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Clinical relevance and functional implications for human leucocyte antigen-g expression in non-small-cell lung cancer

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Abstract

HLA-G has been documented both in establishment of anti-tumour immune responses and in tumour evasion. To investigate the clinical relevance of HLA-G in non-small-cell lung cancer (NSCLC), expression status and potential significance of HLA-G in NSCLC were analysed. In this study, HLA-G expression in 101 NSCLC primary lesions and plasma soluble HLA-G (sHLA-G) from 91 patients were analysed with immunohistochemistry and ELISA, respectively. Correlations between HLA-G status and various clinical parameters including survival time were evaluated. Meanwhile, functional analysis of transfected cell surface HLA-G expression and plasma sHLA-G form NSCLC patients on natural killer (NK) cell cytolysis were performed. Data revealed that HLA-G was expressed in 41.6% (42/101) NSCLC primary lesions, while undetectable in adjacent normal lung tissues. HLA-G expression in NSCLC lesions was strongly correlated to disease stages ($P = 0.002$). Plasma sHLA-G from NSCLC patients was markedly higher than that in normal controls ($P = 0.004$), which was significantly associated with the disease stages (I *versus* IV, $P = 0.025$; II *versus* IV, $P = 0.029$). Patient plasma sHLA-G level (≥median, 32.0 U/ml) had a significantly shorter survival time ($P = 0.044$); however, no similar significance was observed for the lesion HLA-G expression. *In vitro* data showed that both cell surface HLA-G and patient plasma sHLA-G could dramatically decrease the NK cell cytolysis. Our findings indicated that both lesion HLA-G expression and plasma sHLA-G in NSCLC is related to the disease stage and can exert immunosuppression to the NK cell cytolysis, indicating that HLA-G could be a potential therapeutic target. Moreover, plasma sHLA-G in NSCLC patients could be used as a prognosis factor for NSCLC.

Keywords: HLA-G • non-small-cell lung cancer • prognosis

Introduction

Alteration of HLA expression, structure and function represents a frequent event in cancer and serves to circumvent T cell and natural killer (NK) cell responses [1, 2]. The non-classical HLA class I molecule HLA-G has been intensively investigated more than two decades, and postulated as an important immunotolerant which could suppress the functions of various immune cells such as NK cell, both $CD4^+$ and $CD8^+$ T lymphocyte and dendritic cells (DCs) [3].

Unlike classical HLA class I antigens, seven HLA-G isoforms including four membrane-bound (HLA-G1–G4) and three soluble HLA-G isoforms (HLA-G5–G7) are generated [4, 5]. It is noteworthy that another soluble form of HLA-G (sHLA-G) could be generated by shedding of the proteolytically cleaved surface HLA-G1 (sHLA-G1). Therefore, plasma sHLA-G derives from HLA-G5, as well as from the shedding form of HLA-G1 [6]. Increasing evidence indicated that both the membrane-bound and sHLA-G isoforms share similar inhibitory functions *via* binding to their specific receptors, such as ILT2 (CD85j, LIR-1), ILT4 (CD85d, LIR-2) and KIR2DL4 (CD158d) [7].

Apart from initially addressed in development of foetal maternal tolerance during pregnancy, the clinical implication of HLA-G has been involved in a broad spectrum of physio-pathological situations [8]. In normal conditions, HLA-G is expressed in foetal trophoblast

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cells, and in other tissues such as adult thymic medulla, cornea, nail matrix, pancreatic islets, erythroid and endothelial precursors, and mesenchymal stem cells [9–15]. Importantly, an increasing number of studies have highlighted its clinical relevance in certain pathological conditions, such as oncology, autoimmunity, inflammation, infection and transplantation [8]. In the context of tumour biology, since Paul *et al.* [16] described the expression of HLA-G in melanoma for the first time, augmented HLA-G expression *in situ* was observed nearly in 20 types of tumours [17]. HLA-G was preferentially detected in tumour tissue and rarely in the adjacent normal tissue, suggesting its specific association with tumour growth and progression. Aberrant expression of HLA-G in cancer has been implicated both in establishment of anti-tumour immune responses and in tumour evasion which was involved in three essential phases of cancer immunoediting procession: elimination, equilibrium and escape [18]. However, the exact time-point of HLA-G expression in tumour cells remains elusive. Generally, tumour cells are genetically instable, so the activation of HLA-G could take all the way during the tumour formation and development. HLA-G during chronic inflammation would enable the growth of transformed cell beyond recognition of host immunosurveillance. This was supported by the findings that an increased risk for the development of malignancy was observed in the setting of inflammatory conditions of the gut, such as ulcerative colitis and Crohn's disease [19]. HLA-G expression induced by microenvironment cues including cytokines in these conditions might be involved in the anti-inflammation immune responses and malignant transformation [20, 21].

Furthermore, changes in the level of sHLA-G have been detected from patients with solid tumours and lymphoproliferative disorders. However, the clinical relevance of sHLA-G in malignancies remains to be explored. For example, high sHLA-G level is significantly correlated with survival in glioblastoma multiforme but does not appear to be predictive of survival in neuroblastoma [22, 23].

To date, little information was available for the clinical relevance of HLA-G, particularly, both the primary lesion HLA-G expression and patient plasma sHLA-G, in lung cancer. In an effort to gain further insight into the roles of HLA-G in NCSLC, both HLA-G expression in primary lesions and plasma sHLA-G from non-small-cell lung cancer (NSCLC) patients were analysed, and their correlation to clinical parameters were evaluated. Furthermore, functions of the transfected HLA-G cell surface expression in lung cancer cell line A549 and plasma sHLA-G from NSCLC patients on NK cell cytolysis were also determined.

Patients and methods

Study population

Tumour lesions and adjacent normal lung tissues were obtained from 101 patients with NSCLC diagnosed and treated consecutively between 2002 and 2007 at Taizhou Hospital of Zhejiang Province affiliated to Wenzhou Medical College in this study. Patient data collected included age, gender, date of initial diagnosis, histological diagnosis, tumour grade, clinical stage and date of death from NSCLC or last follow-up. The follow-up was done in July, 2008. The median of the follow-up was 20.7 months. Histological diagnosis and tumour grade were determined in accordance with the World Health Organization criteria for lung and pleural tumours [24]. Pathologic stage was based on the revised international system [25]. For the sHLA-G evaluation, 91 plasmas were available from these NSCLC patients and 150 age- and sex-matched, unrelated healthy blood donors were detected.

