Thyroid

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Title: CSPG4 is a Potential Therapeutic Target in Anaplastic Thyroid Cancer

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Abstract:

Background: Anaplastic thyroid cancer (ATC) is a rare cancer with poor prognosis and few treatment options. The objective of this study was to investigate new immune-associated therapeutic targets by identifying ATC-derived, human leukocyte antigen (HLA) class II-presenting peptides. One protein that generated multiple peptides in ATC was chondroitin sulfate -proteoglycan-4 (CSPG4), a transmembrane proteoglycan with increased expression in multiple aggressive cancers but not yet investigated in ATC.

Methods: We applied autologous peripheral blood T cells to ATC patient-derived xenografted mice to examine whether ATC induces a tumor-specific T cell response. We then identified peptide antigens eluted from the HLA-DQ complex in ATC patient derived-cells using mass spectrometry, detecting abundant CSPG4-derived peptides specific to the ATC sample. Next, we analyzed the surface expression level of CSPG4 in thyroid cancer cell lines and primary cell culture using flow cytometry. Additionally, we used immunohistochemistry to compare the expression level and localization of the CSPG4 protein in ATC, papillary thyroid cancer (PTC), and normal thyroid tissue. We then investigated the correlation between CSPG4 expression and clinicopathological features of patients with thyroid cancer.

Results: We found that ATC tissue had a high level of HLA-DQ expression and that the patient's $CD4^+$ T cells showed activation when exposed to the ATC. By eluting the HLA-DQ complex of ATC tissue, we found that CSPG4 generated one of the most abundant and specific peptides. CSPG4 expression at the cell surface of thyroid cancer was also significantly high when determined by flow cytometry, with the majority of ATC cell lines exhibiting approximately 10-fold higher mean fluorescence intensity. Furthermore, most ATC patient cases expressed CSPG4 in the cytoplasm or membrane of the tumor cells. CSPG4 expression was correlated with tumor size, extrathyroidal extension, and intercellular adhesion molecule-1 (ICAM-1) circumferential expression. *CSPG4* mRNA overexpression was associated with worse overall survival in patients with ATC and poorly differentiated thyroid cancer.

Conclusions: CSPG4 expression is significantly elevated in aggressive thyroid cancers, with a strong correlation with a poor prognosis. The vast number of HLA-DQ eluted CSPG4 peptides was identified in ATC, demonstrating the potential of CSPG4 as a novel immunotherapeutic target for ATC.

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Introduction

Anaplastic thyroid cancer (ATC), an aggressive subtype, makes up 1-2% of thyroid cancers and has a median survival of less than 4 months (1, 2). Genomic mutations of thyroid cancers are well studied, with *BRAF* (V600E) mutation being most common in papillary thyroid cancer (PTC) (3), with reported *BRAF* mutational rates of 40% or greater in ATC (4-6). Recently, combination therapy with a BRAF (V600E) inhibitor, dabrafenib, and a MEK inhibitor, trametinib, showed promising results in *BRAF* mutated ATCs (7). However, a significant subset of ATCs do not have *BRAF* mutations, and treatment with kinase inhibitors and chemotherapeutic agents have showed limited response in most ATCs (8-10), other therapeutic targets are needed.

Recent progress in immunotherapy with checkpoint inhibitors has had a tremendous impact on cancer therapy (11); however, fewer than 20% of ATC patients had partial or complete responses to immunotherapy (12). In a recent phase II clinical trial, programmed cell death-1 (PD-1) blockade had an overall response rate of 19% and a 7% complete response rate in ATC (13). Blocking the interaction between checkpoint inhibitors and their ligands promotes T cell activities against tumor antigens. While most cancer immunotherapy efforts have focused on $CD8^+$ T cell responses against peptide antigens presented on the major histocompatibility complex (MHC) class I, recent studies have shown that $CD4^+$ T cells can elicit powerful tumor-control by their specific interactions with tumor antigens presented on MHC II (14-16). By investigating a patient-derived ATC sample for novel MHC II-presented peptide antigens, we identified a large number of chondroitin sulfate-proteoglycan-4 (CSPG4) derived peptides.

CSPG4, a proteoglycan first discovered in melanoma (17-19), is a transmembrane protein that can be expressed on cells either as a N-linked glycoprotein of approximately 250 kDa or as a 450 kDa N-linked glycoprotein with associated proteoglycan (20). The large extracellular domain, with subdomains D1, D2, and D3, functions as a high-affinity receptor for extracellular proteins, growth factors, and integrins (20). Increased levels of CSPG4 expression have been demonstrated in many aggressive cancers, including melanomas (17), gliomas (21), mesotheliomas (22), sarcomas (23) and triple negative breast cancers (24). Moreover, CSPG4 expression has been associated with poor prognosis

(23, 25), hematogenous metastasis (26) and angiogenesis (27). Previous characterization of CSPG4 expression in normal and neoplastic tissues found that normal thyroid tissue did not express CSPG4 (28, 29); however, CSPG4 expression has not yet been determined in thyroid cancer. Previous studies using anti-CSPG4 monoclonal antibodies (mAb) (22, 30) and chimeric antigen receptor (CAR) T lymphocytes against CSPG4 (31-33) have shown promising therapeutic results *in vitro* and in preclinical models of other cancers.

Here we identified multiple CSPG4-derived peptides in ATC patient-derived cells after immunoprecipitation of a human leukocyte antigen (HLA) class II complex by mass spectrometry. We further analyzed CSPG4 expression in normal thyroid, PTC, and ATC patient-derived tissues and cell lines using immunohistochemistry (IHC) and flow cytometry.

Materials and methods

Sample collection and Institutional Review Board approval

Pre-operative informed written consent was obtained from patients who underwent open neck biopsy, hemithyroidectomy or thyroidectomy at Weill Cornell Medicine (WCM) from 2004 to 2020 and the study was approved by the WCM Institutional Review Board (IRB). Patients' demographics and tumor characteristics were collected via review of electronic medical record and pathology reports.

Cell Culture

For thyroid cancer cell lines, BCPAP, 8505C, KHM-5M, and SW1736 were purchased commercially or gifted by researchers. A375-MA2 and T2 were purchased from ATCC (Manassas, VA). B-LCL, MOU and 1331 were from Fred Hutchinson Cancer Research Center (Seattle, WA). The source of the cells, deriviation method for patient-derived ATC cells, and culture condition are described in detail in Supplementary Methods.

