MHC Class I Loss Is a Frequent Mechanism of Immune Escape in Papillary Thyroid Cancer That Is Reversed by Interferon and Selumetinib Treatment In Vitro

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Abstract

Purpose: To evaluate MHC class I expression on papillary thyroid cancer (PTC) and analyze changes in MHC expression and associated immune activation with current and experimental treatments for thyroid cancer using in vitro PTC cell lines.

Experimental Design: MHC class I expression and assessment of tumor-infiltrating leukocyte populations were evaluated by immunohistochemistry. PTC cell lines were analyzed for HLA-ABC expression by flow cytometry following tyrosine kinase inhibitor, IFNα or IFNγ, or radiation treatment. Functional changes in antigenicity were assessed by coculture of allogeneic donor peripheral blood leukocytes (PBL) with pretreated or untreated PTC cell lines and measurement of T-cell activation and cytokine production.

Results: Both MHC class I and β2-microglobulin expression was reduced or absent in 76% of PTC specimens and was associated with reduced tumor-infiltrating immune cells, including effector (CD3+, CD8+, CD16+) and suppressor (FoxP3+) populations. Treatment of PTC cell lines with the MEK1/2 inhibitor selumetinib or IFN increased HLA-ABC expression. This phenotypic change was associated with increased T-cell activation (%CD25+ of CD3+) and IL2 production by PBL cocultured with treated PTC cell lines. Additive effects were seen with combination selumetinib and IFN treatment.

Conclusions: MHC class I expression loss is frequent in human PTC specimens and represents a significant mechanism of immune escape. Increased antigenicity following selumetinib and IFN treatment warrants further study for immunotherapy of progressive PTC. Clin Cancer Res; 20(23); 6034–44. ©2014 AACR.

Introduction

Papillary thyroid cancer (PTC) comprises 85% to 90% of all thyroid malignancies and its incidence has increased 3-fold over the past several decades. Despite an overall good prognosis, 20% to 30% of patients with PTC have persistence or recurrence and 5% to 10% suffer progressive, treatment-refractory disease. For these patients, the adjunctive therapies currently available are often of limited benefit.

Immunotherapy is a potential new treatment strategy for patients with recurrent or progressive PTC. In melanoma and other solid malignancies, including lung, prostate, and renal cell cancers, immunotherapy regimens such as CTLA-4 and programmed death ligand 1 (PD-L1) blockade and IL2 have produced remarkably durable tumor regressions in patients with metastatic disease (5–7). Immunotherapy uses the ability of the body’s own immune cells to recognize and eliminate malignant cells, taking advantage of the inherent specificity and systemic reach of the adaptive immune system. While the host immune system can recognize and be activated to abnormal antigens present on tumors, neoplastic growths frequently evolve mechanisms to escape immune destruction (8, 9). Strategies of immune escape include downregulation of antigen display, and the induction of immune inhibition by tumor expression of inhibitory molecules and the recruitment of suppressor cell populations (9). To eliminate cancer effectively, immunotherapy regimens must reverse the tumor-driven immune dysfunction, restore antitumor immune responses, and induce antigen-specific memory.

MHC class I molecules and their associated proteasomal machinery play a key role in the presentation of peptides, including tumor-associated antigens, expressed on the surface of neoplasms. Expression of these cell antigens concurrent with immune costimulatory signals indicating cell damage induces immune activation and cytotoxic killing of the abnormal cell (9). Downregulation of MHC class I
antigen expression by cancer cells is an important strategy for immune evasion (9–11). HLA-ABC loss has been reported in a number of cancers, including in approximately 70% of head and neck squamous cell carcinomas, 96% of breast carcinomas, 87% of colon carcinomas, 39% of pancreatic carcinomas, and 63% of melanomas (12, 13).

In PTC, immunosuppressive strategies, including tumor expression of immune inhibitory molecules (14–17) and tumor infiltration by suppressive immune cells (17–22), have been described, but the contribution of immune evasion in PTC has not been investigated. In this study, we report in PTC a high proportion of cases with downregulation of MHC class I expression and increased tumor recognition by immune cells in vitro, suggesting MHC class I modulation as a novel immunotherapy approach for patients with advanced PTC.