All histological samples were taken from the primary lesions in the lung. No specimen from metastatic sites was included in the study. All tissue specimens underwent a microscopic confirmation for pathological features prior to their inclusion in the study. This study was performed following an Institutional Ethics Review Board approved protocol to investigate molecular markers relevant to lung cancer pathogenesis.

Immunohistochemistry

Four-micrometre-thick sections of the paraffin-embedded tissue blocks were cut and mounted on polylysine coated slides. They were dewaxed in xylene and rehydrated through a graded series of ethanol. After de-paraffinization, antigen retrieval treatment was performed at 120°C for 5 min. in a 10 mM sodium citrate buffer (pH 6.0). Endogenous peroxidase activity was blocked by using a 3% hydrogen peroxide solution at room temperature for 15 min. Then, anti-HLA-G mAb 4H84 (1:300, Exbio, Prague, Czech Republic) was applied and incubated overnight at 4°C. After that, a thorough washing in a 0.01 M phosphate-buffered saline (PBS) solution was done. Subsequently, binding sites of the primary antibody were visualized using a Dako EnVison kit (Dako, Glostrup, Denmark) according to the manufacturer's instructions. Finally, sections were counterstained with haematoxylin and mounted with glycerol gelatin. HLA-G staining in NSCLC tissues was determined by three pathologists. The pathologists were blinded to any clinical details related to the patients. Membrane or combined membrane and cytoplasmic expression of HLA-G were interpreted as positive. Cytotrophoblast from first trimester human placenta served as a HLA-G^+ and HLA-G^- controls were achieved by including isotype matched IgG1 (1:500, Exbio) in each of the immunostaining. HLA-G expression in part of these NSCLC samples ($n = 20$) was confirmed by Western blot with case-matched fresh lesions.

Staining evaluation

Evaluation of the HLA-G^+ cells was grouped from 0 to 3: negative for 0%, 1 for 1–25%, 2 for 26–50% and 3 for >50%. The percentage of positive cells was assigned a value based on the presence or absence of HLA-G staining, irrespective of staining intensity. First trimester cytotrophoblast sections were served as a HLA-G^+ control. The HLA-G intensity was scored from 0 to 3, with a score of 3 for intensity comparable to the staining of the cytotrophoblast, 0 comparable to the staining of the corresponding negative control using isotype matched IgG1, and 1 and 2 as gradations between the two.

Tissue protein extraction and Western blot analysis

For preparation of protein extracts, 20 case-matched fresh primary NSCLC lesions were crushed with a mortar under liquid nitrogen. Harvested cells

were washed three times with cold PBS. Cell pellets were collected and lysed with lysis buffer (pH 7.4, 50 mM Tris-base, 150 mM NaCl, 1mM ethylenediaminetetraacetic acid, 1% Triton X-100, PMSF 1 mM) with the final concentration of 1×10^7 cells/ml. After centrifugation at 15000 $\times$ *g* at 4°C for 30 min., cell lysate aliquots were separated in 10% SDS-PAGE gel. All samples were heated for 5 min. at 100°C before loading. Proteins were then electro-blotted onto polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA) and blocked by incubation with PBS containing 5% non-fat dry milk for at least 4 hrs. After blocking, membranes were washed in PBS containing 0.2% Tween-20, three times and then probed with the HLA-G specific mAb 4H84 (Exbio) overnight at 4°C and washed in PBS containing 0.2% Tween-20 three times. The membranes were subsequently incubated for 30 min. at room temperature with Peroxidase/DAB+Rabbit/Mouse (Dako), washed thoroughly with 0.1% Tween-PBS. Finally, membranes were developed with Dako REAL™ EnVision™ Detection System (Dako) for 1–3 min. Samples from JEG-3 and JAR cells (ATCC, Rockville, MD, USA) were used as HLA-G$^+$ and HLA-G$^-$ controls, respectively. A mouse IgG1 isotype antibody (1:1000, Exbio) and anti-Calnexin (a house keeping protein, molecular weight: 90 kD) mAb (1:1000, Stressgen, Glanford Ave, Victoria, BC, Canada) were used as internal controls, respectively.

sHLA-G ELISA

sHLA-G concentrations were determined with the sHLA-G specific ELISA kit (sHLA-G kit; Exbio), which measures sHLA-G1 and HLA-G5. Each sample (50 μl) was measured in triplicates. The optical densities were measured at 450 nm (Spectra Max 250, Molecular Devices, Sunnyvale, CA, USA). The final concentration was determined by optical density according to the standard curves (range: 0–125 U/ml). When the concentration exceeds 125 U/ml, diluted samples were used and dilution factors were considered to calculate the sHLA-G concentration. The detection limits were 1 U/ml. Details of the performance were according to the manufacturer's instruction.

HLA-G transfection of the lung adenocarcinoma cell line A549

The A549 cells were transfected with the recombinant pVITRO2-mcs vector (Invivogen, San Diego, CA, USA) containing HLA-G1 using Lipofectamine®2000 reagent (Invitrogen, Grand Island, NY, USA) according to the manual instructions. Details of transfection were described previously [26]. The transfectants were screened with Hygromycin B (Amresco, OH, USA) and cell surface HLA-G expression was monitored by flow cytometry (BD FACSCalibur, San Jose, CA ,USA) with MEM-G/09 and Western blot with mAb 4H84. To test whether the HLA-G1 transfected A549 cells could produce the sHLA-G isoforms, sHLA-G in cell culture supernatants was analysed with ELISA, intracellular sHLA-G detection was performed with flow cytometry (FITC-2A12, IgG1; Exbio) and cell lysates were analysed with Western blot (mAb 5A6G7, IgG1; Exbio), respectively.

Depletion of sHLA-G with immunomagnetic bead immunoprecipitation

Depletion of the plasma sHLA-G from NSCLC patients (with high concentration of sHLA-G) were performed by incubation with sheep-antimouse IgG Dynabeads® M-280 (Invitrogen Dynal AS, Oslo, Norway), coated with the anti-HLA-G-specific antibody MEM-G/9 (5 μg/10^7 beads) (Exbio), according to the manufacturer's instructions. The efficacy of depletion was confirmed with ELISA (sHLA-G kit; Exbio).

