Overexpression of HHLA2 is Markedly Associated with the Poor Prognosis in Medullary Thyroid Carcinoma

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Research Article

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Abstract

Human endogenous retrovirus-H long terminal repeat-associating protein 2 (HHLA2) is a newly identified immune checkpoint molecule that was aberrantly expressed in many malignant tumors. However, its expression in medullary thyroid carcinoma (MTC) is still unclear. This study aimed to investigate the HHLA2 expression in MTC tissues and to evaluate the relationships between its expression and clinicopathologic together with prognostic relevance. Using 51 surgical specimens obtained from MTC patients, the expression levels of the HHLA2 protein in MTC tumor tissues and adjacent noncancerous tissues were measured by immunohistochemistry, and its correlations with clinicopathologic and prognostic features were analyzed. Status of CD8+ tumor-infiltrating lymphocytes (TILs) was also investigated. The results showed that HHLA2 was only detected in tumor tissues, and that 31.4% of the MTC patients had high expression of HHLA2. High HHLA2 expression was significantly associated with lymph node metastasis and advanced AJCC stages (P=0.005). There existed an inverse trend between HHLA2 expression and CD8+ TILs infiltration in MTC tumor samples (P=0.042). The log-rank test showed a shorter disease-free survival in patients with high HHLA2 expression (P=0.002). The disease-free survival rates were also significantly low in cases of MTC with lymph node metastasis, AJCC stages III-IV and multifocality. Multivariate Cox analysis confirmed that HHLA2 acted as an independent predictive factor in the disease-free survival of MTC patients (HR=4.138, 95%CI: 1.027-16.667, P=0.046). Taken together, HHLA2 is highly expressed in MTC patients, and is a poor prognostic biomarker of disease-free survival of MTC patients.

Introduction

Medullary thyroid carcinoma (MTC) is an uncommon neuroendocrine malignancy originating from parafollicular C-cells, and accounts for 3–5% of all thyroid tumors. Although it is relatively rare, MTC leads to 14% of all thyroid carcinoma deaths [1]. About half of the patients are diagnosed with advanced MTC (stages III and IV) at the time of discovery, and the ten-year survival rates for patients with stages III and IV MTC are 71% and 21%, respectively [2]. Surgery is the main curative management for most patients [2, 3]. Unfortunately, approximately 50% of patients have recurrence or metastasis later in the course of the disease [4]. Adjuvant interventions, such as external radiation, thermal ablation or chemotherapy, have limited efficacy [5]. Up to date, the mechanisms of tumorigenesis and development in MTC remain poorly understood. Therefore, there exists an urgent need to reveal the mechanisms of MTC development and progression for investigating novel diagnostic and prognostic markers or seeking effective therapeutic targets.

Tumor immune escape has been reported as a new mechanism of tumor generation and growth. The immune checkpoint B7/CD28 protein family plays an important role in the immune response mediated by T cells as co-signaling molecules [6, 7]. Their abnormal expression is conducive to the formation of an immunosuppressive environment, which prevents tumor cells from surveillance and killing by the immune system, leading to the continuous development of the tumor [8, 9, 10]. Based on the tumor immunological characteristics, immunotherapy for immune checkpoints has become a research focus in the study of

malignant tumors, especially certain advanced cancers [10, 11]. PD-1 and its ligand PD-L1 are important immunosuppressive checkpoints. Drugs targeting anti-PD-1/PD-L1 have been developed, which display significant effects in a variety of solid tumors and lead to great breakthrough in tumor immunotherapy [12–14]. However, the anti-PD-1 drug, nivolumab, is poorly effective in MTC; only one MTC patient's clinical condition was partially alleviated [15].

Human endogenous retrovirus-H long terminal repeat-associating protein 2 (HHLA2) was discovered in 1999 as a newly identified member of B7/CD28 family [16], which plays an important role in immune escape [17]. As a type I transmembrane molecule, HHLA2 shares 10–18% amino acid identity and 23–33% similarity to the other B7 molecules. The unique feature of HHLA2 is that it is expressed in humans but not in mice. It is constitutively expressed on peripheral monocytes and is induced on B cells [18]. HHLA2 serves as the co-inhibitory signal molecule and effectively inhibits TCR-mediated CD4 and CD8 T cell proliferation and cytokine production, thereby participating in tumor immune escape [18]. Recently, systematic studies of HHLA2 in normal human tissues and cancers revealed that HHLA2 was not expressed in most normal tissues, but was widely expressed in many types of malignant tumors, including colon, esophagus, kidney, lung, thyroid, bladder, breast, liver, ovary, pancreas, prostate and melanoma cancers [19]. Therefore, the immune checkpoint protein HHLA2 plays a significant role in the tumorigenesis and development process of malignant tumors. However, the clinical and prognostic significances of HHLA2 expression were not clarified in highly malignant MTC.

