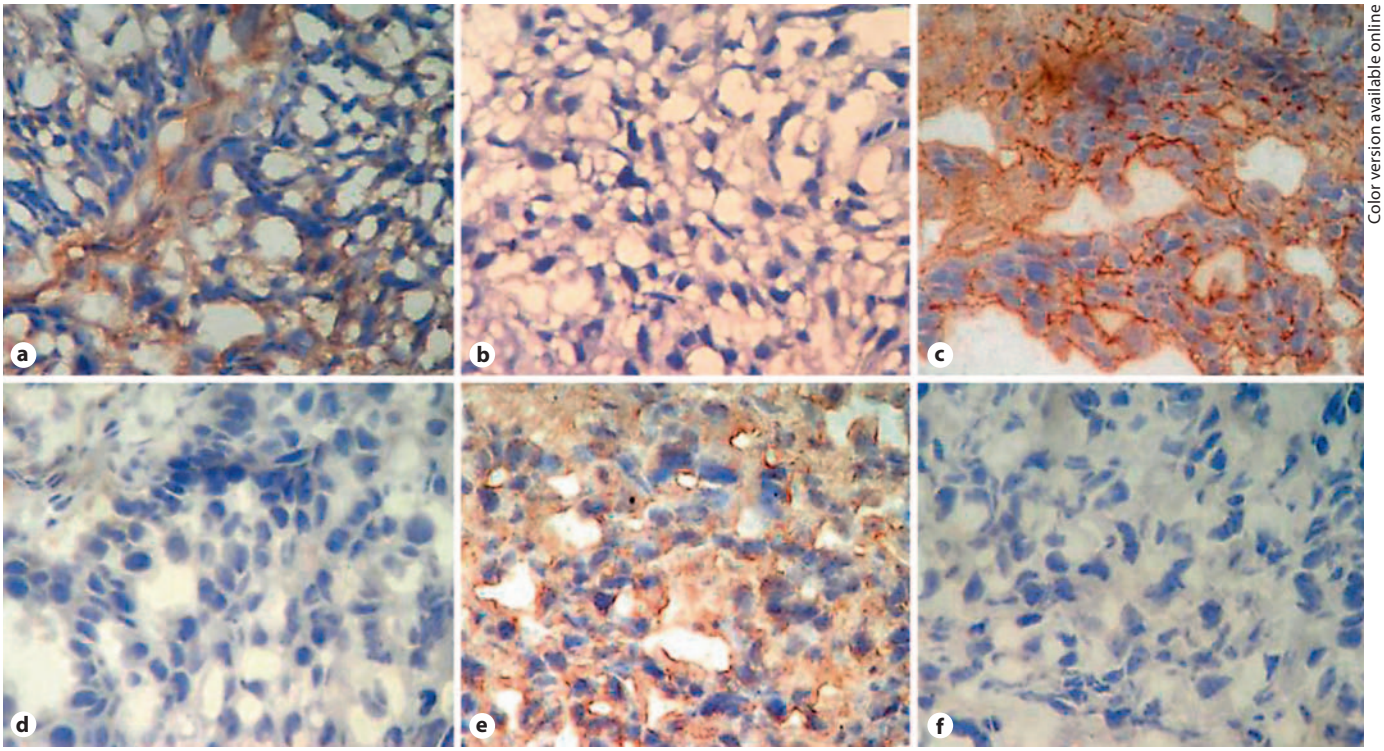

Fig. 3. Immunohistochemistry in cervical biopsies. HLA class I expression (mAb W6/32) from representative positive cervical cancer sample (**a**) and negative control (**b**). HLA-G expression (mAb 4H84) from representative positive cervical cancer sample (**c**) and negative control (**d**). Finally, IL-10 expression (mAb E10) from representative positive cervical cancer sample (**c**) and negative control (**d**).

