Differentiated thyroid carcinomas may elude the immune system by B7H1 upregulation

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Abstract

B7H1 is consistently associated with inhibition of the immune system in many solid tumors. However, there is no report about its impact on differentiated thyroid carcinoma (DTC) presentation, aggressiveness, or evolution. Aiming to investigate the role of B7H1 in DTC and correlate this protein with other tumor-infiltrating immune cells, we studied 407 thyroid nodule tissue samples including 293 from DTC patients, all managed according to a same standard protocol. In addition, we obtained 5 normal and 114 benign thyroid lesions. Eighteen out of the 253 papillary thyroid carcinomas were paired with respective metastatic lymph node tissues. B7H1 (CD274) protein expression was assessed by immunohistochemistry and the gene expression was quantified by real-time PCR. Malignant tissues displayed a more intense B7H1 staining and higher mRNA levels than benign tissues (both $P < 0.0001$). We observed a positive linear correlation between higher age at diagnosis and *B7H1* mRNA levels ($P = 0.02896$). Elevated levels of B7H1 protein were associated with the presence of CD4+, CD8+, CD20+, and FoxP3+ lymphocytes (all $P < 0.05$); tumor-associated macrophages ($P < 0.0001$); and the presence of myeloid-derived suppressor cells ($P = 0.03256$). Stage II–IV patients presented higher *B7H1* mRNA levels than stage I cases ($P = 0.03522$). On the contrary, a decreased expression of B7H1 protein was observed in lymph node metastasis ($P = 0.0152$). In conclusion, our data demonstrate that B7H1 expression is associated with features of aggressiveness, suggesting that this is an immune evasion mechanism of DTC cells.

Introduction

The impact of the immune response on the development and progression of differentiated thyroid cancer remains controversial. We previously observed a less aggressive phenotype associated with evidences of immune response against thyroid tumors (Cunha *et al*. 2011, Cunha & Ward 2012). Conversely, recent studies observed that infiltration of different subsets of lymphocytes may contribute to tumor progression (French *et al*. 2010, Gogali *et al*. 2012), suggesting the occurrence of an immune escape mechanism. Cancers are known to elude the immune system by different mechanisms. One of these mechanisms is the upregulation of B7H1 expression. B7H1 (programmed death ligand-1 (PD-L1) or CD274) is a cell surface glycoprotein normally expressed only by macrophage lineage cells (Dong *et al*. 1999). The molecule may be involved in the regulation of local inflammatory responses, and aberrant tumor expression of B7H1 is consistently associated with inhibition of the immune system (Dong *et al*. 2002, Cao *et al*. 2011*b*, Hua *et al*. 2012). B7H1 is expressed in various tumors, including esophagus and head and neck, colon, ovaries, bladder, breast, and skin, and its expression is further upregulated in responses to the common inflammatory cytokines IFNγ, TNFα, and IL1 (Dong *et al*. 2002). However, its impact on disease presentation, tumor aggressiveness, and patient survival is still confusing in different types of tumors (Hino *et al*. 2010, Wang *et al*. 2010, Gadiot *et al*. 2011, Taube *et al*. 2012). To our knowledge, there is no study correlating B7H1 expression with aggressiveness in differentiated thyroid carcinomas (DTC). As the molecule is transiently induced during T-cell activation but constitutively expressed by exhausted, dysfunctional T-cells, we hypothesized that it would be upregulated in the primary tumor, especially if this tumor displayed features of aggressiveness. Hence, we aimed to investigate the role of B7H1 in the evolution of patients with DTC and correlate this protein with other tumor-infiltrating immune cells.

Materials and methods

Patients

This study was approved by the Research Ethics Committee of the Cancer Hospital AC Camargo, São Paulo, Brazil. We investigated 407 patients whose tissue samples were maintained in the tissue bank of Cancer Hospital AC Camargo. A total of 293 patients were diagnosed with thyroid carcinoma: 253 with papillary thyroid carcinomas (PTCs; 153 cases of the classical form; 80 follicular variants; and 20 tall-cell variants) and 40 with follicular thyroid carcinomas (FTCs; 22 minimally invasive and 18 frankly invasive). In addition, we obtained 5 normal and 114 benign thyroid tissues, including 58 nodular goiters and 56 follicular adenomas. Eighteen out of the 253 PTCs were paired with respective metastatic lymph nodes excised during the initial surgery.