Translational Relevance

While the prognosis for papillary thyroid cancer (PTC) is generally good, a subset of patients suffer significant morbidity and mortality from recurrent or progressive disease. Existing treatments show limited benefit in these cases and new therapies are needed. This study identifies MHC class I downregulation as a frequent mechanism of immune escape in PTC patients that is associated with decreased intratumoral immune cell infiltration. Treatment of PTC cell lines with tyrosine kinase inhibitor selumetinib and IFNs augmented MHC class I expression and increased tumor recognition by immune cells in vitro, suggesting MHC class I modulation as a novel immunotherapy approach for patients with advanced PTC.

Materials and Methods

Tissue specimens

PTC specimens from patients with thyroid cancer with anonymonized clinical data were obtained from the USC Keck Medical Center Tissue Bank (IRB protocol HS-11-00215). When available, contralateral thyroid lobe normal tissue was collected and evaluated in parallel.

Immunohistochemistry

Formalin-fixed paraffin-embedded (FFPE) tissue sections were deparaffinized, rehydrated, and subjected to heat-induced antigen retrieval (0.01 mol/L citrate, pH 6.0) followed by treatment with 3% H2O2 for 10 minutes to block endogenous peroxidase activity. Sections were incubated overnight at 4°C with primary antibodies against CD3 (C14, Santa Cruz Biotechnology), CD8 (C8/144B, Dako), CD16 (O.N.82, Abcam), CD68 (PG-M1, Dako), CD163 (10D6, Abcam), FoxP3 (236A/E7, Novus), HLA-ABC (C-6, Santa Cruz Biotechnology), or β2m (BBM.1, Santa Cruz Biotechnology). Secondary antibody staining and antigen detection with 3,3′-diaminobenzidine was performed using Vectastain ABC Kit (Vector Laboratories). Sections were counterstained with hematoxylin, dehydrated, and mounted. Appropriate positive and negative controls were used for all stains. Hematoxylin and eosin (H&E)-stained sections were provided by the USC Translational Pathology Core. Representative immunohistochemical (IHC) images and stain controls are shown in Supplementary Fig. S1.

Scoring of immune markers

Using an adapted immune infiltrate scoring system to evaluate cancer specimens previously developed in our laboratory (23), areas of tumor and associated tumor-infiltrating leukocytes (TIL), intratumorally or at the invading margin, were identified on H&E-stained sections. Areas of obvious lymphoid follicle arrangement, necrosis, or hemorrhage were excluded. Tumor expression of HLA-ABC or β2m was assessed qualitatively as intact, reduced, or absent. Positively stained leukocytes for CD3, CD8, CD16, CD68, CD163, or FoxP3 were counted in five representative high-power fields (hpf) for each tumor section. Two independent observers scored each section and the results were pooled with rare disagreements resolved by a third evaluator.

BRAF mutational analysis

The gene mutation BRAFV600E encodes for the mutated protein BRAFV600E. For BRAFV600E mutation detection, tumor DNA was isolated from FFPE sections by excision of tumor tissue and DNA purification using a Qiagen QIAmp FFPE Kit (Qiagen). Human BRAF exon 15 was amplified by PCR (forward: TCATAATGCTTGCTCTGAT; reverse: GGCCAAAAATTTAATCAGTGGA; ref. 24). PCR DNA amplicons electrophoresed on 1.5% agarose were amplified by PCR (forward: TCATAATGCTTGCTCTGAT; reverse: GGCCAAAAATTTAATCAGTGGA; ref. 24). PCR DNA amplicons electrophoresed on 1.5% agarose were extracted and purified using a Qiagen MinElute Gel Extraction Kit and sequenced at the USC Genomics Core Facility. Given the rarity of other BRAF mutations, cases without a BRAFV600E substitution were considered wild-type (BRAFWT).

Cell lines and cell culture

PTC cell lines BCPAP (BRAFV600E mutation), K-1 (BRAFV600E mutation, P53 mutation), and TPC-1 (RET/PTC1 translocation, BRAFWT) were obtained from the University of Colorado Tissue Bank in 2013 and authentication was performed by the University of Colorado Cancer Center DNA Sequencing and Analysis Core using DNA profiling of short tandem repeat markers (25). Cells were maintained in a 5% CO2, 37°C, humidified incubator in complete medium (RPMI1640 with 10% fetal calf serum, 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin).