Isolation of peripheral blood mononuclear cells (PBMC) and T cell expansion

Fresh PBMC from an ATC patient were isolated using Ficoll-PaquePLUS density media (GE Healthcare) by gradient centrifugation. T cells were enriched by adding Dynabeads Human T-cell Expander CD3/CD28 (ThermoFisher) in OpTmizer CTS media

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(ThermoFisher) supplemented with 5% human AB serum (Sigma-Aldrich), recombinant human IL7 and IL15 (both cytokines from Peprotech), and cryopreserved until usage.

Mice study

All mouse studies were approved by WCM Institutional Animal Care and Use Committee. For xenograft experiments, we intravenously injected 0.5 million JV-ATC cells expressing fLuc per mouse into 8 to 10-week old NOD-*scid*/IL2Rg^{null} (NSG) (Jackson Laboratory) mice. The patient tumor-derived JV-ATC cells have been described previously (34). After nine days of xenograft, 1 million T cells isolated from the same donor as the tumor were were injected to each xenograft. Tumor growth was tracked *in vivo* via whole-body luminescence imaging (In-Vivo Xtreme 4MP, Bruker). Tumor tissues from the JV-ATC xenograft were isolated and analyzed for the frequency of infiltrating T cells using an antibody cocktail against CD3, CD4, CD8 (Biolegend #319001). Tumor-infilitrating T cells were maintained in the CTS media, supplemented with 5% human AB serum, IL7 and IL15. These T cells isolated from the JV-ATC xenografts were co-cultured with the JV-ATC tumor cells at a 1:1 ratio, and analyzed for CD69 and PD1 expression after 18 hours of co-culture.

MHC-II bound peptide isolation and LC-MS/MS Analysis

Approximately 25 million cells of each type were used for HLA-DQ immunoprecipitation using anti-HLA-DQ antibody (Novus Biologicals, Catalog: NBP2-45041, clone SPV-L3). Methods for peptide elution and mass spectrometry are described in Supplemental Methods.

Immunohistochemistry

Formalin-fixed paraffin-embedded (FFPE) tissue blocks from patient tissue were stained with anti-CSPG4 (Abcam, Catalog: ab139406, clone EPR9195) on a Leica Bond system. The section was pre-treated using heat mediated antigen retrieval with Tris-EDTA buffer (pH9, epitope retrieval solution 2) for 20 minutes prior to antibody addition. See Supplemental Methods for detailed methods.

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Flow cytometry

Key reagents and methods for live cell staining are described in Supplemental Methods. Anti-human CSPG4-APC (Miltenyi Biotec, Catalog: ab130-117-497, clone EP-1) was used to detect CSPG4 expression on live cells. The source and catalog number of the antibodies used in flow cytometry are documented in the Supplemental Methods.

Statistical analysis

Categorical variables were compared using Fisher's exact and chi-squared tests when appropriate, and continuous nonparametric variables were compared using Wilcoxon rank-sum test. Survival curves were generated using Kaplan-Meier method and compared using log-rank test. Analysis was performed using Prism 8.0 (GraphPad) and Stata Version 16.1 (StataCorp). Statistical significance was set at $p < 0.05$.

Results

High expression of HLA-DQ in ATC

Previously, we reported establishing ATC patient-derived xenografts (PDX) and cell line, JV, which maintained pathological and molecular features identical to the patient's original tumor (34). This JV-ATC tumor was *BRAF* wild-type with *NRAS*, *PTEN*, and *TP53* mutations. PBMCs were collected from the patient at time of surgery.

In order to expand tumor-reactive T cells and study their interaction with tumor cells, we administered donor-matched peripheral T cells to the JV-ATC patient-derived xenografts after 9 days of xenograft (Supp. Fig. 1A). Approximately 1 month after xenograft, we noticed that the tumor size was relatively smaller in xenografts administered with autologous T cells relative to no treatment group. This finding suggested a possibility that T cells may be mediating blunted tumor growth.

To further examine whether those tumor-infiltrating T cells were enriched for specific T cell subsets, we isolated the regressing tumor from the JV-ATC xenograft who was injected with the donor-matched T cells, and analyzed the tumor-infiltrating T cells by flow cytometry. Surprisingly, tumor-infiltrating T cells were predominantly $CD4^+$ cells, skewed from the initial CD4:CD8 distribution when we first isolated from the patient's

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peripheral blood. The peripheral blood T cells had 70%:24% ratio of CD4:CD8 frequency, however the tumor-infiltrating T cells were greater than 97% of $CD4^+$ T cells, with barely detectable level of $CD8^+$ T cells (Fig. 1A).

Next, to analyze whether those tumor-infiltrating T cells showed tumor-specific activity, we tested whether these T cells respond to the JV cells *in vitro*. After exposure to JV-tumor cells at a 1:1 ratio, these JV tumor-isolated $CD4^+$ T cells increased expression of CD69, a marker of antigen-dependent T cell activation (Fig. 1B). The expression level of PD-1, an exhaustion marker, was slightly increased. These results suggested that the ATC tumor was antigenic and induced *in vivo* expansion of tumor reactive $CD4^+$ T cells, which were present in the circulating blood of the patient.

We investigated if any specific MHC II complex was expressed on JV-ATC cells, potentially presenting peptides to the $CD4^+$ T cells. Surface expression level of HLA-DQ was the highest, followed by HLA-DP, whereas HLA-DR expression was negligible on the JV tumor cells (Fig. 1C). HLA-DQ expression was increased approximately 8-fold when IFNγ was added to the cells for 48 hours when analyzed with anti-HLA-DQ (1a3) antibody (35) (Supp Fig. 1B). HLA analysis using next generation sequencing determined that the JV cells' HLA-DQ haplotypes were HLA-DQA1*0201, HLA-DQB1*0202/*0302. We examined the HLA-DQ expression level with an anti-HLA-DQ (SPV-L3) antibody (Fig. 1D), which was previously used to profile HLA-DQ-restricted peptides in association with celiac disease (36). Flow cytometric analysis showed that half of JV cells expressed HLA-DQ, a slightly lower percentage of positivity compared to 1a3 antibody (Fig. 1D). However, IFNγ treatment increased the DQ expression to approximately 6-fold, almost to the same level as result observed with 1a3 antibody. HLA-DQ was not detectable in T2 cells, which are known to be defective for MHC II expression. B lymphoblastic cell lines (B-LCL) MOU and 1331 that shared DQ haplotype with JV, displayed close to 100% frequency of DQ expression regardless of IFNγ treatment.

