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Breast and Ovarian Carcinoma Overexpress HLA-G, a Neglected Cancer Immunosuppressive Protein

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Abstract

Purpose: HLA-G binds to the inhibitory receptors of uterine NK cells and plays an important role in protection of fetal cells from maternal NK lysis. HLA-G also mediates tumor escape, but the immunosuppressive role is often neglected. These studies have focused on the examination of HLA-G expression in human breast and ovarian carcinoma and HLA-G immunosuppressive role in NK cytolysis. **Methods:** We examined HLA-G expression in breast and ovarian carcinoma cell lines by real time PCR, ELISA and immunofluorescent staining, and in frozen breast and ovarian carcinoma tissues by immunohistochemistry (IHC). We treated the breast cancer cell lines with anti-human HLA-G antibody or progesterone. Then, NK cytolysis was measured by using MTT assay. **Results:** We find breast and ovarian cancer cell lines increase the expression of HLA-G mRNA and protein, compared to normal cells. IHC shows that 100% of frozen breast and ovarian carcinoma tissues overexpress HLA-G protein. HLA-G IHC scores of breast and ovarian carcinoma are significantly higher than normal breast and ovarian tissues, respectively (both $p < 0.01$). Blocking HLA-G of the breast cancer cells by the antibody increases NK cytolysis. Progesterone upregulates HLA-G mRNA and protein of human breast cancer cell lines. The increased HLA-G expression by progesterone suppresses the NK cytolysis. **Conclusion:** Human breast and ovarian carcinoma overexpress HLA-G immunosuppressive molecules. Blocking HLA-G protein by antibody improves the cytolysis of NK cells against human breast cancer cell lines. In contrast, upregulation of HLA-G expression by progesterone impairs NK cytolytic function. Thus, HLA-G is a new immune checkpoint protein and potential cancer immunotherapeutic target.

Keywords

HLA-G, Breast Carcinoma, Ovarian Carcinoma, NK Cells,

Immunosuppressive Protein

1. Introduction

Human leukocyte antigen (HLA) molecules are essential for the immune recognition and subsequent immunosurveillance. Human immune system uses the HLAs to distinguish own protein from foreign proteins. HLA molecules bind to peptide fragments derived from pathogens or cancer and display them on the cell surface for recognition by the proper T cells. Immune system including T cells, B cells and macrophages is further activated. The consequences are that invaded pathogens and diseases are eliminated [1]. Impaired HLA class I (HLA-I) expression on cell surface is an early and frequent event of carcinogenesis [2] [3]. Total or partial loss of classical HLA class I expression has been reported in various human cancers [4] [5] [6]. Another HLA-mediated immune escape is that cancer cells overexpress non-classical HLA-I molecules such as HLA-G and HLA-E, which function as inhibitor ligands for immune-competent cells [7] [8].

HLA-G was first reported to be restrictedly expressed at the maternal fetal interface on cytotrophoblasts. HLA-G is a ligand for inhibitory receptors of uterine natural killer (NK) cells and associated with maternal-fetal tolerance [9]. In physiology, HLA-G also expressed in the immune-privileged tissues such as cornea, thymic medulla, pancreatic islets, erythroblasts and mesenchymal stem cells [10] [11] [12]. In pathological condition, HLA-G express is found in cancer, inflammatory, autoimmune disease and viral infection [13] [14] [15]. HLA-G gene has alternative splicing of the primary transcript to generate seven different isoforms, 4 membrane bound (HLA-G1 to G4) and 3 soluble (HLA-G5 to G7), all isoforms have a negative regulation on immune cells including NK cells, cytotoxic T lymphocytes (CTLs) and antigen-presenting cells (APCs), by binding to specific receptors [16] [17] [18]. The HLA-G promotor region has regulatory elements such as heat shock, progesterone and hypoxia-responsive elements, and unidentified responsive elements for IL-10, glucocorticoid and other transcription factors [19] [20] [21]. Progesterone is an immunomodulatory steroid hormone secreted both by corpus luteum and placenta and contributes to the immunosuppressive environment of the maternal-fetal interface [22]. The underlying mechanism is primarily mediated by progesterone-binding to an alternative progesterone response element (PRE) in the HLA-G promotor to induce HLA-G expression [19] [20].

Immunohistochemistry (IHC) has shown that many cancers overexpress HLA-G protein in cancer tissues. Lin et al reviewed HLA-G IHC in paraffin embedding tissues of thirty types of tumors and showed that cancers had 30% - 75% HLA-G expression depending on cancer types. HLA-G expression was correlated with clinical advanced disease stage, metastasis and worse prognosis, indi-

cating that HLA-G could promote tumor immune escape [13]. Paraffin embedding is thought to better preserve morphological details but can mask epitopes of antigen. The positive rates of HLA-G expression might be underestimated in paraffin embedding cancer tissues. In comparison to conventional checkpoint proteins such as PD1/PDL, CTLA4/B7, etc., the immunosuppressive role of HLA-G in cancer has been neglected [23]. In the present study, we examined HLA-G expression of frozen breast and ovary carcinoma tissues by IHC. We found 100% breast and ovary carcinoma tissues overexpressed HLA-G protein. HLA-G expression of normal and cancer cell lines was also studied by real time polymerase chain reaction (qPCR), enzyme-linked immunosorbent assay (ELISA) and immunofluorescence. HLA-G mRNA and protein were increased in cancer cell lines. To determine the immune inhibitory role of HLA-G in cancer, we blocked HLA-G protein of breast cancer cell lines by anti-human HLA-G monoclonal antibody and induced HLA-G expression by progesterone, and found the specific antibody improved the NK cytolysis to the breast cancer cells and the upregulation of HLA-G expression impaired the cytolytic function of NK cells.

2. Materials and Methods

2.1. Cell Culture

Human immortalized, untransformed mammary epithelial line MCF-12A was obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). MCF-12A cells were cultured in a 1:1 mixture of Ham's F12 medium and DMEM containing 0.1 μg/mL cholera enterotoxin, 10 μg/mL insulin, 0.5 μg/mL hydrocortisone, 20 μg/mL epidermal growth factor, and 5% horse serum (Sigma Chemical Co., St. Louis, MO, USA). Human primary dermal fibroblasts, breast carcinoma cell lines MCF-7, T47D and MDA-MB-231, and ovarian carcinoma cell lines OVCAR-8 and TOV-112D, and NK-92MI cells were obtained from ATCC. MCF-7 and T47D are estrogen receptor (ER) and progesterone receptor (PR) positive, but MDA-MB-231 is ER and PR negative. Fibroblasts and all cancer cell lines were grown in alpha-MEM supplemented with 10% fetal bovine serum (GIBCO Invitrogen, Carlsbad, CA, USA). NK-92MI is an interleukin 2 (IL-2) independent NK cells derived from the NK-92 cell line by transfection with human IL-2 cDNA. The cell line is cytotoxic against a wide range of malignant cells [24]. NK-92MI cells were grown in alpha-MEM with 0.2 mM inositol, 0.02 mM folic acid, 0.1 mM 2-mercaptoethanol, 12.5% horse serum (Sigma Aldrich, St. Louis, MO, USA) and 12.5% fetal bovine serum. All cell lines were maintained in the media without supplement of any antibiotics.