In vitro treatment of PTC cell lines for HLA modulation

Tumor cell lines were seeded in 6-well tissue culture plates overnight (7.5 × 105 cells/well). For small-molecule inhibitor treatment, tumor cells were treated for 5 days, with refreshment of media and drug every 48 hours. Drugs evaluated included two specific BRAFV600E inhibitors...
vemurafenib and PLX 4720, tyrosine kinase inhibitors sunitinib and sorafenib (Selleck Chemicals; resuspended in DMSO), and a specific MEK1/2 inhibitor selumetinib (MedChem Express; resuspended in DMSO), with drug concentrations selected on the basis of reported drug IC_{50} in human differentiated thyroid cancer cell lines. Tumor cell treatment with IFNγ or α (Sigma-Aldrich) was similarly done for 72 hours, with cytokines refreshed at 48 hours. For radiation treatment, tumor cells were exposed to 30 or 60 Gy using an X-RAD 320 IX irradiator (Precision X-Ray, Inc.). Experiments were performed in duplicate using nonconfluent monolayers. After in vitro treatment, cell lines were analyzed for surface marker expression by flow cytometry or cocultured with healthy donor peripheral blood leukocytes (PBL) to assess their antigenicity, as described below.

**Measurement of immune cell activation**

Functionally relevant changes in HLA expression on PTC cell lines following drug, radiation, or IFN treatment were assessed by a modified mixed lymphocyte reaction in which naïve healthy donor PBL were cocultured with the tumor cell lines and then indicators of immune cell activation were measured. Peripheral blood from healthy donors was obtained by routine venipuncture with IRB approval (protocol HS-06-00579), and PBL were isolated by differential density gradient centrifugation. After in vitro pretreatment of tumor cell lines with drug, radiation, cytokine, or vehicle control, the medium was replaced and tumor cells were cocultured with freshly isolated CFSE-labeled PBL (10^6 cells/well). Coculture experiment controls included single donor PBL alone (i.e., without allogeneic tumor cell lines) in the presence or absence of anti-CD3/CD28 stimulation (Invitrogen; Supplementary Fig. S2). After 72 hours, PBL were collected from cocultures and analyzed for immune cell markers as described below. In addition, coculture supernatants were collected and analyzed for levels of the T-cell cytokine IL2 by cytometric bead array (BD Biosciences) as per manufacturer’s instructions. In two independent experiments, IL2 production by K-1, BCPAP, and TPC-1 cell lines alone was undetectable.

**Flow cytometry**

MHC class I molecule expression on tumor cell lines and immune markers on PBL from tumor cell line cocultures were evaluated by flow cytometry. Tumor cells were collected from wells using Detachin (Genlantis) to minimize cell surface protein digestion. Cell washing and staining was performed as described previously (11) using fluorescein-conjugated monoclonal antibodies against CD25 (4E3, Miltenyi Biotec), HLA-ABC (G46-2.6, BD Biosciences), PD-L1 (MIH11, BD Biosciences), PD-L2 (MIH1B, BD Biosciences), HLA-G (MEM-G/9, Life Technologies), CD3 (UCHT1, BD Biosciences), or iotype-matched controls (BD Biosciences). Samples were run (≥20,000 live events) in duplicated on an Attune flow cytometer (LifeTechnologies), or a BD LSRII flow cytometer using FACSDIVA software (BD Biosciences) for acquisition and compensation, and analyzed using FlowJo software (FlowJo).