Next, we aimed to identify HLA-DQ-associated peptides enriched in JV-ATC cells and compare the number of peptides eluted in B-LCL MOU cells and T2 cells (Fig. 1E). Approximately 25 million cells of each group were used for eluting HLA-DQ-bound peptides using the SPV-L3 antibody. JV-ATC cells were stimulated with IFNγ to maximize

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the quantity of peptide elution. While both JV and MOU cells yielded a significant number of peptides (n = 920 for JV; n = 1239 for MOU), there were very few peptides eluted from T2 cells (n = 35) concordant with a lack of HLA-DQ expression (Supp. Table 1). The length of the peptides varied between 6 and 30 amino acids with a peak in distribution at 14 and 15 amino acids for MOU and JV, respectively.

Peptides eluted from HLA-DQ cells were specific to ATC

We searched the immune epitope database (IEDB) for HLA-DQA1*0201/DQB1*0202 (HLA-DQ2.2) peptides (37). Similar to MOU and JV cells, the peptides eluted from the HLA-DQ2.2 complex showed the highest peaks at position 14 and 15 (Supp. Fig. 2). We found specific peptides eluted in either JV or MOU cells or present in both cells (Supp. Table 1). Among the top 20 proteins with the most eluted peptides in each cell type, there was little overlap between MOU and JV, except for UBC, HSPA8, and HLA-B, proteins with function in homeostasis and antigen presentation (Tables 1 and 2, Supp. Table 2). S100A8 and S100A9, Ca^{2+} binding proteins overexpressed in ATC cells (38), produced the most abundant peptides in JV-ATC cells, with the highest area among eluted peptides but were not eluted in MOU cells. We isolated identical peptides, including β_2-microglobulin, CD74, serglycin and ubiquitin, from MOU cells as previously reported (36), validating our experimental procedure to identify DQ-restricted peptides presented in cells. Peptides eluted from both JV and MOU (39) exhibited a strong preference for threonine at position 6 and 7 (Fig. 1F). After these threonine residues, the next four positions were enriched with alanine followed by glutamic acid in the peptides isolated from JV, whereas charged amino acids such as aspartic acid and glutamic acid were enriched in the peptides eluted from MOU. IEDB-registered peptides eluted from the HLA-DQ2.2 complex showed remarkably similar sequence motif to the motif generated from MOU cells (Supp. Fig. 2B). Overall, there were similarities in the DQ-eluted peptide length and composition but with different types of proteins that were processed and presented between JV and MOU cells.

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CSPG4-derived peptides are presented in the HLA-DQ complex of an ATC

CSPG4 generated the highest peptide spectrum matches that were unique to JV-ATC cells (Supp. Table 1). The average length of the CSPG4-derived peptides was 15 amino acids in JV cells but undetectable in MOU and T2 cells (Fig. 2A). All 20 unique peptides belonged to the 3 distinct extracellular domains (20) of CSPG4 (Fig. 2B). To assess overall performance of eluted CSPG4 peptides, we estimated the affinity of eluted peptides to the respective HLA molecules by predicting binding affinities (K_d) using NetMHCII pan-4.0 prediction program (40). We analyzed the CSPG4 peptides' binding affinity in the context of DQ2.2 and DQA1*0201/ DQB1*0302, two types of possible DQ haplotypes expressed in the JV tumor cells (Table 3, Fig. 2C). The top eluted peptide, DPDSAPGEIEYEVQ, appeared as variable lengths with multiple occurrences (Table 3), with predicted weak binding to both DQ haplotypes. The third most abundant peptide, VAGPQTSEAFAITVR, was predicted to have strong binding affinity in both DQ haplotypes (Fig. 2C). Overall, CSPG4 peptide binding affinities for the DQ2.2 haplotype were weaker than DQA1*0201; DQB1*0302. The predicted median binding affinities, K_d, of CSPG4 peptides were 2.39 μM and 0.57 μM for DQ2.2 and DQA1*0201; DQB1*0302, respectively (Table 3). Taken together, multiple HLA-DQ-restricted, CSPG4-derived peptides were isolated from ATC with strong signals, implicating high expression and antigen processing of CSPG4 in ATC cells.

CSPG4 expression is correlated with ATC histology and extrathyroidal extension (ETE)

Based on the abundance of CSPG4 eluted peptides from JV-ATC cells on mass spectrometry, we evaluated CSPG4 expression histologically in surgical specimens of PTC, ATC and adjacent normal thyroid tissue (Fig. 3). In normal thyroid tissue, staining was rare and only cytoplasmic (Table 4A). Compared to normal thyroid tissue and PTC, expression was significantly increased in ATC samples (Table 4A). For ATCs, 86% of tumor specimens displayed CSPG4 staining compared to 36% of PTCs. Patterns of CSPG4 staining included perinuclear, cytoplasmic and membranous (Fig. 3A-D). Most PTCs (Supp. Fig. 3A-B) showed negative or weak staining while the majority of ATCs were positive for CSPG4 with weak, intermediate and strong staining (Fig. 3E).

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Our group has previously reported the association of intercellular adhesion molecule-1 (ICAM-1) and program death-ligand-1 (PD-L1) expression with aggressive thyroid cancers (34, 41, 42). To assess for an association with CSPG4, all tumor samples also underwent IHC for ICAM-1 and PD-L1. CSPG4 expression was correlated with circumferential ICAM-1 expression (Table 4B), a finding we previously reported as having significant correlation with poorly differentiated thyroid cancer (PDTC) and ATC tumors (41). There was no association between PD-L1 and CSPG4 expression. Clinicopathologically, CSPG4 expression was significantly associated with extrathyroidal extension (ETE), lymphovascular invasion and tumor size (Table 4B). Moreover, all 4 PTC samples that were positive for CSPG4 expression had ETE on pathology report while none of 7 PTC that were negative for CSPG4 expression had ETE. *BRAF* (V600E) mutational status on our ATC samples was limited as our institution started routine *BRAF* (V600E) testing in 2010, and there was no significant correlation with CSPG4 expression (Table 4B).