Table 2. Analysis of LOH in 6p21.3 and 15q21 in cervical cancer patients with normal HLA class I expression

Tumor	IHC	6q	6p25	LOH 6p21								LOH 15q		
	HLA-I expression	D6S311	D6S1617	D6S291	D6S1618	D6S1583	D6S273	D6S265	D6S105	D6S276	Total OH	D15S209	D15S126	Total LOH
17	+	ND	R	R	H	R	ND	R	H	R	R	ND	R	R
21	+	R	H	R	H	R	R	R	R	R	R	H	R	R
25	+	ND	R	H	R	R	R	H	R	ND	R	ND	R	R
63	+	ND	R	R	R	L	R	R	R	R	L	ND	L	L
31	+	L	ND	ND	L	L	R	L	L	L	HL	L	ND	L
35	+	L	ND	L	R	L	L	H	L	L	HL	L	H	L
53	+	R	L	H	L	L	R	H	L	ND	HL	R	R	R
37	+	L	L	H	L	ND	L	H	L	H	HL	L	R	L
41	+	L	ND	H	L	L	L	L	L	L	HL	H	L	L

Immunohistochemical staining (IHC) of HLA class I (W6/32) and LOH on chromosomes 6p and 15q of 9 invasive cervical cancers. R = Retention (no LOH); L = LOH; HL= haplotype loss; H = homozygous (not informative); ND = not determined.

Table 3. Analysis of LOH in 6p21.3 and 15q21 in cervical cancer patients with altered HLA class I expression

Tumor	IHC	6q	6p25	LOH 6p								LOH 15q		
	HLA-I	D6S311	D6S1617	D6S291	D6S1618	D6S1583	D6S273	D6S265	D6S105	D6S276	Total LOH	D15S209	D15S126	Total LOH
54	–	R	ND	H	R	R	R	H	R	H	R	ND	R	R
23	–	R	H	R	R	R	R	H	H	R	R	R	R	R
13	–	ND	R	H	R	R	R	H	H	R	R	ND	R	R
22	–	ND	L	R	R	R	R	R	H	H	R	ND	R	R
8	–	H	R	H	H	H	R	R	R	R	R	ND	R	R
38	–	ND	H	R	R	R	R	R	R	R	R	ND	ND	ND
7	–	ND	ND	H	R	R	R	R	H	ND	R	ND	H	ND
11	–	ND	R	R	R	R	H	H	R	R	R	L	ND	L
26	–	R	R	R	R	R	R	R	R	R	R	H	L	L
3	–	ND	R	R	R	R	ND	L	R	L	L	ND	ND	ND
4	–	L	R	R	R	L	ND	R	R	ND	L	ND	L	L
61	–	L	R	R	R	H	R	R	L	H	L	ND	R	R
30	–	ND	ND	R	ND	R	R	R	L	H	L	R	H	R
34	–	ND	ND	H	ND	L	R	H	L	R	L	H	ND	ND
55	–	R	R	H	L	H	R	R	H	R	L	R	H	R
15	–	R	R	H	R	L	R	L	R	ND	L	R	R	R
33	–	R	R	H	R	L	R	R	R	R	L	R	R	R
40	–	R	R	R	R	R	H	L	R	R	L	L	ND	L
32	W	R	ND	ND	L	R	H	R	R	H	L	R	ND	R
5	W	L	R	H	L	H	H	H	H	L	L	H	H	H
6	W	ND	ND	H	L	R	H	H	R	ND	L	ND	R	R
59	W	L	ND	H	R	R	R	H	L	R	L	R	H	R
46	W	R	R	H	R	R	R	R	L	R	L	ND	R	R
12	W	R	L	H	R	R	R	L	H	H	L	R	L	L
49	W	R	L	ND	ND	H	L	L	H	H	L	ND	ND	ND
47	W	R	H	L	L	H	L	L	L	L	HL	H	H	H
44	W	R	L	H	L	L	L	L	L	L	HL	ND	H	ND
28	W	L	L	R	L	L	L	L	L	L	HL	ND	ND	ND
45	W	ND	ND	H	L	R	L	L	R	H	HL	H	H	H
2	–	L	L	H	ND	L	L	L	H	H	HL	ND	L	L
10	–	R	R	L	L	H	R	L	L	L	HL	ND	R	R
18	–	R	H	H	L	L	R	L	L	H	HL	ND	R	R
20	–	L	H	L	L	L	H	L	L	L	HL	R	H	H
24	–	R	R	L	L	L	L	H	L	L	HL	H	R	R
27	–	L	ND	L	H	L	H	H	L	L	HL	H	L	L
29	–	L	L	L	L	H	L	H	L	L	HL	R	H	H
36	–	R	L	L	H	ND	H	H	L	L	HL	R	ND	R
42	–	H	H	L	ND	L	L	H	L	ND	HL	ND	ND	ND
43	–	L	L	H	R	L	L	L	L	H	HL	R	L	L
48	–	L	L	H	L	L	L	L	L	L	HL	L	ND	L
56	–	L	H	L	H	H	L	L	L	L	HL	R	H	R
58	–	ND	L	L	H	L	H	L	L	L	HL	R	ND	R
60	–	H	L	R	R	L	L	H	H	L	HL	R	ND	R
62	–	L	L	H	H	L	L	L	L	H	HL	R	R	R
16	–	L	H	L	ND	L	ND	L	L	H	HL	ND	ND	ND
39	–	L	H	H	L	L	R	L	L	L	HL	R	H	R
50	–	ND	L	L	R	L	ND	H	L	ND	HL	ND	R	R
52	–	L	ND	L	ND	L	L	R	ND	H	HL	R	ND	R
14	–	L	L	H	R	L	L	L	L	ND	HL	ND	H	ND
51	–	L	L	R	L	L	H	L	R	L	HL	R	R	R
1	ND	R	L	H	L	R	R	L	L	R	HL	R	R	R
57	ND	ND	H	H	ND	L	L	L	ND	R	HL	R	ND	R
9	ND	ND	H	L	R	R	R	R	R	H	L	ND	R	R
19	ND	L	H	R	ND	L	H	R	R	H	L	L	R	L