Lactic dehydrogenase releasing cytotoxicity and blocking assay

Cytotoxicity was performed with CytoTox96® Non-Radioactive Cytotoxicity Assay Kit (Promega, Madison, WI, USA). Details of the performance were as described previously [26]. The percentage of specific lysis was determined as follows: (experimental release – effector spontaneous release – target spontaneous release) / (target maximum release – target spontaneous release) $\times$ 100.

Statistical analysis

Statistical analysis was performed with SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA). Correlations between the degree of staining and clinical parameters were calculated with Pearson chi-square test. Overall patient survival was evaluated from the date of diagnosis to the date of last follow-up (censored) or date of patient death (event). Survival probabilities were calculated using the Kaplan–Meier method. Difference of sHLA-G between groups was analysed with Mann-Whitney U-test. A $P < 0.05$ was considered to be significant.

Results

HLA-G expression in primary NSCLC lesions

Overall, 41.6% (42/101) tumour lesions were classified as HLA-G$^+$ (Table 1). In the malignant tumour sections, the intensity of staining varied from tumour to tumour and from one area to another within the same tumour. Heterogeneous staining was noted in all histological types of NSCLC. Some tumours showed focal patchy positive staining, and others displayed uniform staining pattern in tumour nests. Positive staining was observed in both the cell membrane and the sub-membranous cytoplasm region. The cytotrophoblasts were used as internal positive control for HLA-G expression. No staining was detected in corresponding adjacent normal lung tissue and tissue sections incubated with irrelevant mouse IgG1 (Fig. 1A).

Specificity of the antibodies for detecting HLA-G raises concerns as Apps *et al.* mentioned [27]. Here, HLA-G expression in partial samples was test by Western blot. Our data showed that immunohistochemistry results were highly consistent with that of the Western blot analysis (Fig. 1B).

Plasma sHLA-G expression in NSCLC patients

Plasma sHLA-G levels in 91 NSCLC patients and 150 normal healthy individuals were determined by ELISA. Concentration of

Table 1 Association of HLA-G expression in primary NSCLC lesions with clinicopathological parameters

Variables	No. of cases	HLA-G expression†				*P**
		Negative	1–25%	26–50%	>50%	
Histological type						
Squamous	52	33	6	5	8	0.510
Adenocarcinoma	47	25	7	10	5	
Large cell	2	1	0	1	0	
Tumour location						0.944
Left	58	33	8	10	7	
Right	43	26	5	6	6	
Gender						
Male	80	51	7	13	9	0.051
Female	21	8	6	3	4	
Age						
≤Median (58 years)	52	28	6	10	8	0.608
>Median	49	31	7	6	5	
Tumour grade						
I	5	4	1	0	0	0.432
II	62	33	9	13	7	
III	34	22	3	3	6	
Tumour stage						
I	34	23	6	1	4	0.002‡
II	28	20	4	3	1	
III/ IV	39	16	3	12	8	

*Comparison of HLA-G expression status between or among each variable using the Pearson chi-square test.
†The percentage of positive cells was assigned a value based on the presence or absence of HLA-G staining, irrespective of staining intensity.
‡The overall *P*-value among Tumour stage I, II and III/ IV is 0.002 ($X^2_6 = 21.3$); *P*-values for the comparison between I and II, between I and III/IV, between II and III/IV are 0.691 ($X^2_3 = 1.46$), 0.001 ($X^2_3 = 16.3$) and 0.020 ($X^2_3 = 9.89$), respectively.

the plasma sHLA-G was with the median of 34.0 U/ml (range: 3.13–275.5) for NSCLC patients, 20.4 U/ml (range: 0.97–270.6) for normal controls, respectively. Data revealed that sHLA-G expression in NSCLC patients was significantly higher than that in normal controls ($P = 0.004$) (Fig. 2A).

HLA-G expression in NSCLC relative to clinicopathological parameters

HLA-G expression was observed in 32.35% (11/34) in tumour stage I, 28.57% (8/28) in stage II, and 58.97% (23/39) in stage III/IV, respectively. HLA-G expression in primary NSCLC lesions was strongly associated with disease stage with an overall *P*-value of 0.002. When sub-grouped, significant difference was found for tumour stage I *versus* III/IV ($P = 0.001$), stage II *versus* III/IV ($P = 0.020$), while no significance was observed between stage I and II ($P = 0.691$) (Table 1). However, HLA-G expression in NSCLC lesions was not significantly associated with clinical parameters such as patient age, gender, tumour histological type, grade and location (Table 1). Furthermore, significance was also observed for the plasma sHLA-G expression in NSCLC patients, where sHLA-G level in stage IV was markedly higher than that in stage I ($P = 0.025$) or in stage II ($P = 0.029$) (Fig. 2B).