2.2. qPCR

We used qPCR to measure mRNA expression of HLA-G. The detailed protocol was published previously [25]. In brief, total RNA was isolated by PurLink RNA Kit (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized by the High Capacity RNA-to-cDNA kit (Applied Biosystems, Grand Island, NY, USA). Gene ex-

pression quantification was performed with TaqMan Gene Expression Assay, a proven 5' nuclease-based real-time PCR chemistry. Primers and probes were synthesized by Integrated DNA Technologies (IDT, Coralville, Iowa, USA). Sequences of HLA-G primers and probe are as follows: CCTGACCCTGACCGAGA (forward), GTAGCCCATGGCGATGAA (reverse) and ACTCCATGAGGTATTTCAGCGCCG (probe). β-actin (ACTB) was used as endogenous gene control to normalized PCRs for the amount of RNA added to the reverse transcription reactions. The sequences of ACTB primers and probes are AGAAAGGGTGTAACGCAACTAA (forward), GGATCAGCAAGCAGGAGTATG (reverse) and TCGTCCACCGCAAATGCTTCTAGG (probe). Probes contain at the 5' end the FAM (6-carboxy fluorescein) as a fluorescent reporter dye, and internal and at 3' end the ZEN™/Iowa Black FQ as fluorescent double quenchers. The qPCR reaction was performed with 7900 HT real time PCR system (Applied Biosystems, Grand Island, NY, USA). Standard mode ran as 2 minutes at 50˚C and 10 minutes at 95˚C, and 40 cycles (15 seconds at 95˚C and 1 minute at 60˚C). Gene expression was analyzed by RQ Manager 1.2 software (Applied Biosystems, Grand Island, NY, USA) and calculated as the ratio of mRNA of the cancer cell lines to that of the primary fibroblasts or MCF-12A. All the experiments were carried out in triple wells and repeated four times independently.

2.3. Treatment of MCF-12A and Breast Cancer Cell Lines with Progesterone

1×10^6 cells of MCF-12A, MCF-7, T47D and MDA-MB-231 were cultured in 25 cm^2 culture flasks overnight. At second day, culture media were replaced by fresh media with 0.64 μM progesterone for MCF-12A, MCF-7 and T47D, and 3.2 μM progesterone for MDA-MB-231. At 2-, 24- and 48-hours post-treatment, cells were harvested. Cell homogenates and total RNA were obtained. Protein concentration of homogenates was measured by BCA protein assay (Thermo Scientific, Rockford, IL, USA). Levels of HLA-G mRNA were determined by qPCR. HLA-G protein in homogenates was measured by HLA-G ELISA kit (Biomatik, Delaware, USA). The procedure was referred to the protocol of kit provided by manufactory. HLA-G concentration (pg/ml) was calibrated by total protein concentration (mg/ml). The final HLA-G concentrations were presented as pg/mg protein [HLA-G concentration by ELISA (pg/ml) ÷ total protein concentration (mg/ml)]. All the experiments were carried out in triple wells and repeated four times independently.

2.4. Immunofluorescent Staining

10,000 cells were cultured on 35 mm glass bottom cell culture dishes (Stellar Scientific, Owings Mills, MD, USA) overnight. At next day. The cells were fixed 15 minutes by 4% paraformaldehyde solution in phosphate-buffered saline (PBS). The staining procedure was as follows: washing 3 times with PBS, blocking 1 hour at room temperature (RT) with 5% goat serum PBS, washing 2 times,

incubating cells with 1:200 rabbit anti-human HLA-G polyclonal antibody (Sigma Aldrich, St Louis, MO, USA) at 4˚C overnight, washing 4 times, incubating cells with 1:500 goat anti-rabbit IgG (H + L)-Alexa 488 (Invitrogen, Carlsbad, CA, USA) for 60 minutes at RT, and washing 4 times. Antibody diluent without HLA-G antibody was added into the dishes as blank control. The cells were observed by Olympus IX83 fluorescent microscope (Tokyo, Japan). Five pictures were taken in different areas of each dish. All pictures were taken with 1 second exposure. Fluorescent intensity was determined by software Cellsense 1.16. All the experiments were repeated three times independently.

2.5. IHC of Frozen Tissues

Frozen slides of human breast and ovarian normal and carcinoma tissues were purchased from BioChain (IRB# IORG0006917, Newark, CA, USA). Slides were fixed with pre-chilled acetone for 15 minutes, dried completely for 30 minutes at RT under airflow, and rinsed 3 times with PBS. HLA-G staining procedure was referred from the protocol of HRP/DAB (ABC) Detection IHC kit (Abcam, Cambridge, MA, USA). The procedure was briefly as follows: blocking non-specific antibody binding and endogenous peroxidases with 5% goat serum PBS and hydrogen peroxide blocking reagent respectively, washing with PBS, incubating tissues with 1:200 rabbit anti-human HLA-G polyclonal antibody at 4˚C overnight, washing with PBS, incubating slides with 1:2000 goat anti-rabbit IgG (H + L)-biotin (Invitrogen, Carlsbad, CA, USA) at RT for 60 minutes, washing with PBS, incubating with strapavidin-perioxidase complex for 10 minutes at RT, washing with PBS, and developing stain with DAB reagent for 3 minutes at RT. Blank controls were covered by antibody diluent without rabbit anti-human HLA-G. The slides were counterstained with hematoxylin, dehydrated and covered with coverslips. HLA-G staining was evaluated by Klein et al semi-quantitative scoring system [26] [27] (**Table 1**).

2.6. Cytolysis of NK Cells against Human Breast Cancer Cell Lines

We modified MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay to examine the NK cytolysis against cancer cells [28]. 20,000 breast cancer cells were cultured in 100 μl media with or without the addition of 10 μg/ml HLA-G monoclonal antibody (87 G) (Invitrogen, Carlsbad, CA, USA) or

Table 1. IHC Scoring system used by Klein *et al.* [26] [27].