CSPG4 is expressed on the cell surface of ATC cell lines

Because membranous CSPG4 expression was observed predominantly in ATC, we assessed surface expression of CSPG4 in multiple thyroid cancer cell lines and a primary culture of ATC by flow cytometry. As expected, expression in a melanoma cell line, A375-MA2, was high in the majority of cells. The JV cell line had an average of 97.9% expression with an average mean fluorescence intensity (MFI) 33.1-fold higher. Our cell line derived from an ATC, RM, had an average of 94.3% cells expressing CSPG4 with an average MFI 18-fold higher. Interestingly, in the patient's formalin-fixed, paraffin-embedded (FFPE) sample, 1 of 2 ATCs was negative for CSPG4 IHC expression (Supp. Fig 3C-D), while the cell line created from this tumor showed expression by flow cytometry. The percentage of 8505C cells expressing CSPG4 ranged from 67-90% with an average MFI 12.2-fold increase. Other ATC cell lines, KHM-5M and SW1736, had lower CSPG4 expression. Our primary culture of a recent ATC showed an average of 55.4% of tumor-associated cells stained with an average MFI 9.2-fold increase. The PTC line, BCPAP, had 52-53% expression of CSPG4 with an average MFI 2.5-fold increase (Fig. 4). Overall, the majority of ATC cells tested exhibited CSPG4 at the cell surface with approximately 10-fold increase in MFI.

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Patients with increased tumoral CSPG4 expression have worse prognosis in ATC

CSPG4 expression has been associated with worse prognosis in other cancers (25, 43, 44). To determine whether CSPG4 overexpression is correlated with survival rates in thyroid cancer, we analyzed RNA expression and clinical data in patients with PDTC and ATC from a previously published study (45). In this microarray data, there were two probes that matched to CSPG4, 204736 and 214297. When we compared the overall survival (OS) for patients categorized into the top 50% and bottom 50% of *CSPG4* expression for each probe, we found that patients with higher level of *CSPG4* expression had decreased overall survival (Fig. 5) (*p* values were 0.006 and 0.056 for probe 204736 and 214297, respectively, when determined by log-rank test). Median survival was markedly decreased with elevated *CSPG4* expression. These data demonstrate that PDTC and ATC patients with increased tumoral *CSPG4* mRNA expression were associated with worse overall survival.

Discussion

ATC is a deadly cancer with limited treatment options in need of further therapeutic targets. A potential target, CSPG4, has not previously been studied in ATC, although it is a well-described antigen in other aggressive cancers. Given its elevated expression in malignant tissues but restricted expression in normal tissue, CSPG4 is an attractive molecular target for cancer therapy. In this study, we demonstrated CSPG4 overexpression in ATC using our own and publicly available data from patient samples and thyroid cancer cell lines using several methods, including IHC, flow cytometry, mass spectrometry and mRNA expression analysis.

Data regarding peptide antigens presented by the MHC II, in particular HLA-DQ, have been limited because of its highly polymorphic gene structure, which each allelic variant exhibiting different peptide binding affinities. Here, we provide mass spectrometry-based characterization of HLA-DQ-restricted peptides eluted from an ATC patient-derived cell line, JV. We selected HLA-DQ to study immunopeptidomes because JV-ATC cells elicited robust activation by autologous $CD4^+$ T cells and these cells showed the strongest expression of HLA-DQ among the MHC II complexes. We identified 920 HLA-DQ-eluted

peptides isolated uniquely from the JV cells, which need further validation to characterize their potential as tumor-associated antigens.

CSPG4 generated one of the strongest peptide signals in JV cells. The most abundant CSPG4 peptide overlapped 10 amino acids with an epitope predicted to bind HLA-DRB1*10:01 allele (epitope ID 1188394) (46), verifying its presence among immunopeptidomes. Most of the CSPG4-derived peptides we identified were novel. The addition of $CD4^+$ T cells has been shown to potentiate T cell therapies against CSPG4 in preclinical experiments (47). Many treatments against CSPG4 have been developed using hybridoma technology for mAb targeting (48, 49) or generating chimeric antigen receptor-T cell based therapies (31, 50). The HLA-DQ peptides eluted from CSPG4 in our patient sample are prospective immunogenic agents that may help develop T cell receptor-based immune therapies (51). While we discovered significant levels of CSPG4 expression in most ATC, it will be important to demonstrate whether CSPG4-directed adoptive T cell therapy or blockade of CSPG4 signaling will eliminate tumor growth *in vivo*. In addition, understanding the functional role of CSPG4 will give insight into devising the optimal strategy to target CSPG4 in ATC effectively. Given the complexity and heterogeneity of ATC tissue, CSPG4 blockade in addition to inhibitors of BRAF or MAPK/ERK signaling may achieve improved efficacy for targeting ATC (52, 53).

Our IHC data show that the majority of ATC express CSPG4, although most scored an intermediate level of staining. More ATC stained cytoplasmic than membranous; however, flow cytometry confirmed cell surface expression in the majority of ATC samples and cell lines. Curiously, 1 of the 2 ATC specimen negative for CSPG4 using IHC on an FFPE slide had strong CSPG4 expression on flow cytometry using our cell line, RM, derived from the patient's tumor. Given this discordant data, it is possible our IHC antibody may have technical limitations in detecting weaker or denatured CSPG4 protein expression in the FFPE tissue. Alternatively, it is possible that the tumor tissues showed heterogeneous CSPG4 expression, and the tumor cells that sustained in culture were selected for CSPG4-expressing and potentially more aggressive tumor cells. Nevertheless, given our expression data, CSPG4 is a potential target for mAb as well as T cell-based therapies in ATC.

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Limitations to our study include our small sample size, as well as our limited analysis of expression including IHC, mass spectrometry, and mRNA data from public sources. A previous study on head and neck squamous cell carcinoma correlated CSPG4 protein expression by IHC analysis and its mRNA expression level (43). For survival analysis, our institutional data was limited, thus we analyzed survival using previously published RNA expression data (45). Importantly, this data showed a significant shorter overall survival with increased *CSPG4* expression, similar to the prognostic value of CSPG4 expression in other cancers (43, 44).

In conclusion, we have identified CSPG4 as a new potential therapeutic target in ATC, an aggressive cancer with poor prognosis and limited treatment options. Our data shows that ATCs have higher CSPG4 expression and should be further studied for therapeutic potential with mAb and T cell-based therapies. Moreover, the CSPG4 peptides eluted from MHC II offer additional specific targets for designing T cell receptor-based therapies. Ultimately, developing therapeutic strategies to target CSPG4 may provide new treatment options for ATC.

