Immunology Letters 161 (2014) 13–19

Contents lists available at ScienceDirect

Immunology Letters

journal homepage: www.elsevier.com/locate/immlet

Human leukocyte antigen G is associated with esophageal squamous cell carcinoma progression and poor prognosis

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ARTICLE INFO

Article history:
Received 23 January 2014
Received in revised form 10 April 2014
Accepted 11 April 2014
Available online 21 April 2014

Keywords:
Esophageal squamous cell carcinoma
HLA-G
IL-10, Clinicopathological parameters
Prognosis

ABSTRACT

Human leukocyte antigen G (HLA-G) is a non-classical HLA class I molecule thought to play a key role in maternal-fetal tolerance and cancer immune evasion. This study aimed to investigate the HLA-G expression in lesion sections and plasma sHLA-G levels of primary esophageal squamous cell carcinoma (ESCC) patients and its clinical significance in diagnosis and prognosis of ESCC. 60 ESCC patients and 28 healthy controls were recruited, and the positive expression of HLA-G in ESCC lesions and adjacent normal tissues were 70% (42/60) and 8.6% (5/60) ($P < 0.05$), respectively, while no expression was found in normal controls. HLA-G1 and HLA-G5 were determined to be dominating isoforms measured by RT-PCR. There was a significant difference in plasma sHLA-G levels between patients with ESCC (15.04 U/ml, range 4.33–250.00 U/ml) and healthy controls (6.81 U/ml, range 0–29.27 U/ml) ($P < 0.01$). The plasma IL-10 level was higher in ESCC patients than the controls (23.86 pg/ml vs. 12.81 pg/ml, $P < 0.01$). HLA-G expression in lesion tissues was correlated with cancer cell differentiation ($P = 0.033$), lymph node metastasis ($P = 0.035$) of ESCC. However, no obvious correlations were demonstrated between the plasma sHLA-G levels and the clinicopathological parameters. There was a significant correlation between sHLA-G and IL-10 expression ($r = 0.353$, $P = 0.006$) in patients with Esophageal squamous cell carcinoma. HLA-G positive expression showed poorer prognosis of ESCC. HLA-G positive expression might serve as a potential marker in the diagnosis or prediction of ESCC.

1. Introduction

Esophageal cancer ranks 8th in incidence and 4th in mortality of malignant cancers wordwide [1]. The number of patients with esophageal cancer is increasing in response to changes in many inherent and environmental factors. Accounting for about 90% of esophageal cancer, esophageal squamous cell carcinoma (ESCC) predominates in eastern Asia and eastern and southern Africa [2,3]. Generally, the development of ESCC undergoes several phases, including atypical squamous cell hyperplasia, carcinoma *in situ* and infiltrating carcinoma. Patients are nearly always diagnosed with later-stage ESCC because of the hidden initial symptoms and limitations in detecting the cancer [4]. To date, the main factors determining the clinical outcome of ESCC depend on clinicopathology parameters and nodal status, which are not very informative. Therefore, special biomarkers associated with different clinical phases of the disease are required to predict the occurrence and survival rate of ESCC.

Human leukocyte antigen G (HLA-G) may serve as a biomarker of ESCC development. First described in 1987 by Geragthy et al. [5], HLA-G was unveiled to occur at the maternal-fetal interface on the extravillous cytotrophoblast and was recognized as a non-classical major histocompatility I b antigen. It is widely accepted that HLA-G is indispensable in inducing and sustaining maternal immunotolerance of the fetus [6]. Nowadays, interest in HLA-G is gradually shifting from fetal immunotolerance to its abnormal expression in malignant cancers, including colon, gastrointestinal, breast and kidney cancer [7–9]. Shang-rong Ye et al. reported that HLA-G protein expression was observed in 64.6% (130/201) of primary colorectal carcinomas, but not in normal colorectal tissues or benign adenomas [10]. Kurman found that 45 (61%) out of 74 ovarian serous carcinoma samples and 22 (25%) invasive ductal

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http://dx.doi.org/10.1016/j.imlet.2014.04.007
0165-2478/

breast carcinoma samples demonstrated HLA-G immunoreactivity, which ranged from 2 to 100% of the tumor cells [11]. Likewise, accumulating data indicate that HLA-G may be involved in tumor cell initiation and evolution. Emerging evidence suggests that HLA-G employs similar strategies in mediating the escape of cancer cells from immune surveillance as those used during maternal immunotolerance of the fetus. HLA-G executes its functions by suppressing alloreactive $CD4^+$ T cell proliferation, inhibiting NK- or T-cell-mediated cytolysis and impeding maturation and function of dendritic cells (DC) [9,12]. HLA-G occurs as seven isoforms derived from seven alternatively spliced transcripts from a single mRNA, of which four are membrane-bound (-G1, -G2, -G3 and -G4) and three soluble (-G5, -G6 and -G7) [13].

Interleukin-10 (IL-10), which is a type of immunosuppressive cytokine, is thought to promote tumor evasion and can be secreted by tumor and tumor-infiltrating cells [14]. Previous studies have shown that IL-10 can upregulate the expression of HLA-G in tumor cells and that there is a mutual increase in IL-10 and HLA-G expression, which can then initiate a vicious cycle to promote immune evasion [15]. However, the expression levels of soluble HLA-G (sHLA-G) and IL-10 in the peripheral blood of patients with ESCC is still unclear. Although HLA-G expression has been explored in many tumors, little information is currently available for interpretating the correlation between HLA-G expression and ESCC growth and development. In this study, we aimed to determine the role of HLA-G played in the occurrence, development and prognosis of ESCC and the effect of IL-10 on sHLA-G expression.