In this study, the levels of HHLA2 and CD8 + tumor-infiltrating lymphocytes (TILs) infiltration in MTC tissues were analyzed by the immunohistochemical method, and the relationships between HHLA2 expression and clinicopathological characteristics and prognosis were discussed.

Materials And Methods

Patients

MTC patients were included in this study if they underwent surgical resection in the Affiliated Hospital of Qingdao University between March 2015 and January 2020. All patients were treated initially, and histopathological diagnoses were performed by two professional physicians. Formalin-fixed, paraffin-embedded tumor tissues and adjacent tissues were collected for histopathological analysis, and clinicopathological and follow-up information were retrieved from the patient's medical records. This study was approved by the Ethics Committee of the Affiliated Hospital of Qingdao University, and informed patient consent was obtained.

Immunohistochemistry

The rabbit anti-human HHLA2 polyclonal antibody (1:200 dilution, ab214327, Abcam, Cambridge, UK) was used to detect HHLA2 expression in tumor cells and adjacent noncancerous cells. CD8 mouse monoclonal antibody (diluted at 1:200, GB13068-2, Servicebio, Wuhan, China) was used to assess CD8 expression on tumor-infiltrating T lymphocytes. The paraffin-embedded tumor samples and

corresponding adjacent tissues were deparaffinized and rehydrated with decreasing ethanol concentrations, and citrate buffer (pH 6.0) was used for antigen retrieval. Endogenous peroxidase activity was blocked by incubating with 3% H_2O_2 at room temperature in the dark for 25 min. Nonspecific binding of IgG was blocked with 3% BSA for 30 min at room temperature. The sections were individually incubated with the rabbit anti-human HHLA2 polyclonal antibody or CD8 mouse monoclonal antibody overnight at 4 ^{o}C, followed by incubation with the HRP-labeled secondary antibodies directed against rabbit or mouse IgG for 50 min at room temperature. Immunoreactivity was visualized using DAB. The sections were counterstained with hematoxylin, dehydrated, and mounted. Primary antibodies were replaced with phosphate buffered saline as a negative control, and HHLA2 expression in rectal cancer tissues was chosen as the positive control.

Evaluation of immunohistochemical staining

The immunohistochemical results were reviewed independently by two experienced pathologists. The positive cell staining intensity score (IS) criteria was adapted as follows: no staining (similar to the background color) was 0 points, mild staining (light yellow) was 1 point, moderate staining (tan) was 2 points, heavy staining (brown) was 3 points. On the HHLA2-positive cell rating score (AP): 0% is 0 points, 1–25% is 1 point, 26–50% is 2 points, 51–75% is 3 points, and > 75% is 4 points. The final H-score was calculated by the following equation: H-score = IS × AP. The median score was used to determine the cutoff point between a high or low expression [20, 21]. Additionally, the numbers of CD8 + TILs in five high-power (×400) visual fields for each sample were counted, and the average value was used for statistical analysis [22].

Statistical analysis

All statistical analyses were performed using SPSS 22.0 statistical software (SPSS, Inc., Chicago, IL, USA). Continuous variables are represented as mean ± standard deviation, and categorical variables are represented by frequency (%). The relationships between HHLA2 expression and clinicopathological characteristics were evaluated using the χ^2 test or Fisher's exact test, as appropriate. The Mann-Whitney U-test was used for comparison between two groups. The Kaplan-Meier method was used to estimate disease-free survival analysis and the log-rank test was used to compare curves. A multivariate Cox proportion hazard regression model was established to assess the independent predictors of disease-free survival. All *P*-values < 0.05 were considered statistically significant.

