

# Classical HLA class I antigen downregulation and abnormal HLA-G expression in ovarian cancer

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**Objective:** To elucidate the potential role of HLA-G and classical HLA class I molecules in ovarian cancer, we researched their patterns of expression in benign and malignant ovarian tumors. **Methods:** In 10 benign and 33 malignant ovarian tumor tissues, HLA-G expression was determined both at the mRNA level by reverse transcriptase-polymerase chain reaction and immunohistochemical staining. The expression of classical HLA class I heavy chains were determined immunohistochemically. **Results:** Immunohistochemical analysis revealed the expression of HLA-G molecules in 27 of the 33 (81.8%) ovarian cancers but in none of the benign ovarian tumors. Classical HLA class I antigen expression was down-regulated in 25 out of the 33 (75.8%) ovarian cancers and in only 1 of the 10 benign ovarian tumors. HLA-G expression and classical HLA class I antigen down-regulation were related to disease stage (Spearman's rho = 0.468,  $P = 0.001$ ; Spearman's rho = -0.392,  $P = 0.005$ ). **Conclusion:** Our results reveal that abnormal expression of HLA-G and down-regulation of classical HLA class I antigen in ovarian cancer may be one of the mechanisms by which cancer cells may escape host's immune system.

## Keywords

Ovarian cancer; Immune escape; Human leukocyte antigen class I molecule; Human leukocyte antigen-G

## 1. Introduction

Ovarian cancer is one of the most intractable gynecologic malignancies. Unfortunately, ovarian cancer does not commonly show any signs or symptoms and it is diagnosed as being already progressed in approximately 75% of patients. Despite aggressive treatment, such as cytoreductive surgery followed by adjuvant chemotherapy, the prognosis of advanced ovarian cancer remains poor. The primary reasons why ovarian cancer is difficult to treat are the imperfect understanding of its etiology and the very few available diagnostic molecular markers and therapeutic targets. To date, various modalities for the early diagnosis and treatment of ovarian cancer have been examined [1–3].

Human leukocyte antigen (HLA) bind-antigenic peptides derived mostly, but not exclusively from endogenous proteins. Following their expression on cell surface, HLA class I antigen complexes interact with T-lymphocyte receptors

and thereby activates the immune system. HLA molecules present tumor-specific antigens, non-self antigens from the interior of the cell, as well as antigens from invaders outside the cell. These antigens are recognized by the immunosurveillance system and then removed. Because HLA molecules present tumor-antigens, the phenotypic transformation and debilitation of the immunosurveillance system seems to be mandatory for the development and growth of the tumor [4, 5].

Defects in classical HLA class I molecule expression have been reported in many types of tumor cells [5, 6]. Once classical HLA class I antigen expression has been completely lost, cancer cells are not recognized and lysed by cognate cytotoxic lymphocytes (CTLs). However, this suppression activates natural killer (NK) cells and thereby triggers NK cell-mediated lysis [7]. Therefore, the survival of the tumor cells requires additional mechanisms to prevent NK cell-induced lysis. Recent studies have shown that HLA-G, one of the non-classical HLA class I molecules, protects the cells from the lysis by NK cells. Moreover, it does not mediate the autologous immune responses by T lymphocytes present in peripheral blood [8].

HLA-G was first identified as being expressed in extravillous cytotrophoblast cells, and has been reported to play a key role in providing the fetus, a semi-allograft, with immune tolerance from the maternal immune system [9]. According to recent studies, HLA-G is also expressed in various types of malignant cancers, including breast cancer, colorectal cancer, lung cancer, melanoma, and leukemia [10–14]. In terms of HLA-G expression in ovarian cancer, Malmberg *et al.* [15] reported that HLA-G expression gradually decreased in cells forming ovarian cancer tissues when cultured for a short-term period. Moreover, Singer *et al.* [16] reported that 61% of ovarian serous carcinomas demonstrated HLA-G immunoreactivity on the basis of immunohistochemistry. To date, however, no studies have examined the potential clinical relevance of HLA-G expression in combination with classical HLA class I antigens in ovarian cancer. In the present

study, HLA-G and classical HLA class I antigen expression was examined at the transcript levels in benign and malignant ovarian tumors. In addition, an analysis was performed to identify correlations between their expression levels and clinical characteristics to examine whether HLA-G expression could be further used to clarify the immune escape mechanisms utilized by tumor cells in ovarian cancer patients.

## 2. Materials and methods

### 2.1 Ovarian cancer tissue

Benign ovarian tumor tissue and malignant ovarian tumor tissue were sampled from 10 and 33 patients, respectively, who underwent surgery to treat ovarian tumors at Seoul St. Mary's Hospital from March 2012 to December of 2016. The tissue samples were stored in liquid nitrogen (-70 °C) until further use. None of the patients underwent preoperative radiotherapy or anti-cancer chemotherapy. The histological classification of the tumors was based on the World Health Organization (WHO) system [17]; the staging of the disease was based on the International Federation of Gynecologists and Obstetricians (FIGO) staging system [18].

The age distribution of the 10 patients with benign ovarian tumors ranged from 16-76 years. Based on histopathological findings, 1 patient had a Brenner tumor; 5, serous cystadenoma; 1, serous cystadenofibroma; and 3, mucinous cystadenoma. The 33 patients with malignant ovarian tumor had an age distribution ranging from 25 to 73 years. Histopathologically, 20 patients had serous cystadenocarcinoma; 5, mucinous cystadenocarcinoma; 5, endometrioid carcinoma; 1, a mixed germ-cell tumor, 1, a clear-cell carcinoma, and 1, a small-cell carcinoma. Based on the degree of histological differentiation [19], there were 3 well-differentiated cases, 13 moderately differentiated cases, and 17 poorly differentiated cases. Meanwhile, based on the FIGO staging system, 9 cases of Ia, 1 case of Ib, 1 case of Ic, 1 case of IIc, 1 case of IIIa, and 20 cases of IIIc were observed. In this study, the choriocarcinoma cell line JEG-3 served as the positive control because HLA-G is expressed in this cell line, whereas the human leukemia cell line K562 was used as the negative control because it lacks HLA-G expression.