2. Materials and methods

2.1. Study populations

60 ESCC patients underwent operative treatment and were enrolled between January 2008 and January 2009 at the Department of Thoracic Surgery in TangDu Hospital, Xian, Republic of China. The mean age of the patients at the time of diagnosis was 60 years (SD = 10.45 years); 47 males and 13 females were included. All the patients were primary ESCC cases without history of other tumors and none had preoperative chemotherapy or radiotherapy. At the same time, 28 normal patients without tumors but undergoing gastroscopy examination were used as a matched group. The ESCC and surrounding pericarcinomatous tissues obtained after surgery were fixed in 10% formalin, and the rest were frozen in liquid nitrogen and stored at −80 °C. This study was approved and monitored by the ethics committee at TangDu Hospital.

2.2. Immunohistochemical staining

Staining of streptavidin/peroxidase (S–P) complexes was performed to detect the expression of HLA-G. The cut 5-μm-thick paraffin-embedded esophageal ectopic tissue sections were mounted on polylysine-coated slides, dewaxed in xylene and rehydrated using a graded ethanol series, before being washed 3 times with phosphate buffered saline (PBS). Deparaffinized tissue sections were subjected to antigen retrieval treatment at 100 °C for 10 min in 0.01 M citrate–sodium buffer (pH 6.0 ± 0.1) and then cooled to room temperature. The section was then placed in 0.3% H_2O_2 for 20 min to block esophageal peroxidase activity and nonspecific binding was prevented by applying 30% of non-immuno serum for 20 min before staining with the primary anti-HLA-G mAb MEMG-1 (Biovendor, Czech) (1:60) for 3 h at 37 °C. A thorough washing in 0.01 mol/l PBS was then performed. Immunostained sites were visualized using a DAKO EnVision kit (DAKO, Glostrup, Denmark), in accordance with the manufacturer's instructions. Subsequently, the tissue sections were lightly counterstained with hematoxylin for about 40 s and then fixed with neutral balata for observation. Matched sections that were treated with PBS buffer instead of primary anti-HLA-G mAb MEMG-1 were set as blank controls, while normal esophageal squamous tissues without HLA-G expression were used as negative controls. Gastric gland carcinoma tissue, known to express HLA-G, was employed as a HLA-G-positive control.

2.3. Analyzing tissue staining

HLA-G-positive staining was determined by the occurrence of yellow particles in the cell cytoplasm and membrane of the stained ESCC or normal tissues. At least 5 random fields were observed and the mean estimated percentage of HLA-G-positive cells was obtained. Finally, the staining results were categorized into 4 grades: negative (tissue specimen without staining); 1+ (percentage of HLA-G-positive ESCC cells <25%); 2+ (percentage of positive ESCC cells is between 25 and 50%); and 3+ (percentage of positive ESCC cells >50%). Accordingly, the negative and 1+ groups were assigned as HLA-G negative, and the 2+ and 3+ HLA-G positive. All tissue sections were analyzed by two experienced pathologists separately and a third one was used if there was a difference in opinion.

2.4. RT-PCR

The total RNA of normal and ESCC tissues were extracted and purified using the Rneasy mini kit, according to the manufacturer's instructions (Qiagen, SantaClarita, CA). After measuring RNA purity, 1 μg of RNA was used as the template for PCR. DNA sequences of HLA-G1 and HLA-G5 were obtained from GeneBanK and their primers were designed using Primer5.0 and synthesized by Beijing AuGCT DNA-SYN Biotechnology Co., Ltd., as follows. HLA-G (988 bp) sense primer: 5′-GGA AGA GGA GAC ACG GAA CA-3′, antisense primer: 5′-TGAGAC AGA GAC GGA GAC AT-3′; HLA-G5 (449 bp) sense primer: 5′-ACCGACCCTGTTAAAGGTCTT-3′, antisense primer: 5′-CAATGTGGCTGAACAAAGGAGAG-3′; β-actin (315 bp) sense primer: 5′-GAAGCATTTGCGGTGGACGAT-3′, antisense primer: 5′-TCCTGTGGCATCCACGAAACT-3′. The proliferation reaction was performed at 94 °C for 30 s after 3 min of initial denaturation, then annealing at 56, 54 and 55 °C for 30 s for HLA-G1, HLA-G5 and β-actin, respectively, followed by target DNA elongation at 72 °C for 45 s. Thirty cycles were executed with a final elongation at 72 °C for 5 min. PCR products were subjected to agarose gel electrophoresis analysis and scanned.

2.5. sHLA-G and IL-10 determination

3 mL of blood were obtained from these patients before the operation, kept at room temperature for 2 h and then centrifuged for 15 min. Plasma samples were stored at −80 °C until analyzed. The sHLA-G and IL-10 levels were measured with a sHLA-G ELISA kit (Biovendor, CZ) and IL-10 ELISA kit (BioSource, USA), respectively. The cut-off values for plasma sHLA-G and IL-10 levels were 3 U/ml and 0.2 ng/ml, respectively, according to the manufacturer's instructions.

2.6. Statistical analysis

All statistical analyses were carried out using the SPSS 13.0 software program (SPSS, Chicago, IL). For comparisons among groups, the Chi-square test was used for categorical variables, and the unpaired Student's *t*-test or the one-way analysis of variance was applied for continuous variables. The levels of sHLA-G and IL-10