Immunohistochemical staining (IHC) of HLA class I (W6/32) and LOH on chromosomes 6p and 15q of 54 invasive cervical cancers. R= Retention (no LOH); L= LOH; HL= haplotype loss; H= homozygous (not informative); ND= not determined.

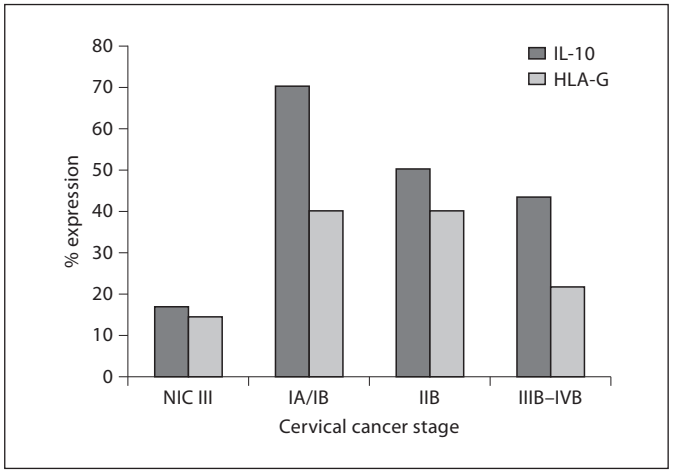

Fig. 4. HLA-G and IL-10 expression according to tumor stage of cervical cancer patients.