### 2.2 Monoclonal antibodies

The monoclonal antibody (mAb), HC-10 [20], which recognizes  $\beta$ 2m-free HLA-A3, -A10, -A28, -A29, -A30, -A31, -A32, -A33, and all  $\beta$ 2m-free -HLA-B (excluding -B5702, -B5804, and -B73) and -HLA-C heavy chains was developed and characterized as described [21–23]. mAb was purified from ascitic fluid by affinity chromatography on Protein A. Activity and purity of mAb preparations were monitored by binding assays and SDS-PAGE. mAb MEM-G1 [24] which recognizes denatured HLA-G heavy chain was purchased from Abcam (Cambridge, MA, USA).

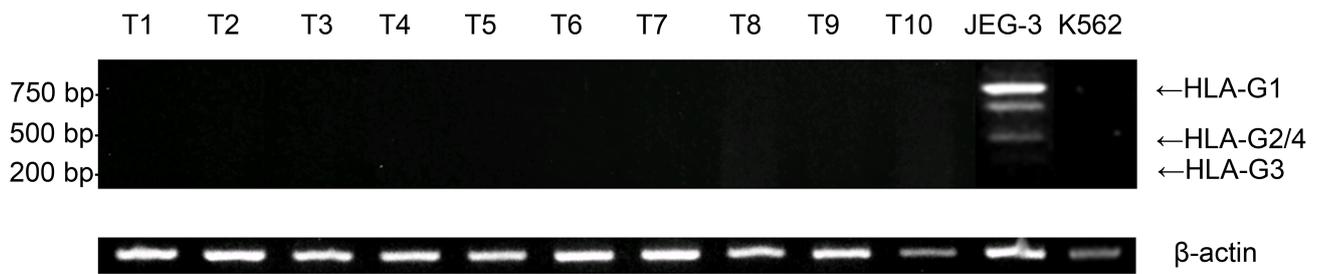
### 2.3 RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

RNA was extracted using the RNeasy mini kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. First-strand cDNA synthesis was performed using 2  $\mu$ g of RNA and avian myeloblastosis reverse transcriptase (first-strand synthesis kit for RT-PCR, Mannheim, Germany). For the synthesis of cDNA, annealing was performed at 25 °C for 10 minutes, and an extension was performed at 42 °C for 90 minutes. For inactivating the reverse transcriptase, the sample was maintained at 94 °C for 5 minutes in a Takara MP thermal cycler (Shiga, Japan). The Pan-HLA-G primer set was used for the amplification of HLA-G alternative transcripts, while the  $\beta$ -actin primer set was used to amplify  $\beta$ -actin as an internal standard [25]. Both primer sets were purchased in a customized order from Genotech (Seoul, Korea) for the current experimental procedures. For the amplification of each cDNA, 1  $\mu$ L of cDNA generated through the RT-PCR was added to a mixture containing 10  $\mu$ L of 1  $\times$  reaction buffer (10 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.1% Triton X-100; Boehringer Mannheim), 2.5  $\mu$ L of 2.5 mM dNTPs, 0.5  $\mu$ L of sense primer, 0.5  $\mu$ L of antisense primer, and a 1.25 U of Taq DNA polymerase (Boehringer Mannheim). The reaction volume was brought to a total volume of 25  $\mu$ L using distilled water. The PCR conditions included denaturation for 1 minute at 94 °C, annealing for 1 minute at 62 °C for  $\beta$ -actin and for 2 minutes at 63 °C for HLA-G, and an extension for 2 minutes at 72 °C, with the respective cycles repeated 25 times for  $\beta$ -actin and 35 times for HLA-G. The PCR products were separated on a 2% agarose gel at 3–4 V/cm, stained using 0.5  $\mu$ g/mL ethidium bromide, and photographed using a UV transilluminator.

### 2.4 Immunohistochemical staining

Immunohistochemical staining was done with a CAP-Plus Detection Kit (ZYMED Laboratories Inc., San Francisco, CA, USA) using methods involving Streptavidin-biotin (sABC)/horse radish peroxidase (HRP) complexes. The samples were fixed using 10% neutral buffered formalin, and prepared paraffin-embedded tissue was sequentially sectioned at a thickness of 3  $\mu$ m. The sections were then placed on ProbeOn Plus slides (Fisher Scientific, Pittsburgh, PA, USA) previously coated with poly-L-lysine and dried. The slides were deparaffinized in 2 rinses (5 min each) of a mixture of xylene and Histo-clear II (National diagnostics, Atlanta, GA, USA) and then rinsed twice (5 minutes each) in 100% ethanol. Next, the slides were treated with a TBS solution (ScyTek Lab., Logan, UT, USA) with 3% H<sub>2</sub>O<sub>2</sub> at 45 °C for 10 minutes to remove endogenous peroxidase activity. The samples were rinsed with TBS, placed in 10 mM citrate buffer (pH 6.0; DakoCytomation), and autoclaved for 15 minutes.

For observing HLA-G expression, the slides were incubated with a 1 : 100 dilution of MEM-G1 at 4 °C for 24 hours. The samples were rinsed with TBS and then treated with biotinylated antimouse immunoglobulin antibody (ZYMED Laboratories Inc.) for 30 minutes at 45 °C. After being rinsed



**Fig. 1. Reverse transcription-polymerase chain reaction analysis of HLA-G in benign ovarian tumor tissues from patient T1-10.** HLA-G alternative transcripts were detected by amplification using pan-HLA-G primers, G-257 and G-1004. As a positive control, JEG-3 cell line were used. Beta-actin gene amplification was used as an internal control.

