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Human leukocyte antigen-E alleles and expression in patients with serous ovarian cancer

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Human leukocyte antigen-E (HLA-E) is one of the most extensively studied non-classical MHC class I molecules that is almost non-polymorphic. Only two alleles (*HLA-E*0101* and *HLA-E*0103*) are found in worldwide populations, and suggested to be functional differences between these variants. The HLA-E molecule can contribute to the escape of cancer cells from host immune surveillance. However, it is still unknown whether *HLA-E* gene polymorphisms might play a role in cancer immune escape. To explore the association between *HLA-E* alleles and the susceptibility to serous ovarian cancer (SOC), 85 primary SOC patients and 100 healthy women were enrolled. Here, we indicated that high frequency of *HLA-E*0103* allele existed in SOC patients by the allele-specific quantitative real-time PCR method. The levels of HLA-E protein expression in SOC patients with the *HLA-E*0103* allele were higher than those with the *HLA-E*0101* allele using immunohistochemistry analysis. The cell surface expression and functional differences between the two alleles were verified by K562 cells transfected with *HLA-E*0101* or *HLA-E*0103* allelic heavy chains. The *HLA-E*0103* allele made the transfer of the HLA-E molecule to the cell surface easier, and HLA-E/peptides complex more stable. These differences ultimately influenced the function of natural killer cells, showing that the cells transfected with *HLA-E*0103* allele inhibited natural killer cells to lysis. This study reveals a novel mechanism regarding the susceptibility to SOC, which is correlated with the *HLA-E*0103* allele.

Major histocompatibility complex class I molecules may be subdivided into two families, MHC class Ia (classical) and MHC class Ib (non-classical). The MHC class Ia molecules, including human leukocyte antigen (HLA)-A, B, and C, play a central role in adaptive immunity. Downregulation of MHC class Ia molecules expression is a widespread mechanism used by tumor cells to escape antitumor T-cell-mediated immune responses.[1,2] Why, then, can natural killer (NK) cells not lyse these MHC class Ia-deficient tumor targets? Previous studies reported that the aberrant expression of MHC class Ib molecules in tumors provided the required inhibitory signal to NK cells.[3] In humans, the MHC class Ib family members include HLA-E, -F, -G, and HFE (HLA-H). Of all the MHC class Ib molecules, the function of HLA-E has been the most completely elucidated through its interaction with CD94/NKG2 receptors.[4] HLA-E can be expressed on the cell surface of nucleated cells and presents a limited variety of peptides to CTLs and NK cells.[5] HLA-E has low cell surface expression in normal tissues, whereas upregulation of HLA-E recognized by CTLs and NK cells may lead to either an inhibitory or a stimulatory response, depending on subsequent receptor binding. Binding to CD94/NKG2A receptor leads to an inhibitory response, whereas binding to CD94/NKG2C receptor leads to a stimulatory response.[6] In most conditions, the binding of HLA-E to CD94/NKG2A is much stronger than that to CD94/NKG2C, suggesting a subordinate role of this latter interaction.[7] Growing evidence on HLA-E expression and function on cancer biology are revealed. The overexpression of HLA-E is a common finding across a variety of malignancies, including lymphoma,[8] glioblastomas,[9,10] colorectal cancer,[11,12] and gynecological tumors.[13]

HLA-E has the least polymorphisms of all the MHC class I genes, presenting only 11 alleles encoding three distinct proteins (International Immunogenetics Database, version 3.12.0). Overall, three allele groups (*HLA-E*0101*, *HLA-E*0103*, and *HLA-E*0104*) have been described in diverse populations. However, only two allele groups (*HLA-E*0101* and *HLA-E*0103*) are found in worldwide populations.[14] An A/G variation at codon 107 defines *HLA-E*0101* and *HLA-E*0103* (http://hla.alleles.org/data/txt/e_nuc.txt), resulting in an arginine at position 107 in *HLA-E*0101* ($HLA\text{-}E^{107R}$) being replaced by a glycine in *HLA-E*0103* ($HLA\text{-}E^{107G}$).[15] Both allele groups are found at nearly equal frequencies, suggesting that balancing selection may be acting on this gene, with putative functional differences between these variants.[14] To our knowledge, published reports concerning *HLA-E* polymorphisms and susceptibility to cancer are controversial and still very limited. This study is the first to explore the association between *HLA-E* alleles and ovarian cancer. We analyzed the implication of the two confirmed *HLA-E* alleles in serous

ovarian cancer (SOC) susceptibility in a Chinese Han population. HLA-E expression levels in tissues with different genotypes were detected, and the functional differences between the two alleles were also investigated in the present study.