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Contributions:

C. E. Egan: Acquisition, analysis, or interpretation of data for the work, Manuscript writing and revision, Final approval of the version to be published, Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

D. Stefanova: Acquisition, analysis, or interpretation of data for the work, Manuscript revision, Final approval of the version to be published, Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

A. Ahmed: Acquisition, analysis, or interpretation of data for the work, Manuscript revision, Final approval of the version to be published, Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

V. J. Raja: Acquisition, analysis, or interpretation of data for the work, Manuscript revision, Final approval of the version to be published, Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Figure Legends

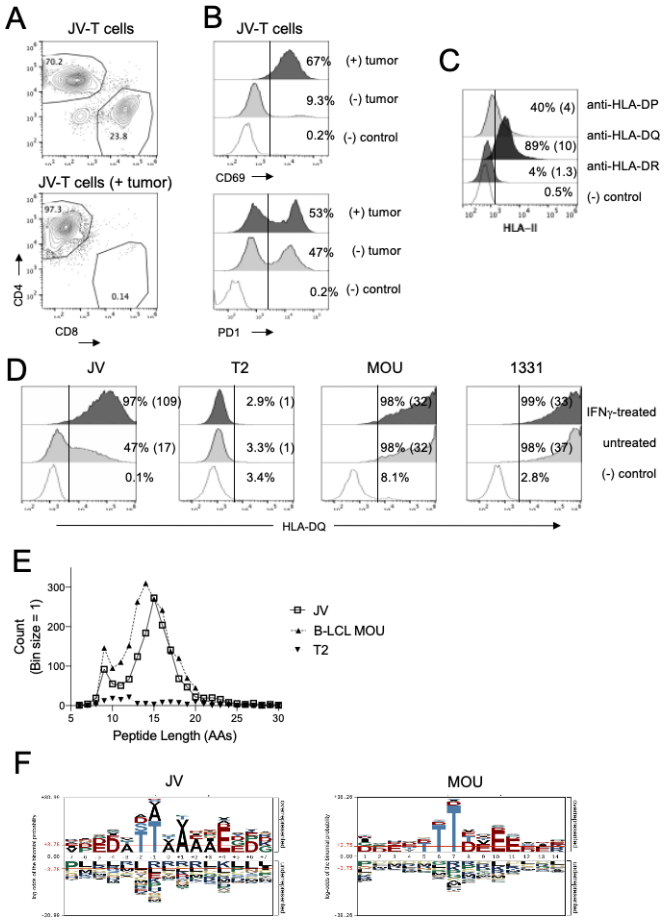
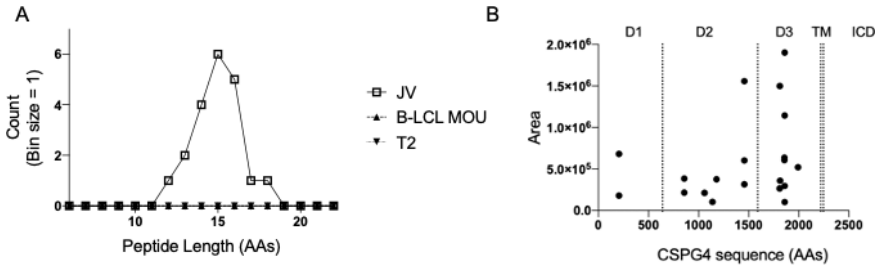


Figure 1. HLA-DQ-eluted peptides in JV anaplastic thyroid cancer cells.

A, B. Characterization of peripheral T cells isolated from JV patient as determined by flow cytometry. **A.** Frequencies of CD4:CD8 in peripheral JV-T cells (top) or *in vivo* expanded JV-T cells after exposure to autologous tumor cells *in vitro* (bottom). **B.** Expression of CD69 (top) or PD1 (bottom) in JV-T cells prior to or after exposure to autologous tumor cells. **C.** Surface expression of MHC II molecules in JV tumor cells was determined by flow cytometry. The gates for positively stained cells were determined by staining with secondary antibody only and were marked with vertical lines within the plots. The numbers in the parenthesis are the fold increases in MFI of each MHC II expression. **D.** The expression levels of HLA-DQ in uninduced and IFNγ-induced conditions (2e4 IU/mL for 48 hours) were determined in JV, T2, B lymphoblastic cell line (B-LCL) MOU, and 1331 cells using SPV-L3 antibody. HLA-DQ histograms analyzed with 1a3 antibody presented a similar profile to SPV-L3 (Supp. Fig. 2). JV cell HLA-DQ haplotypes were HLA-DQA1*0201, HLA-

DQB1*0202/HLA-DQB1*0302. MOU and 1331 B-LCL lines had DQ2.2 (HLA-DQA1*0201/HLA-DQB1*0202) and DQ8.1 (HLA-DQA1*0301/HLA-DQB1*0302), respectively. T2 cells were deficient in MHC II expression including HLA-DQ. **E.** Peptide length distribution of HLA-DQ-eluted peptides from MOU, T2, and IFNγ-induced JV cells were shown as a histogram. **F.** Sequence logos of eluted peptides isolated from JV-ATC (15 a.a., left) and MOU (14 a.a., right) were generated using pLogo generator (38).



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ID	MHC	Peptide	Core_peptide	% Rank_EL	% Rank_BA	Affinity, K_d, (μM)	Bind Level
1	DQA1*02:01/DQB1*02:02	DPDSAPGEIEYEVQ	SAPGEIEYE	12.54	29.76	3.91	-
1	DQA1*02:01/DQB1*03:02	DPDSAPGEIEYEVQ	SAPGEIEYE	6.54	49.17	2.87	WB
2	DQA1*02:01/DQB1*02:02	SHPVAFTVTVLPVNDQPF	AFTVTVLPV	4.33	5.03	1.27	WB
2	DQA1*02:01/DQB1*03:02	SHPVAFTVTVLPVNDQPF	AFTVTVLPV	3.57	1.85	0.44	WB
3	DQA1*02:01/DQB1*02:02	VAGPQTSEAFAITVR	PQTSEAFAI	1.43	7.87	1.61	SB
3	DQA1*02:01/DQB1*03:02	VAGPQTSEAFAITVR	PQTSEAFAI	0.91	0.67	0.33	SB

Figure 2. HLA-DQ-eluted CSPG4 peptides in JV ATC cells.