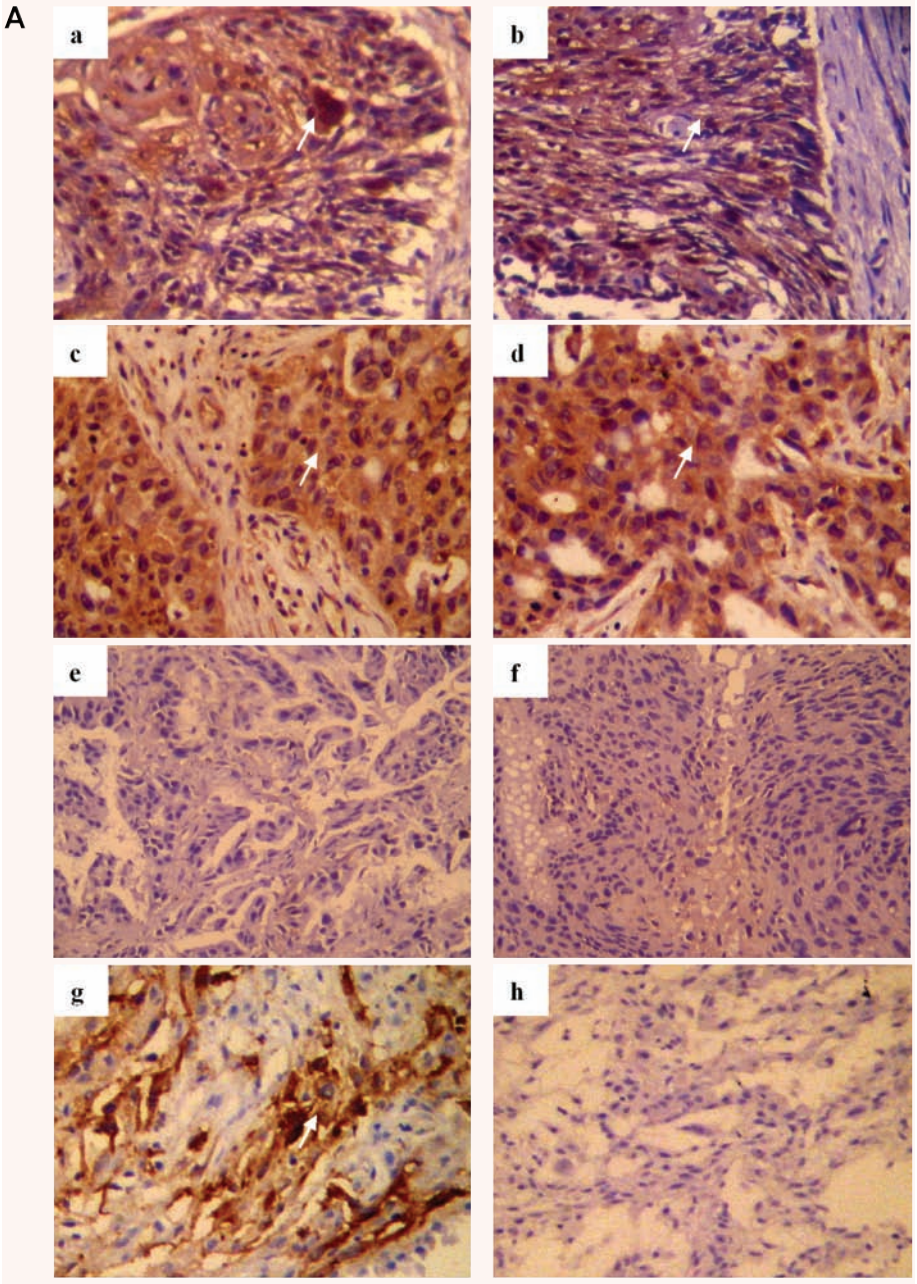

Fig. 1 (**A**) Immunohistochemical staining of HLA-G expression in primary NSCLC lesions. (a, b) lung squamous cancer with HLA-G expression. (c, d) Lung adenocarcinoma with HLA-G expression. (e) HLA-G$^-$ adenocarcinoma. (f) HLA-G$^-$ squamous cancer (original magnification: 100×). Cytotrophoblast tissues were used as internal controls (g, with mAb 4H84) and (h, with an IgG1 matched isoytpe antibody), respectively. HLA-G mAb 4H84 (1:500) was used to detect the HLA-G expression. Arrows indicate the specific staining of HLA-G expression. (**B**) Western blot analysis of HLA-G expression. The analysis was performed with the HLA-G mAb 4H84 (1:1000). M, molecular weight ladder; samples of patient (P1, 3, 5) were from HLA-G$^-$, and patient (P2, 4, 6) was from HLA-G$^+$ NSCLC patients. The degree of HLA-G expression was shown in brackets according to the case-matched immunohistochemistry data. JEG-3 and JAR lysates were used as HLA-G$^+$ and HLA-G$^-$ controls, respectively. Isotype, a mouse IgG1 isotype antibody (1:1000) was used as internal control for JEG-3 lysates. The house keeping protein Calnexin was also used as an internal control (molecular weight: 90 kD).

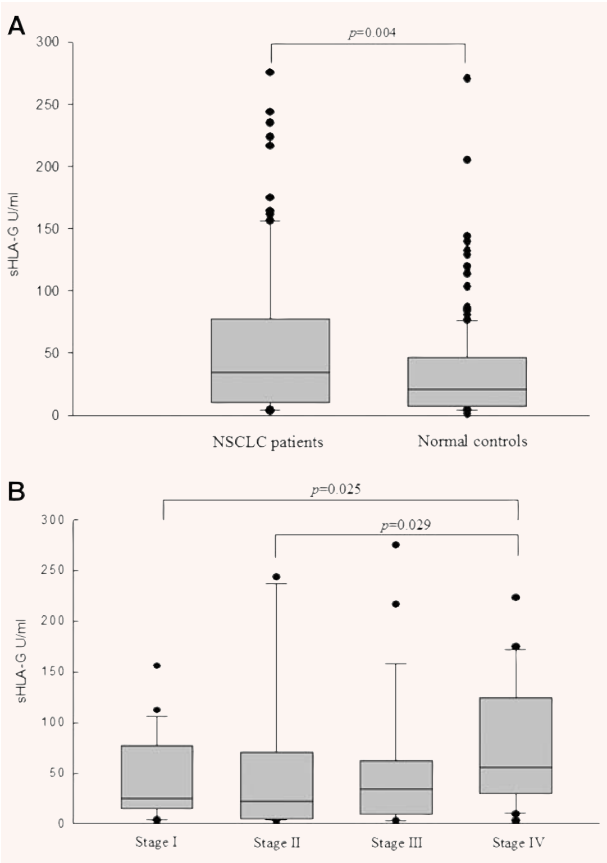

Fig. 2 Plasma sHLA-G levels in normal and NSCLC patients, and its correlation with clinical stage. (**A**) Comparison of plasma sHLA-G between NSCLC patients and normal controls. Plasma sHLA-G concentrations from 91 NSCLC patients (median: 34.0 U/ml, range: 3.13–275.5) were significantly higher ($P = 0.004$) than those detected in 150 age-matched healthy controls (median: 20.4 U/ml, range: 0.97–270.6). (**B**) Correlation between patient plasma sHLA-G levels and clinical disease stage. Plasma sHLA-G level in different clinical stage of NSCLC patients is 25.3 U/ml (range: 3.13–155.9) for Stage I, 22.3 U/ml (range: 3.27–243.6) for Stage II, 34.3 U/ml (range: 3.46– 275.5) for Stage III and 56.6 U/ml (range: 3.13–223.5) for Stage IV, respectively. NSCLC patient plasma sHLA-G level in Stage IV was significantly higher than that in Stage I ($P = 0.025$) and Stage II ($P = 0.029$), respectively.