A: % of IHC + labeled cells	B: Intensity of IHC staining	Final score
0 = 0%	0 = no staining (blank control, background)	A × B = range from 0 to 9
1 = <30%	1 = weak	
2 = 30% - 60%	2 = mild	
3 = >60%	3 = strong	

progesterone in 96-well plate for 24 hours. There was the Control (blank control, cells in media without any treatment), the Isotype antibody control [cancer cells treated with 10 μg/ml mouse IgG2a (Invitrogen, CA)], and the HLA-G blocking control [cancer cells cultured with the mixture of progesterone and 10 μg/ml HLA-G monoclonal antibody (87 G) in the media]. At next day, the media were removed. 200 μl of NK-92MI cells (effector) were added to the breast cancer cells (target) as effector/target cell ratios of 2.5:1, 5:1 and 10:1. The control wells contained either breast cancer cells or NK-92MI cells alone. The final volume is 200 μl. The plate was then incubated for 4 hours in 5% CO_2 at 37˚C. After 4 hours of incubation, plate was shaken for 2 minutes by orbital shaker at 200 rpm. Then the media containing dead cells, lysed cancer cells and NK-92MI cells were carefully aspirated without disturbance of attached cancer cells. 100 μl of complete alpha-MEM and 10 μl of 5 mg/ml of MTT solution were added to each well. The plate was incubated for another 4 hours in 5% CO_2 at 37˚C. The media were aspirated and 150 μl DMSO was pipetted into each well. The plate was shaken for 10 minutes by orbital shaker at 200 rpm. The optical density (OD) was measured at a wavelength of 492 nm. NK cytolysis was calculated as follows: Cytolysis (%) = [OD of the control wells with breast cancer cells − (OD of experimental wells with cancer cells and NK cells − OD of the control wells with NK cells)] ÷ (OD of the control wells with breast cancer cells) × 100. All the experiments were carried out in triple wells and repeated four times independently.

2.7. Statistical Analysis

Data were analyzed by t-test and presented as mean ± standard deviation (SD) (N, sample size). Findings were considered significant at $p < 0.05$.

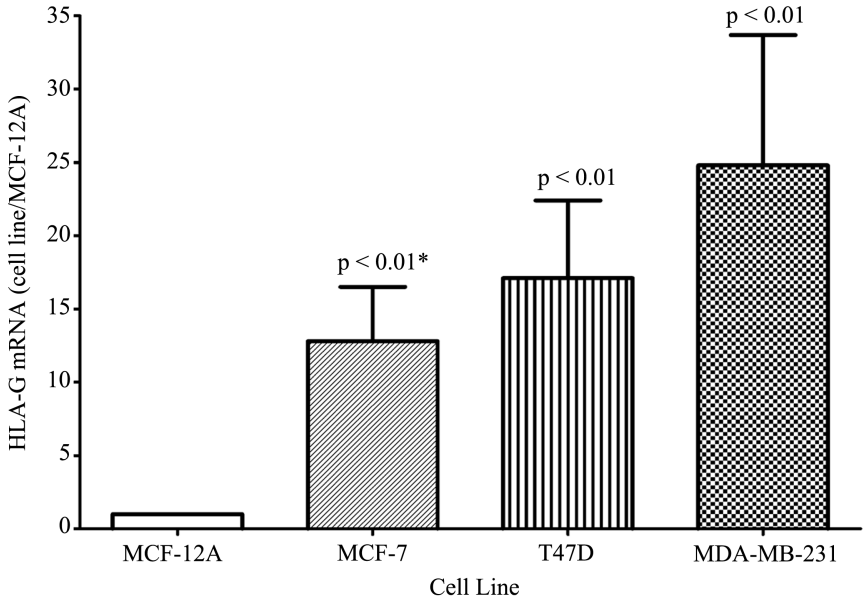
3. Results

3.1. Cancer Cell Lines Overexpress HLA-G mRNA and Protein

qPCR has been used to measure mRNA expression in MCF-12A and breast cancer cell line MCF-7, T47D and MDA-MB-231. The mRNA levels are calculated as the ratio of mRNA of cell lines to that of MCF-12A. HLA-G mRNA levels are 1, 12.8 ± 3.7, 17.1 ± 5.3 and 24.8 ± 8.9 in MCF-12A, MCF-7, T47D and MDA-MB-231 cell lines, respectively. Breast carcinoma cell lines significantly upregulate HLA-G mRNA expression (all $p < 0.01$, compared to MCF-12A, unpaired t-test, N = 12) (**Figure 1**). The ELISA shows that HLA-G protein of MCF-7 (2300 ± 250 pg/mg protein), T47D (2680 ± 270 pg/mg protein) and MDA-MB-231 (3100 ± 456 pg/ml protein) are significantly higher than that of MCF-12A (1470 ± 135 pg/mg protein) (all $p < 0.01$, compared to MCF-12A, unpaired t-test, N = 12) (**Figure 2**).

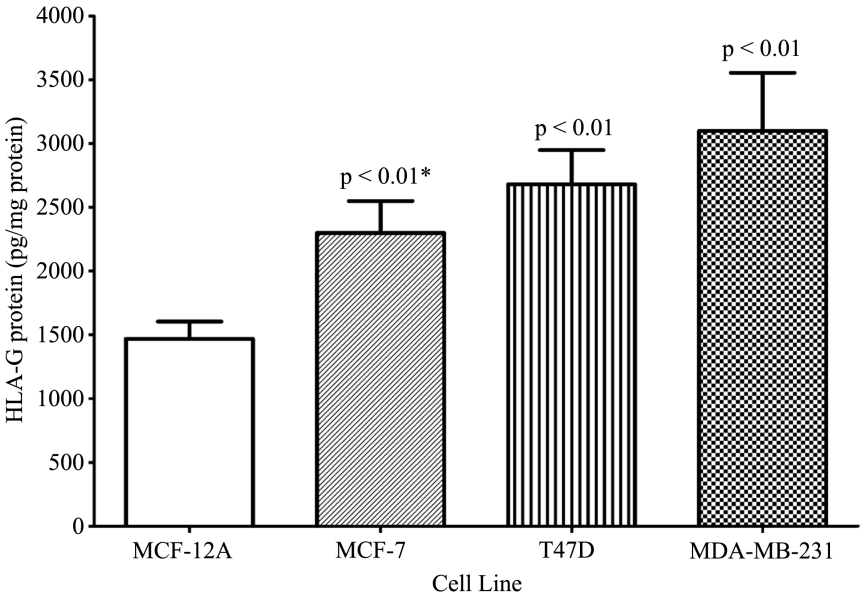
HLA-G protein in cell lines is also detected by immunofluorescent staining. There is no positive fluorescence in the primary fibroblasts and MCF-12A cells. Positive HLA-G staining in the cytoplasm is observed in breast carcinoma cell

lines MCF-7, T47D and MDA-MB-231, and ovarian carcinoma cell lines OVCAR-8 and TOV-112D. The fluorescent intensity of cancer cell lines is significantly higher than primary fibroblasts and MCF-12A (Table 2, Figure 3).