A. Peptide length distribution of HLA-DQ-eluted CSPG4 peptides from MOU, T2, and IFNγ-induced JV cells were shown as a histogram. **B.** Eluted CSPG4 peptide count areas (closed circles) were displayed along the CSPG4 sequence. Extracellular domains 1-3 (D1-3), transmembrane (TM), and intracellular domains (ICM) were shown on the top of the plot. **C.** The predicted binding affinities of the top 3 CSPG4 peptides for HLA-DQA1*02:01/DQB1*02:02 and HLA-DQA1*02:01/DQB1*03:02 were determined using the NetMHCIIpan-4.0 (38). The 'Core peptide' identifies the binding core of the peptide to each 'MHC' allele. '% Rank_EL' indicates the percentile rank of eluted ligand prediction score. '% Rank_BA' is calculated as a percentile rank of the predicted peptide affinity compared to a set of 100.000 random natural peptides." Affinity is the predicted binding affinity, K_d, in μM. Threshold for strong (SB) and weak binding (WB) peptides was set at <2% and <10%, respectively.

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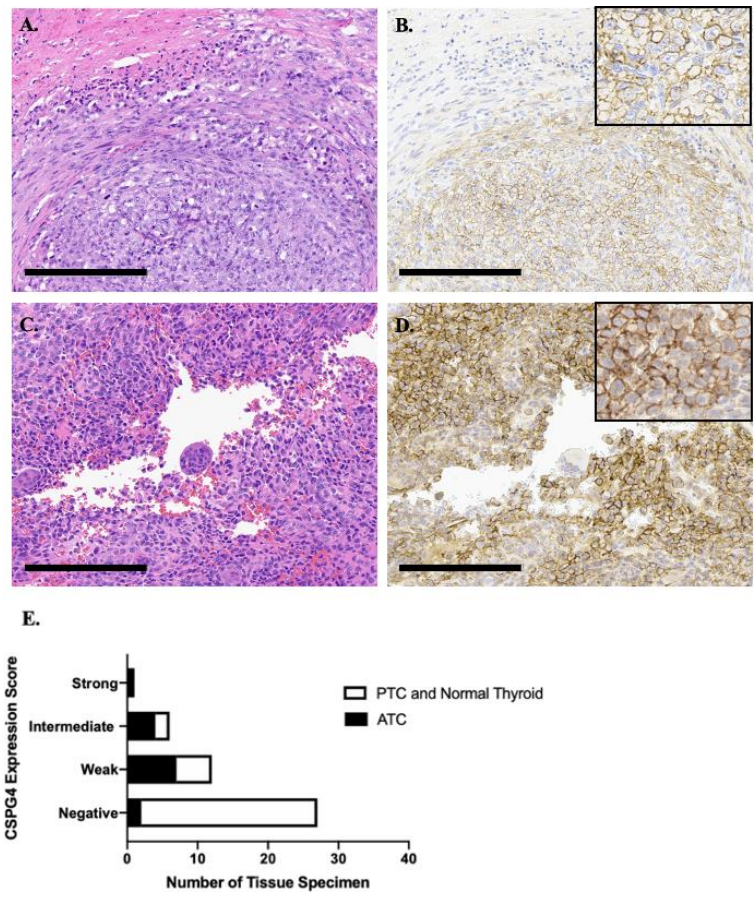

Figure 3. CSPG4 is expressed predominantly in the cytoplasm and/or membrane of ATC.

A, B. Hematoxylin and eosin (H&E, left) and membranous CSPG4 (right) staining in ATC. Scale bar = 200 µm. **C, D.** H&E (left) and membranous and cytoplasmic CSPG4 (right) staining in ATC. Scale bar = 200 µm. **E.** IHC tissue sides were scored for percentage of tumor cells stained (0=negative, 1=1-29%, 2=30-59%, 3=60-100%) and intensity of staining (0=negative, 1=weak, 2=intermediate, 3=strong) by an endocrine pathologist in a blinded review. Intensity (0-3) and percentage stained (0-3) were added together for a possible total of 0-6 points (0=negative, 1-2=weak, 3-4=intermediate, 4-6=strong). Scores compared ATC to PTC and normal thyroid tissue using Fisher's exact test (p<0.001).

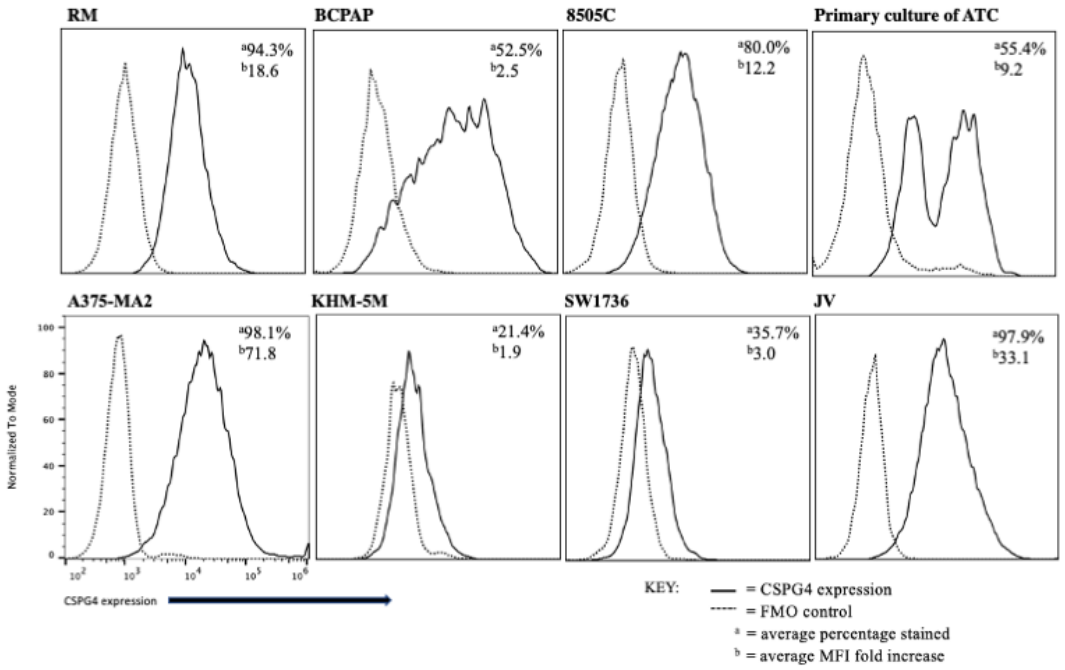

Figure 4. Some ATC cells show membranous expression of CSPG4.