Materials and Methods

Study subjects. This study was carried out on 85 primary SOC patients at FIGO stage I–III (median age, 55 years; range, 38–61 years). The peripheral blood samples were obtained from the patients prior to any treatment, and these patients were all from the Department of Oncology, Fudan University Shanghai Cancer Center (Shanghai, China) between 2012 and 2014. All patients underwent surgery and the histopathologic diagnosis was carried out by pathologists. After surgical resection, tumor specimens were fixed in formalin, embedded in paraffin, and stored. Additionally, the peripheral blood from 100 healthy women (median age, 49 years; range, 31–58 years) and ovary tissues from patients with benign gynecological diseases were regarded as control groups in HLA-E genotyping and immunohistochemistry staining, respectively. For the use of these clinical specimens for research purposes, all participants gave their informed consent. The study's protocol was reviewed and approved by the Ethics Committee of Shanghai Cancer Center, Fudan University (certification no. 050432-4-1212B).

HLA-E genotyping. Genomic DNA was extracted from peripheral blood samples using the TIANamp Blood DNA Kit (Tiangen Biotech, Beijing, China). Genotyping was accomplished by allele-specific quantitative real-time PCR (qRT-PCR) with two forward primers respectively discriminative of *HLA-E*0101* and *HLA-E*0103* alleles: E*0101F (5′-GCG-AGC-TGG-GGC-CCG-CCA-3′) and E*0103F (5′-GCG-AGC-TGG-GGC-CCG-CCG-3′). Each of the forward primers was combined with a common HLA-E-specific reverse primer: 5′-CCG-CCT-CAG-AGG-CAT-CAT-TTG-3′. Two PCR reactions for each sample were carried out in a 10 μL reaction solution containing 20 ng genomic DNA, 0.2 μmol/L allele-specific forward primer, 0.2 μmol/L common reverse primer, and 5 μL 2× SYBR Premix Ex Taq (TaKaRa, Shiga, Japan). The PCR amplification was carried out at 95°C for 10 min, 40 cycles at 95°C for 15 s, and 65°C for 40 s, followed by a final stage of product dissociation cycle, using Mastercycler ep realplex (Eppendorf, Hamburg, Germany). Allele discrimination was manually performed according to the different PCR amplification efficiencies for different alleles, which can be shown by the cycle of threshold (C_t). Generally, ΔC_t (the difference in C_t between two PCR reactions for each sample) ≥ 6 may be considered to be a homozygote, whereas ≤ 1 may be considered to be a heterozygote.[16]

Immunohistochemical staining and evaluation. Formalin-fixed and paraffin- embedded sections (4-μm thickness) were mounted on glass slides. Each section was dewaxed in xylene and hydrated in grade alcohol, followed by inhibition of endogenous peroxidase activities with methanol containing 0.3% H_2O_2. After boiling in 10 mmol/L of citrate buffer (pH 6.0) for antigen retrieval and cooling down, the sections were blocked with 1% BSA and incubated overnight at 4°C with primary mouse monoclonal anti-HLA-E antibody (MEM-E/02, 1:1000; Abcam, Cambridge, USA). On the second day, these sections were incubated for another 45 min at 37°C. After washing with PBS, the sections were incubated with HRP-conjugated secondary antibody (Shanghai Long Island Biotech, Shanghai, China) for 1 h at room temperature, followed by reaction with diaminobenzidine, and counterstaining with hematoxylin. The evaluation of the immunohistochemical staining was carried out double-blinded by two pathologists. The scoring system proposed by Ruiter *et al.*[17] was used. The percentage of positive tumor cells was scored as: 0, <1%; 1, 1–5%; 2, 6–25%; 3, 26–50%; 4, 51–75%; and 5, >75%. The intensity of staining was scored using the following scale: 0, negative; 1, weak; 2, moderate; 3, strong. A final score was calculated by totaling the scores for percentage and intensity, resulting in a score from 0 to 8. Final scores indicated: 0, negative expression; 2–4, weak expression; 5–6, moderate expression; and 7–8, strong expression.

