**Inhibition of BRAF Sensitizes Papillary Thyroid Carcinoma to Immunotherapy by Abrogating the TGF-β1/SMAD3 Tumor Specific MHC-II Mediated Immune Escape**

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**RUNNING TITLE: Combination of Immunotherapy and BRAF Inhibitor in PTC**

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# **Abstract**

**Purpose:** Multiple mechanisms play roles in restricting the ability of T-cells to recognize and eliminate tumor cells. This work aims to identify immune escape mechanisms involved in papillary thyroid carcinoma (PTC) to optimize immunotherapy.

**Experimental Design:** iTRAQ analysis was conducted to identify proteins differentially expressed in PTC samples with or without BRAFV600E mutation. Proteomic results were verified by immunohistochemistry (IHC), real-time PCR, Western blot, and flow-cytometry with particular emphasis on antigen processing and presenting molecules. Molecular regulatory mechanisms for tumor cell evasion were investigated by modulating BRAF-MAPK and related pathways *in vitro*, and the pathological significance of identified tumor-specific major histocompatibility complex class II (tsMHCII) molecules in mediating tumor cell immune escape and targeted immune therapy were further evaluated in a transgenic mouse model of spontaneous thyroid cancer.

**Results:** Proteomic analysis showed that tsMHCII level was significantly lower in BRAFV600E-associated PTCs and negatively correlated with BRAF mutation status. Constitutive activation of BRAF decreased tsMHCII surface expression on tumor cells which inhibit activation of CD4+ T-cells and led to immune escape, and vice versa. Pathway analysis indicated that the TGF-β1/SMAD3 pathway mediated repression of tsMHCII through BRAF and could be reversed via BRAF inhibition (BRAFi). Targeting this pathway with a combined therapy of BRAF inhibitor PLX4032 and anti-PD-L1 antibody efficiently blocked tumor growth via CD4+ T-cell infiltration and elimination in a transgenic PTC mouse model.

**Conclusions:** BRAFV600E impairs the expression of tsMHCII through the TGF-β1/SMAD3 pathway to enhance immune escape of tumors. Combination treatment of PLX4032 and anti-PD-L1 antibody promotes recognition and elimination of PTC by the immune system and offers an effective therapeutic strategy for patients with advanced PTC.

# **Introduction**

The incidence of papillary thyroid cancer (PTC) has increased since the early 1980s and is now the fastest-growing cancer in most countries ([1](#_ENREF_1), [2](#_ENREF_2)). Although the overall prognosis of PTC is good, 20-30% of patients experience recurrence, and 5-10% have progressive and treatment-refractory disease ([3](#_ENREF_3)). These patients normally go through multimodal therapy including surgery, radioactive iodine therapy, external beam radiation, watchful waiting, and experimental trials ([4](#_ENREF_4)). In spite of the improvement of each module, the treatment effect remains unsatisfactory such that the 10-year overall survival (OS) of stage IV PTC is only 19.7-23.5% ([5](#_ENREF_5), [6](#_ENREF_6)). Therefore, developing systemic and reliable adjuvant therapies to improve disease-free survival (DFS) in patients with advanced PTC is necessary.

Tumor cells have developed various immune escape mechanisms to manipulate the host immune system and the immune cell microenvironment to avoid recognition and elimination. Strategies employed include up-regulation of immune-inhibitory molecules, down-regulation of antigen display, or recruitment of suppressive cell populations ([7](#_ENREF_7), [8](#_ENREF_8)). In PTCs, high levels of immune checkpoint Programmed Death Ligand 1 (PD-L1) and loss of major histocompatibility complex class I (MHCI) surface expression correlate with tumor-associated macrophages (TAMs), CD8+ and CD4+ T-cells, and T-reg lymphocytic infiltration though the regulatory mechanisms for these observations have not been addressed ([8-10](#_ENREF_8)).

As the most common genetic alteration associated with PTC (40%–80%), BRAFV600E mutation is associated with the suppressive immune microenvironment of PTC and recurrence/persistence ([11-14](#_ENREF_11)). In murine models of thyroid carcinoma, tumors harboring BRAFV600E displayed a high M2-like TAM infiltration ([15](#_ENREF_15)). Human samples with BRAFV600E have a low intratumoral CD8+/Foxp3+ ratio and high expression of indoleamine 2,3-dioxygenase (IDO), cytotoxic T-lymphocyte antigen 4 (CTLA-4), and PD-L1 ([16-18](#_ENREF_16)). In melanoma, BRAF inhibition improves tumor recognition and elimination by the immune system; therefore, BRAF inhibition may induce a similar response in patients with advanced PTC ([19](#_ENREF_19), [20](#_ENREF_20)). Understanding the immune regulatory events induced by BRAFV600E-induced constitutive activation of the BRAF-MAPK pathway may lead to the development of more effective treatments for PTC.