Representative histogram plots of CSPG4 expression and FMO controls in multiple thyroid tumor cell lines and primary tumor culture were determined by flow cytometry. These experiments were repeated at least 3 times independently.

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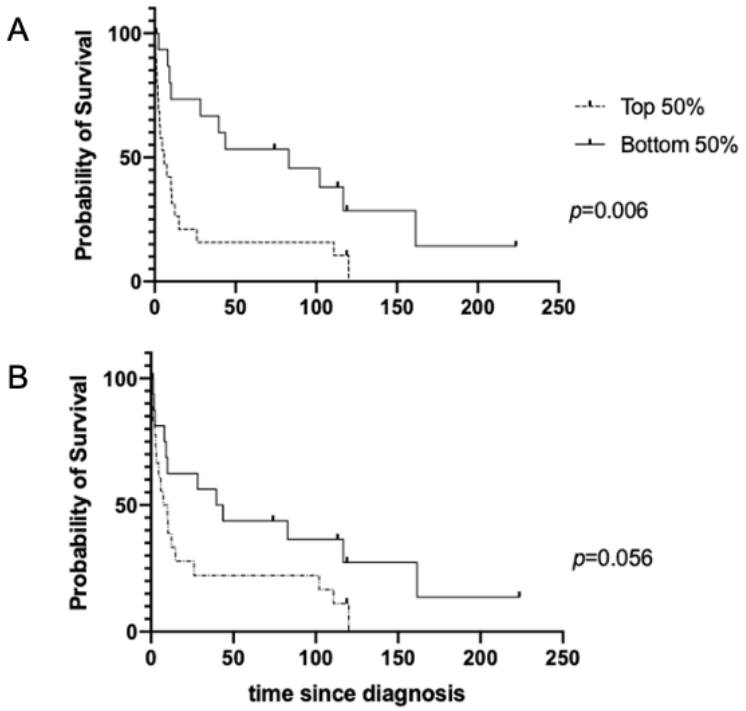

Figure 5. **Advanced thyroid tumor patients with higher *CSPG4* mRNA expression is correlated with a shorter overall survival rate.**

A. Kaplan-Meier survival curves were generated using the overall survival and mRNA expression data available from a published study on PDTC and ATC patients (43). Statistical difference was analyzed by comparing two cohorts with *CSPG4* mRNA expression (top 50% and bottom 50%) using log-rank test with data obtained with *CSPG4* probe 204736 (p=0.006), with median survival of 5.92 months and 82.83 months for top and bottom 50% of *CSPG4* expression, respectively. **B.** Another *CSPG4* probe 214297 was also used to analyze OS (p=0.056) using log-rank test with median survival of 8.78 and 41.68 months for the top and bottom 50%, respectively.

Table 1. Twenty most abundant proteins that generated peptides eluted with HLA-DQ specific antibody (SPV-L3) in JV cell line compared to MOU and T2.

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			Cell lines		
Uniprot[a]	**gene symbol**	**description**	**MOU[b]**	**T2[b]**	**JV[b]**
P05109	*S100A8*	Protein S100-A8, N-terminally processed	0	0	23999080 0
P06702	*S100A9*	Protein S100-A9	0	0	9387110 0
P0CG48	*UBC*	Polyubiquitin-C; Ubiquitin	3684509 3	8213 6	5505380 0
P04406	*GAPDH*	Glyceraldehyde-3-phosphate dehydrogenase	1173260 0	0	5151655 3
P36941	*LTBR*	Tumor necrosis factor receptor superfamily member 3	0	0	3399100 0
P18464	*HLA-B*	HLA class I histocompatibility antigen, B-51 alpha chain	1679600	0	3187000 0
P01040	*CSTA*	Cystatin-A, N-terminally processed	0	0	3009780 0
Q6UVK1	*CSPG4*	Chondroitin sulfate proteoglycan 4	0	0	1730460 0
P31151	*S100A7*	Protein S100-A7	0	0	1611304 9
P01903	*HLA-DRA*	HLA class II histocompatibility antigen,	1539290 0	0	1508286 2

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		DR alpha chain			
P20930	*FLG*	Filaggrin	14160287	0	14160287
P11142	*HSPA8*	Heat shock cognate 71 kDa protein	65869600	96885	13812492
Q9NZT1	*CALML5*	Calmodulin-like protein 5	0	0	13770187
P07093	*SERPINE2*	Glia-derived nexin	0	0	12985300
P23381	*WARS*	Tryptophan--tRNA ligase, cytoplasmic; T1-TrpRS; T2-TrpRS	0	0	10930469
P00441	*SOD1*	Superoxide dismutase [Cu-Zn]	0	0	10864709
P35527	*KRT9*	Keratin, type I cytoskeletal 9	9880967	6290028	10417904
Q10589	*BST2*	Bone marrow stromal antigen 2	14997772	0	9795100
P08865	*RPSA*	40S ribosomal protein SA	189000	0	8996000
P60174	*TPI1*	Triosephosphate isomerase	0	0	8915073

[a]Protein amino acid sequences were derived from Uniprot database.

[b]Protein area is derived from summing intensities of all peptides that have been matched to amino acid sequences of a particular protein.

Table 2. Twenty most abundant proteins that presented peptides with HLA-DQ complexes in MOU cells and their quantities in JV and T2 cells.