Cell lines. The tumor cell lines K562R and K562G (transfected with red fluorescence protein and EGFP, respectively), along with HLA-E expression lentivirus vectors pLL3.7 with *HLA-E*0101* or *HLA-E*0103* allele, were all kindly provided by Dr. Shengli Song (Shanghai Institute of Immunology, Shanghai Jiaotong University School of Medicine, Shanghai, China). The lentivirus was produced by transfection in HEK293T cells using Lipofectamine 2000 transfection reagent (Life Technologies, Carlsbad, USA). The K562G cells were infected with the virus in the presence of 6 μg/mL polybrene (Sigma-Aldrich, St. Louis, USA). The stable transfected cells were screened by 400 μg/mL hygromycin B (Life Technologies) in complete RPMI-1640 medium (Gibco, Mauricio Minotta, USA).

Western blot analysis. Cells (5×10^5) were collected and lysed, then the proteins were separated and incubated with anti-HLA-E mAb (MEM-E/02, 1:500; Abcam) or anti-GAPDH antibody (rabbit polyclonal, 1:3000; Abcam) at 4°C for 12 h, followed with secondary antibody conjugated to HRP incubated at room temperature for 2 h. ECL reagent (Gene Tech, Shanghai, China) was used to detect the results.

Peptide binding and HLA-E cell surface analysis. The signal sequence of HLA-B7 (B7sp, VMAPRTVLL) was synthesized by GL Biochem (Shanghai, China). Cells were cultured at 26°C for 16 h with 100 μM peptides. For the thermal stability assay, these cells binding with peptides were further incubated at 37°C for 2 h with or without 1000 ng/mL Brefeldin A (Selleck Chemicals, Burlington, USA). Surface HLA-E expression in cultured cells was detected by flow cytometry with 3D12-APC mAb (eBioscience, San Diego, USA). The data were acquired on a FACSCalibur (BD Biosciences, New Jersey, USA), and analyzed using FlowJo software (Treestar, Ashland, OR, USA).

Natural killer lysis assay. Natural killer cells were obtained from the peripheral blood of healthy donors using an NK cell isolation kit (Miltenyi Biotech, Bergisch Gladbach, Germany) according to the manufacturer's instructions. The purity of the enriched NK cells was evaluated by flow cytometry using fluorochrome-conjugated antibodies (CD3-FITC and CD56-PE). The antibodies were purchased from Miltenyi Biotech. Target cells (all transfected with EGFP) were loaded with B7sp peptide at 26°C for 16 h, then these cells (2×10^5/well) were co-cultured with graded numbers of NK cells in 96-well plates at 37°C for 2 h. The cells in each well were collected into a FACS tube, and reference cells K562R (K562 cells transfected with red fluorescence protein) were added (2×10^5/well). Thus, the change in the ratio of target cells to reference cells was detected by flow cytometry. The percentage of NK lysis was determined as follows: % of lysis = [(ratio of targets to references without NK − ratio of targets to references with

NK) / ratio of targets to references without NK] × 100. The experiment was carried out in triplicate and repeated three times.

Statistical analysis. Genotype frequencies were tested for the Hardy–Weinberg equilibrium for both patients and controls using the χ^2-test. The same test was applied to compare the allele and genotype frequencies between the two groups. Odds ratios with 95% confidence intervals were calculated to estimate the relative risk. Other data were compared between two groups using an independent Students' *t*-test (two-tailed). Statistical analysis was carried out with SPSS version 18.0 software (IBM, New York, USA). Differences were considered significant when $P < 0.05$.