**Table 1. Clinical, histological and immunochemical data in 10 benign ovarian tumor patients.**

No	Age	Tumor Histology	RT-PCR (HLA-G transcripts)	IHC* (HLA-G Protein)	IHC* (HLA class I)
B1	57	Brenner tumor	-	0	3
B2	55	mucinous cystadenoma	-	0	4
B3	36	mucinous cystadenoma	-	0	4
B4	75	mucinous cystadenoma	-	0	4
B5	24	serous cystadenoma	-	0	4
B6	65	serous cystadenoma	-	0	4
B7	76	serous cystadenofibroma	-	0	4
B8	24	serous cystadenoma	-	0	4
B9	16	serous cystadenoma	-	0	4
B10	48	serous cystadenoma	-	0	4

RT-PCR, reverse transcription-polymerase chain reaction; IHC, immunohistochemistry; \*Scoring of immunohistochemical staining was performed as follows: 0, no reactivity; 1, staining confined to  $\leq$  5% of cell; 2, 6-25% of cell; 3, 26-50% of cell; 4, > 50% of cells were positive.

with TBS, the samples were incubated with streptavidin-HRP complex for 30 minutes. After the samples were rinsed with TBS, the color was developed using an aminoethylcarbazole (AEC) solution and counterstained with hematoxylin. For observing HLA class I expression, the samples were incubated with HC-10 (1 : 100 dilution) at 4 °C for 24 hours. The immunohistochemical staining was then performed in the same manner as that for HLA-G expression.

The immunohistochemical staining was recorded as light-microscopy images, and the findings were interpreted by one pathologist. For the exclusion of false-positive results resulting from the staining of areas adjacent to the tissue sample, the interpretation of the staining patterns was performed using the central area of the tissue sample. The staining properties were evaluated based on a 5-grade scoring system as follows: 0 points (< 1%), 1 point (1-5%), 2 points (6-25%), 3 points (26-50%), and 4 points (51-100%).

### 2.5 Statistical analysis

A correlation analysis of clinical parameters and the expression pattern of HLA class I and HLA-G molecules was performed using Spearman's rho, with the statistical significance evaluated using SPSS (version 13 for Window, SPSS Inc., Chicago, IL, USA). A value of  $P < 0.05$  was considered statistically significant.

### 2.6 Ethical approval

Written informed consent was obtained from all study subjects, and the study was approved by the Institutional Review Board of our institution on April 1st 2012 (KC12TISI0213).

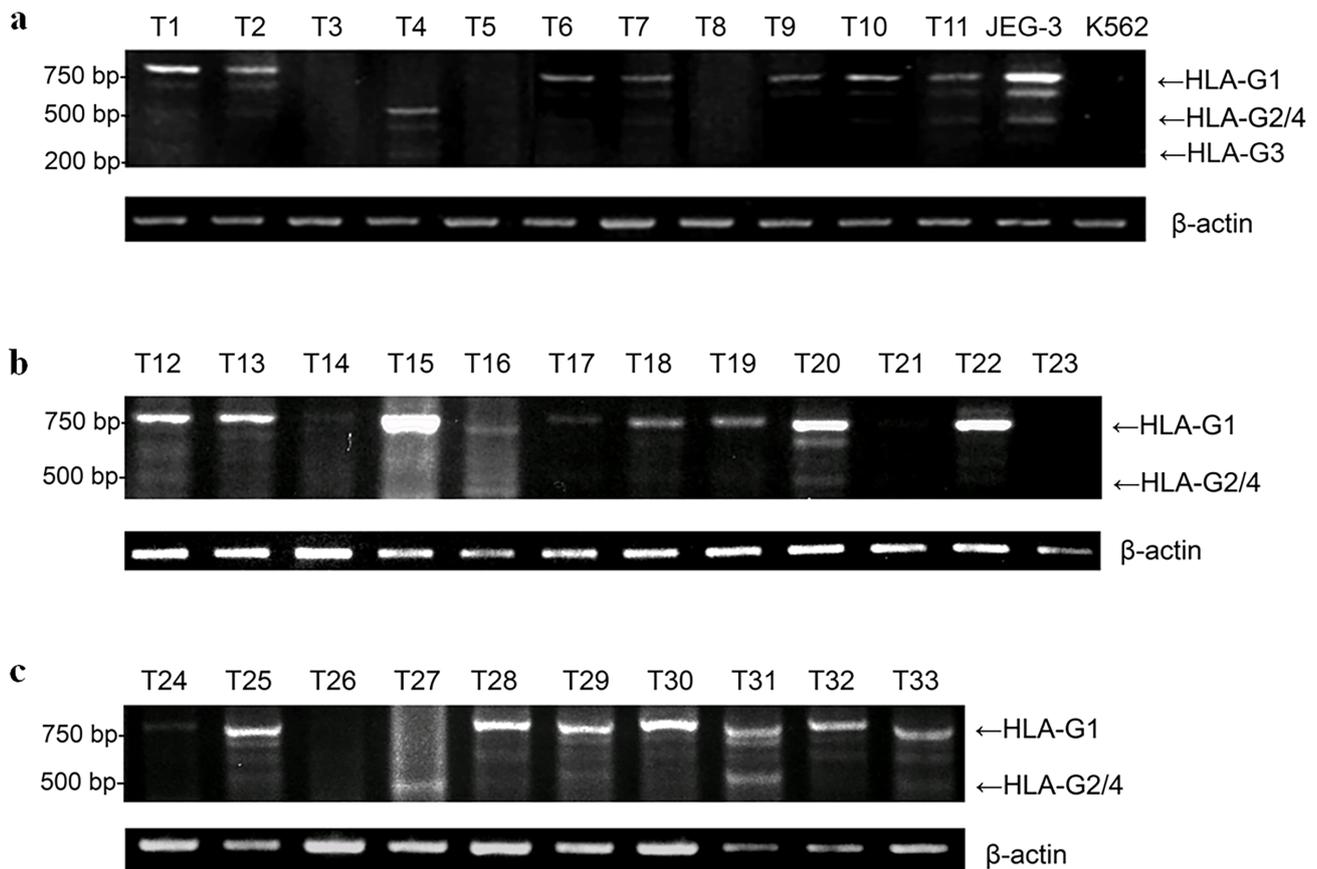
## 3. Results

### 3.1 The expression of HLA-G mRNA and HLA class I molecules in benign ovarian tumors

RT-PCR analysis was performed to examine the expression of HLA-G in benign ovarian tumors through the detection of the alternative transcripts HLA-G1, -G2, -G3 and -G4 at the previously reported sizes of 764 bp, 488 bp, 491 bp, and 215 bp, respectively [25, 26]. No HLA-G mRNA was detected in benign ovarian tumor tissue (Fig. 1), and immunohistochemical staining confirmed this lack of HLA-G mRNA expression. In terms of the expression of HLA class I protein, the Brenner tumor showed a moderate staining intensity and was assigned a score of 3 points, whereas the remaining cases were given a score of 4 points, corresponding to a higher degree of expression (Table 1).