**Quantitative reverse transcriptase PCR**

Thyroid cancer cell lines BCPAP, K-1, and TPC-1 were treated with selumetinib 10 μmol/L or vehicle alone for 48 hours in triplicate and then evaluated for gene expression of MHC class I molecules, antigen-processing machinery, and cytokines by quantitative reverse transcriptase PCR (qRT-PCR), as reported previously (23). Briefly, RNA was isolated from tumor cell lines using RNeasy Micro Kit with on-column DNase treatment (Qiagen). For real-time RT-PCR, 100 ng of DNase-treated RNA was amplified with gene-specific primers using one-step Power SYBR green RNA-to-Ct kit (Applied Biosystems) and an MX3000P Stratagene thermocycler in duplicate. Primer sequences were from the validated NIH qRT-PCR database (http://primerdepot.nci.nih.gov), are listed in Supplementary Table S1, and were synthesized by the USC Microchemical Core Facility. Gene expression was normalized to housekeeping gene GAPDH and reported as a mean fold change in expression for each gene in selumetinib-treated thyroid cancer cell lines relative to expression in vehicle-treated cells.

**Statistical analysis**

Statistics are shown as mean ± SD or SEM as indicated. Unpaired student t tests with Bonferroni correction for multiple comparisons was used to compare differences in mean positively stained immune cells/hpf between tumors with absent or reduced versus intact MHC class I (HLA-ABC or β2m) expression. Differences in HLA expression among treated and untreated tumor cell line groups, differences in the mean fraction of activated T cells, and mean cytokine levels from PBL in tumor cell line cocultures among treated and untreated groups were evaluated by ANOVA followed by pairwise comparisons with Dunnett test or Bonferroni corrected t test. Gene expression differences by qRT-PCR between selumetinib or vehicle control-treated PTC cell lines were compared by student t test with correction for multiple comparisons by the Holm–Sidak method, with α = 0.05. Statistical tests were performed using GraphPad Prism 6.0 software and graphs and figures were produced using GraphPad and Adobe Photoshop.

**Results**

**Patient characteristics**

Tumor specimens and clinical data from 33 PTC patients were retrospectively obtained from the Keck Medical Center Tissue Bank, as summarized in Supplementary Table S2. Female patients constituted 29 of 33 (87.8%) of the sample, and the median patient age was 49 years (range 22–75). Primary tumors were noninvasive (TNM 1 or 2) in 26 (78.8%) and invasive (TNM 3 or 4) in 7 (21.2%) cases. Lymph node metastases were present at initial surgery in 12 of 33 (35.2%) patients. No patient had known distant metastases at the time of the initial surgery. Evaluation revealed the BRAF^V600E mutation in 17 of 33 (51.5%) of tumor samples.
Loss of MHC class I expression in PTC

Expression of MHC class I molecules by tumor specimens was evaluated by staining of FFPE sections for HLA-ABC by immunohistochemistry. As shown in Fig. 1, HLA-ABC expression was decreased or absent in 29 of 33 (87.9%) tumor specimens compared with normal thyroid tissue, with only four specimens showing intact cellular membrane staining. Expression of β2m by immunohistochemistry was similar, though demonstrating more cases (7/33, 21.2%) with intact expression. When considering only cases with congruous HLA-ABC and β2m results, 25 of 33 cases (76%) had "absent or reduced" expression of both of these MHC class I markers. Of cases with intact expression of either marker, 6 of 8 (75%) were BRAFV600E positive, compared with only 11 of 25 (44%) in BRAFWT tumors, though the difference between these proportions was not statistically significant, possibly attributable to small sample size.

Because MHC class I molecules facilitate immune recognition, we hypothesized that those tumors retaining strong HLA-ABC and/or β2m expression would demonstrate greater tumor leukocyte infiltration. For these studies, tumor specimens were grouped as "intact" if either HLA-ABC or β2m expression was intact (n = 8) or "reduced/absent" if both HLA-ABC and β2m were reduced and/or absent (n = 25) compared with normal thyroid tissue. As shown in Fig. 2, intact expression of HLA-ABC or β2m by tumor cells was associated with a greater number of intratumoral immune cells. The mean number of CD3⁺ T cells/hpf was 51.47 ± 15.67 compared with 15.15 ± 1.88 in tumors with intact versus reduced/absent HLA-ABC expression, respectively (P = 0.0011). Similarly, tumors with intact compared with reduced/absent HLA-ABC or β2m demonstrated a higher mean number of tumor-infiltrating CD8⁺ T cells of 13.28 ± 4.08 cells/hpf compared with 5.67 ± 0.91 cells/hpf, respectively (P = 0.013). Linear regression analysis between mean CD3⁺ cells/hpf infiltration and increasing HLA-ABC expression score demonstrated a significant positive correlation (r² = 0.29, P = 0.0011; Fig. 2B). All other immune cell populations examined, namely CD16⁺ natural killer cells, FoxP3⁺ regulatory T cells, CD68⁺ pan-macrophages, and CD163⁺ M2 macrophages, were found to be more abundant in HLA-ABC/β2m intact tumors, but these differences did not reach statistical significance (Fig. 2C). Representative IHC staining of tissue sections is shown in Supplementary Fig. S1.