Patient clinical information was obtained from their records. Aggressiveness at diagnosis was ascertained using the tumor node metastasis and stage classification systems for DTC, as recommended by the American Thyroid Association guidelines (American Thyroid Association Guidelines Taskforce on Thyroid *et al*. 2009). We grouped patients classified as stages II–IV in one class named 'higher stages' for statistical purposes. Patients were followed with periodic total body scans and serum TSH and thyroglobulin (Tg) measurements, according to a standard protocol that included X-ray, ultrasonography, computed tomography scan, and other eventual procedures to detect distant metastasis for a period of 12–298 months (43.50 ± 33.29 months). Patients presenting high non-stimulated serum Tg levels (>2 mg/dl) were submitted to a through image search. We defined tumors as persistent/recurrent and/or presenting long-distance metastasis, according to the aforementioned parameters.

Formalin-fixed paraffin-embedded tissues from all 407 cases were reviewed for diagnostic confirmation, aiming to select the most representative areas designed to build a tissue microarray (Beecher Instruments, Silver Springs, MD, USA) for immunohistochemical analysis.

We also obtained fresh thyroid tissue samples from the same cases of formalin-fixed, paraffin-embedded tissues, including 85 thyroid carcinomas (56 classical PTCs, 27 follicular variants of PTCs, one tall-cell variant of PTC, and one follicular carcinoma). In addition, we used 18 nodular goiters and 13 follicular adenomas collected at surgery, immediately snap-frozen in liquid nitrogen, and stored at $-80\,°C$ for mRNA quantification.

Chronic lymphocytic thyroiditis, investigated in nonmalignant thyroid parenchyma of the tumor contralateral lobe, was characterized by extensive lymphocytic infiltration with lymphoid follicles, scarring, and follicular regenerative activity in the form of numerous small follicles, frequently lined by Hurthle cells (Kasagi *et al*. 1996). The clinical diagnosis of Hashimoto's thyroiditis was confirmed by patient serum thyroperoxidase and Tg antibody titers.

Immunohistochemistry

Five micrometer tissue microarray sections were placed on electrically charged slides, deparaffinized, and rehydrated in decreasing concentrations of alcohol. The endogenous peroxide activity was blocked with H_2O_2 for 15 min. All tissue sections were subjected to heat-induced antigen retrieval using 10% citrate buffer (10 mM, pH 6.0) in a steamer (90 °C for 30 min). Tissue sections were then incubated overnight at 6 °C, with rabbit anti-B7H1 polyclonal antibody (prediluted; ab82059; Abcam, Cambridge, UK). We also investigated the presence of different tumor-infiltrating lymphocytes (CD3-, CD4-, CD8-, CD20-, and FoxP3-positive lymphocytes), tumor-associated macrophages (CD68+ cells), and myeloid-derived suppressor cells (colocalization of CD33 and CD11b), as described previously (Cunha *et al.* 2012*a*). The advanced biotin-free polymer detection system was used (DAKO, Carpinteria, CA, USA). DAB (3.3-diamino-benzidine tetrahydrochloride; Sigma) was applied as chromogen for 5 min at room temperature. Sections were counterstained with hematoxylin. Positive and negative controls were run in the same batch of reaction.