### 3.2 Expression of HLA-G mRNA and protein and of HLA class I heavy chains in malignant ovarian tumor tissue

As shown by RT-PCR, HLA-G transcript was detected in 78.8% (26/33) of the ovarian cancer tissue samples. Of these



**Fig. 2. Reverse transcription-polymerase chain reaction analysis of HLA-G in ovarian cancer tissues from patient T1-33 (a, b and c).** HLA-G alternative transcripts were detected by amplification using pan-HLA-G primers, G-257 and G-1004. JEG-3 and K562 cell line were used as positive and negative controls, respectively. Beta-actin gene amplification was used as an internal control.

26 samples, 4 showed the expression of both HLA-G1 and HLA-G2/G4. Meanwhile, only HLA-G1 was observed in 20 cases, while only HLA-G2/G4 was detected in 2 cases. By contrast, none of the cases showed the expression of HLA-G3 (Table 2, Fig. 2). Neither the alternative transcripts (i.e., HLA-G1 and HLA-G2/G4) nor their synchronous expression showed any relationship with the histologic classification (tumor histology), tumor grade, or surgical stages. HLA-G protein was expressed at differing levels in 81.8% (27/33) of the malignant ovarian tumor tissue samples, but was not detected in 6 cases (Table 2, Fig. 3). With the exception of 1 tumor showing no HLA-G mRNA transcripts but protein expression, these results were in agreement with those of RT-PCR analysis.

The loss of HLA class I expression in the malignant ovarian tumor tissue samples showed a heterogeneous pattern (Table 2, Fig. 4). Of the 33 tumors tested, 6 (18.2%) had complete loss of expression, where 19 cases (57.6%) showed a loss of > 50%.

Next, the scores for the expression of HLA-G were averaged according to the histologic grade of the tumor and the surgical stage (Fig. 5a,b). The malignant tumors were classified as early (Ia-IIc) and advanced (> IIIa). For both the

histologic grade and the surgical stages, the scores for HLA-G expression in the malignant tumors were increased relative to those in the benign tumors. Moreover, among the malignant tumors, the scores were significantly increased in cases in which the degree of histologic differentiation was poor (Spearman's rho = 0.489,  $P = 0.001$ ) (Fig. 5c) or the tumor stage was advanced (Spearman's rho = 0.468,  $P = 0.001$ ) (Fig. 5d).

The scores for the expression of HLA class I were also averaged according to the histologic grade of the tumor and the surgical stages (Fig. 6a,b). For both the histologic grade and the surgical stages, the scores for the expression of HLA class I molecules were meaningfully decreased in the malignant tumors compared to those in the benign ones. Among the malignant tumors, the scores were significantly decreased in cases in which the degree of histologic differentiation was poor (Spearman's rho = -0.347,  $P = 0.015$ ) (Fig. 6c) or the disease stage was more advanced (Spearman's rho = -0.392,  $P = 0.005$ ) (Fig. 6d).

Next, we examined whether the decreased expression of HLA class I proteins was correlated with the increased expression of HLA-G in cases in which the degree of tumor differentiation was poor or the surgical stage was advanced.

**Table 2. Clinical, histological and immunochemical data in 33 ovarian cancer patients.**

No	Age	Tumor Histology	Tumor grade	Stage	RT-PCR	IHC*	IHC*
					(HLA-G transcripts)	(HLA-G Protein)	(HLA class I)
C1	73	SCC	PD	Ib	+(G1)	1	2
C2	59	SCC	PD	IIIc	+(G1)	1	3
C3	50	SCC	MD	Ia	-	0	2
C4	45	SCC	PD	IIc	+(G2/G4)	4	4
C5	47	CCC	MD	Ia	-	0	3
C6	53	SCC	PD	IIIc	+(G1)	3	3
C7	72	SCC	MD	IIIc	+(G1)	1	2
C8	64	SCC	MD	IIIc	-	2	2
C9	53	SCC	MD	IIIc	+(G1)	4	3
C10	65	MCC	MD	IIIc	+(G1)	4	4
C11	61	MCC	WD	IIIc	+(G1, G2/G4)	1	2
C12	48	EC	MD	Ia	+(G1)	3	3
C13	44	SCC	WD	IIIc	+(G1)	2	3
C14	50	SCC	PD	Ia	-	0	4
C15	56	SCC	PD	IIIc	+(G1)	2	4
C16	51	EC	MD	Ia	+(G1)	1	4
C17	54	SCC	PD	Ia	+(G1)	4	3
C18	65	SCC	PD	IIIc	+(G1)	1	3
C19	44	SCC	PD	IIIc	+(G1)	2	3
C20	45	MCC	MD	Ia	+(G1, G2/G4)	1	4
C21	60	EC	PD	Ia	-	0	0
C22	46	MCC	MD	Ic	+(G1)	2	2
C23	66	SmCC	PD	IIIc	-	0	0
C24	25	MGT	WD	Ia	+(G1)	3	0
C25	39	SCC	PD	IIIc	+(G1)	1	3
C26	45	MCC	MD	IIIc	-	0	4
C27	58	EC	PD	IIIa	+(G2/G4)	3	3
C28	38	EC	PD	IIIc	+(G1)	2	0
C29	59	SCC	PD	IIIc	+(G1)	1	0
C30	66	SCC	PD	IIIc	+(G1)	3	1
C31	45	SCC	MD	IIIc	+(G1, G2/G4)	4	4
C32	61	SCC	PD	IIIc	+(G1)	1	1
C33	69	SCC	MD	IIIc	+(G1, G2/G4)	3	0