Effect of tyrosine kinase inhibitors on PTC cell line HLA expression

Tyrosine kinase inhibitors are clinically available drugs used in thyroid cancer treatment and some have been shown to modulate PTC expression of differentiated thyroid antigens (26, 27). Using papillary thyroid cancer cell lines as an in vitro model of disease, several kinase inhibitors previously examined in thyroid cancer were evaluated for their respective effects on MHC class I expression and potential as immunotherapeutic reagents. HLA-ABC expression was measured by flow cytometry following incubation of each cell line with drug or vehicle control. As shown in Fig. 3, baseline HLA-ABC expression of cell lines BCPAP, K-1, and TPC-1 in culture was similar and treatment with...
JAK/STAT inhibitor sunitinib or MEK1/2 inhibitor selumetinib produced significant, dose-responsive increases in HLA-ABC expression in all three PTC cell lines. Treatment with sorafenib, another tyrosine kinase inhibitor, yielded modest and nonsignificant increases in HLA-ABC expression. Two specific BRAFV600E inhibitors, vemurafenib and PLX 4720, each generated a modest increase in HLA-ABC expression on the K-1 cells, and no significant change in HLA-ABC expression on BCPAP cells (Supplementary Fig. S3A).

The kinase inhibitors sunitinib and selumetinib showed the greatest efficacy in upregulating HLA-ABC across all PTC cell lines, but sunitinib also produced significant upregulation of immunosuppressive ligands PD-L1 (Fig. 3B), PD-L2 (data not shown), and HLA-G (Fig. 3C), making it a less attractive candidate for immunotherapy. Therefore, selumetinib was selected for further evaluation.

PTC cell lines included greater T-cell activation (CD25+ fraction of CD3+ T cells) and cytokine production (IL2; Fig. 3D and E). Selumetinib (10 μmol/L) pretreated cells of all three PTC cell lines caused a statistically significant increase in IL2 production by cocultured PBLs (P < 0.01 for BCPAP, P < 0.05 for K-1 and TPC-1), with significant (BCPAP) or near-significant increases seen after pretreatment with 1 μmol/L, compared with untreated (vehicle alone) PTC cells. This was accomplished by modest but not significant increases in the proportion of CD3+ T cells expressing the CD25+ activation marker among cocultured PBL following PTC cell line pretreatment with 1 or 10 μmol/L selumetinib for BCPAP, K-1, and TPC-1 models.

Effect of radiation on PTC cell line HLA expression
To evaluate the effect of radiation treatment on MHC class I expression, PTC cell lines were exposed to 30 or 60 Gy and then evaluated for changes in HLA-ABC expression by flow cytometry after 48 hours. The level 30 Gy was chosen to approximate the effects of radioactive iodine (131I) exposure (28) and because of data demonstrating increased MHC I expression at similar doses in a nonthyroid cancer mouse model (29), with the 60 Gy dose added in anticipation of possible radioactivity resistance of PTC cell lines. As shown in Supplementary Fig. S3B, radiation produced modest increases in HLA-ABC expression in PTC cell lines, with only a trend toward significance for PTC-1 at the 60 Gy dose (P = 0.09) and no significant increases in the K-1 or BCPAP cell lines. In regard to changes in immune activation of PBL,
there was a trend toward increased CD25+CD3+ T cells when PTC cell lines were pretreated with 30 or 60 Gy, though these increases were statistically significant only for BCPAP (Supplementary Fig. S3C). Similarly, IL2 production by PBL cocultured with radiation-treated BCPAP cells was increased modestly, and no appreciable increase was noted for PBL cocultured with K-1 or TPC-1 (Supplementary Fig. S3D).