Results

Frequency of *HLA-E*0103* allele is increased significantly in SOC patients. To explore the association between HLA-E alleles and SOC, 85 patients and 100 age-matched healthy women were recruited to detect allelic variations in the 107 Arg/Gly codon. The genotypic distributions were consistent with the Hardy–Weinberg equilibrium in both controls and patients. The frequency of the *HLA-E*0101*0101* homozygote in patients was much lower compared with healthy controls. If both heterozygote and homozygote for *HLA-E*0103* are combined, then the difference between patients and controls becomes more significant ($P = 0.004$). Alleles of *HLA-E*0101* and *HLA-E*0103* displayed close frequencies in healthy controls: *HLA-E*0101*, 47%; *HLA-E*0103*, 53% (Table 1), which was comparable to those previously reported in a Chinese Han population.[18] However, *HLA-E*0103* showed much higher frequency than *HLA-E*0101* in the group of cases ($P = 0.017$, odds ratio = 1.668 [1.096–2.540]; Table 1). These results suggested that the *HLA-E*0103* allele was associated with susceptibility to SOC.

Table 1. Human leukocyte antigen-E (HLA-E) genotype and allele frequencies in serous ovarian cancer (SOC) patients and healthy controls

HLA-E polymorphism	SOC patients ($n = 85$)	Healthy controls ($n = 100$)	*P*-value	OR (95% CI)
Genotypes				
HLA-E*0101*0101	8	26	0.015	–
HLA-E*0101*0103	43	42		–
HLA-E*0103*0103	34	32		–
Alleles (%)				
HLA-E*0101	59 (34.7)	94 (47)	0.017	0.599 (0.394–0.913)
HLA-E*0103	111 (65.3)	106 (53)		1.668 (1.096–2.540)

–, Not compared; CI, confidence interval; OR, odds ratio.

HLA-E expression in different *HLA-E* genotypes of SOC. HLA-E is frequently overexpressed in many types of tumor, including ovarian cancer,[13] but whether *HLA-E* polymorphism will have an impact on HLA-E expression is unknown. As analyzed above, the *HLA-E*0103* allele was a risk factor that may induce susceptibility to SOC, so we compared the level of HLA-E expression among patients with different *HLA-E* genotypes including eight homozygotes for *HLA-E*0101*, 10 heterozygotes for *HLA-E*0103*, and 10 homozygotes for *HLA-E*0103*. These patients were all chosen from the identified patients above, and they were at FIGO stage IIIc. Correspondingly, three HLA-E genotypes of normal ovary tissues were collected from patients with benign gynecological diseases. Table 2 summarizes the HLA-E expression in different groups. The normal ovary tissues were all negative for HLA-E expression, and the SOC all showed positive to different extents. Among the three genotypes, all the *HLA-E*0103*0103* homozygotes in SOC showed the strongest HLA-E expression, and 60% (6/10) of samples of *HLA-E*0101*0103* heterozygotes had stronger expression than *HLA-E*0101*0101* homozygotes. Examples of negative and positive HLA-E expression patterns are depicted in Figure 1. These results showed that the *HLA-E*0103* allele might promote HLA-E protein expression in SOC tissues.

Effects of *HLA-E*0103* allele on HLA-E cell surface expression and peptide binding. Previous evidence had indicated that cell surface expression of HLA-E molecules contributed to immune escape of tumor cells.[19] Therefore, we examined the *HLA-E* alleles for differential surface expression levels of HLA-E when they were transfected into K562 cells. A human erythroleukemic cell line K562 was used because of it expresses neither classical nor non-classical MHC class I molecules. Considering the follow-up assay by flow cytometry, EGFP was also transfected into K562 cells to indicate living cells. As shown in Figure 2(a–c), HLA-E expression in the transfected cells (K562G-E*0101 and K562G-E*0103) was detected by qRT-PCR and Western blot, showing that the overall levels of the two allelic heavy chains of HLA-E were roughly equal. HLA-E cell surface expression was analyzed by flow cytometry with the 3D12-APC mAb. Figure 2(d,e) shows that, in the absence of MHC class I-derived peptides, the cell counts of K562G-E*0103 with HLA-E surface expression were much higher than those of K562G-E*0101. Under physiological conditions, cell surface expression of HLA-E depends on the binding of nonamer peptides derived from the signal sequence of MHC class I molecules.[20] In this study, signal peptides of HLA-B7 (B7sp), the predominant peptides presented by HLA-E, were used as exogenous peptides. When enough B7sp peptides were available at 26°C, enhancement of cell surface HLA-E expression was seen with no differences between the