*unpaired t-test, compared to MCF-12A

Figure 1. Breast cancer cell lines overexpress HLA-G mRNA (all $p < 0.01$, compared to MCF-12A, unpaired t-test, $N = 12$). The mRNA levels of HLA-G were measured by qPCR. All the experiments were carried out in triple wells and repeated four times independently.



*unpaired t-test, compared to MCF-12A

Figure 2. Breast cancer cell lines increase HLA-G protein (all $p < 0.01$, compared to MCF-12A, unpaired t-test, $N = 12$). The levels of HLA-G were measured by ELISA. All the experiments were carried out in triple wells and repeated four times independently.

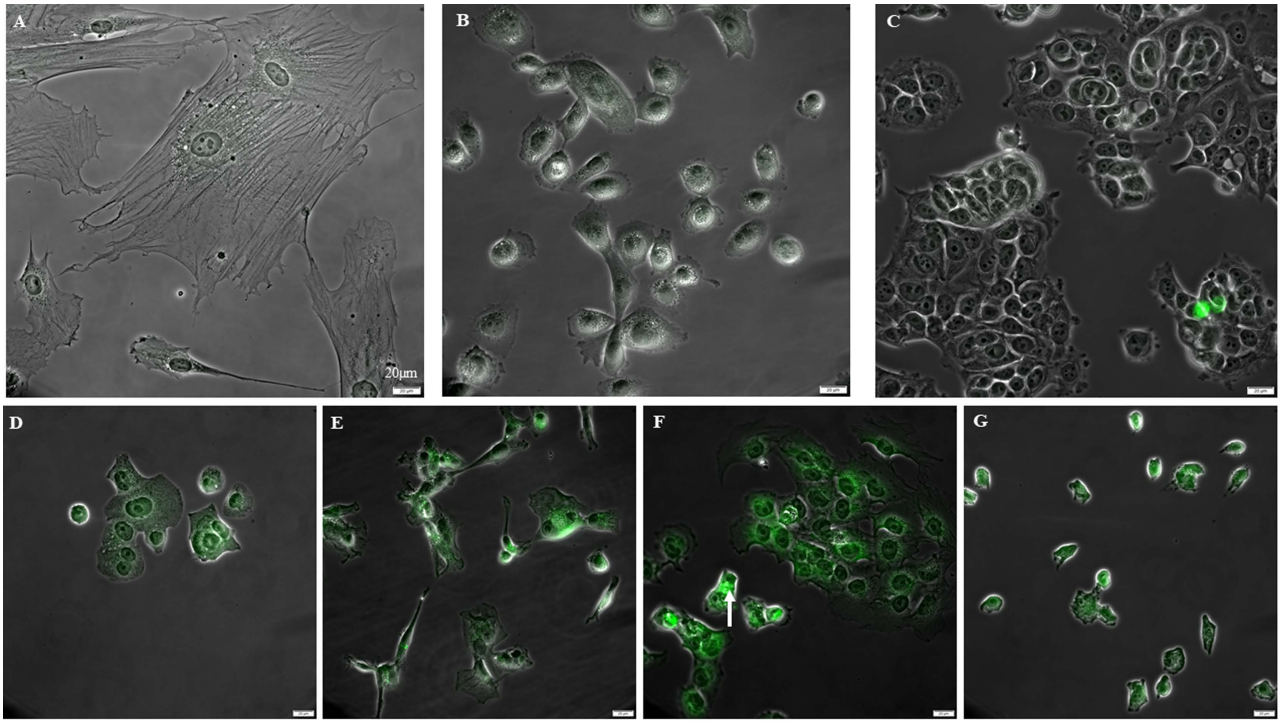


Figure 3. Increased HLA-G immunofluorescent staining (green fluorescence, pointed by arrow, phase contrast—fluorescent image) in human carcinoma cell lines. (A) Human fibroblasts; (B) Human mammary epithelial MCF-12A; (C): Breast carcinoma MCF-7; (D) Breast carcinoma T47D; (E) Breast carcinoma MDA-MB-231; (F) Ovarian carcinoma OVCAR-8; (G) Ovarian carcinoma TOV-112D.

Table 2. Cancer cell lines overexpress HLA-G protein. Fluorescent intensity was measured by Olympus IX83 fluorescent microscope with software Cellsense 1.16.

Cell lines	Fluorescent intensity	Probability (p)[b]
Primary fibroblasts	3.5 ± 1.8 (15)[a]	
MCF-12A	4.7 ± 2.8 (15)	>0.05[c]
MCF-7	9.2 ± 4.5 (15)	<0.01[c]; <0.01[d]
T47D	15.9 ± 5.7 (15)	<0.01; <0.01
MDA-MB-231	35.9 ± 10.2 (15)	<0.01; <0.01
OVCAR-8	45.8 ± 13.5 (15)	<0.01; <0.01
TOV-112D	26.6 ± 9.8 (15)	<0.01; <0.01

[a]Mean ± SD (N). [b]Unpaired t-test. <0.05 is considered significant. [c]Compared to primary fibroblasts. [d]Compared to MCF-12A.