Effect of IFN treatment on PTC cell line HLA expression

The effect of IFNγ and IFNα on antigen expression by PTC cell lines was evaluated by in vitro treatment over 72 hours followed by measurement of HLA-ABC surface expression by flow cytometry. In response to IFNγ treatment at 50 or 100 U/mL, all three PTC cell lines showed strong upregulation of MHC class I molecules, as shown in Fig. 4 (P < 0.05 for BCPAP and TPC-1, trend for K-1). IFNα similarly induced a significant and dose-related increase in HLA-ABC expression in BCPAP and TPC-1 PTC cell lines at doses of 100 and 500 U/mL, with a trend toward greater expression in K-1 cells, though the changes in expression were more modest. As shown in Fig. 4B and C, the greater expression of MHC class I on PTC cell lines following IFNγ pretreatment produced a significant increase in T-cell activation and IL2 production in all three PTC cell lines in a dose-responsive fashion. IFNα treatment of cell lines...
yielded significant but smaller increases in cytokine IL2 production by PBL in PTC cell line cocultures.

**Additive effect of combination selumetinib and IFN therapy**

As both MEK1/2 inhibitor selumetinib and IFN therapy produced significant increases in MHC class I expression and antigenicity, as indicated by greater T-cell activation and cytokine production by cocultured PBL for all three PTC cell lines, combinations of selumetinib (10 μmol/L) and IFNα (100 U/mL) or IFNγ (500 U/mL) were investigated. As shown in Fig. 5, the addition of IFNα or IFNγ to selumetinib treatment produced further increases in HLA-ABC expression in all three PTC models. Pretreatment of PTC cell lines with the combination of selumetinib and IFNα produced a trend toward increased donor PBL T-cell activation compared with pretreatment with either selumetinib or IFNα alone. IL2 production by these cocultured T cells was statistically greater for combination therapy than for IFNα treatment alone for all cell lines, and selumetinib treatment alone in some of the cell lines. Selumetinib and IFNγ combination pretreatment of PTC cell lines was similarly found to be superior to single-agent therapy, yielding increased T-cell activation and significantly greater IL2 production (Fig. 5C). While the mechanism of IFNs on MHC class I molecules and antigen-processing machinery is unclear, these results suggest that combination therapy with selumetinib and IFN might offer additional therapeutic benefits.
has been evaluated previously, the mechanism of selumetinib to increase antigenicity has not been studied in detail. Furthermore, the observed effect of selumetinib to increase MHC class I expression was intrinsic, occurring in the absence of infiltrating immune cells. To study this further, expression of common antigen-processing machinery (APM), cytokines, STAT, and MHC class I molecule genes in thyroid cancer cell lines after selumetinib treatment was evaluated by quantitative RT-PCR. These preliminary results showed upregulation in all cell lines of TAP1, STAT1, STAT6, and LMP2 with selumetinib treatment, though these differences did not meet statistical significance for all cell lines (Supplementary Fig. S4). Increases in the expression of APM genes were greatest in K-1, the cell line showing the greatest phenotypic response to selumetinib treatment.

Discussion

Both recurrent and metastatic thyroid cancer remain difficult to treat, often necessitating additional therapies such as radioactive iodine ablation, surgical resections, and/or tyrosine kinase inhibitor therapy, all of which may cause substantial morbidity. Novel therapeutic approaches like immunotherapy may be of benefit in these cases. Barriers to tumor clearance by the host immune system include evasion and inhibition strategies adopted by the cancer during its evolution. Antigen expression by MHC class I molecules on the surface of tumor cells is critical to immune recognition and loss of these proteins is a well-recognized mechanism of tumor immune escape (9, 10, 23). In this retrospective analysis of PTC tumor specimens, we report a striking loss of MHC class I expression in PTC, with 76% of cases demonstrating reduced or absent expression of both HLA-ABC and β2m. Autoimmune thyroid disease and thyroiditis associated with immunotherapy for other cancer types are common, and may suggest a particular susceptibility of thyroid tissue to immune activation and destruction (7, 30). MHC class I-mediated tumor antigen display may induce an early immune response in PTC, possibly explaining the overall good prognosis observed. During the process of tumor immunoediting by host immune cells (31), highly antigenic tumor cells may be eliminated, selecting for survival of PTC with low MHC class I expression. Low antigen expression by thyroid cancer cells, in concert with upregulation of immunosuppressive ligands (14–17), may effectively hide the tumor cells from host immune surveillance and help explain the difficulty of achieving disease-free status in persistent PTC. This suggestion is supported by the present findings of a global decrease