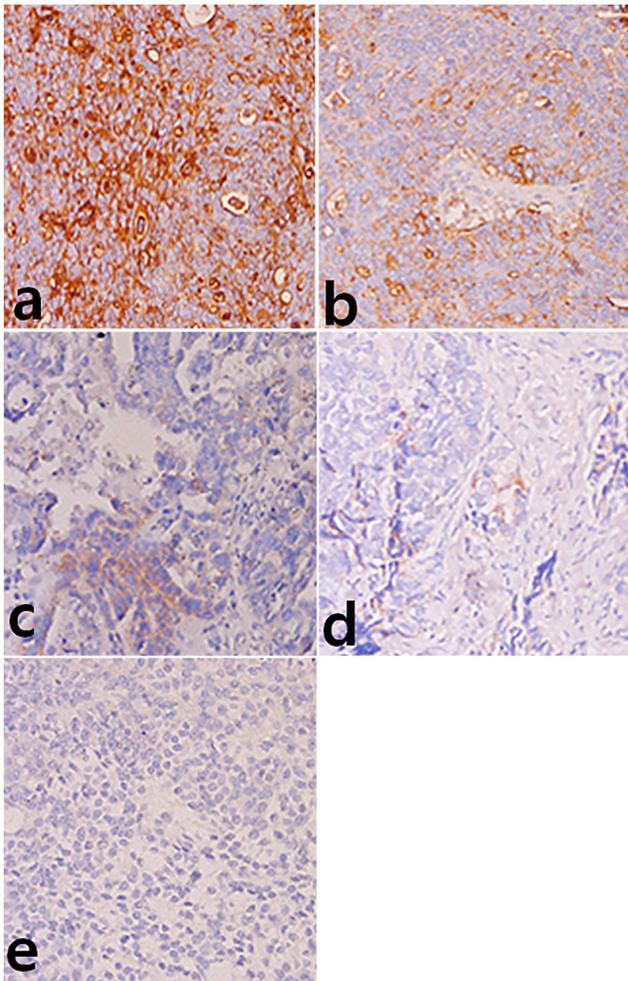
RT-PCR, reverse transcription-polymerase chain reaction; IHC, immunohistochemistry; SCC, serous cystadenocarcinoma; MCC, mucinous cystadenocarcinoma; EC, endometrioid carcinoma; CCC, clear cell carcinoma, MGT, mixed germ cell tumor. SmCC, small cell carcinoma; WD, well differentiated, MD, moderately differentiated, PD, poorly differentiated; \*Scoring of immunohisto-chemical staining was performed as follows: 0, no reactivity; 1, staining confined to  $\leq$  5% of cell; 2, 6-25% of cell; 3, 26-50% of cell; 4, > 50% of cells were positive.

To do this, a direct correlation analysis was performed between the expression of HLA class I and HLA-G molecules. No statistical significance was observed (Spearman's rho = -0.109,  $P = 0.255$ ). However, upon exclusion of the cases in which the degree of differentiation was poor, the degree of correlation was increased, but was not statistically significant (Spearman's rho = -0.330,  $P = 0.062$ ) (Fig. 7).

#### 4. Discussion

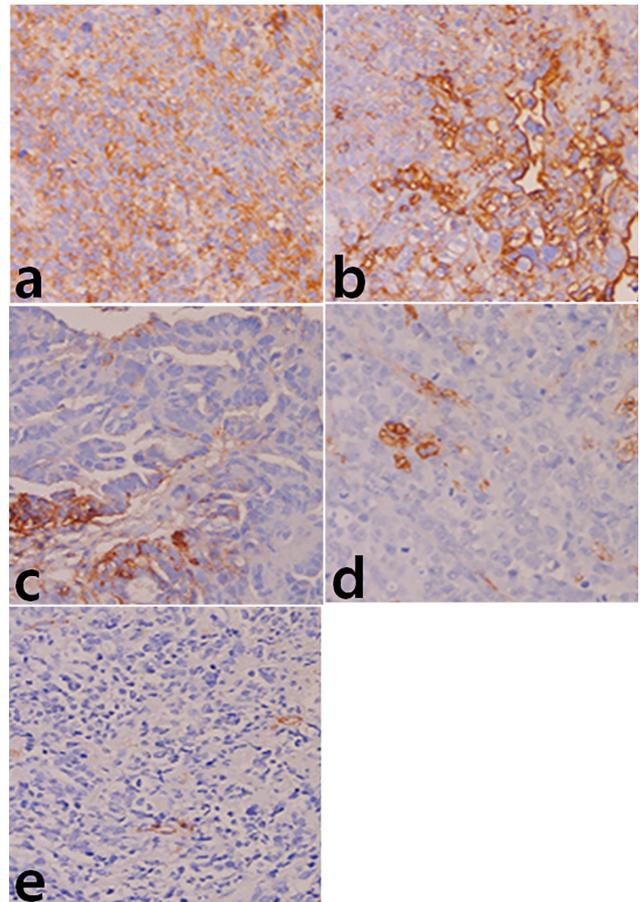
HLA plays an essential role in the human immunosurveillance system, and changes in its expression are essential in the suppression and formation of tumors. The abnormal expression of HLA class I molecules enables the immune escape of tumor cells through the failure to present tumor-specific

antigens to CTLs. In various tumors, such as breast cancer, head-and-neck cancers, lung cancer, colorectal cancer, uterine cervical cancer, and melanoma, a loss in the expression of HLA class I molecules has been reported to occur in 39%-88% of patients [6, 27]. In the current study, a decreased expression of HLA class I molecules was almost not observed in the benign ovarian tumors but was completely lost in 18.2% of the malignant cancers. Moreover, HLA class I expression was lost by more than 50% in 57.6% of the malignant cancers. In total, 75.8% of the malignant tumor cases showed abnormal expression of HLA class I molecules. These results are in agreement with those by Vitale *et al.* [28], who observed a loss of HLA class I expression in 37.3% of patients with primary ovarian tumor. Several authors have examined



**Fig. 3. Immunohistochemical analysis of HLA-G expression in ovarian cancer tissues.** In a, > 50% of tumor cells within ovarian cancer cell lesion of patient T4 were stained using MEM-G1 monoclonal antibody. In b, c, and d, 26-50% of tumor cells from patient T6, 5-25% of tumor cells from patient T8, and < 5% of tumor cells from T16 were stained, respectively. In contrast, tumor cells from patient T23 (e) were not stained. All photographs were taken at magnification of  $\times 200$ .

whether the expression of HLA class I molecules decreased as the tumors progressed from a benign state to a malignant one, but the results have differed among studies. According to Lee *et al.* [29], HLA class I expression in metastatic ovarian cancers was decreased relative to that in normal tissue. However, these authors noted that the decreased expression was not statistically significant. By contrast, Vitale *et al.* [28] reported that the expression of HLA class I molecules was decreased in advanced tumors with a poor degree of differentiation in a high-risk group of patients when compared to that in tumors in a low-risk group of patients. In the current study, depending on the degree of differentiation and the progression of the disease stage, HLA class I expression was significantly decreased. These results indicate that decreased HLA class I expression might play a key role in the infiltrative progression of ovarian tumor cells and may thereby function as one of the possible mechanisms of immune escape [30].