Table 2. Human leukocyte antigen-E (HLA-E) expression in serous ovarian cancer (SOC) and the controls (non-SOC) tissues with different *HLA-E* genotypes

HLA-E expression	SOC			Non-SOC		
	HLA-E*0101*0101	HLA-E*0101*0103	HLA-E*0103*0103	HLA-E*0101*0101	HLA-E*0101*0103	HLA-E*0103*0103
Negative	0	0	0	10	10	10
Weak	5	1	0	0	0	0
Moderate	3	3	0	0	0	0
Strong	0	6	10	0	0	0

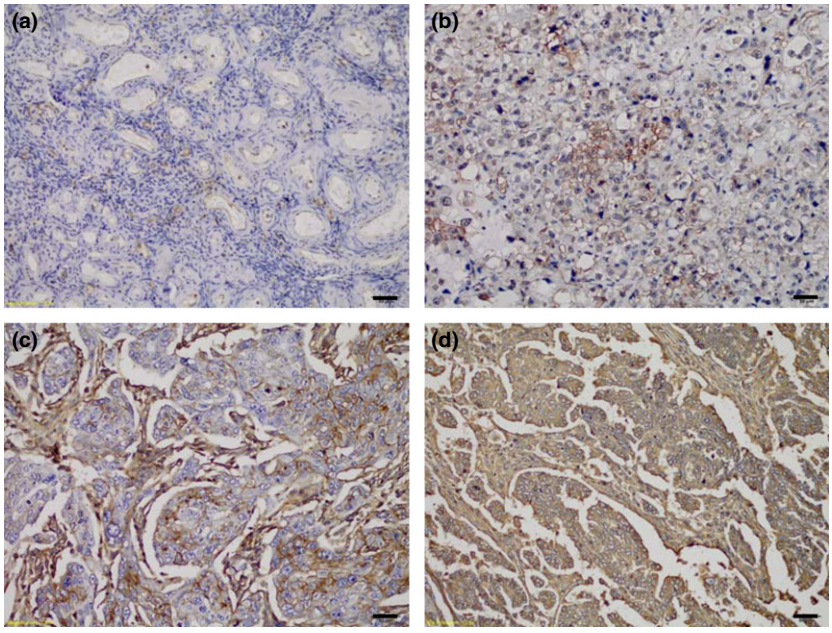

Fig. 1. Samples with different cytoplasmic staining intensities against human leukocyte antigen-E (HLA-E) protein by immunohistochemical analysis. (a) Negative HLA-E expression in the control tissue. (b) Weak HLA-E expression in serous ovarian cancer (SOC) with the *HLA-E*0101*0101* genotype. (c) Moderate HLA-E expression in SOC with the *HLA-E*0101*0103* genotype. (d) Strong HLA-E expression in SOC with the *HLA-E*0103*0103* genotype. Bar = 50 μm.