Immunohistochemical evaluation

Slides were quantified by at least two of the authors (L L Cunha and/or E C Morari and M A Marcello) and then submitted to other two independent score evaluations performed by two experienced pathologists (J Vassallo and F A Soares), both blinded to tumor features. B7H1 staining was found in the cytoplasm of tumor cells (Fig. 1); hence, cells were considered positive for B7H1 when a clear-cut brown staining was observed in cytoplasm. Visual evaluation was made for each tissue spot, estimating the percentage of positive tumor cells and the intensity of staining. The percentage of positive cells was graded as follows: 0, no positive cell; 1, up to 10% positive cells; 2, 10–30% positive cells; 3, more than 30% positive cells. For statistical purposes, cases scored as 0 were considered negative and cases scored from 1 to 3 were grouped as positive. In addition, we analyzed the immunohistochemical expression of B7H1, using the Automated Cellular Imaging System III (ACIS-III; Chroma Vision Medical Systems, Inc., San Juan Capistrano, CA, USA). Briefly, the system attributes a numerical value proportional to the intensity and extension of brown staining of digitalized images using the following formula: score=(intensity ×brown area)/(brown area+blue area). Each spot is assessed in triplicate and the final value is given by the
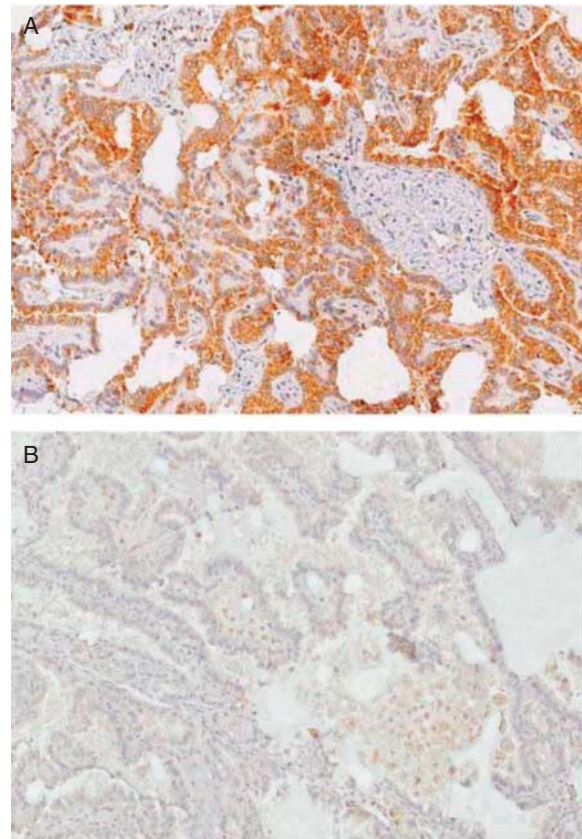
Figure 1

B7H1 staining in papillary thyroid carcinoma. (A) Primary tumor (patient #5996) presenting a strong diffuse B7H1 staining. (B) Paired lymph node metastases (patient #5996). A decreased expression of B7H1 is observed. Magnification, 200×.

mean of triplicates, as previously reported (Minot *et al.* 2009, Morari *et al.* 2010). Aiming to perform survival analysis, staining ≤median was considered negative and staining >median was considered positive, as described previously (Cunha *et al.* 2012*b*).

Quantitative real-time PCR

Total RNA was extracted from pulverized frozen thyroid tissues using TRIzol reagent (Invitrogen Life Technologies, Inc.), according to the manufacturer's instructions. The samples were digested with DNase I amplification grade (Life Technologies) and RT, using SuperScript III reverse transcriptase (Invitrogen Life Technologies, Inc.). Gene transcript abundance was assessed by quantitative real-time PCR (qRT-PCR), with the use of commercially available TaqMan gene expression assays (Applied Biosystems) for *B7H1* (Hs01125299_m1) relative to the internal reference gene *GAPDH* (Hs02758991_g1). Reactions were

prepared with TaqMan gene expression master mix (Applied Biosystems), according to the manufacturer's protocol. Analysis was performed with a 7500 RT-PCR system (Applied Biosystems), using a four-stage program: 50 °C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. Each sample was assayed in triplicate. Threshold cycle (Ct) was obtained using the sequence detection software (Applied Biosystems SDS v1.3 Software). We used $\Delta\Delta CT$, in which the amount of target normalized to an endogenous reference and relative to calibrator is given by $2^{-\Delta\Delta CT}$.

Statistical analysis

The statistical analysis was carried out using the Winstat Software (Cambridge, MA, USA). Disease-free survival was calculated using Kaplan–Meier curves with log-rank comparison. Nonparametric analysis was performed using the χ^2 or Fisher's exact test, as indicated. A multivariate logistic regression model was applied using clinical risk factors, including gender and age as explicative variables. Mann–Whitney U tests were used to compare continuous or arranged measures between two groups whose variables did not present Gaussian distribution; Kruskal–Wallis test was used to compare three or more groups whose variables did not present Gaussian distribution. Spearman's test was used to compare quantitative variables. Paired t-test was used to infer changes in B7H1 expression in primary tumor and metastatic lymph nodes. Sensitivity, specificity, and predictive values were evaluated. Quantitative data were expressed as mean $\pm$ 95% confidence. The accuracy of quantification of B7H1 expression to predict malignancy was evaluated using receiver operating curve analysis, based on predicted probabilities from logistic regression models. All tests were conducted at a 0.05 significance level.