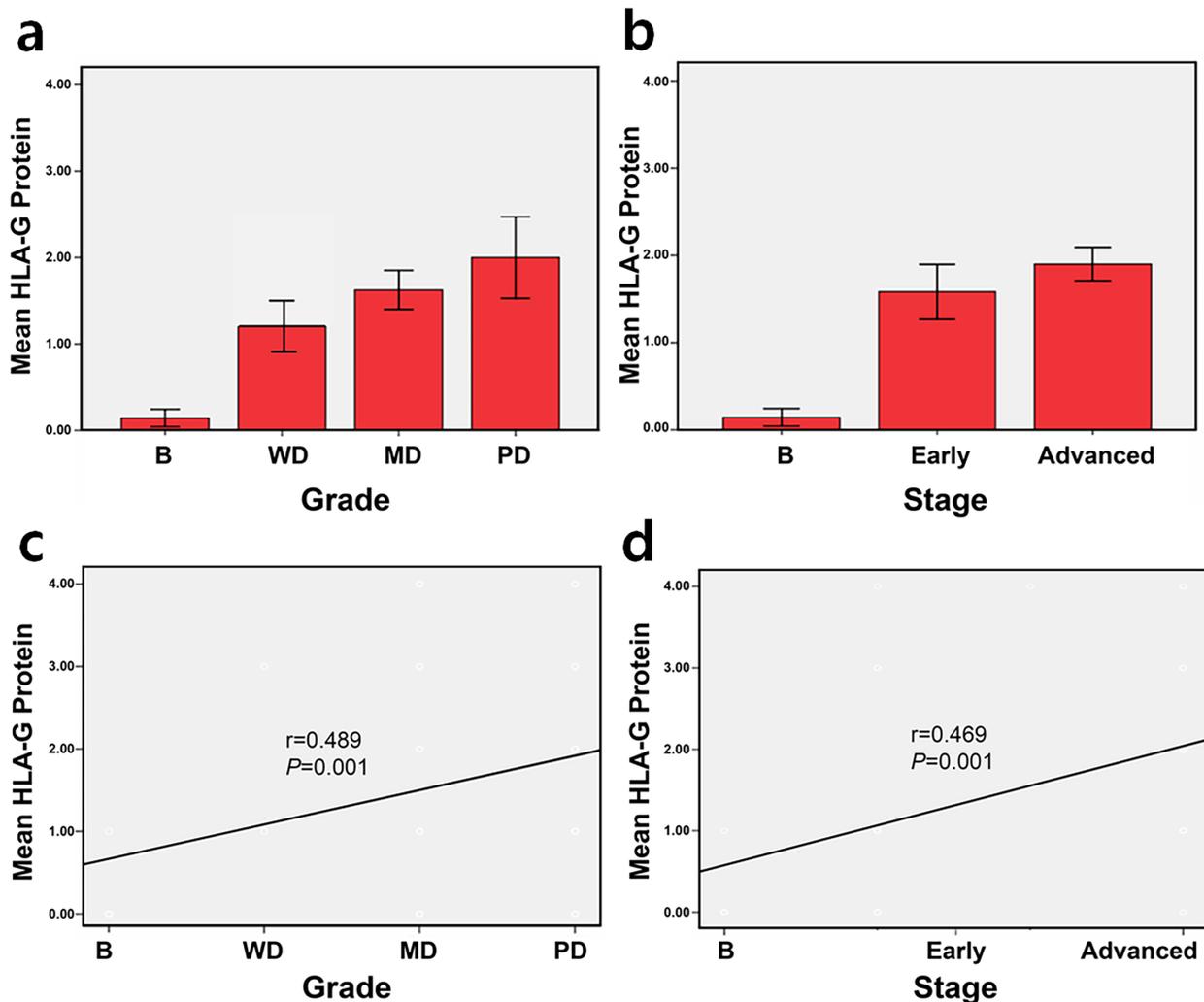


**Fig. 4. Immunohistochemical analysis of HLA class I expression in ovarian cancer tissues.** In a, > 50% of tumor cells within ovarian cancer cell lesion of patient T4 were stained using HC-10 monoclonal antibody. In b, c, and d, 26-50% of tumor cells from patient T6, 5-25% of tumor cells from patient T8, and < 5% of tumor cells from T32 were stained, respectively. In contrast, tumor cells from patient T23 (e) were not stained. All photographs were taken at magnification of  $\times 200$ .

Through the abnormal expression of HLA class I molecules, tumor cells can avoid the immune responses resulting from CTLs. Nevertheless, a complete loss of HLA class I expression would enhance the sensitivity of tumor cells to NK cells, since HLA class I proteins are ligands effectively bound by killer inhibitory receptors on the surface of NK cells [7].

Despite the immune surveillance functions of NK cells, tumor cells show a metastasizing tendency, implying that tumor cells have additional mechanisms for resisting NK-mediated cell cytolysis during the natural course of their growth. That is, the progression and metastasis of tumors occurs through the immune selection of tumor cells that newly express other types of killer inhibitory receptor ligands synchronously with the decreased HLA class I expression.