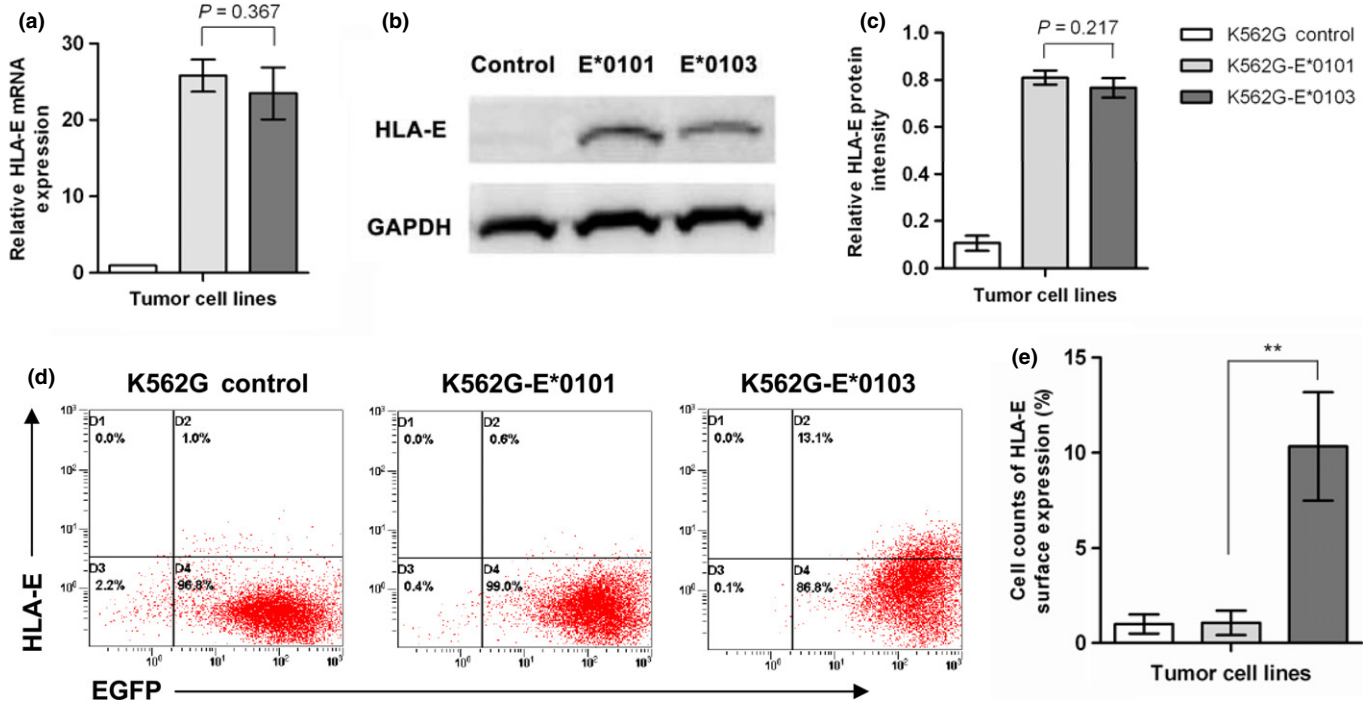

Fig. 2. Expression levels of human leukocyte antigen-E (HLA-E) in transfected K562 cells. (a) Relative HLA-E mRNA expression was assessed using quantitative real-time PCR assay. The data were normalized to the gene expression of K562G using the standard $2^{-\Delta\Delta C_t}$ method. (b) Examples of HLA-E protein intensity assayed by Western blot. (c) Expression level of HLA-E protein was normalized against that of GAPDH. (d) Examples of HLA-E cell surface expression detected by flow cytometry. Cells were stained with 3D12-APC mAb after incubation for 16 h at 26°C. Both HLA-E and EGFP positive cells represent living cells expressing HLA-E molecule on the cell surface. (e) Percentage of cells with HLA-E surface expression in the transfected cells. All results were expressed as mean ± SD for three replicate experiments. $^{**}P < 0.01$. K562G Control, K562 cells transfected with EGFP and pLL3.7; K562G-E*0101, K562 cells transfected with EGFP and pLL3.7-HLA-E (*HLA-E*0101* allele); K562G-E*0103, K562 cells transfected with EGFP and pLL3.7-HLA-E (*HLA-E*0103* allele).

two alleles. However, the thermal stability of HLA-E/B7sp complex on the surface of K562G-E*0103 was significantly higher than that of K562G-E*0101 cells. As shown in Figure 3, when the cells binding with B7sp were incubated at 37°C for 2 h with or without Brefeldin A, HLA-E surface expression on K562G-E*0101 cells was decreased significantly, whereas there were no differences on the surface of K562G-E*0103 cells.

Roles of HLA-E molecule with HLA-E*0103 allele in protecting tumor cells from NK lysis. As the *HLA-E*0103* allele made