Results

As expected, most (83.6%) patients were female. Individuals with benign (49.2 ± 15.1 years old) and malignant (43.6 ± 15.9 years old) thyroid lesions presented similar age at diagnosis. Ninety-nine (33.8%) tumors were classified as encapsulated and 194 (66.2%) as non-encapsulated. Multifocality was observed in 130 (51.4%) of the PTC cases whereas 123 (48.6%) were unifocal PTC tumors. One hundred and nineteen (40.6%) presented extrathyroidal invasion and 174 (59.4%) tumors did not. DTC patients were classified according to the pathological tumor node metastasis staging system (Shaha 2007) as I (173 cases, 59%), II (31 cases, 10.6%), III (44 cases, 15.0%), and IV (45 cases, 15.4%). A retrospective analysis of evolution of patients with thyroid cancer demonstrated that 61 cases (20.6%) presented recurrence, whereas 232 evolved free of disease (79.4%).

Semiquantitative analysis of B7H1 protein expression

Positivity for B7H1 was detected in 2/5 (33.3%) of normal thyroid tissues, 45/58 (78.4%) of goiters, 47/56 (84.3%) of follicular adenomas, 209/254 (82.5%) of PTCs, and 35/40 (87.5%) of FTCs. We did not find any correlation between B7H1 protein expression and clinical characteristics that could be related to prognosis such as gender ($P=0.4635$) and age at diagnosis ($P=0.7911$). We also did not find B7H1 expression to be associated with any tumor feature that could be related to aggressiveness including the presence of multifocality ($P=0.4014$), the presence of a capsule ($P=0.2571$), extrathyroidal invasion ($P=1.000$), concurrent chronic lymphocytic thyroiditis ($P=0.1336$), metastasis at diagnosis ($P=0.5090$), tumor size ($P=0.6274$), or stage ($P=0.2945$), suggesting that the visual analysis of B7H1 was not clinically useful in terms of prognosis prediction. In fact, a log-rank test failed to demonstrate B7H1 positivity as a prognostic marker ($P=0.97748$). However, we observed that lymph node metastasis presented a decreased expression of B7H1 protein ($P=0.0158$).

Quantitative analysis of B7H1 protein expression

We further investigated B7H1 protein expression using a quantitative method. A Kruskal–Wallis test evidenced that different tissues presented distinct B7H1 expression levels (Fig. 2A; $P=0.0002$). There was no difference in the levels of B7H1 expression in different types of thyroid carcinomas ($P=0.1991$). Malignant tissues had a more intense B7H1 staining (44.7235 ± 5.11891) than benign tissues (25.5799 ± 4.54097; Fig. 2B; $P<0.0001$). PTC samples presented a more intense staining (37.7859 ± 6.42821) than goiters (21.0166 ± 5.24818; Fig. 2A; $P=0.0004$). However, B7H1 expression presented relatively low sensitivity (0.6000), specificity (0.6150), accuracy (60.50%), positive predictive (75.90%), and negative predictive (43.20%) values.