3.2. Progesterone Upregulates HLA-G mRNA and Protein of Normal Breast and Cancer Cell Lines

Progesterone binds to progesterone receptor (PR) and glucocorticoid receptor (GR). The affinity of progesterone to PR is much higher than to GR [29]. Cell lines MCF-12A, MCF-7 and T47D are PR positive, but MDA-MB-231 is PR negative. Progesterone binds to GR of MDA-MB-231 cells to function. In the

pre-experiment, we titrated the progesterone concentrations by the measurement of cell growth and found the maximum concentrations without the inhibition of cell growth were 0.64 μM in MCF-12A, MCF-7 and T47D, and 3.2 μM in MDA-MB-231. Thus, we treated MDA-MB-231 cells with high concentration of progesterone (3.2 μM) and other cell lines with 0.64 μM progesterone. The HLA-G mRNA reaches to the peak at 2 hours post-treatment and remains to increased levels up to 48 hours post-treatment (**Figure 4**). At 2 hours, HLA-G mRNA levels of MCF-12A, MCF-7, T47D and MDA-MB-231 are increased as folds of 2.84 ± 0.23, 7.71 ± 0.56, 4.83 ± 0.35 and 19.40 ± 0.96 as the levels before treatment, respectively. Progesterone significantly upregulates HLA-G mRNA in the 4 cell lines (all $p < 0.01$ at 2 hours post-treatment, unpaired t-test, compared to the level before treatment, N = 12). HLA-G protein increases slower than HLA-G mRNA. At 24 hours post-treatment, HLA-G protein increases to the peak levels which are 5100 ± 530, 4800 ± 480, 5220 ± 368, and 6500 ± 475 pg/mg protein in MCF-12A, MCF-7, T47D and MDA-MB-231, respectively. The levels of HLA-G protein are significantly higher than that before treatment (all $p < 0.01$ at 24 hours post-treatment, unpaired t-test, compared to the level before treatment, N = 12) (**Figure 5**). These results show that progesterone upregulates the expression of HLA-G of normal mammary epithelial and breast carcinoma cell lines.

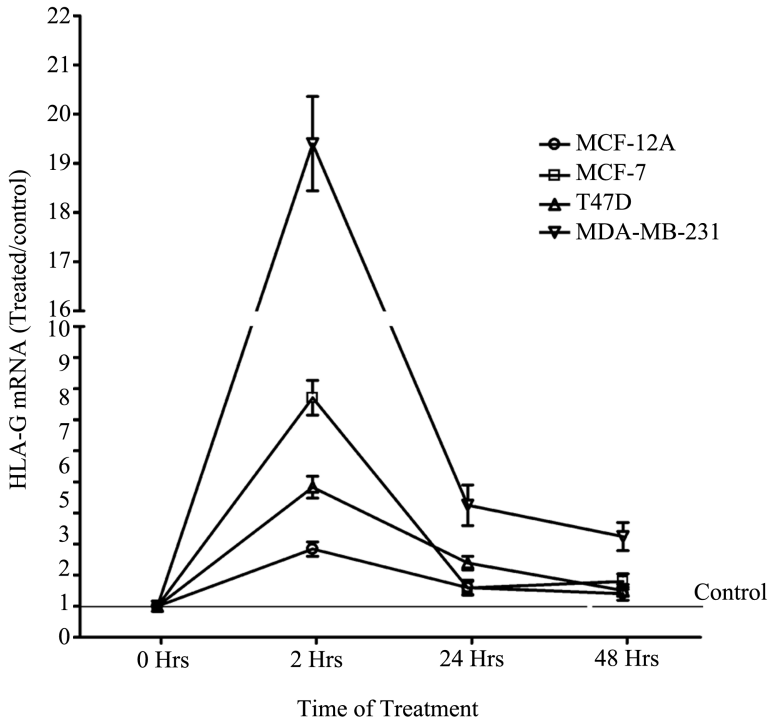


Figure 4. Progesterone upregulates HLA-G mRNA of normal mammary epithelium MCF-12A and breast cancer cell lines MCF-7, T47D and MDA-MB-231. MCF-12A, MCF-7 and T47D were incubated in media with 0.64 μM progesterone. MDA-MB-231 was treated in media containing 3.2 μM progesterone. Levels of HLA-G mRNA were measured by qPCR.

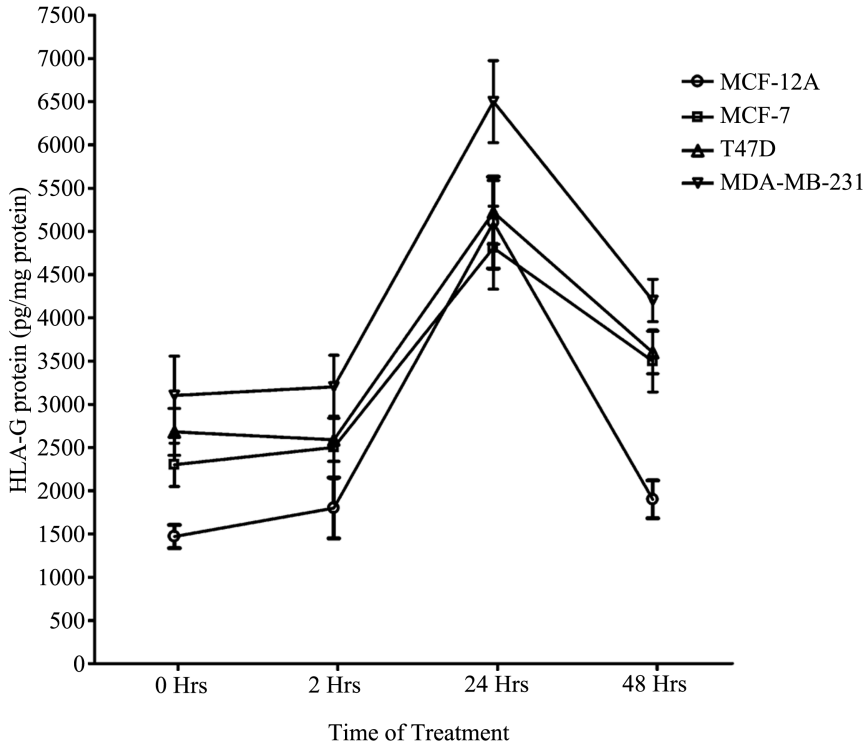


Figure 5. Progesterone increases HLA-G protein of normal mammary epithelium MCF-12A and breast cancer cell lines MCF-7, T47D and MDA-MB-231. MCF-12A, MCF-7 and T47D were incubated in media with 0.64 μM progesterone. MDA-MB-231 was treated in media containing 3.2 μM progesterone. HLA-G protein was measured by ELISA.

3.3. Breast and Ovarian Carcinoma Tissues Overexpress HLA-G Protein

HLA-G protein is detected by IHC in 15 frozen normal breast and 44 breast carcinoma tissues. Except the pathological diagnosis, other patient clinical information has not been released by the tissue suppliers. All breast carcinoma tissues (100%) have positive HLA-G brown staining in the cytoplasm. Breast carcinoma increases HLA-G expression (**Figure 5**). HLA-G staining scores of breast carcinoma tissues are significantly higher than that of normal breast tissues ($p < 0.01$) (**Table 3**). HLA-G protein was also examined in 11 frozen normal ovarian tissue and 35 frozen ovarian carcinoma tissues. 100% of frozen ovarian carcinoma tissues show positive HLA-G staining. Ovarian carcinoma tissues significantly upregulate HLA-G expression, compared to normal ovarian tissues ($p < 0.01$) (**Table 3**, **Figure 6**).