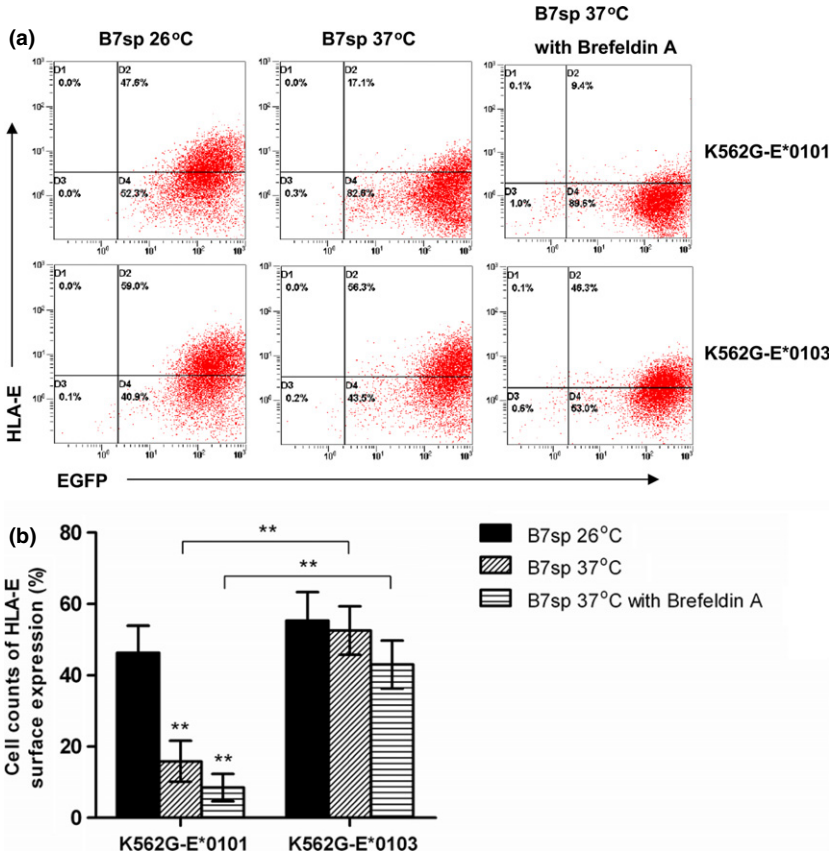

Fig. 3. Peptide binding and complex stability were analyzed by flow cytometry. (a) To detect the ability of human leukocyte antigen-E (HLA-E) to bind with peptides, cells were stained with 3D12-APC mAb after incubation with 100 μM B7sp peptides for 16 h at 26°C. For HLA-E/B7sp complex thermal stability assay, cells were incubated with 100 μM B7sp peptides for 16 h at 26°C, then further incubated at 37°C for 2 h with or without 1000 ng/mL Brefeldin A. Antibody 3D12-APC mAb was also used for staining. (b) Results were expressed as mean ± SD for three replicate experiments. $^{**}P < 0.01$.

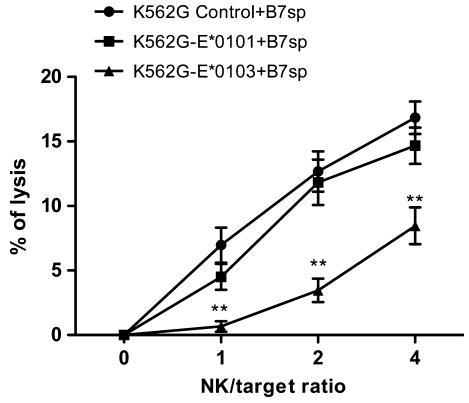

Fig. 4. Cells transfected with the *HLA-E*0103* allele escaped from natural killer (NK) lysis. Target cells were cocultured with NK cells at 37°C after incubation for 16 h at 26°C with B7sp peptides. After another 2 h, the percentage of NK lysis was calculated. Results were expressed as mean ± SD for three replicate experiments. $^{**}P < 0.01$ *versus* K562G-E*0101 + B7sp.

the stability of HLA-E surface expression higher than the *HLA-E*0101* allele, we hypothesized whether different levels of surface expression of HLA-E could alter its ability to protect cells from NK lysis. As shown in Figure 4, in the presence of exogenous B7sp peptides, K562G control cells without HLA-E protein expression were sensitive to NK lysis. HLA-E molecules with the *HLA-E*0103* allele could inhibit NK lysis more obviously than the molecules with the *HLA-E*0101* allele. These results indicated that high levels of HLA-E surface expression binding with proper peptides enhanced the ability to protect tumor cells from NK lysis.