Results

Clinical characteristics of patients with MTC

Among the 51 patients included in the study, 24 (47.1%) were men and 27 (52.9%) were women, with a mean age of 48.1 ± 13.5 (range, 16–71 years). The maximum diameter of the tumors ranged from 0.7–7.5 cm, with an average of 2.6 ± 1.5 cm. As defined in the 8th AJCC staging system, 21 patients were classified as stages I-II and 30 as stages III-IV. 30 patients were diagnosed as neck lymph node

metastasis and 21 patients were not neck lymph node metastasis. 3 cases were extrathyroid invasion and the others were no extrathyroid invasion. There were 34 cases with unifocality and 17 cases with multifocality (Table 1).

Table 1
Patient characteristics (N = 51)

Characteristic	Sub-characteristic	Value (%)
Age		48.1 (range 16–71)
Gender	Male	24 (47.1%)
	Female	27 (52.9%)
Tumor size (cm)		2.6 (range 0.7–7.5)
Depth of invasion	T1 + T2	38 (74.5%)
	T3 + T4	13 (25.5%)
Lymph node metastasis	Yes	30 (58.8%)
	No	21 (41.2%)
AJCC stages	I-II	21 (41.2%)
	III-IV	30 (58.8%)
Extrathyroid invasion	Yes	3 (5.9%)
	No	48 (94.1%)
Multifocality	Yes	17 (33.3%)
	No	34 (66.7%)
Smoking history	Yes	18 (35.3%)
	No	33 (64.7%)
Alcohol history	Yes	12 (23.5%)
	No	39 (76.5%)
Diabetes history	Yes	7 (13.7%)
	No	44 (86.3%)
Total		51 (100%)

HHLA2 expression in MTC and noncancerous tissues

The expression of HHLA2 protein in MTC tissues is currently unknown. In the present study, HHLA2 expression was not detected in the adjacent noncancerous tissues, whereas HHLA2 immunostaining was

observed in MTC tissues (Fig. 1). In tumor tissues, HHLA2 is located in the cytoplasm of tumor cells (Fig. 2). According to the HHLA2 scoring standard, the H-score of HHLA2 expression ranged from 0.0–4.0, with a median of 2.0. The median was used to determine the cutoff point between high or low expression of HHLA2, with an H-score > 2 regarded as high expression. In 51 MTC tissues, 16 cases (31.4%) showed high HHLA2 expression, and 35 cases (68.6%) had low HHLA2 expression (H-score ≤ 2).

Correlations between HHLA2 expression and clinicopathological features

The associations between HHLA2 expression and clinicopathological features in MTC were investigated using the χ^2 test or Fisher's exact test (Table 2). High HHLA2 levels were significantly correlated with lymph node metastasis and advanced AJCC stages (III-IV) ($P = 0.005$); HHLA2 expression was not significantly associated with other clinical features, including age, gender, tumor size, depth of invasion, extrathyroid invasion, and multifocality. There were no significant differences in smoking history, alcohol history, or diabetes history in patients with MTC ($P > 0.05$).

Table 2
Correlations between HHLA2 expression and clinicopathological parameters of patients with MTC

Characteristic	N	HHLA		
		Low (%)	High (%)	*P*-value
Age				
49 years	25	17 (33.3%)	8 (15.7%)	0.925
≥ 49 years	26	18 (35.3%)	8 (15.7%)	
Gender				
Male	24	16 (31.4%)	8 (15.7%)	0.776
Female	27	19 (37.3%)	8 (15.7%)	
Tumor size				
> 2 cm	29	21 (41.2%)	8 (15.7%)	0.503
≤ 2 cm	22	14 (27.5%)	8 (15.7%)	
Depth of invasion				
T1 + T2	38	27 (52.9%)	11 (21.6%)	0.730
T3 + T4	13	8 (15.7%)	5 (9.8%)	
Lymph node metastasis				
Yes	30	16 (31.4%)	14 (27.5%)	0.005
No	21	19 (37.3%)	2 (3.9%)	
AJCC stages				
I-II	21	19 (37.3%)	2 (3.9%)	0.005
III-IV	30	16 (31.4%)	14 (27.5%)	
Extrathyroid invasion				
Yes	3	2 (3.9%)	1 (2.0%)	1.000
No	48	33 (64.7%)	15 (29.4%)	
Multifocality				
Yes	17	10 (19.6%)	7 (13.7%)	0.286
No	34	25 (49.0%)	9 (17.6%)	
Smoking history				

