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Association between the HLA-G molecule and lymph node metastasis in papillary thyroid cancer

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ABSTRACT

Papillary thyroid carcinoma (PTC) is the most common type of thyroid cancer and can present as lymph node metastasis in 30 to 65% of cases when initially diagnosed. High frequency recurrence, distant metastasis and treatment resistance can be found in cases of PTC so early diagnosis and treatment are critical for improved prognosis and better survival rates. The characterization of new biomarkers has proved useful for the diagnosis and follow-up of these patients. HLA-G is a non-classical HLA class I molecule whose expression in cancer cells has been associated with tumor evasion of immune response. Therefore, the aim of this study was to investigate the HLA-G expression and its clinical significance in PTC. Paraffin-embedded thyroid biopsies of 70 PTC patients (40 of whom had presented with metastasis) were evaluated. HLA-G-staining was observed in tumor cells in PTC, and the HLA-G expression was significantly associated with an increased occurrence of lymph node metastasis ($p = 0.0006$) and capsular invasion ($p = 0.02$). This preliminary data shows the HLA-G expression in thyroid carcinoma specimens for the first time and suggests that this expression could impair efficient anti-tumor immunity in PTC. This would indicate that HLA-G could have an independent prognostic value in PTC, principally for tumor recurrence.
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1. Introduction

Papillary thyroid cancer (PTC) is a malignant epithelial tumor characterized by the formation of papillae or many different nuclear aspects [1]. It is the most common form of thyroid tumor, and represents approximately 1% of malignancy in Western societies and 80% of all thyroid carcinomas. Compared with other human cancers, the incidence of this form has increased, mainly among women [2]. Even though PTC is one of the most curable cancers, a papillary thyroid tumor may present lymph node metastases in 30 to 65% of cases, when initially diagnosed. In 15% of cases the tumor is aggressive and local invasion, distant metastases and treatment resistance can be found. There is also an increased risk of dying as a consequence of the disease and a high frequency of recurrence [3]. Thus, early diagnosis and treatment of thyroid cancers is crucial for improved prognosis and a better survival rate. The characterization of new biomarkers has proved useful, not only for the early detection of thyroid cancer, but also for detecting recurrent and persistent diseases and for predicting the effectiveness of surgical removal, radioiodine ablation, and chemotherapy [4].

HLA-G is a non-classical HLA class I molecule initially described as being selectively expressed at the maternal fetal interface on cytotrophoblast cells, where it appears to participate in maternal tolerance towards the fetus. Although HLA-G is not expressed in most adult tissues, its ectopic expression has been seen in diseases, such as viral infections, autoimmune disorders, and in cancer in particular [5].

The HLA-G molecule differs from the other HLA class I molecules as it presents limited polymorphism, restricted tissue

Abbreviations: PTC, papillary thyroid cancer; HLA, human lymphocyte antigen; NK, natural killer cell; CTLs, cytotoxic T lymphocytes; APCs, antigen-presenting cells; TNM, tumor–node–metastasis; ROC, receiver operating curve; AUC, area under the ROC curve; IHC, immunohistochemistry.

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expression, seven isoforms (HLA-G1 to G7) and exerts an overall negative immune function inhibiting the activity of natural killer cells (NKs), cytotoxic T lymphocytes (CTLs) and antigen-presenting cells (APCs), all of which are essential to the development of a cytotoxic anti-tumor immune response [6]. Thus, the HLA-G expression could be an additional mechanism used by tumor cells to resist an efficient anti-tumor immune response by impairing the function of host immune effector cells [7,8].

There is evidence that HLA-G is induced in various cancers, including ovarian, gastric, endometrial, breast, renal cell and lung carcinomas, coetaneous melanoma, hematopoietic tumors, mesothelioma, and trophoblastic tumors [9–15]. Several studies have demonstrated a correlation between the HLA-G expression and patient's clinical outcome, including overall survival and the risk of developing metastatic diseases [16].

Considering the importance of the characterization of new biomarkers in thyroid cancer and the fact that HLA-G immunoreactivity could prove useful in predicting clinical behavior in cancer patients and provide oncologists with a new molecular approach for improved management of their cancer patients, the aim of this study was to investigate whether the HLA-G expression could be activated in PTC and thus constitute a marker of tumor aggressiveness. To date there have been no other studies carried out on the HLA-G expression of *in situ* thyroid cancer.