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			Cell lines		
Uniprot[a]	**gene symbol**	**description**	**MOU[b]**	**T2[b]**	**JV[b]**
P61769	*B2M*	Beta-2-microglobulin; Beta-2-microglobulin form pI 5.3	8917824758	0	1302700
P04233	*CD74*	HLA class II histocompatibility antigen gamma chain	1291969600	1376700	0
P0CG48	*UBC*	Polyubiquitin-C; Ubiquitin	368450983	82136	55053800
Q9NPF2	*CHST11*	Carbohydrate sulfotransferase 11	136873400	0	0
P10124	*SRGN*	Serglycin	110363488	0	0
P30457	*HLA-A*	HLA class I histocompatibility antigen, A-66 alpha chain	81047700	0	0
P62	*YWHAE*	14-3-3 protein epsilon	770022	0	0

258			35		
P61073	*CXCR4*	C-X-C chemokine receptor type 4	69844548	0	0
P11142	*HSPA8*	Heat shock cognate 71 kDa protein	65869600	96885	13812492
P11836	*MS4A1*	B-lymphocyte antigen CD20	60944700	0	0
Q02818	*NUCB1*	Nucleobindin-1	60418500	0	0
P07108	*DBI*	Acyl-CoA-binding protein	36739789	0	0
P01857	*IGHG1*	Ig gamma-1 chain C region	36209718	23296 00	0
P11279	*LAMP1*	Lysosome-associated membrane glycoprotein 1	31294400	0	7941915
Q07	*HLA-C*	HLA class I histocompatibility	268730	0	0

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000		antigen, Cw-15 alpha chain	00		
Q15758	*SLC1A5*	Neutral amino acid transporter B (0)	25598800	0	5772797
Q9Y287	*ITM2B*	Integral membrane protein 2B; BRI2, membrane form; BRI2 intracellular domain; BRI2C, soluble form; Bri23 peptide	24537353	0	1027300
P61981	*YWHAG*	14-3-3 protein gamma;14-3-3 protein gamma, N-terminally processed	24525256	0	0
Q7Z4F1	*LRP10*	Low-density lipoprotein receptor-related protein 10	24398500	0	277800
P03989	*HLA-B*	HLA class I histocompatibility antigen, B- 27 alpha chain	23919000	0	0

[a]Protein amino acid sequences were derived from Uniprot database.

[b]Protein area is derived from summing intensities of all peptides that have been matched to amino acid sequences of a particular protein.

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Table 3. Unique CSPG4 peptides eluted from HLA-DQ complexes in JV-ATC tumors.

Gene symbol	Peptide	Peptide Length	Area	Count	Predicted affinity, K_d (μM)[a] DQA1*02:01 DQB1*02:02	Predicted affinity, K_d (μM)[a] DQA1*02:01 DQB1*03:02
CSPG4	DPDSAPGEIEYEVQ	14	1899000	1	3.91	1.72
CSPG4	SHPVAFTVTVLPVNDQPP	18	1556000	2	1.27	0.44
CSPG4	VAGPQTSEAFAITVR	15	1498000	2	1.61	0.33
CSPG4	DPDSAPGEIEYEV	13	1143000	1	4.95	4.48
CSPG4	GRPTSAFSQFQIDQ	14	520800	1	2.00	1.14
CSPG4	FSGPHSLAAFPAWGT	15	680500	1	2.79	0.27
CSPG4	VVDPDSAPGEIEYEV	15	635600	1	3.10	2.10
CSPG4	VVDPDSAPGEIEYEVQ	16	606600	1	3.18	2.09
CSPG4	SHPVAFTVTVLPVNDQ	16	603000	2	0.70	0.29
CSPG4	VTYGATARASEAVED	15	383800	2	1.96	0.26
CSPG4	DPDSAPGEIEYE	12	295400	2	7.40	5.94
CSPG4	GPQTSEAFAITVR	13	359900	2	2.88	0.89

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CSPG4	AGQPATAFSQQDLLD	15	375100	1	0.81	0.69
CSPG4	SHPVAFTVTVLPVN	14	315500	1	0.98	0.41
CSPG4	VTYGATARASEAVE	14	216100	1	2.47	0.38
CSPG4	KDLLFGSIVAVDEPTRP	17	211800	2	0.47	0.26
CSPG4	SVAGPQTSEAFAITVR	16	266400	2	1.55	0.33
CSPG4	FSGPHSLAAFPAWGTQ	16	178700	2	3.10	0.30
CSPG4	DPDSAPGEIEYEVQR	15	101900	1	3.73	2.59
CSPG4	GGQGTIDTAVLHLDTN	16	104500	1	2.31	1.02

[a]MHCII binding affinity was predicted using the prediction program described by Reynisson et al. (38).

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Table 4A. CSPG4 expression and pattern in thyroid cancer tissue samples including adjacent normal thyroid.

	n	CSPG4 0%	CSPG4 ≥ 1%	*p*[a] value
Histology				0.0001
PTC	11	7 (64%)	4 (36%)	
			1 nuclear	
			2 cytoplasmic	
			1 membranous	
ATC	14	2 (14%)	12 (86%)	
			0 nuclear	
			7 cytoplasmic	
			5 membranous	
Normal thyroid	21	18 (86%)	3 (14%)	
			0 nuclear	
			3 cytoplasmic	
			0 membranous	

[a]*p* value was determined using chi-squared test.

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Table 4B. Clinical characteristics and IHC staining of thyroid cancer samples used in this study.

	CSPG4 0% (n=9)	CSPG4 ≥ 1% (n=16)	*p* value
Histology			0.0168[a]
PTC	7 (78%)	4 (25%)	
ATC	2 (22%)	12 (75%)	
ICAM1 Expression			>0.9999[a]
>1%	9 (100%)	16 (100%)	
ICAM1 Pattern			0.0168[a]
Apical	7 (78%)	4 (25%)	
Circumferential	2 (22%)	12 (75%)	
PDL1 Expression			0.4341[a]
>1%	4 (44%)	10 (63%)	
Clinical Characteristics			
Age	44 (16-62)	61 (26-86)	0.0736[b]
Male sex	0/9 (0%)	5/16 (31%)	0.0613[a]
Tumor size (cm)	1.6 (0.5-4.8)	3.7 (1.1-7.3)	0.0035[b]
Lymph node metastases	3/5 (60%)	9/12 (75%)	0.6000[a]
Distant metastases	2/9 (22%)	4/13 (31%)	>0.9999[a]
Lymphovascular invasion	2/9 (22%)	11/13 (85%)	0.0073[a]
Extrathyroidal extension	1/7 (14%)	15/15 (100%)	<0.0001[a]

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***BRAF*^{V600E} mutation**[c]	7/8 (88%)	6/10 (60%)	0.3137[a]

[a] *p* values were determined using Fisher's exact test.

[b] *p* values were determined using Wilcoxon rank-sum test.

[c] *BRAF*^{V600E} mutational status was not assessed until 2010 at our institution.

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