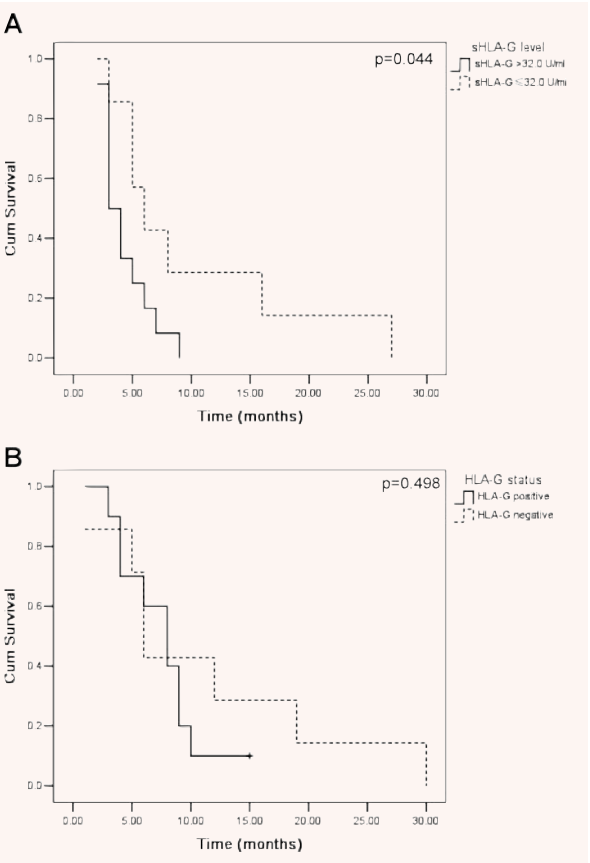

Fig. 3 Kaplan–Meier estimates the survival curves for NSCLC patients with HLA-G expression in primary lesions ($n = 51$) or plasma sHLA-G ($n = 67$). (**A**) Patients with the sHLA-G above median level (32.0 U/ml) had a significantly shorter survival time than that with lower sHLA-G expression ($P = 0.044$). (**B**) No statistical significance was observed for the HLA-G expression in primary lesions and survival ($P = 0.498$).

Relation between HLA-G expression and patient survival

We next identified potential effects of both plasma sHLA-G ($n = 67$) and lesion HLA-G status ($n = 51$) on patient survival using the Kaplan–Meier method. Patients with the sHLA-G above median level (32.0 U/ml) had a significantly shorter survival time than those with lower sHLA-G expression ($P = 0.044$) (Fig. 3A). However, for the lesion HLA-G expression, no statistical significance was reached for the survival analysis ($P = 0.498$) (Fig. 3B).

Inhibition of NK-92 cytolysis against A549 cells by both plasma sHLA-G and cell surface HLA-G *in vitro*

To test the biological function of plasma sHLA-G from NSCLC patients, we analysed its ability to influence NK-mediated cytolysis. In the current study, the interleukin (IL)-2 activated NK-92 cells were introduced as the effector. NK-92 cells were pre-treated with medium alone, or plasma from NSCLC patients (sHLA-G in pooled plasma 1: 118–174 U/ml; plasma 2: 216–243 U/ml) either untreated or sHLA-G depleted. Expression of both cell surface and total HLA-G in A549 or A549-G cells was confirmed by flow cytometry and Western blot (Fig. 5A, B). No sHLA-G was detected in the A549-G cells with ELISA, Western blot and immunocytochemistry (Fig. S1), and flow cytometry (Fig. 5B, C). These data

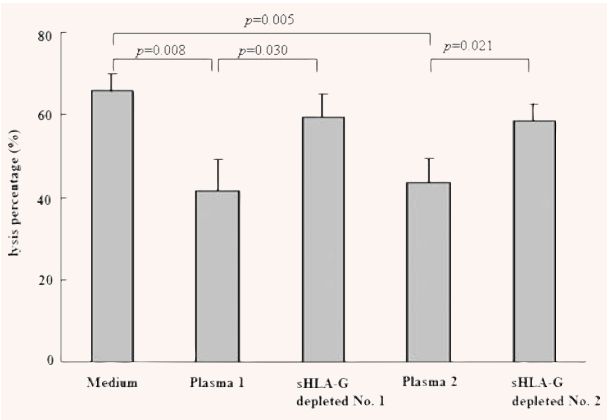

Fig. 4 Function of plasma sHLA-G from NSCLC patients in inhibition of NK cell cytolysis. Cytolysis of the A549 lung adenocarcinoma cell line by IL-2 activated NK-92 cells following 1 hr pre-incubation of the effector cells with medium (control), or pooled plasma from three NSCLC patients (pooled plasma 1, sHLA-G range: 118–174 U/ml; pooled plasma 2, sHLA-G range: 216–243 U/ml), either unmanipulated or depleted of sHLA-G by immunomagnetic beads coated with MEM-G/9. Cytotoxicity was tested in a lactic dehydrogenase releasing assay.

suggested that only the cell surface HLA-G1 was expressed in A549-G cells. Cytotoxic effector cells were then challenged with target lung cancer A549 cells by the LDH release assay. As shown in Fig. 4, plasmas with high sHLA-G expression from NSCLC patients were able to inhibit NK cell cytolysis and the decreased cytotoxicity could be significantly restored with the sHLA-G depleted (specific lysis 59.53 $\pm$ 5.52 *versus* 41.67 $\pm$ 7.55, $P = 0.030$; 58.5 $\pm$ 4.10 *versus* 43.72 $\pm$ 5.62, $P = 0.021$).

By transfection of HLA-G1 in the lung adenocarcinoma cell line A549, the cytolysis capability of the NK-92 cells was also significantly inhibited (specific lysis 67.11 $\pm$ 4.11 *versus* 41.3 $\pm$ 5.37, $P = 0.003$, Fig. 5C), which could also be restored partially by the functional HLA-G specific antibody 87G in a manner of dosage dependent (Fig. 5D).