HLA-G is an effective ligand for various types of NK inhibitory receptors, and has been reported to interfere with cytolysis by NK cells *in vitro* [8, 21, 31]. For this reason, the presence of HLA-G expression in tumor cells has been of in-



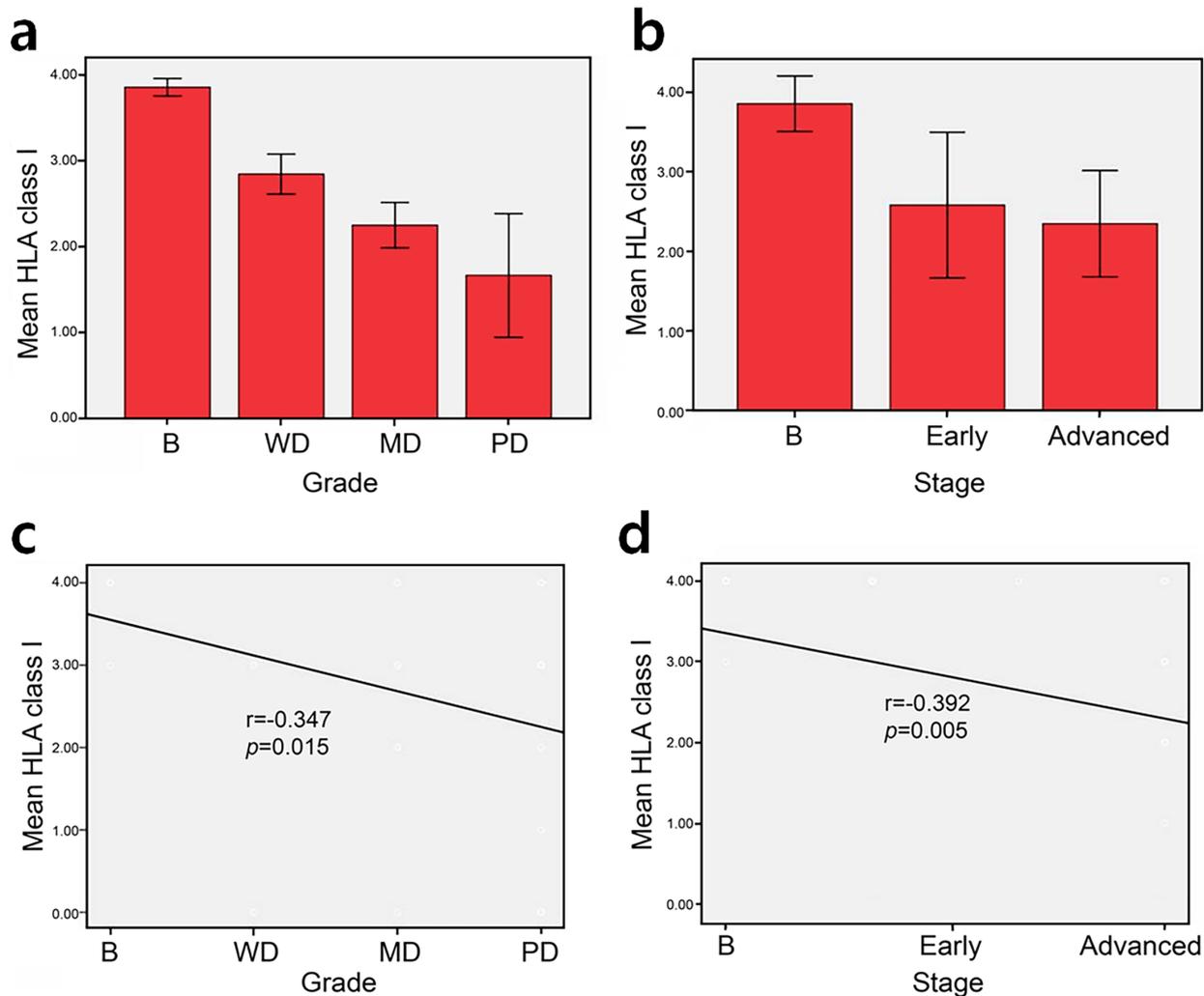
**Fig. 5. The degree of HLA-G expression expressed in mean of value according to tumor grade (a) and clinical stage of disease (b).** Scoring of immunoreactivity of benign and malignant tumor cells with anti-HLA-G monoclonal antibody are described in Materials and Methods. Bars represent mean standard deviation of the mean. HLA-G expression were correlated either with tumor grade (c) or with disease stage (d). The data were analyzed by Spearman's rho (A, Spearman's rho = 0.489,  $P = 0.001$ ; B, Spearman's rho = 0.468,  $P = 0.001$ ). B, benign; WD, well differentiated, MD, moderately differentiated, PD, poorly differentiated.

creasing interest to researchers. Indeed, some cell lines derived from blood tumors or solid tumors feature the expression of HLA-G transcripts [13, 22]. Further, in many cases of malignant melanoma, a higher degree of HLA-G mRNA expression has been found [14, 23]. In comparison with the expression of HLA class I genes, however, the degree of HLA-G transcription has been reported to be relatively lower and more variable, and controversy exists in terms of the protein expression. For example, in cases of malignant melanoma, Paul *et al.* [32] reported the expression of HLA-G protein in a biopsy sample of malignant melanoma. Meanwhile, Real *et al.* [33] the expression of HLA-G mRNA in malignant melanoma but observed a lack of protein expression. In addition, Pangault *et al.* [34] has reported a lack of protein expression in other types of solid tumor. These discrepancies might be due to the difference in the methods for examining the expression of HLA-G mRNA and in the sensitivities of

the monoclonal antibodies that were used.

HLA-G forms 7 types of alternative transcripts from the primary transcript through alternative splicing. These transcripts have been classified as encoding the membranous forms (HLA-G1, -G2, -G3 and -G4) and the soluble forms (HLA-G5, -G6 and -G7). Of the isoforms encoded by these alternative transcripts, only HLA-G1 and HLA-G5 form a stereoscopic structure consisting of 3 extracellular domains and a b2-microglobulin in an equivalent manner to other typical types of HLA class I molecules and thereby preserving a binding loop for CD8 [35]. These structures bind to the killer inhibitory receptors and thereby suppress the functions of NK cells, CTLs, and dendritic cells. However, little is known about the role of other types of HLA-G alternative transcripts, and the functions of HLA-G in regulating the alternative splicing remain to be elucidated.