2. Materials and methods

2.1. PTC biopsies

Paraffin-embedded thyroid biopsies from 70 patients presenting with PTC from 2000 to 2005 were retrospectively selected from the archives of the Department of Pathology, Anatomopathology Division at the Araújo Jorge Hospital, Goiás Combat Cancer Association (HAJ/ACCG), Goiânia, Goiás, Brazil. Thyroid biopsies were obtained from 40 patients presenting with cervical lymph node and/or distant metastases and from 30 metastasis-free patients. The paraffin-embedded tumor specimens were stained with hematoxylin–eosin–safranin for histological re-examination and tumor grading. Tumors were staged according to the tumor–node–metastasis (TNM) classification of malignant tumors by the International Union against Cancer [17].

Clinical and epidemiological data (sex, age, tumor size, number of committed lymph nodes and the presence of thyroid capsular invasion and sites of distant metastasis) were obtained from medical archives.

The study protocol was approved by the local Research Ethics Committee (protocol 049/2010)

2.2. Immunohistochemistry

Four-micrometer sections were cut from the paraffin-embedded specimens. The Universal HRP-Polymer MACH 4 detection system (Biocare Medical, Concord, CA, USA) was used. After rinsing the sections in phosphate buffered saline with 0.1% saponin, endogenous peroxidases were inhibited using H_2O_2. Samples were initially incubated with specific or irrelevant antibodies for 1 hour at room temperature and subsequently with a solution containing a MACH 4 Mouse Probe for 15 minutes. Diaminobenzidine plus a chromogen-substrate were used to develop antibody fixation. The specific monoclonal antibody MEM-G/2 (Exbio, Praha, Czech Republic), which recognizes the free heavy-chain of all HLA-G isoforms, was used. An identical IgG1 isotype anti-desmin antibody which was run simultaneously with each sample served as a negative control. Invasive (intermediate) cytotrophoblasts from a third-trimester human placenta served as a positive protein control.

2.3. Evaluation of stained sections

An immunohistochemical analysis of tumor tissue was undertaken. Immunoreactivity was scored by evaluating the percentage of positive tumor cell, using a semi-quantitative scoring method. The cut-off score for determining the positivity of HLA-G detected by immunohistochemistry was obtained by receiver operating characteristic (ROC) curve analysis. ROC curve analysis was performed for the HLA-G expression in both metastatic and non-metastatic samples. The score with the shortest distance from the curve to the point with both maximum sensitivity and specificity (i.e. the point (0.0, 1.0) was selected as the cut-off score, leading to the greatest number of tumors correctly classified as either having or not having metastasis. The percentage of intra-tumoral inflammatory cells was scored as follows: 0, no inflammatory cells; 1, <25% of inflammatory cells; and 2, $\geqslant$25% of inflammatory cells. All sections were blindly analyzed using a light microscope with high-power fields ($\times$400).

2.4. Statistical analysis

The ROC curve of staining performance for determining the cut-off score of the HLA-G expression was done. The immunostaining scores were compared with the Mann–Whitney *U* test and the correlations of the immunostaining scores were tested using the Spearman correlation analysis. Comparative analyses between the groups were performed using the two-sided Fisher exact test. The odds ratio (OR) was used to estimate the metastasis and capsular invasion relative risk when patients expressed HLA-G. A p value of less than 0.05 was considered significant. All statistical analyses were performed using the GraphPad Instat (version 5.0).

3. Results

3.1. Clinical and epidemiological findings

The main clinical features of this series of 70 patients presenting with PTC are summarized in Table 1. The patient group consisted of 48 (68.6%) females and 22 males (31.4%), ranging in age from 13 to 81 (mean: 35.8 years).

Forty patients presented metastases, of who 38 presented with lymph node metastases alone while 2 presented with distant metastases as well (brain or mediastinal lymph nodes). Females predominated in this group (72.5%).

Table 1
Main clinical findings of patients with metastatic PTC (n = 40) and non-metastasis PTC (n = 30)

	Metastatic	Non-metastatic
Gender		
Female	72.5%	63.3%
Male	27.5%	36.7%
Mean age in years	33	41
Tumor size (*mean*)	2.33 cm	2.4 cm
Mean of committed lymph nodes	4.2 out of 10	–
Distant metastasis	5.4%	–
Presence of thyroid capsular invasion	85%	30%
PTC Staging		
I	67.5%	76.7%
II	2.5%	13.3%
III	0	6.7%
IV	30%	3.3%

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