Discussion

Ovarian cancer is the deadliest of the gynecological malignancies. The high mortality rate is thought to result from delayed diagnosis owing to generally vague presenting symptoms, advanced stage at diagnosis, and adverse underlying biologic features of the tumor.[21,22] HLA-E is frequently overexpressed in ovarian cancer and associated with poor prognosis.[13,23] The primary cause is that the HLA-E molecule can present peptides to inhibitory CD94/NKG2A receptors on NK cells, providing a mechanism for tumor cells to escape from immune surveillance.[24] In this study, we found the association between *HLA-E* polymorphisms and ovarian cancer. The frequency of the *HLA-E*0103* allele was much higher in SOC patients than in healthy women (Table 1). This result is comparable to the study in nasopharyngeal carcinoma, which reported that the frequency of the *HLA-E*0103* allele was increased in nasopharyngeal carcinoma patients.[25] In this regard, the HLA-E expression differences in three genotypes of SOC patients were verified. Our study showed a correlation between the *HLA-E*0103* allele and high levels of HLA-E protein expression in SOC tissues (Table 2). Thus, this result may be an explanation for the association between the *HLA-E*0103* allele and genetic susceptibility to SOC. However, a study reported that *HLA-E* polymorphisms did not have an impact on HLA-E expression in colorectal cancer.[26] The authors did not detect the differential expression levels of HLA-E on the surface of tumor cells, therefore it is still

unknown whether *HLA-E* polymorphisms play a role in colorectal cancer. Strong *et al.*[27] showed that the HLA-E*0103 molecule has a higher affinity for most peptides compared to the HLA-E*0101 molecule. Differences of over two orders of magnitude were apparent between the two alleles when B7sp was used. In our study, an excessive B7sp concentration was used, so the two HLA-E molecules achieved maximum assembly with no differences (Fig. 3). The arginine-to-glycine substitution resulted in the elimination of a hydrogen bond between the side chains of Arg^{107} and His^{3} and in the apparent, slight rearrangement of the hydrogen bond network around the residues surrounding position 107 in the E^{R} *versus* E^{G} structures. The E^{G} complex has a significantly higher melting temperature over E^{R} regardless of the peptide bound.[27] In this study, the thermal stability of the HLA-E/B7sp complex on the cell surface was measured and showed that the HLA-E*0103 molecule produced more stable binding (Fig. 3). The differences in thermal stability were consistent with and could elucidate the functional differences in NK lysis (Fig. 4).

Downregulation of HLA-A, -B, and -C has been viewed as a major subterfuge to deceive T cells, but it is unlikely to provide a comprehensive explanation of immune evasion, as it impairs ligand donation to HLA-E and the direct engagement of inhibitory NK receptors. Under physiological conditions, the surface expression of HLA-E depends on the binding of sequence peptides derived from newly synthesized MHC class Ia molecules, otherwise it will be degraded in the endoplasmic reticulum.[20] Recently, some investigations reported that tumor cells could produce alternative peptides showing homology with signal peptides of MHC class Ia molecules.[28,29] In this study, the HLA-E transfected cells with the *HLA-E*0103* allele had high levels of HLA-E cell surface expression in the absence of MHC class Ia molecules (Fig. 2). It was possible that some unknown peptides would be presented by HLA-E molecules, and the *HLA-E*0103* allele had a higher affinity for these peptides. The difference was apparently not due to higher overall levels of HLA-E protein in these cells, as evidenced by Western blot analysis measuring relative levels of the two allelic heavy chains as roughly equal (Fig. 2). However, CD94/NKG2A expression can be induced in response to cytokines such as interleukin-15[30] and transforming growth factor β.[31,32] These cytokines are frequently present in the tumor microenvironment. Hence, the overexpression of HLA-E is a mechanism for tumor cells to evade immunosurveillance even in the absence of MHC class I molecules.

In summary, the *HLA-E*0103* allele is associated with susceptibility to SOC. One possible mechanism is that the elevated expression of total and cell surface HLA-E molecule with the *HLA-E*0103* allele increases the inhibitory effect on NK cell lysis, allowing tumor cells to escape from NK immune surveillance.

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Disclosure Statement

The authors have no conflict of interest.

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