Discussion

At the beginning of primary tumours expansion, the cells are less invasive and metastatic. During cancer immune editing, host immune system plays in eliminating tumour cells and in facilitating the emergence of their immunoresistant variants. Due to genetic alterations present in each cancer cell, metastatic cells acquire particular genetic, phenotypic and biological characteristics that are not present in the primary tumours [1]. Among these alterations, partially or totally down-regulate the expression of MHC class I antigens is one of the most common events in the tumour progression. A variety of altered HLA phenotypes have been defined in human tumours, including HLA total loss, HLA haplotype loss, HLA-specific locus down-regulation, HLA allelic losses and a combination of these phenotypes [28]. Consequently, HLA expression abnormalities not only affect the presentation of tumour antigen (TA)-derived peptides (HLA class I–TA peptide complex) to HLA class I antigen-restricted TA-specific cytotoxic T lymphocytes (CTLs), but also influence the susceptibility of tumour cells to lysis by NK cells. The latter phenomenon reflects the loss of NK cell inhibitory signals triggered by the interaction of HLA class I antigens with NK cell inhibitory receptors [29, 30]. In the scenario of NSCLC, the ratio of down-regulation of HLA class I expression varies widely from 25% to 90% among different studies [31–34], and the clinical relevance of HLA class I expression status in disease prognosis remains controversial [32, 34]. Be noted, expression of HLA class I antigens is not always associated with the susceptibility of tumour cells to CTL lysis which is partially due to the lack of TA-derived peptide presentation by a given HLA class I allospecificity [35]. But when HLA class I-defective tumour variants appear, T-cells cannot recognize these targets, and these tumour clones acquire a growth advantage that allows them to take over the other clonal tumour populations with the expression of immunosuppressive molecules such as HLA-G [36].

Since the first address of HLA-G expression by tumour cells in 1998, numerous studies have been performed and HLA-G *in situ* expression was observed in various malignancies such as colorectal cancer, retinoblastoma, ovarian carcinoma, breast carcinoma, haematopoietic tumours, renal cell carcinoma and lung cancer [18]. In addition to HLA-G expression *in situ*, sHLA-G molecules have been found circulating at high concentrations in certain cancer patients including glioblastoma multiforme, breast and ovarian cancers, lymphoblastic and monocytic acute leukaemia, malignant melanoma and neuroblastoma [7, 23, 37, 38]. Among these previous studies, the potential clinical relevance of HLA-G was also addressed.

In the present study, for the first time, we analysed both plasma sHLA-G and primary malignant lesion HLA-G expression in NSCLC patients, and their potential significance was also discussed. Our data revealed that HLA-G expression was detected in 41.6% NSCLC primary lesions. HLA-G expression status was significantly associated with tumour stage, which is preferentially observed in patients with more advanced tumour stage. The frequency of HLA-G expression in our study was similar to that (mAb 4H84, 24.2%, 8/33) in the study by Urosevic *et al.* [39] but remains discrepant to the recent report for NSCLC by Yie *et al.* [40], where HLA-G expression was observed in 75.0% (79/106) of the lesions analysed. Foremost, the difference between our data and that study may contribute to the staining evaluation method used in the studies. It is stated that the (+) designation refers to tissue specimens with less than 25% of the cancer tissues and weakly stained; however, it is likely that in actuality they mean to specimens in which less than 25% of the cancer tissues are weakly stained [40]. A more objective method of quantifying the amount of cancer cells expressing HLA-G would be beneficial. Secondly, the different antibodies used may contribute to the