Characteristic	N	HHLA		
		Low (%)	High (%)	*P*-value
Yes	18	13 (25.5%)	5 (9.8%)	0.683
No	33	22 (43.1%)	11 (21.6%)	
Alcohol history				
Yes	12	9 (17.6%)	3 (5.9%)	0.730
No	39	26 (51%)	13 (25.5%)	
Diabetes history				
Yes	7	6 (11.8%)	1 (2%)	0.410
No	44	29 (56.8%)	15 (29.4%)	

Relationship between HHLA2 expression and CD8 + TILs infiltration

To investigate the relationship between HHLA2 and CD8 + TILs infiltration, the MTC patients were divided into two groups based on HHLA2 levels (high or low), then CD8 expression was analyzed. In the high HHLA2 expression group, the numbers of CD8 + TILs infiltration ranged from 0.0 to 5.5, while for the low group, the numbers of CD8 + TILs were from 1.0 to 25.0. The mean value of CD8 + TILs infiltration (± SD) in high and low HHLA2 groups was 2.67 ± 0.407 and 6.71 ± 1.096, respectively. The CD8 + TILs levels were significantly lower in tumor tissues of the high HHLA2 expression group compared with the low group (Fig. 3, P = 0.042).

Survival analysis

The median follow-up time was 36 months (IQR: 12–57 months). During the follow-up, only one patient died and 12 patients had disease progression (recurrence and/or metastasis). Kaplan-Merier survival analysis illustrated that MTC patients with high HHLA2 expression had inferior disease-free survival rates than those with low HHLA2 expression ($P = 0.002$). The disease-free survival were also significantly shorter in patients with lymph node metastasis, AJCC stages III-IV and multifocality (P 0.05) (Fig. 4). The multivariate Cox regression analysis showed that high expression of HHLA2 (HR = 4.138, 95%CI: 1.027–16.667, $P = 0.046$) was an independent prognostic factor in the disease-free survival of patients with MTC (Table 3).

Table 3
Multivariate Cox regression analysis for the prediction of disease-free survival of MTC patients

Variable	Disease-free survival		
	HR	95%CI	*P*-value
HHLA2 (high vs. low)	4.138	1.027–16.667	0.046
Lymph node metastasis (Yes vs. No)	1.386	0.215–8.936	0.731
AJCC stages (III-IV vs. I-II)	1.386	0.215–8.936	0.731
Multifocality (Yes vs. No)	2.658	0.811–8.707	0.106

Discussion

HHLA2, as an immune checkpoint molecule of the B7 family, plays a critical role in tumor immune evasion [23]. HHLA2 serves as a negative costimulatory molecule in several malignant tumors [24]. Recently, Janakiram et al. have found that HHLA2 was elevated in many human cancers [19]. Subsequently, other researchers also reported a high upregulation of HHLA2 in various malignant tumor tissues, such as colorectal cancer [20], gastric cancer [21], lung cancer [7, 25, 26], osteosarcoma [27], bladder cancer [28], liver cancer [29] and clear cell renal cell carcinoma [30]. As a highly malignant tumor, MTC is prone to local spread or distant metastasis. However, the HHLA2 expression in MTC was still not very clear. This research investigates the relationships between HHLA2 expression and clinical relevance in MTC.

In our study, HHLA2 was located in the cytoplasm of tumor cells by immunohistochemistry, with nearly one third of the cases exhibiting high expression. Besides, HHLA2 expression was not detected in the normal thyroid tissues around the corresponding tumors, in agreement with the aforementioned result that normal thyroid tissue was negative for HHLA2 protein [19]. Furthermore, statistical analysis indicated that high HHLA2 levels were significantly related to lymph node metastasis and advanced AJCC stages, which was in accordance with HHLA2 expression in other tumors. In patients with triple-negative breast cancer, 56% of tumor tissues exhibited high expression of HHLA2, which was significantly associated with local lymph node metastasis and advanced stages at diagnosis [19]. In addition, high HHLA2 expression in gastric cancer tissues was positively correlated with advanced clinical stage, tumor invasion, lymph node metastasis and distant metastasis [21]. In bladder urothelial carcinoma, HHLA2 expression had positive association with tumor size, stage, grade and lymph node metastasis [28]. Besides, high HHLA2 expression levels were related to lymph node metastasis and clinical staging in intrahepatic cholangiocarcinoma [29]. As for clear cell renal cell carcinoma, HHLA2 overexpression had positive correlation with tumor size, clinical stage and histological grade [30]. These studies demonstrate that overexpression of HHLA2 was closely related to tumor evolution and can serve as an important indicator in tumor clinical progression.