Figure 5. Combination selumetinib and IFN treatment of PTC cell lines produces additive increases in HLA-ABC expression and antigenicity. A, the effect of selumetinib and IFNγ or IFNo treatment on PTC HLA-ABC expression. Data shown are mean ± SEM, with statistically significant differences from vehicle control indicated by *, P < 0.05; **, P < 0.01; †††, P < 0.001; ††††, P < 0.0001. Representative flow cytometry histograms for HLA-ABC expression are shown to the right of the respective graph. B, T-cell activation measured as the fraction CD3+ of CD8+ T cells or IL2 production in PBL-PTC cocultures (C) after PTC were pretreated with interferon, selumetinib, or combination therapy. B and C, data shown are mean (n = 4) ± SEM, with significant differences from single reagent therapy indicated by *, P < 0.05; ††, P < 0.01; †††, P < 0.001.

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in immune cell infiltration in PTC specimens associated with loss of HLA-ABC and β2m compared with those with intact expression. The relationship between high MHC class I expression and greater antitumor immune responses has been established in a number of other solid malignancies, including colorectal cancer (32), head and neck squamous cell carcinoma (23), and experimental tumor models (11). Specific to thyroid cancer, Cunha and colleagues (33) recently showed improved outcomes in differentiated thyroid cancer patients with greater TILs, and better clinical prognosis has been demonstrated in patients with thyroid cancer who have concurrent Hashimoto’s thyroiditis (34).

Recognition of low MHC class I expression as a frequent mechanism of immune escape in papillary thyroid cancer identifies HLA augmentation as a target for immunotherapy protocols.

Garrido and colleagues (35) proposed classification of HLA tumor loss into two categories based upon the functional ability to recover or upregulate HLA expression following immunotherapy (e.g., cytokine treatment). They proposed that irreversible defects in HLA expression, or those that do not reverse with cytokine treatment, arise secondary to structural genetic problems. The most common etiologies of these structural changes are loss of heterozygosity (LOH) or mutations/deletions affecting HLA or β2m genes on chromosomes 6 and 15, respectively. In contrast to this, they propose that reversible MHC class I alterations are primarily defects in the gene regulation of HLA class I heavy chain genes, β2m gene, and components of the antigen-processing machinery. This framework provides insight to the mechanisms underlying MHC class I defects in a tumor and the likely responsiveness to immunotherapy treatment. Garrido and colleagues evaluated clinical responses to immunotherapy in patients with melanoma showing MHC class I loss, and found that disease regression was more frequent in those patients with reversible HLA expression (i.e., regulatory gene defects) than in those with irreversible HLA defects (i.e., structural defects). Preliminary in vitro data in the present study suggests that the MHC class I defects observed in thyroid cancer may be primarily reversible by cytokine therapy and likely amenable to immunotherapy. Using tumor cell lines as models of PTC, we demonstrate that HLA-ABC expression can be increased by treatment with the MEKI1/2 inhibitor selumetinib and IFNs. Furthermore, this phenotypic change correlates with increased antigenicity, as evidenced by an increase in markers of T-cell activation (CD25 positivity) and IL2 production by allogeneic donor PBL cocultured with the tumor cell lines. These data are consistent with the physiologic function of IFNs and previous findings showing increased antigen expression by cancer cells following IFN therapy. IFNs are lymphocyte-produced cytokines critical in cell-mediated immune responses with pleiotropic effects on antigen presentation through MHC class I, including upregulation of MHC class I expression directly, and enhancement of antigen processing and loading through upregulation of proteasomal subunits LMP2 and LMP7, transporters associated with antigen processing (TAP) proteins, and the proteasome regulator PA28 (36–39). Previous studies demonstrated enhancement of HLA class I antigen expression and immunogenicity following IFNα or IFNβ treatment in melanoma, embryonal carcinoma, and glioblastoma multiforme (36, 38, 39). Of interest, the induction of thyroiditis, including immune cell-mediated follicular cell destruction, is a common secondary effect of IFNβ treatment for hepatitis (40) and cancer immunotherapy (41). In addition to MHC class I changes, IFNα has also been shown to upregulate immune costimulatory molecules B7.1 and HLA-DR on thyroid follicular cells that could contribute to thyroid-directed immune reactions (42, 43). In this study, IFNγ produced greater increases in HLA-ABC expression and antigenicity of PTC cell lines than IFNα.