For cases of ovarian cancer, Mamberg *et al.* [15] reported



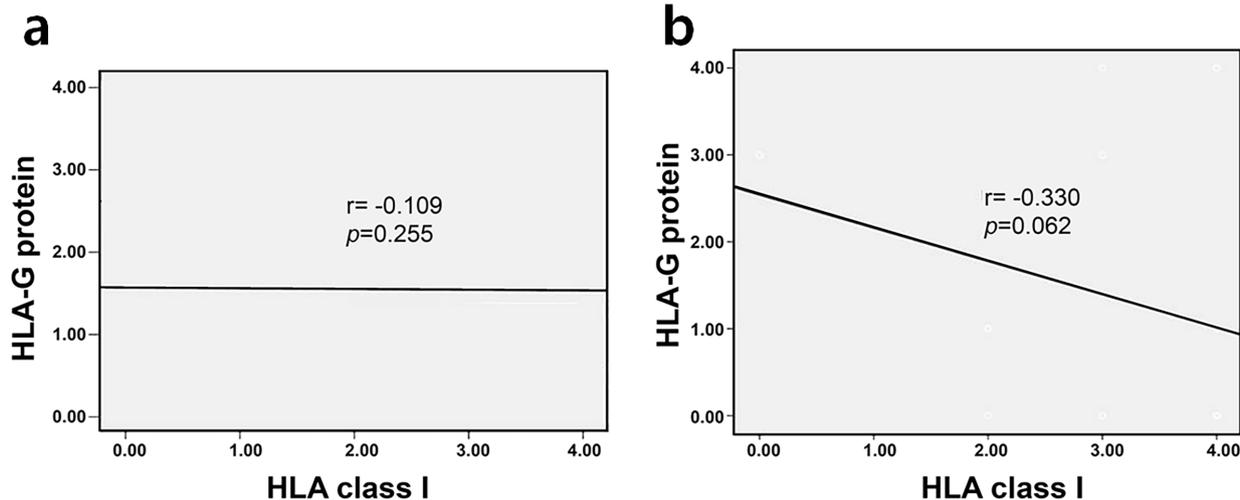
**Fig. 6. The Degree of HLA class I expression expressed in mean of value according to tumor grade (a) and clinical stage of disease (b).** Scoring of immunoreactivity of benign and malignant tumor cells with anti-HLA class I monoclonal antibody are described in Materials and Methods. Bars represent mean standard deviation of the mean. HLA class I down-regulation were correlated either with tumor grade (c) or with disease stage (d). The data were analyzed by Spearman's rho (A, Spearman's rho = -0.347,  $P = 0.015$ ; B, Spearman's rho = -0.392,  $P = 0.005$ ). B, benign; WD, well differentiated, MD, moderately differentiated, PD, poorly differentiated.

that the expression of HLA-G gradually decreased in ovarian cancer cells cultured for a short-term period. In the current study, the expression of HLA-G transcript was mostly observed in malignant ovarian tumor tissues. In particular, the expression of HLA-G2/G4 was seen in 6 cases; however, the expression of HLA-G3 was not observed in any cases. Further studies are warranted to clarify the significance of the difference in the expression of HLA-G alternative transcripts. In addition, the expression of one of the soluble forms, HLA-G5, was not examined in the current study but deserves further studies to understand the overall pattern of expression for the alternative transcripts.

Several potential mechanisms might be responsible for the discrepancy for cases in which HLA-G transcript but not protein is detected. First, some of the antibodies might lack the sensitivity to detect HLA-G protein when expressed at low levels. Second, HLA-G protein may be translated but might

be very unstable, so that its expression cannot be detected. Third, there are factors that suppress the translation of HLA-G mRNA. Fourth, mutations could have developed in the coding or non-coding regions of the gene [36, 37]. Mutations can develop in HLA-G genes through epigenetic mechanisms, including DNA methylation and histone deacetylation. These changes can alter gene splicing or transcriptional activity and thereby result in reduced gene expression in an *in vitro* setting. Such epigenetic changes have been reported to cause an impairment in the expression of HLA-G mRNA [38, 39]. To date, however, it has not been definitely clarified whether the differences in the mRNA transcription and protein expression of HLA-G originates from these mechanisms. This must be examined through further experimental studies in an effort to draw definitive conclusions.

No significant correlation has been reported between the clinical and histopathological characteristics of most types of



**Fig. 7.** The staining patterns by anti-HLA-G monoclonal antibody (mAb) did not correlated with that by anti-HLA class I mAb in 10 benign and 33 malignant specimens (a), and in cases excluded poorly differentiated type (b). The data were analyzed by Spearman's rho (a, Spearman's rho = -0.109,  $P = 0.255$ ; b, Spearman's rho = -0.330,  $P = 0.062$ ).

tumors and the expression of HLA-G. However, in the current study, the expression of HLA-G was significantly increased in the progression of the cancer from a benign tumor to a malignant one. In cases in which the tumor was poorly differentiated, the expression of HLA-G was also significantly increased. This correlation with the clinical characteristics was in agreement with the study by Urosevic *et al.* [40], which found a significant difference in the expression of HLA-G depending on the histologic grade of the tumors in patients with lung cancer. Moreover, correlations between HLA class I and HLA-G expression were examined in cases in which the degree of histologic differentiation was poor and the surgical stage was advanced. The results showed no statistical significance. However, excluding cases in which the degree of histologic differentiation was poor, the statistical significance was shown to increase, indicating an increase in HLA-G expression and the loss of HLA class I molecules plays a key role in the mechanisms by which tumor cells escape immune detection in cases of poor histologic differentiation.

## 5. Conclusions

Our results provide the basis for a mechanism by which the immune selection of tumor cells occurs in the avoidance of the cytotoxic anti-cancer tumor response in the ovarian cancer tissues. This suggests that HLA-G plays an important role in avoiding the immunosurveillance system of NK cells in patients with ovarian cancer. In cases of ovarian cancer tissue lacking HLA-G protein expression, however, other mechanisms may interfere with the activity of NK cells. Hence, further studies are warranted to examine whether the expression of HLA-G is correlated with that of HLA class II as well as that of HLA class I. Finally, our results may provide a clue for understanding the mechanisms by which immunological abnormalities occur in patients with ovarian cancer.

## Author contributions

SYH conceived and designed the experiments; SIJ, HJY and EYK performed the experiments; SYH and MJS analyzed the data; SIJ and JML contributed reagents and materials; MJS wrote the paper.

## Ethics approval and consent to participate

Written informed consent was obtained from all study subjects, and the study was approved by the Institutional Review Board of our institution on April 1st 2012 (KC12TISI0213).

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## Conflict of interest

The authors declare that they have no conflicts of interest.

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