Further analysis revealed that disease-free survival was significantly poor in patients with lymph node metastasis, AJCC stages III-IV and multifocality. At the same time, the disease-free survival of patients with high expression of HHLA2 was also lower than that of patients with low expression, and high HHLA2 expression was an independent risk factor for disease-free survival, which was consistent with previous studies in human colorectal carcinoma, gastric cancer, lung adenocarcinoma, osteosarcoma, bladder cancer, liver cancer and clear cell renal cell carcinoma [20, 21, 26–30].

HHLA2 inhibited CD8 + T cell functions by binding to the corresponding receptor [31]. Our results revealed that the numbers of CD8 + TILs in the high HHLA2 group were significantly lower than those of the low HHLA2 group. Consistent with intrahepatic cholangiocarcinoma, high HHLA2 expression was correlated with lower intratumoral CD8 + TILs counts [29]. Similar result was also reported in colorectal carcinoma [20]. These results indicate that HHLA2 mainly acts as a T cell co-inhibitory ligand. It binds to putative receptors on the surface of a variety of immune cells, including T cells and antigen-presenting cells. Subsequently, HHLA2 inhibits CD4 + and CD8 + T cell proliferation and cytokine production, including IFN-γ, TNF-α, IL-5, IL-10, IL-13, IL-17A, and IL-22 [18, 24, 31], which consequently weakens the body's anti-tumor immune response, thereby allowing tumor immunity to escape. The HHLA2 pathway may represent a novel immunosuppressive mechanism and provide an attractive target for the development of new immunotherapies in MTC.

There are some limitations in our study. The low incidence of MTC limits the number of available tissue samples. The role and specific mechanisms of HHLA2 in immune system regulation also need to be explored in future studies. In addition, the relationship between HHLA2 expression and immunotherapy should attract more attention.

In conclusion, HHLA2 was upregulated in MTC tissues. High HHLA2 expression was closely associated with tumor progression and can serve as an independent prognostic factor for the disease-free survival of patients with MTC. HHLA2 expression is negatively correlated with CD8 + TILs infiltration in MTC tumor samples, which is attributed to the co-inhibitory effect for T cell.

Declarations

Funding This study was funded by Start-Up Funds from the Affiliated Hospital of Qingdao University.

Compliance with ethical standards

Conflict of interest The authors declare that there are no conflict of interests in this study.

Ethical approval This study was approved by Ethics Committee of the Affiliated Hospital of Qingdao University and was conducted in accordance with the ethical principles.

Informed consent Informed patient consent was obtained in this study.