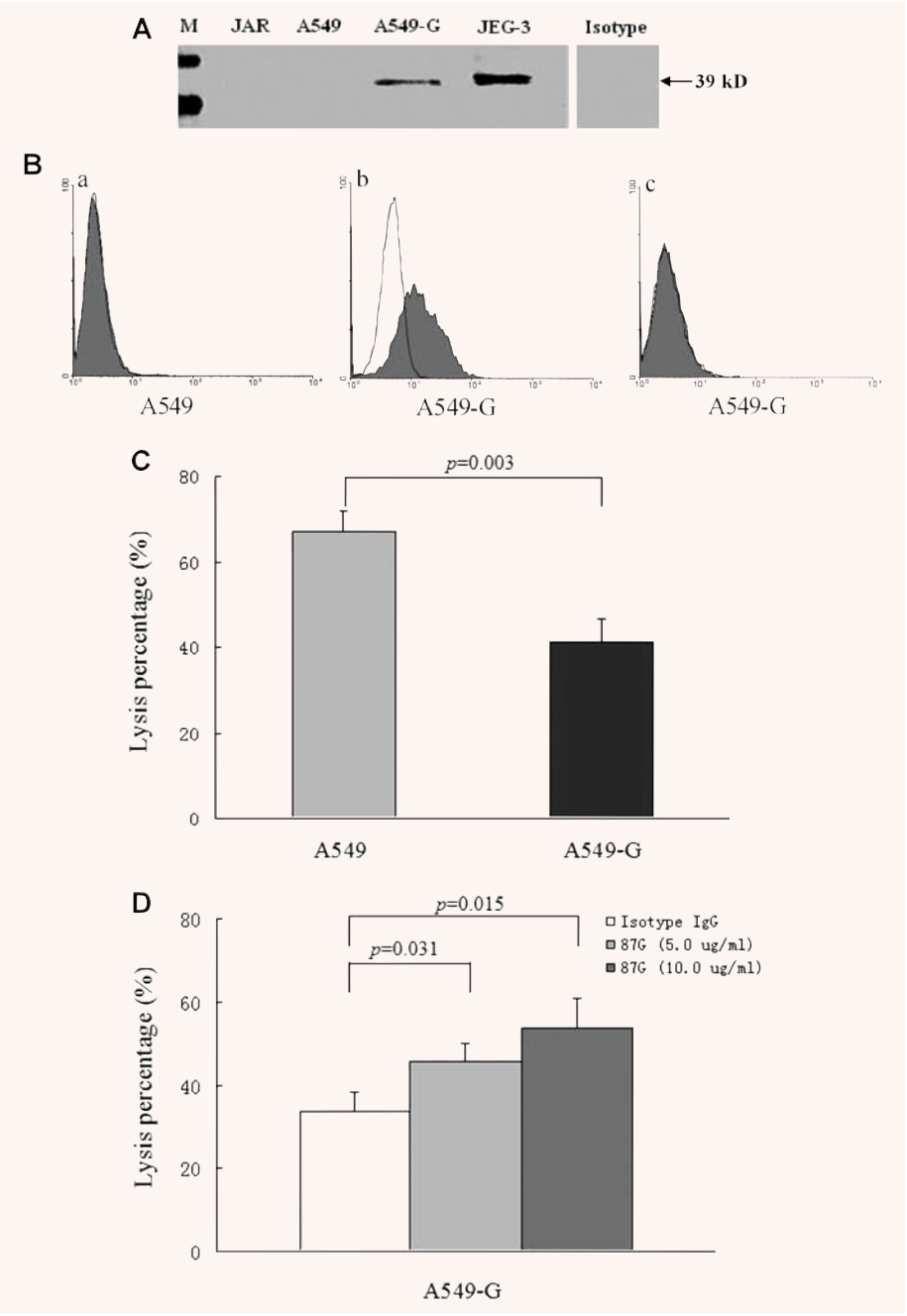

Fig. 5 NK-92 cytotoxicity inhibition induced by HLA-G expression. (**A**) Western blot analysis of HLA-G expression in A549 and A549-G cells with mAb 4H84. Choriocarcinoma cell line JAR and JEG-3 were used as HLA-G$^-$ and HLA-G$^+$ controls, respectively. Isotype, a mouse IgG1 isotype antibody (1:1000) was used as internal control for JEG-3 lysates. (**B**) Flow cytometry analysis of cell surface HLA-G expression on A549 cells (a) and A549-G cells (b) with mAb MEM-G/9; intracellular sHLA-G staining in A549-G cells with mAb 2A12 (c). (**C**) Comparison of the mean cytolytic percentage of NK-92 to A549 and A549-G. (**D**) Restoration of cytotoxicity by HLA-G specific mAb 87G antibody blockade. Target cell A549-G was pre-incubated with 5 μg/ml and 10 μg/ml 87G, respectively, or an isotope IgG1 as a control. Experiments were performed in quadruplicate with an effector / target ratio of 20:1, and the results were expressed as percentage of specific lysis ± S.D.

discrepancies. In that study, an HGY antibody was used to detect HLA-G expression. The antibody HGY was also applied to evaluate the HLA-G expression in lung cancer [40] – other malignancies such as oesophageal squamous cell carcinoma (HLA-G$^+$ staining: 110/121, 90.9%), gastric cancer (113/160, 71%) and colorectal cancer (130/201, 65%) were also investigated by the same group [41–43]. HLA-G$^+$ percentage in these studies was prone to be higher than that in other studies such as colorectal cancer (mAb 4H84, 52/100, 52%, $P = 0.03$) [44], gastric cancer (mAb MEM-G/1, 52/115, 45%, $P = 2.2 \times 10^{-5}$) [45]. Though similar affinity and specificity to mAb 4H84 was addressed in the detection of HLA-G expression in lung and colorectal cancers, more detailed information for mAb HGY is absolutely needed [40].

We then analysed the HLA-G expression status relative to various clinicopathological parameters. Lesion HLA-G expression was only significantly associated with stage of the disease.

Meanwhile, our study showed that plasma sHLA-G from NSCLC patients was dramatically increased compared to that in normal controls, and sHLA-G levels were significantly associated with the disease stage, where sHLA-G in stage IV was much higher than that in stage I or in stage II. We also found that there is no correlation between the lesion HLA-G expression and plasma sHLA-G level in the same patient. Data from a study on melanoma indicated that sHLA-G molecules were preferentially released by peripheral blood rather than melanoma cells, and this was supported by the fact that peripheral blood monocytes are the predominant cells secreting HLA-G5 [6, 38], and the fact that some healthy controls in this study exhibit high levels of sHLA-G. sHLA-G expression in NSCLC lesions is not a frequent event (2/30) when this was tested with the sHLA-G mAb 5A6G7 in our study. Furthermore, probing with the mAb 2A12 showed that, high level of intracellular sHLA-G staining was observed in peripheral blood $CD45^+$ nucleated cells ($n = 10$, Fig. S2). These data indicated that plasma sHLA-G is mainly produced from the peripheral blood cells, but not from the malignant cells. Several variables have been addressed to influence the expression of sHLA-G. One of them is the gender of the donors; the study indicated that the level of sHLA-G is higher in women than in men [46]. Other important variables represented by the HLA-G polymorphisms such as the 14bp insertion/deletion variation in the exon 8 of the HLA-G gene could also alter the expression of sHLA-G [47].

In tumour patients, high levels of systemic sHLA-G may synergistically help malignant cells to overcome the host immune system. Whether the increased plasma sHLA-G levels detected in NSCLC patients reflects increased shedding of sHLA-G by tumour cells and/or up-regulation of surface HLA-G or secretion of sHLA-G by peripheral blood monocytes through tumour-derived cytokines such as IL-10 and transforming growth factor-β_1, remains to be determined [38, 48, 49]. The latter mechanism was indicated by the induction of *in vitro* sHLA-G production by monocytes following incubation with tumour-cell-derived supernatants [50]. Given the widely acknowledged tolerogenic properties of HLA-G in cancer immune escaping, our data raised the hypothesis that HLA-G expression could play a critical role in dampening anti-NSCLC immune response and in contributing to tumour progression.