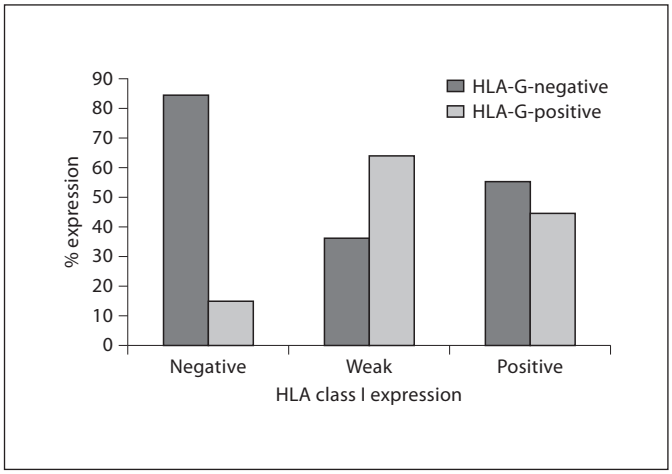

Fig. 5. Association of HLA-G expression and downregulation of HLA class I expression.

Table 4. Association of IL-10 expression with HLA class I expression and HLA-G expression

	IL-10 expression		
	negative, n	positive, n	p
HLA class I expression			
Negative (n = 38)	25 (80.6%)	13 (48.1%)	0.028*
Weak expression (n = 11)	4 (12.9%)	7 (25.9%)	
Positive (n = 9)	2 (6.5%)	7 (25.9%)	
Total (n = 58)	31 (100%)	27 (100)	
HLA-G expression			
Negative (n = 42)	29 (69.0%)	13 (31.0%)	0.000**
Positive (n = 16)	2 (12.5%)	14 (87.5%)	

$p < 0.05$ indicates significant differences. * Pearson's χ^2 test. ** Fisher's exact test.

grade and tumor stage [38]. Figure 4 shows HLA-G and IL-10 expression at different stages of cervical cancer. Although HLA-G expression was observed even in the early stage of cervical cancer, it did not show a significant association with the tumor stages.

Analysis of HLA Class I, HLA-G and IL-10 on Survival

We analyzed the role of IL-10, HLA-G and HLA class I expression on overall survival. Although the patients that showed HLA-G and IL-10 expression had a shorter survival rate compared to those that did not have expression, this difference was not significant ($p > 0.5$) (data not shown). Similarly, a non-significant association was observed between total loss or HLA class I downregulation and decreased overall survival.

Association of HLA-G Expression and Downregulation of HLA Class I Expression

When the association between HLA-G and HLA class I expression was analyzed, we observed that among the patients who had a total loss of HLA class I expression, 15.4% (6/39) expressed HLA-G. Among patients with HLA class I weak expression, 63.6% (7/11) expressed HLA-G. The expression of HLA-G in HLA class I-positive cases was 44.4% (4/9; Pearson's χ^2, $p = 0.004$; fig. 5).

Discussion

Lymphoproliferative responses to specific HPV 16 E6 and E7 peptides appear to be associated with the clearance of HPV infection and with the regression of cervical lesions [39]. However, an increase of HPV-16 E7-specific T-helper type 1 response in peripheral blood of cervical cancer patients after radiotherapy is not enough for controlling tumor cell regression [40]. This observation underlines the importance of several mechanisms that could influence the anti-tumoral immune response. A shift from a Th1 to a Th2 cytokine profile with subse-

quent IL-10 expression has been associated with impairment of tumor immune surveillance and progression of cervical cancer [23, 24]. Furthermore, other cancer models indicate that IL-10 expression may contribute to impaired anti-tumor response through downregulating HLA class I and the selective HLA-G expression allowing tumor progression [30–32, 41]. In this study, we analyzed the relationship between LOH in the 6p21 and 15q21 regions and HLA class I, HLA-G and IL-10 expression in biopsies of cervical cancer patients in order to comprehend how these alterations could contribute to evasion of immune surveillance control in cervical cancer.