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Figures

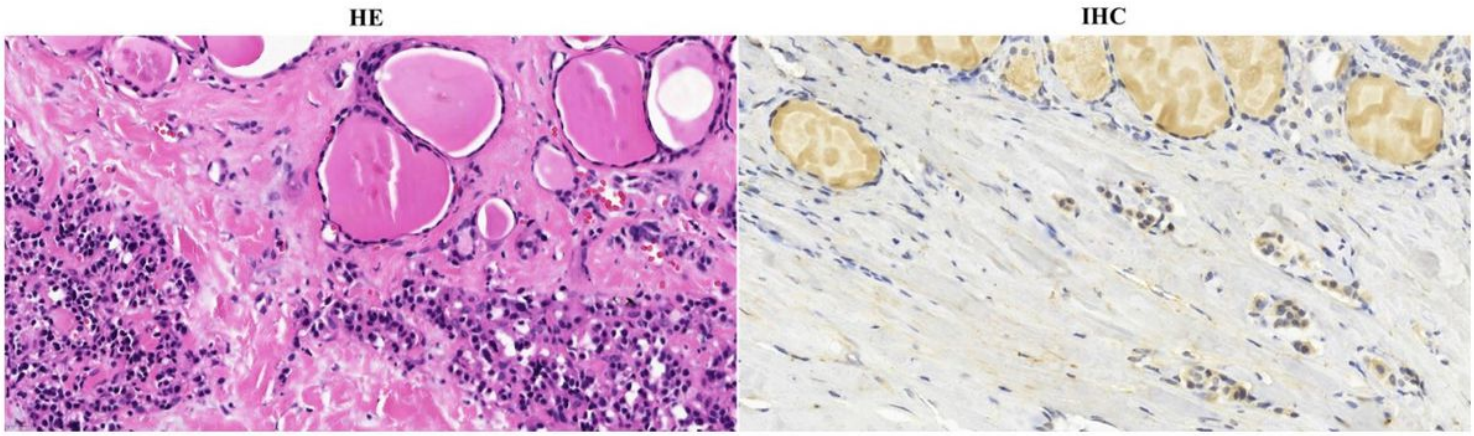

Figure 1

Histopathological analysis of HHLA2 expression in medullary thyroid carcinoma patients. Medullary thyroid carcinoma tissues show positive expression, but no expression of HHLA2 is detected in the adjacent noncancerous tissues (magnification 400×).