We were not able to find any association between B7H1 protein expression and multifocality ($P=0.33888$), capsule ($P=0.70109$), extrathyroidal invasion ($P=0.90799$), metastasis at diagnosis ($P=0.2454$), tumor size ($P=0.41403$), gender ($P=0.1607$), age at diagnosis ($P=0.06498$), and tumor stage ($P=0.98352$).
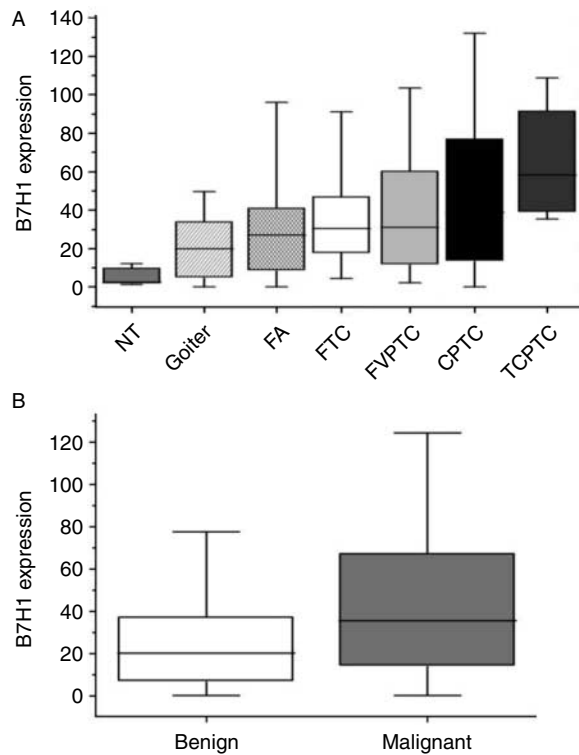
Figure 2
(A) B7H1 immunohistochemical expression in different thyroid tissues.
(B) B7H1 immunohistochemical expression in benign and malignant
thyroid tissues. NT, normal thyroid; FA, follicular adenoma; CCPTC, classic
papillary thyroid carcinoma; FVPTC, follicular variant of papillary thyroid
carcinoma; TCPTC, tall-cell variant of papillary thyroid carcinoma; FTC,
follicular thyroid carcinoma.

However, tumors with concurrent chronic lymphocytic thyroiditis presented higher B7H1 levels (61.0188 $\pm$11.3663) than tumors without concurrent autoimmunity (43.7773$\pm$7.86066; $P=0.00647$). We were not able to find any association between B7H1 protein expression and circulating thyroid antibodies titers. In addition, a decreased expression of B7H1 protein was observed in most lymph node metastasis, confirming semiquantitative data (Fig. 3; $P=0.0152$). Only four cases presented upregulated B7H1 expression in lymph node metastasis regarding primary tumor. These four cases were high-risk patients; however, only one of them presented a recurrence, whereas the three other patients evolved free of disease.

High levels of B7H1 protein were associated with the presence of CD4+ lymphocytes ($P=0.04942$), CD8+ lymphocytes ($P=0.0003$), CD20+ lymphocytes ($P=0.01283$), FoxP3+ lymphocytes ($P=0.00626$), tumor-associated macrophages ($P<0.0001$), and myeloid-derived suppressor cells ($P=0.03256$).
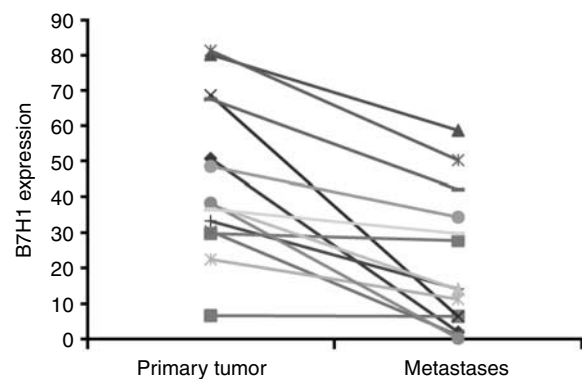
B7H1 mRNA expression

B7H1 mRNA levels were higher in malignant (52 952.3 $\pm$13 277.4) than in benign nodules (18 771.4$\pm$5591.97; $P<0.0001$). B7H1 was not upregulated in benign nodules associated with autoimmune thyroiditis. B7H1 mRNA levels were not correlated with the presence of multifocality ($P=0.66989$), capsule ($P=0.1666$), extrathyroidal invasion ($P=0.32219$), concurrent chronic lymphocytic thyroiditis ($P=0.28912$), metastasis at diagnosis ($P=0.97781$), tumor size ($P=0.64948$), or gender ($P=0.56153$).

Tumors classified as 'higher stages' (stages II–IV) presented higher B7H1 mRNA levels (84 009.6 $\pm$44 837.7) than tumors classified as stage I (44 454.3 $\pm$15 479.3; $P=0.03522$). We observed a positive linear correlation between age at diagnosis and B7H1 mRNA levels (Fig. 4; correlation coefficient $r=0.2065$; $P=0.02896$).