3.4. Blocking HLA-G Protein Increase Natural Killer Cytolysis

The blocking of HLA-G on melanoma cells transfected with HLA-G1 cDNA with anti-human HLA-G (87 G) restores specific lysis of human polyclonal NK cells by using the isotype mouse IgG2a as the control [https://www.thermofisher.com/antibody/product/HLA-G-Antibody-clone-87G-Monoclonal/MA1-10356].

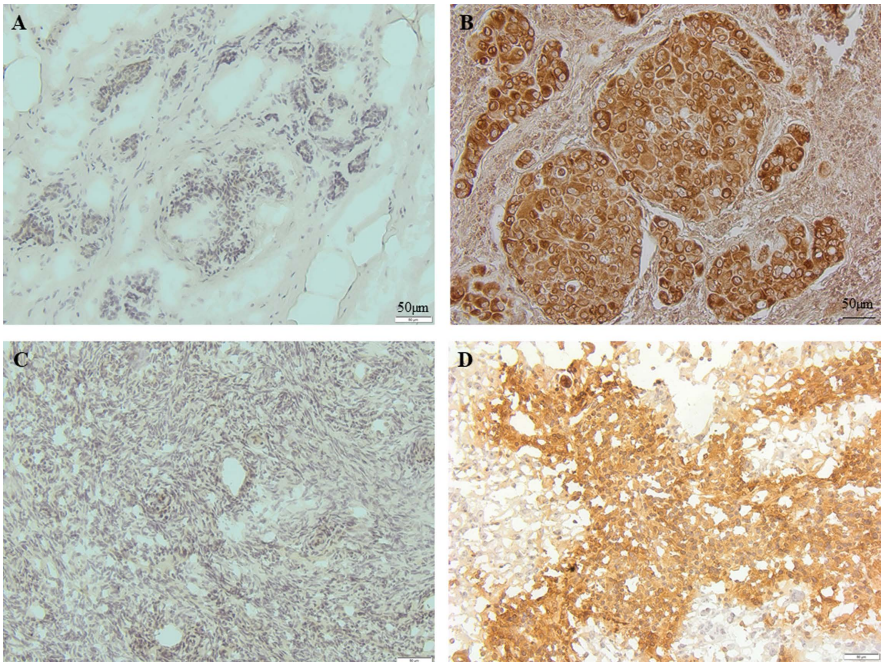


Figure 6. Breast and ovarian carcinoma upregulate HLA-G expression. IHC shows that positive HLA-G brown staining in the cytoplasm on frozen tissue slides. (A) Normal breast; (B) Breast carcinoma; (C) Normal ovary; (D): Ovarian carcinoma.

Table 3. Breast and ovarian carcinoma tissues upregulate HLA-G expression. HLA-G protein was examined by immunohistochemistry on frozen tissue slides. The detailed protocols are seen in the Materials and Methods.

Tissues	HLA-G Score	p[b]
Normal breast	1.67 ± 1.59 (15)[a]	
Breast carcinoma	6.13 ± 2.46 (44)	<0.01[c]
Normal ovary	1.09 ± 0.79 (11)	
Ovarian carcinoma	5.8 ± 2.8 (35)	<0.01[d]

[a]Mean ± SD (N). [b]Unpaired t-test. <0.05 is considered significant. [c]Compared to normal breast tissues. [d]Compared to normal ovarian tissues.

In this study, 3 human breast cancer cell lines are pre-treated by 10 μg/ml anti-human HLA-G (87 G) antibody for 24 hours at 37˚C and 5% CO_2 to block HLA-G expression. Then, the breast cancer cell lines are co-incubated with NK-92MI cells with 3 different ratios of effective cells (NK-92MI) to target cells (cancer cells). The 10 μg/ml antibody doesn't change the growth of breast cancer cells. The blocking of HLA-G by anti-human HLA-G (87 G) antibody significantly increases the cytolysis of NK-92MI, compared to the controls without pre-treatment (Table 4). 10 μg/ml of control isotype mouse IgG2a doesn't significantly change the NK cytolysis in all 3 breast carcinoma cell lines, compared to controls without pre-treatment (all $p > 0.05$, unpaired t-test, N-12) (Figure 7).

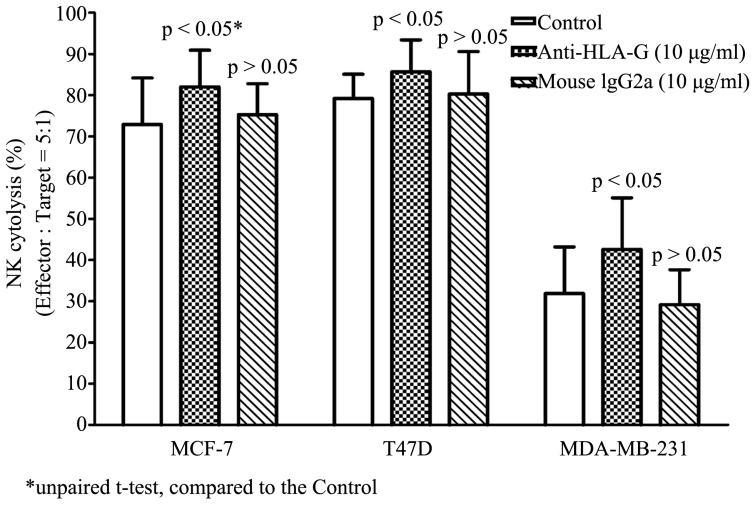


Figure 7. Blocking of HLA-G on the breast carcinoma cell lines with 10 μg/ml anti-human HLA-G (87 G) restores specific lysis of NK-92MI cells. The isotype mouse IgG2a (10 μg/ml in media) doesn't significantly change the NK cytolysis, compared to the controls without any pre-treatment (all $p > 0.05$, unpaired t-test, N = 12). Effector cells: NK-92MI. Target cells: human breast carcinoma MCF-7, T47D or MDA-MB-231.

Table 4. Blocking HLA-G of breast cancer cells improves the cytolysis of natural killer cell line NK-92MI. The NK cytolysis was measured by MTT assay.