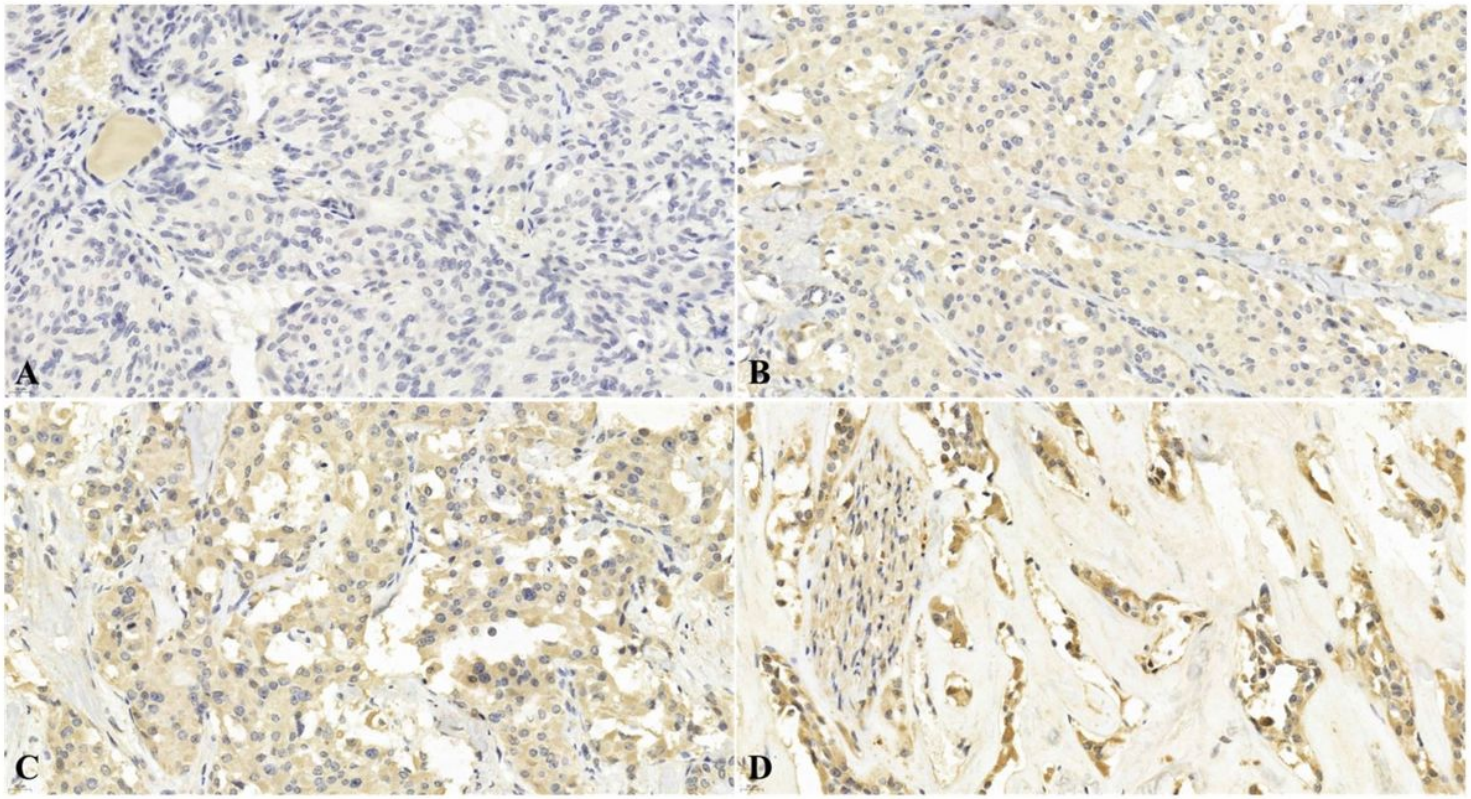

Figure 2

Representative photomicrographs of HHLA2 expression in medullary thyroid carcinoma tissues. (A) no expression, (B) weak expression, (C) moderate expression, (D) strong expression, nerve invasion (+) (magnification 400×).

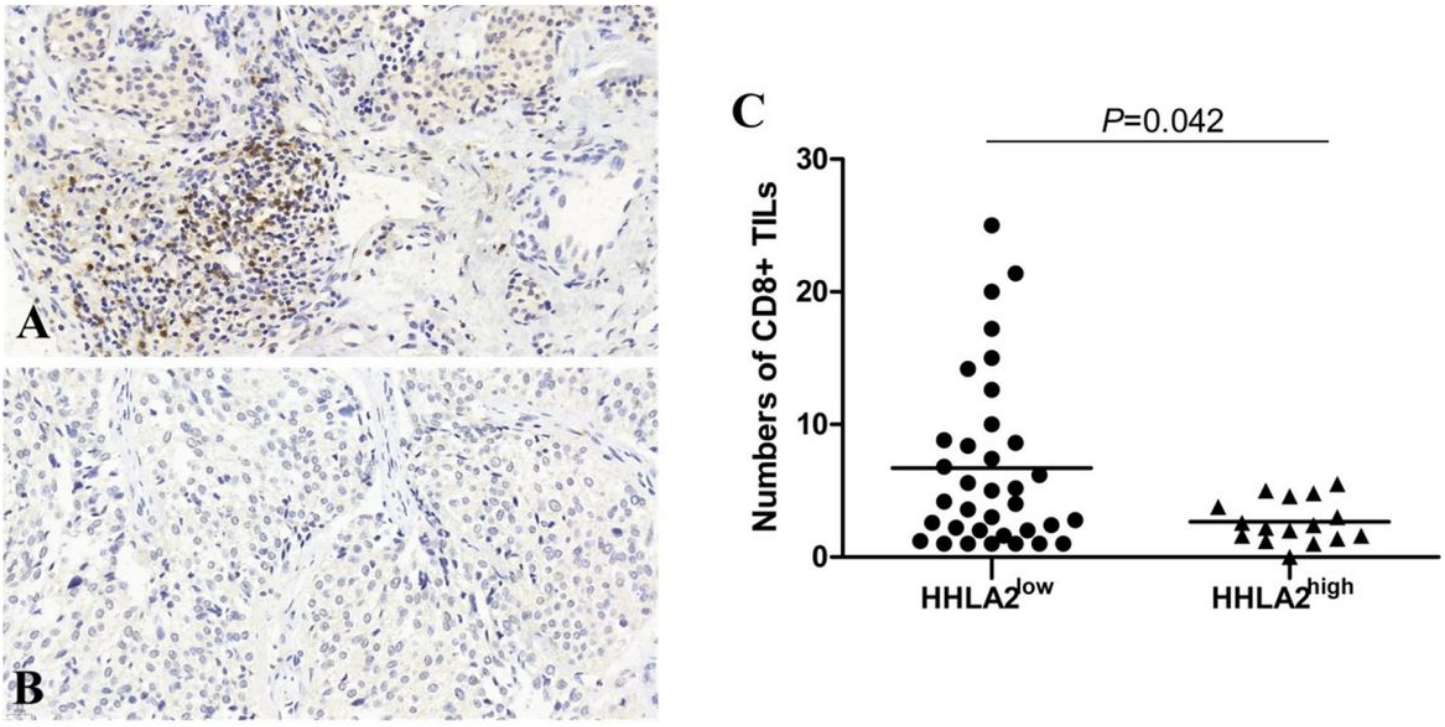

Figure 3

HHLA2 expression and CD8+ TILs infiltration exhibited an inverse trend in tumor tissues. (A) CD8 positive expression in HHLA2-low tumor tissues, (B) CD8 negative expression in HHLA2-high tumor tissues (magnification 400×), (C) Numbers of CD8+ infiltration in tumor tissues with HHLA2 low or high expression