In addition, we observed a positive association between upregulated B7H1 mRNA levels and the presence of CD3+ lymphocytes ($P=0.00838$), CD4+ lymphocytes ($P=0.01049$), CD8+ lymphocytes ($P=0.03472$), and FoxP3+ lymphocytes ($P=0.01193$).

Because the increased expression of B7H1 could be just an epiphenomenon related to the frequent presence of lymphocytic infiltration, especially in PTC, we performed a multiple regression analysis, which confirmed an independent association between B7H1 staining intensity and the presence of concurrent chronic lymphocytic thyroiditis ($P=0.01818$), as well as with a more advanced age at diagnosis ($P=0.00319$).

Figure 3
Comparison between B7H1 expression in primary tumor and corresponding lymph node metastases. Paired t-test; $P=0.0152$.
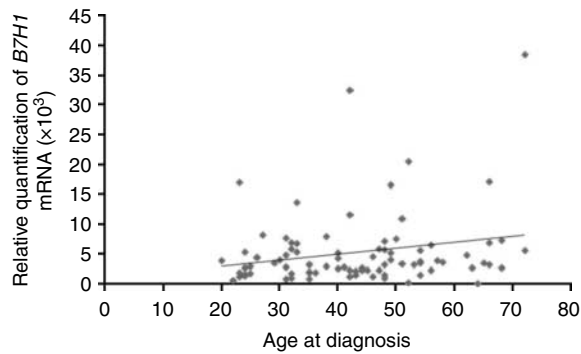
Figure 4
Age at diagnosis and *B7H1* mRNA expression. Spearman's test; $P = 0.02896$.

Discussion

We demonstrated that both B7H1 protein and *B7H1* mRNA are upregulated in DTC, contrasting with the low levels displayed by benign tissues. Tumor-associated B7H1 is implicated in immune escape mechanisms and, in this scenario, emerges as a potent molecule involved in the tumorigenic process. In fact, some reports indicate that B7H1 has a key role in the conversion of normal cells into tumor cells (Cao *et al.* 2011*a,b*). Hence, our findings reinforce the current oncologic concept that avoiding immune system response is one of the hallmarks of cancer (Hanahan & Weinberg 2011). Unfortunately, the similarity of B7H1 distribution among the different thyroid tissues studied and the relatively small number of samples did not allow us to state B7H1 as a diagnostic marker.

We also observed an association between high *B7H1* mRNA levels and the presence of some characteristics of tumor aggressiveness, such as higher stages at presentation and increased age at diagnosis, suggesting that high levels of B7H1 expression may identify individuals who need more aggressive management. Wang *et al.* (2010) found that B7H1 significantly correlated with the pathological grade of pancreatic carcinoma. Yao *et al.* (2009) found a correlation between the expression of B7H1 and the malignancy grade of human gliomas, suggesting that B7H1 is implicated in tumor aggressiveness determination. Routh *et al.* (2008) observed that B7H1 was more likely to occur in anaplastic Wilms tumor, reinforcing the idea that B7H1 may determine cancer aggressiveness. However, our survival analysis failed to correlate B7H1 with outcome. In fact, at difference from some other human tumors, an appropriate initial management is a very important modifiable prognostic factor and

even aggressive differentiated thyroid tumors may be controlled and remain stable even when metastatic at presentation for long periods, especially in young patients (Morari *et al.* 2011).

We observed that tumors with concurrent chronic lymphocytic thyroiditis presented higher B7H1 levels than tumors without concurrent autoimmunity, suggesting that the chronic inflammation process may contribute to B7H1 upregulation in tumors cells. B7H1 expression was associated with the presence of a mixture of immune cells infiltrating tumor samples including FOXP3+ lymphocytes, which we recently demonstrated associated with DTC features of aggressiveness (Cunha *et al.* 2012*b*). These lymphocytes may provoke a molecular mimicry that enables immune evasion (Hinz *et al.* 2007) and modulates patterns of molecule expression in tumor cells, thus favoring an aggressive phenotype (Merlo *et al.* 2009). Many reports have shown that B7H1 frequently avoids cancer cell immune destruction despite the presence of different immune cells infiltrating the tumor microenvironment (Chen *et al.* 2012, Hua *et al.* 2012, Taube *et al.* 2012). Taube *et al.* (2012) suggested that B7H1 expression may represent a host response to tissue inflammation. In fact, our data showed that B7H1 was higher in tumors that also presented concurrent chronic lymphocytic thyroiditis. This apparent paradox may be explained by the fact that B7H1 is engaged in the immune exhaustion process. Concurrent thyroiditis may provide a microenvironment enriched with IFNγ, IL10, IL6, and common g-chain cytokines that are able to increase B7H1 expression (Kinter *et al.* 2008, Wölfle *et al.* 2011, Taube *et al.* 2012).