Cancer cells	Effector: Target[a]	NK cytolysis (%)[b]		p[c]
		Control (Not pre-treated)	Pre-treated by 10 μg/ml Anti-HLA-G	
MCF-7	10:1	80.3 ± 10.5 (12)	89.8 ± 8.5 (12)	<0.05
	5:1	72.9 ± 11.3 (12)	82.0 ± 8.9 (12)	<0.05
	2.5:1	50.2 ± 10.4 (12)	60.4 ± 11.9 (12)	<0.05
T47D	10:1	86.3 ± 8.5 (12)	90.1 ± 2.3 (12)	NS[d]
	5:1	79.2 ± 5.9 (12)	85.7 ± 7.7 (12)	<0.05
	2.5:1	68.1 ± 4.4 (12)	76.3 ± 2.7 (12)	<0.01
MDA-MB-231	10:1	47.7 ± 14.3 (12)	60.3 ± 13.7 (12)	<0.05
	5:1	31.9 ± 11.3 (12)	42.6 ± 12.5 (12)	<0.05
	2.5:1	15.1 ± 11.8 (12)	26.8 ± 12.0 (12)	<0.05

[a]NK-92MI: Cancer cells (MCF-7, T47D or MDA-MB-231). [b]Mean ± SD (N). [c]Unpaired t-test, compared to the control, <0.05 is considered significant. [d]Not significant.

3.5. Upregulated HLA-G of Breast Cancer Cells by Progesterone Inhibits the Cytolysis of NK-92MI

We pre-treated MCF-7 and T47D with 0.64 μM progesterone, and MDA-MB-231 with 3.2 μM progesterone for 24 hours. We tested the cytolysis of NK-92MI to these 3 cell lines. Progesterone 0.64 μM and 3.2 μM don't alter the growth of breast cancer cells. Table 5 shows pre-treatment with progesterone inhibits the cytolysis of NK-92MI to the breast cancer cell lines, compared to controls without pre-treatment (all $p < 0.05$, paired t-test). The pre-treatment with the mix-

ture of progesterone and 10 μg/ml anti-HLA-G antibody doesn't significantly increase or decrease the NK cytolysis to the 3 breast cancer cell lines (all $p < 0.05$, unpaired t-test, compared to the non-treated cells, N = 12). The blocking HLA-G protein by 10 μg/ml anti-HLA-G can neutralize the inhibitory effect of progesterone on NK cytolysis (Figure 8). These suggest the increased HLA-G by progesterone inhibits the cytolytic function of natural killer cells to breast cancer cells.

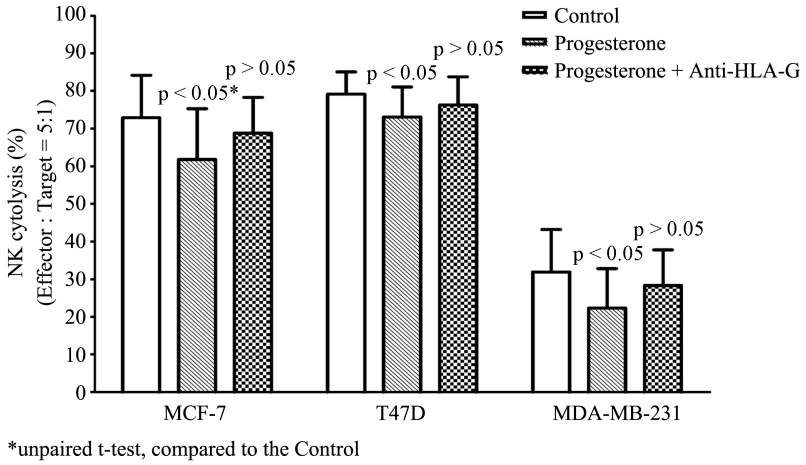


Figure 8. Increased HLA-G of breast cancer cells by progesterone inhibits the cytolysis of NK-92MI. Blocking HLA-G protein by 10 μg/ml anti-HLA-G can neutralize the inhibitory effect of progesterone on NK cytolysis. Effector cells: NK-92MI. Target cells: human breast carcinoma MCF-7, T47D or MDA-MB-231 cells. Cancer cells were pre-treated with progesterone, or the mixture of progesterone and 10 μg/ml HLA-G monoclonal antibody (87 G) in the media for 24 hours. The control was cultured in media only.

Table 5. Pre-treatment of breast cancer cells by progesterone inhibits the cytolysis of natural killer cell line NK-92MI. The NK cytolysis was measured by MTT assay.

Cancer cells	Effector: Target[a]	NK cytolysis (%)[b]		p[d]
		Control (Not pre-treated)	Pre-treated by progesterone[c]	
MCF-7	10:1	80.3 ± 10.5 (12)	70.2 ± 12.9 (12)	<0.05
	5:1	72.9 ± 11.3 (12)	61.8 ± 13.5 (12)	<0.05
	2.5:1	50.9 ± 10.4 (12)	40.2 ± 11.8 (12)	<0.05
T47D	10:1	86.3 ± 8.5 (12)	78.9 ± 8.0 (12)	<0.05
	5:1	79.2 ± 5.9 (12)	73.1 ± 8.0 (12)	<0.05
	2.5:1	68.1 ± 4.4 (12)	62.6 ± 6.5 (12)	<0.05
MDA-MB-231	10:1	47.7 ± 14.3 (12)	36.3 ± 12.5 (12)	<0.05
	5:1	31.9 ± 11.3 (12)	22.3 ± 10.5 (12)	<0.05
	2.5:1	15.1 ± 11.8 (12)	6.5 ± 3.5 (12)	<0.05

[a]NK-92MI: Cancer cells (MCF-7, T47D or MDA-MB-231). [b]Mean ± SD (Number). [c]MCF-7 and T47D were pre-treated with 0.64 μM progesterone, and MDA-MB-231 with 3.2 μM progesterone for 24 hours. [d]Unpaired t-test, compared to the control, <0.05 is considered significant.