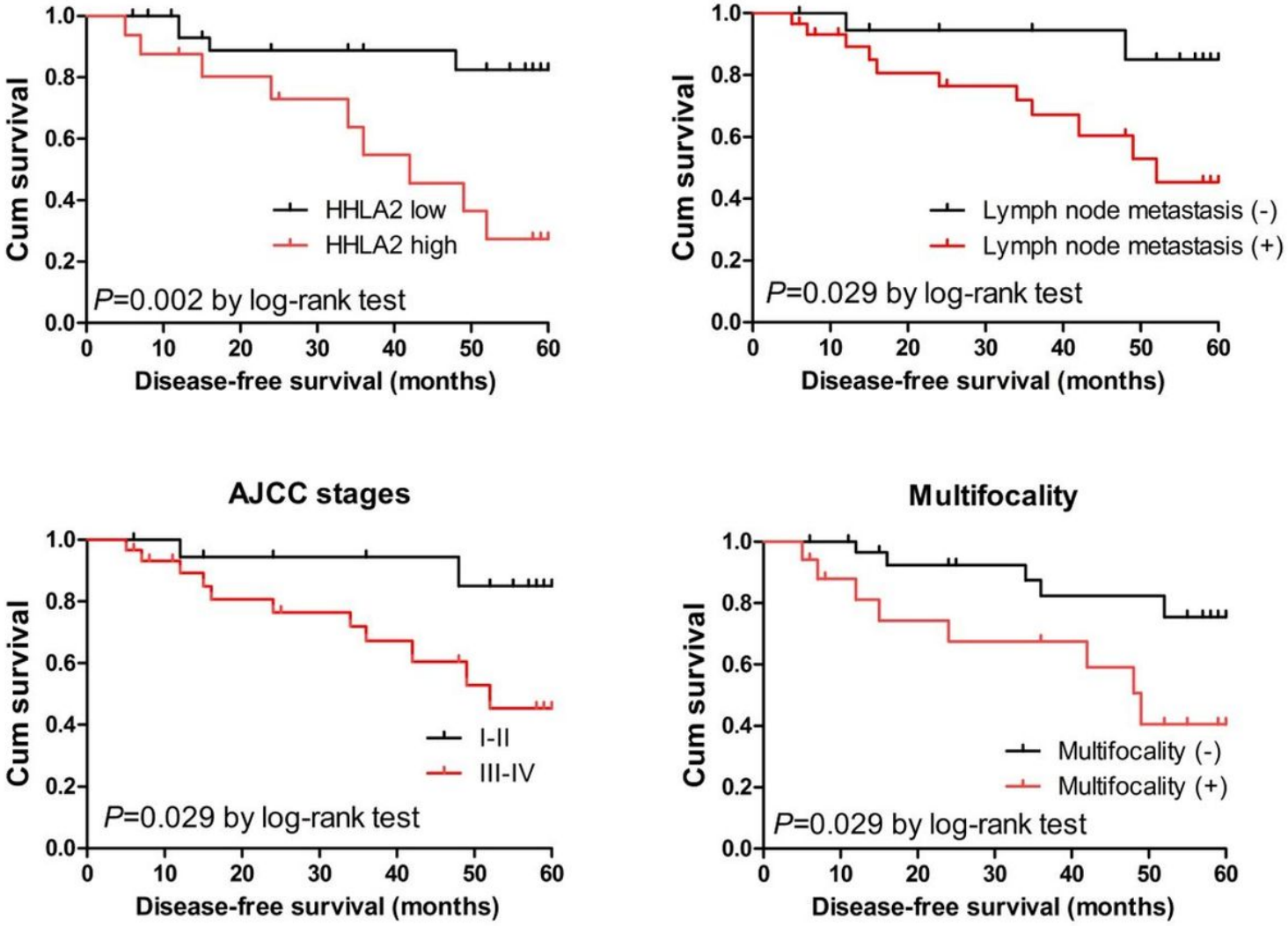

Figure 4

Kaplan-Meier curves for disease-free survival in patients with medullary thyroid carcinoma. (A) HHLA2 expression, (B) Lymph node metastasis, (C) AJCC stages, (D) Multifocality.