We cannot rule out the possibility that the observed increase in B7H1 expression is an epiphenomenon. In fact, the multiple regression analysis confirmed an association between B7H1 staining intensity and the presence of concurrent chronic lymphocytic thyroiditis, suggesting that both events might be associated. On the other hand, B7H1 was not upregulated in benign thyroid nodules associated with autoimmune thyroiditis and we observed an independent association between B7H1 staining and a more advanced age at tumor diagnosis indicating that, even correlated with concurrent thyroiditis, B7H1 expression is important to determine aggressiveness. In fact, Waeckerle-Men *et al.* (2007) demonstrated that B7H1 has a negative effect on CD8+ lymphocyte activation. Blank *et al.* (2006) demonstrated that the blockade of B7H1 on human tumors resulted in enhanced cytolytic activity of tumor-infiltrating lymphocytes and cytokine production of T-helper cells when interacting directly

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with the tumor, explaining the aggressiveness associated with B7H1 expression in DTC even that these tumors are frequently associated with signals of antitumor immune response (Cunha *et al.* 2011, 2012*a*, Cunha & Ward 2012). Recently, Kaiser *et al.* (2012) found that chronically stimulated CD8+ lymphocytes become sensitive to B7H1-mediated functional inhibition upon low antigen detection, suggesting that immune escape may be orchestrated by both B7H1 mechanism and tumor antigen density. In fact, Kaiser's results are coherent with our previous data, demonstrating that the pattern of immune cells infiltrating DTC is closely associated with molecular markers of these tumors (Cunha *et al.* 2012*a*).

Lymph node metastatic tissues had lower levels of B7H1 than the primary tumor, suggesting an exhaustion of T-cell activation. French *et al.* (2012) recently described high levels of IFN+/CD8+T cells in 12 metastatic lymph node tissues excised during the initial surgery, at patient presentation. The authors found that proliferating lymphocytes were evident in tumor-involved lymph node metastases that, in opposition to our findings, were enriched with PD-1+ lymphocytes (French *et al.* 2012). They hypothesized that the presence of metastases does not arrest and may, on the contrary, promote an IFN+ response, suggesting the generation of an antitumor response, which may impair tumor evasion. In fact, this could explain the decrease in B7H1 expression that we observed in our matched metastases, suggesting that the exhaustion of T-cell activation is a mechanism of tumor progression.

In conclusion, our data demonstrate that B7H1 expression is upregulated and associated with features of aggressiveness in DTC, suggesting that this is a mechanism of tumor evasion from the immune surveillance system. Further studies are necessary to better understand the B7H1 role in thyroid tumor development; however, our observations indicate that therapies designed to block this pathway may benefit B7H1+ patients, offering a new perspective of immunotherapy for thyroid carcinoma patients.

Declaration of interest

All authors state that there is no financial interest in or arrangement with a company whose product was used in a study. In addition, there is no financial interest in or arrangement with a competing company, and there is no other direct or indirect financial connections, or other situations that might raise the question of bias in the work reported or the conclusions, implications, or opinions stated – including pertinent commercial or other sources of funding for the individual author(s) or for the associated department(s) or organization(s), personal relationships, or direct academic competition.

Author contribution statement

L L Cunha participated in conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing, and final approval of manuscript. M A Marcello and F F Conte participated in data analysis and interpretation, qRT-PCR analysis, and final approval of manuscript. E C Morari and S Nonogaki participated in data analysis and interpretation, histopathological analysis, and final approval of manuscript. R Gerhard, F A Soares, and J Vassallo participated in histopathological analysis and final approval of manuscript. L S Ward participated in conception and design, data analysis and interpretation, manuscript writing, and final approval of manuscript.

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