Downregulation of HLA class I expression has been reported in many tumors of different origin and represents an important mechanism for tumors to escape from immune surveillance [8, 9]. It eventually leads to accumulation of new tumor variants with low immunogenicity and high capability for metastatic progression [7, 42, 43]. In addition, it has been reported that loss of a 6p haplotype reduces the cell's heterozygous advantage to present a wide variety of tumor antigens in the context of HLA, for recognition by CTLs [44]. In cervical cancer, LOH very frequently occurs in the 6p21.3 region and represents an important common mechanism by which HLA genes and their products are abolished [45]. In this study, we first analyzed LOH in the 6p21.3 region in 63 cancer patients. As we had observed previously [35], the frequency of this HLA alteration varied from 24.1 to 62% depending on the STR marker. Patients with advanced stages of cervical cancer had greater frequencies of LOH than patients with CIN III. Nevertheless, the only significant difference was observed when we compared the CIN III group with invasive stage IV (data not shown). These results are in accordance with previous studies and extend the data reported by Vermeulen and coworkers [13, 45] and Koopman et al. [14], who suggested that LOH in the 6p21.3 region occurs early and is frequently observed in cervical carcinogenesis. Moreover, a higher haplotype loss was observed in advanced stages of cervical cancer, supporting the concept that the 6p21.3 region, which carries HLA genes, is unstable during malignant transformation of cells, contributing to the mechanism of tumor escape and metastatic progression [46].

It has been observed that LOH in the 6p21.3 region is a frequent mechanism that leads to HLA class I abnormalities in other cancer models [47]. In this study, no correlation between LOH in the 6p21.3 region and HLA class I downregulation was found. Although a previous study reported that LOH in the 6p21.3 region is a frequent event occurring in tumors with downregulation of HLA class I expression compared to those that have a normal HLA class I expression [36], in our study a high percentage of tumors with normal HLA class I expression showed LOH and haplotype loss. It can be explained by the fact that W6/32 mAb used in this study recognizes surface-expressed HLA class I molecules associated with β_2m, but not allele-specific molecules of HLA class I, and there are still a number of important HLA allele specificities that cannot be defined in tissue because of the lack of an appropriate anti-HLA mAb [34, 37]. On the other hand, some cases (5) with total loss of HLA class I expression did not have LOH. These results could also suggest that decrease of HLA class I expression may be due to mechanisms other than LOH in the 6p21.3 region.

In cervical cancer, it has been observed that the expression of anti-inflammatory cytokines such as IL-4, IL-10 and TGF-β correlates with disease severity [48]. Moreover, the expression of anti-inflammatory cytokines in a cervical tumor microenvironment can lead to local immunosuppression, which is associated with deregulation of several molecules of the immune response [49] including downregulation of MHC class I expression [50–52]. In this study, loss or downregulation of HLA class I expression among patients who expressed IL-10 was found, supporting the concept that the alteration of HLA class I expression could be caused by the presence of IL-10 in the tumor environment [30]. Nevertheless, we found some cases without IL-10 expression that showed a downregulation of MHC class I expression. It has been reported that E6 and E7 oncoproteins of high-risk HPVs can disturb cell-cycle control and downregulate surface HLA class I antigens by decreasing components of the antigen presentation pathway [53]. In this study, most of the patients were HPV 16 infected, and it is possible that this event could contribute to the HLA downregulation in the tumor milieu.

HLA-G is a non-classical MHC molecule detected on extravillous cytotrophoblasts which plays an important role in immune tolerance by inhibiting the cytotoxic functions of T cells and NK cells [54], and in tumor cells its expression contributes to evasion of immune surveillance [27, 28, 32, 55]. In the present study, cervical lesions of different stages were evaluated for HLA-G expression. Contrary to what was reported by Zhou et al. [56], who observed a strong and uniform HLA-G expression in normal epithelium, while only a small proportion of CINs and squamous cell carcinoma samples showed reduced expression of HLA-G, our results show upregulation of HLA-G from the early stages of cervical cancer