Prior clinical evaluations of recombinant IFNα therapy in patients with cancer in the mid-1980s to 1990s produced modest, if any, clinical benefit and were accompanied by frequent side effects of pyrexia and malaise during treatment (44, 45). Compared with these earlier studies, we now recognize that cancers evolve different strategies to escape host immune destruction (11), and that immunotherapy is effective in producing tumor regression only when the regimen is matched to the specific mechanisms of immune escape present in that tumor (23, 46, 47). The efficacy of IFNα treatment in cancers with marked MHC class I loss, like PTC (76%), may be greater than for other solid tumor types with lower prevalence of HLA downregulation. We previously developed chNTN-3/muIFNγ, a tumor-targeted fusion protein consisting of recombinant IFNγ and the tumor necrosis–targeted monoclonal antibody chNTN-3 to overcome systemic toxicity by targeting IFNγ to the tumor site (48). In mice transplanted with the metastatic MAD109 lung carcinoma tumor model, which we demonstrated to have low MHC class I expression in vivo (11), treatment with chNTN-3/muIFNγ produced a significant increase in intratumoral infiltration by leukocytes and a decrease in the number of metastatic foci without causing observable toxicity (48). An analogous human IFNα tumor-targeted fusion protein, chNTN-3/huIFNγ (49), was also generated, and may be an ideal immunotherapy reagent for immunotherapy of progressive PTC.

Tyrosine kinase inhibitors are an important group of clinically available adjuvant therapies for PTC. In this study, selumetinib effectively increased MHC expression on PTC cell lines, a finding supported by similar effects seen in melanoma cell lines (50). The mechanism by which MEKI1/2 inhibitor selumetinib increases MHC class I expression is less well understood. Preliminary evaluation in this study suggested that selumetinib produces increases in TAP1, STAT1, STAT6, and proteasomal component LMP2 gene expression, with variable downregulation of IFNα and/or IFNβ in PTC cell lines. These preliminary data suggest that the intrinsic effects of selumetinib to increase HLA expression on PTC cell lines are not mediated through upregulation of IFNs. Together, with the additive effect of combination treatment with selumetinib and IFNα or IFNβ on increasing PTC cell line expression of HLA-ABC and subsequent PBL immune activation, these data suggest two
complementary mechanisms of action augmenting MHC class I expression.

Other kinase inhibitors were evaluated in this study, though their effects on antigenicity were not significant or were accompanied by increases in immunosuppression. The JAK/STAT inhibitor sunitinib, for example, augmented HLA-ABC expression by PTC cell lines but this change was concurrent with upregulation of PD-L1, PD-L2, and HLA-G, which are likely to hinder effective antitumor immune responses. In addition, in some tumor models, radiation treatment has been shown to augment endogenous tumor-antigen priming (8) but was not found to significantly alter HLA-ABC expression in this study. One reason for this difference may be that enhanced antigenicity of tumor cells following radiation is thought to be due in part to the indirect effects of lymphocyte-derived IFNs secreted in the tumor microenvironment (31), which was not present in our in vitro experiments.

In conclusion, we identified frequent MHC class I molecule downregulation as a unique feature of PTC tumors that is associated with low T-cell infiltration and suggestive of a highly immunodedited cancer. Furthermore, recovery of MHC class I expression on PTC cell lines following treatment with selumetinib and IFNs improved antitumor immune responses and may be a promising immunotherapy approach in patients with progressive PTC.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References
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