Indeed, correlation between poor clinical outcome and *in situ* HLA-G expression, such as gastric and colorectal tumours, leukaemia, has been addressed [42, 43, 51, 52]. Moreover, patients with acute leukaemia, breast cancer and ovarian cancer show high levels of sHLA-G in plasma and in malignant ascites supernatants, respectively, suggesting possible correlations between the secretion of this molecule (sHLA-G) and disease progression [53, 54]. Be noted, types of sample used for sHLA-G detection may affect the data interpretation. Rudstein-Svetlicky *et al.* pointed out that plasma HLA-G values were almost invariably higher than those from serum for the same individual because HLA-G might be trapped and/or consumed during clot formation and the amount trapped within the clot is variable and inconsistent, and recommended that sHLA-G should be determined in the plasma which reflects the true biological levels [55]. Here, we found that plasma sHLA-G above the median level in NSCLC patients is strongly associated with a shorter survival time. Previous studies indicated that high serum sHLA-G level in neuroblastoma patients was strongly correlated with relapse, and glioblastoma multiforme patients with high plasma sHLA-G levels had a significantly shorter survival than those with low sHLA-G levels [23, 50]. However, in our study, relation between primary lesion HLA-G status and patient survival did not reach statistical difference. More recently, Yie *et al.* [40] evaluated HLA-G expression in NSCLC patients and pointed out that NSCLC primary lesion HLA-G expression was significantly associated with the poor prognosis and shorter survival time. However, as mentioned above, the immunohistochemistry HLA-G staining evaluation criteria in that study may have a pit fall and accounts for the discrepancy.

It is now widely acknowledged that both membrane bound HLA-G and sHLA-G could protect tumour cells from immune competent cell-mediated anti-tumour responses. New findings were also addressed, such as the effects of sHLA-G on angiogenesis, inducing resting NK cells to produce chemokines and cytokines and impairing NK/DCs crosstalk *via* inhibition of DCs [56–58]. Furthermore, HLA-G involved suppressor cells such as HLA-G induced regulatory T cells, DCs, and the HLA-G bearing APC, MSCs, T and NK cells or even the $HLA\text{-}G^+$ tumour cells, has a long-term immune modulatory function and can induce the generation of suppressive/regulatory cells, and can block the immune effectors [59]. Thereby, the immune clearance of tumour cells might be enhanced by blocking HLA-G. In the current study, we finally analysed the potential roles of both cell surface HLA-G generated artificially on A549 cells and plasma sHLA-G from NSCLC patients on NK cell mediated cytolysis. In HLA-G transfected A549 cells (A549-G), only the cell surface HLA-G isoform was expressed which was supported with the fact that no sHLA-G detected both in cell culture supernatants and in intracellular staining. Data showed that both cell surface and sHLA-G isoforms could inhibit the cytotoxic activities of activated NK cells *in vitro*. The cell surface HLA-G mediated NK cell cytolysis inhibition was confirmed by using the HLA-G conformational blocking mAb 87G, which could be dramatically recovered when HLA-G was blocked. In a similar scenario, a study by Bukur *et al.* indicated that cytotoxicity of lymphokine activated killer against the $HLA\text{-}G^+$ renal carcinoma cell line MZ2733RC could be restored with the pan anti-HLA class I mAb W6/32 [60]. The restoration of NK cell cytolysis was also observed in our previous studies on ovarian cancer and acute myeloid leukaemia when HLA-G was blocked by HLA-G specific mAb 87G [26, 52]. Furthermore, data revealed that plasma sHLA-G in NSCLC patients could inhibit the cytotoxic activities of activated NK and the specificity of the observed sHLA-G mediated NK cytolysis inhibition was supported by the depletion experiment.

Studies discussed above suggest that HLA-G mediated immune suppression appear as an attractive molecular target for developing new anti-tumour interventions. Given the fact that the inhibitory effects mediated by HLA-G are mainly through the interactions with cell inhibitory receptors such as ILT2 and ILT4, it is likely that blocking the interactions would have clinical benefit in treating HLA-G expressing cancers. Along this line, antibodies against ILT2

and ILT4 are able to restore the T-cell proliferating activity [61]. These data support a potential application of using HLA-G blockers, such as HLA-G neutralizing antibodies, soluble recombinant ILT2 and ILT4, to abrogate HLA-G mediated inhibitory effects in immune cells. As to the tumour cell surface expressed HLA-G molecules, HLA-G antibody alone or in combination with other antibodies can be used to decorate the surface of liposomes or nanoparticles that are packed with anti-cancer drugs, which are expected to specifically target cancer cells to maximize the therapeutic effects and to minimize the untoward side effects that are frequently associated with anti-cancer reagents. Be noted, a recent report indicated that HLA-G derived peptide HLA-$G_{146\text{-}154}$ could effectively induce peptide-specific CTLs, and HLA-$G_{146\text{-}154}$ peptide-stimulated PBMCs exhibited cytotoxic activity against HLA-G-expressing HLA-$A24^+$ RCC cells, indicating that HLA-G peptide-based anti-cancer immunotherapy is possible [62].

In summary, our study demonstrated that both lesion HLA-G and plasma sHLA-G expression in NSCLC are significantly related to the disease stage and exert immunosuppressive to the NK cell cytolysis, indicating that HLA-G could be a potential therapeutic target. Moreover, plasma sHLA-G in NSCLC patients could be used as a prognosis factor for NSCLC.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Analysis of sHLA-G production in A549-G cells. (**A**) Analysis of sHLA-G in cell culture supernatants with ELISA. Culture supernantant from JAR-G5 (JAR cells transfected with HLA-G5) was used as an HLA-$G5^+$ control. Medium Con., Medium controls. The dash indicates the background level. sHLA-G was undetectable in A549-G, A549 cell culture supernantants. (**B**) Western blot analysis of sHLA-G expression. The analysis was performed with the sHLA-G mAb 5A6G7 (1:1000). M, molecular weight ladder; JAR-G5 lysates were used as HLA-$G5^+$ control. Isotype, a mouse IgG1 isotype antibody (1:1000) was used as internal control for JAR-G5 lysates. (**C**) Analysis of sHLA-G with immunocytochemistry. The analysis was performed with the sHLA-G mAb 5A6G7 (1:1000). JAR and JAR-G5 cells were used as HLA-$G5^-$ and HLA-$G5^+$ controls, respectively. No intracellular sHLA-G staining was detected in A549-G cells.

Fig. S2. Flow cytometry analysis of HLA-G expression in peripheral blood $CD45^+$ nucleated cells. (**A**) An IgG isotype was used as an internal control. (**B**) Analysis of cell surface HLA-G expression in $CD45^+$ peripheral blood cells with mAb FITC-MEM-G/09. (**C**), (**D**) Analysis of intracellular sHLA-G expression in $CD45^+$ peripheral blood cells with FITC-2A12.

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