which increased in a malignant lesion; however, a diminution was observed in the more advanced stage. These results correlate with the report by Yoon et al. [33], who found high HLA-G mRNA expression associated with early-stage cervical cancer and support a probable role for HLA-G in early carcinogenesis. In another study, Goncalves et al. [57] reported similar results: HLA-G5 isoform molecules were detected by immunohistochemistry in 25 cases of 74 cervical cancer biopsies (31.6%), 17 (32.7%) without metastasis and 8 (29.6%) with metastasis. Moreover, a low expression of the HLA-G5 isoform was observed in the majority of HPV-related cases [58]. In this report the authors suggest that HPV may be involved in the modulation of HLA-G surface expression by using similar mechanisms to those observed in HLA class I downregulation by HPV oncoproteins; however, it is well-known that the HLA-G-immunosuppressive effect is mediated by upregulation of this molecule.

On the other hand, in another report, Dong et al. [59] found that in cervical cancer lesions, HLA-G is associated not only with disease progression but also with HPV infection. They observed a significantly higher HLA-G expression in CIN lesions infected with HPV 16/18 and cervical cancer than in HPV-negative patients. In our study, most of the patients were HPV 16 infected, and it is possible that this event could contribute to the HLA-G expression; however, the molecular mechanisms of HPV involved in the modulation of HLA-G expression are unknown. In addition, the mechanisms underlying the differential expression of HLA-G within a tumor type and among different tumors remain to be determined, but it may be influenced by tumor microenvironment as well as by the pathogenesis underlying the malignant transformation of the cells such as surrounding cytokine profile, epigenetic modifications and stress such as hypoxia [22, 60].

In human cancer, IL-10 expression appears to be one of the factors responsible for the upregulation of HLA-G [32, 41]. In a previous report, Yoon et al. [33] reported that expression of both HLA-G and IL-10 in cervical cancer might play an important role in cervical cancer progression. However, in their study there was no significant correlation between HLA-G and IL-10 expression both at the levels of mRNA and protein. In this study, we found that IL-10 was expressed in 46.6% of cases while HLA-G was expressed in 27.6% of cases. Moreover, we also observed that most of the HLA-G-positive cases (14/16, 87.5%) exhibited upregulation of the IL-10 cytokine ($p = 0.000$). These results are in agreement with those reported by Urosevic and Dummer [61] in lung cancer where 77% of HLA-G-positive cases overexpressed IL-10 cytokine. Given the fact that the tumors themselves as well as tumor-infiltrating cells often produce IL-10 [62], and that IL-10-producing cells often localize in the vicinity of HLA-G-expressing cells, it is conceivable that IL-10 might be one of the factors responsible for HLA-G upregulation in cancer [61]. Thus, our results extend the previous findings reported by Urosevic et al. [32] suggesting that IL-10 might be one of the factors responsible for the upregulation of HLA-G and suggest, like other cancer models, that HLA-G could be another mechanism involved in immune escape in cervical cancer.

Finally, HLA-G expression has been proposed as a highly specific marker for malignant transformation [63] and can be useful in predicting the clinical outcome in some cancer patients [64–66]. However, in this study there were no significant differences in overall survival rate between patients with or without HLA-G expression. Moreover, HLA-G expression was not directly associated with the tumor stage. In accordance with other cancer models [67], it is possible that in cervical cancer, as suggested by Yoon et al. [33], HLA-G may be involved earlier in the course of malignant transformation, decreasing in the latter invasion stages, but may not be a prognostic factor [33]. Although our study is limited due to the small number of patients, further studies are necessary to resolve the clinical implications of HLA-G expression in cervical cancer.

Overall, our results suggest that in cervical cancer, the tumor microenvironment characterized by IL-10 secretion may induce an immunosuppressive environment by upregulating HLA-G expression and downregulating HLA class I expression, which can conduce to a low susceptibility to specific CTL-mediated lysis, and decreased NK susceptibility. These results could have important implications for identification of patients who could benefit from new therapeutic strategies, particularly in those selected for T-cell-based immunotherapies.

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