4. Discussion

The reestablishment and maintenance of anti-tumor immunity are the goal of cancer immunotherapy. Tumor cells escape host anti-tumor immunity by immunosuppressive molecules called checkpoint proteins. These checkpoints are mediated by the interaction between ligand molecules on tumor cells and their receptors on immune cells mainly T cells. Exampled checkpoints include CTLA4/B7 interaction which specifically inhibits the induction of the T cell response while PD1/PDL plans a prominent role in the effector phase of T cell response [30] [31]. Other checkpoints have been described and may also significantly contribute to tumor immune escape, such as CD47/SIRP1a, TIGIT/PvR, LAG3/MHC-II, BTLA/HVEM, CD200/CD200R, B7-H3, B7-H4, VISTA, CD39/CD38/CD73/CD203a/CD157/ADOR, TIM-3/Galectin [32]. Blocking checkpoints by monoclonal antibodies targeting to PD1 or PDL1 has shown the survival benefit in cancer patients [33] [34]. Despite these positive results, these anti-checkpoint monotherapies are usually inefficient in the majority of patients. The possible reasons include the involvement of several checkpoints which regulates distinct inhibitory pathways through non-overlapping mechanisms. HLA-G is a neglected immune checkpoint protein which inhibits many types of immune cells through interaction with its receptors, ILT2 (LILRB1/CD85j) and ILT4 (LILRB2/CD85d), on immune cells. ILT2 is expressed on monocytes/macrophages, dendritic cells, B cells, and T and NK cells, while ILT4 is mainly on surface of neutrophils and myeloid cells. The interaction of HLA-G and its ILTs receptors inhibits the function of these immune cells including T, B and NK cells and induces immunosuppressive cells such as Tregs and myeloid suppressive cells [7] [23] [30]. The immunosuppressive role of HLA-G has also been demonstrated in mice. In a tumor-implantation model, control HLA-G-negative tumors were rejected, whereas HLA-G-expressing tumors grew. Blocking of HLA-G by a specific neutralizing antibody prevented the growth of HLA-G-expressing tumors, providing the proof of concept for new antitumor therapeutic strategy [35]. In the present study, we confirm breast and ovarian carcinoma cell lines overexpress HLA-G mRNA and proteins, compared to human primary fibroblasts and normal mammary epithelia (**Table 2**, **Figure 1** and **Figure 2**). In frozen tissues, 100% of human breast and ovarian carcinoma overexpress HLA-G. HLA-G is significantly upregulated in breast and ovarian carcinoma tissues, compared to normal breast and ovarian tissues, respectively (**Table 3** and **Figure 5**). Lin et al reviewed HLA-G immunohistochemistry studies in paraffin-embedding tissues of thirty types of tumors. There were 30% - 75% HLA-G expression depended on cancer types [13]. The rate of HLA-G expression was apparently underestimated in paraffin-embedding tissues. The process of paraffin-embedding of tissues might cause the loss of HLA-G antigenicity or mask the epitopes of HLA-G.

Breast carcinoma is associated with progesterone. In a case-cohort study of postmenopausal women, elevated circulating progesterone levels were associated with a 16% increase in the risk of breast cancer [36]. The underlying mechanism

might include upregulation of HLA-G by progesterone on normal mammary epithelia. Progesterone binds to an alternative progesterone response element (PRE) in the HLA-G promotor to induce HLA-G expression [19] [20]. We find progesterone increases the HLA-G expression of normal mammary epithelial MCF-12A and 3 breast cancer cell lines (**Figure 4** and **Figure 5**). The upregulation of HLA-G expression may lead to immune suppression which promotes breast tumorigenesis and cancer prognosis. Progesterone hormone therapy has been used to treat symptoms of menopause and some types of cancer [37] [38]. Our results in the present study suggest that caution should be taken to observe the effect of progesterone on HLA-G and host anti-tumor immunity when we give progesterone hormone therapy for cancer patients.

NK cells play major roles in first-line innate immunity against viral infections, tumorigenesis, and tumor growth and progression. NK cells possess a combination of activating and inhibitory receptors. In humans, major activating receptors involved in target cell killing are the natural cytotoxicity receptors (NCRs) and NKG2D. Activating receptors recognize ligands that are overexpressed or expressed de novo upon cell stress, viral infection, or tumor transformation. The HLA-I-specific inhibitory receptors constitute a fail-safe mechanism to avoid unwanted NK-mediated damage to healthy cells [39]. HLA-G binds to the inhibitory receptors on NK cells to inhibit NK cytolysis in human pregnancy and cancers [9] [30] [40]. We find the blocking HLA-G on breast cancer cells improves anti-tumor function of NK-92MI. In contrast, increased HLA-G expression of breast cancer cells by progesterone impeded the tumor lysis of NK-92MI. At least *in vitro*, these data provide the proof of principle of blocking HLA-G for new antitumor therapeutic strategy. On the other side, blockade of inhibitory receptors, the HLA-G ligands, on NK and T cells is another new cancer immunotherapeutic strategy. It has been reported that blocking and downregulating ILT2, an HLA-G ligand, by Lenalidomide restore NK cell function in chronic lymphocytic leukemia [41].

5. Summary

Human breast and ovarian carcinoma overexpress HLA-G. Blocking HLA-G of breast cancer cells by antibody restores the tumor lysis of NK-92MI cells. In contrast, upregulation of HLA-G on breast cancer cells by progesterone inhibits the anti-tumor function of NK-92MI cells. Therefore, HLA-G is a new cancer immunotherapeutic target. Several further studies remain to be done: 1) evaluation of blood HLA-G and progesterone levels and the expression of HLA-G and its ligands in tumor microenvironment before cancer immunotherapy including conventional checkpoint inhibitors; 2) development of humanized antibodies which specifically block HLA-G and inhibitory receptors (HLA-G ligands) on cancer and immune cells; 3) antisense oligonucleotides which downregulate the expression of HLA-G and its ligands and directly suppress tumor proliferation; 4) silent progesterone antagonists which may inhibit the expression of HLA-G

on cancer cells.

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Availability of Data and Material

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' Contributions

Xian-Peng Jiang designed the study, developed the methodology, performed the experiments, analyzed the data and wrote the manuscript, reviewed and revised the manuscript. Catherine C. Baucom designed the study and reviewed the manuscript. Toby Jiang performed the experiment. Robert L. Elliott conceived and approved the study.

Ethics Approval

Human tissues were purchased from company BioChain (Newark, CA, USA) which has the approved tissue collection IRB# IORG0006917.

Consent for Publication

All authors have read, reviewed and approved the manuscript.

Conflicts of Interest

No conflicting financial interest exists.

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Abbreviations

HLA	Human Leukocyte Antigen
HLA-G	Human Leukocyte Antigen G
NK	Natural Killer
PRE	Progesterone Response Element
IHC	Immunohistochemistry
qPCR	Real Time Polymerase Chain Reaction
ELISA	Enzyme-Linked Immunosorbent Assay
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
ER	Estrogen Receptor
PR	Progesterone Receptor