Major histocompatibility complex class II (MHCII) molecules are mainly expressed on antigen presenting cells (APC), such as dendritic cells (DC), macrophages, and B cells, and primarily present 12–16 amino acid peptides to CD4+ T-cells ([21](#_ENREF_21)). In addition to the well-known localization on APCs, tumor-specific MHCII (tsMHCII) is also expressed on various tumor cells and related to intratumoral immune cell infiltration, superior prognosis, and improved response to immune checkpoint inhibition in humans ([22](#_ENREF_22)). In mouse models of sarcoma, breast, lung, and colon cancer, overexpression of tsMHCII increased tumor rejection and resistance to challenge with parental tsMHCII-negative tumor cells ([23-25](#_ENREF_23)). In lung and colon cancer, depletion of DCs or macrophages had no effect on the ability of mice to reject tsMHCII expressing tumor cells ([25](#_ENREF_25)). The above reports raise the possibility that tsMHCII-expressing tumor cells may act as APCs to prime CD4+ T-cells. Whether tsMHCII is playing a role in PTC immune escape, especially in BRAFV600E-related advanced PTC, needs further investigation.

Immunotherapy could potentially be a new treatment strategy for recurrent or refractory PTC. Through blocking immune checkpoints, mainly including cytokine T-lymphocyte antigen-4 (CTLA-4), PD-L1, and programmed death receptor-1 (PD-1), immunotherapy cause the body’s own immune cells to recognize and eliminate malignant cells and have produced remarkable treatment effects in lung, prostate, melanoma, and other cancers ([26](#_ENREF_26), [27](#_ENREF_27)). Other possible strategies to increase tumor immunogenicity are stimulating the expression of MHC molecules and increasing immune cell recognition of tumor-associated antigens to achieve an ideal anti-cancer immune state.

# In the current study, we analyzed the immune regulatory mechanisms of PTC and found that PTCs with BRAFV600E mutation have down-regulated tsMHCII expression. This condition leads to decreased recognition of tumor cells by CD4+ T-cells and enhances immune escape. Mechanistic studies of PTC activity in culture determined that elevated TGF-β1 functions through the classical SMAD-dependent pathway to mediate immune cell evasion in an autocrine manner. Combined therapy of anti-PD-1 antibody with BRAF inhibitor blocked tumor growth *in vitro* and *in vivo* by improving T-cell infiltration and tumor cell elimination via increased tsMHCII expression.

# **Result**

## BRAFV600E related PTC is associated with decreased tsMHCII

To identify differentially expressed factors between PTC samples expressing BRAFV600E versus wildtype BRAF and to understand the underlying mechanisms of immune evasion, we performed a proteomics analysis on two matched groups of clinical samples. The process of iTRAQ-labeling LC-MS/MS analyses is shown in **Fig. 1A**, and about 4,722 peptides were identified (**Fig. 1B** and **Fig. S1A-B**)with approximately 480 proteins showing 1.2-fold or higher expression in the BRAFV600E samples relative to the wildtype BRAF PTC samples as summarized in **Fig. 1C**. Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis indicated that factors involved in multiple biological signaling pathways were altered. Specifically, MHCI antigens HLA-A and HLA-B and MHCII antigen HLA-DPA1, which are involved in antigen processing and presentation, were significantly decreased in BRAFV600E PTC samples (**Fig. 1D-E**). A few other MHC I and II antigens were also downregulated in the BRAFV600E group, indicating that infiltration of immune cells or tumor immune environment could be modulated by BRAFV600E and induce PTC tumorigenesis. In a previous study, downregulation of MHC I was shown to decrease the capability of CD8+ T cells to recognize and kill PTC cells and considered to be associated with MAPK pathway activation ([10](#_ENREF_10)). These findings support a plausible biological mechanism for variation MHC II surface expression in PTCs expressing BRAFV600E.

To have a global visualization of tsMHCII expression within PTC tissues, IHC staining was conducted on 185 PTC samples (**Fig. 1F**). We observed the distribution of tsMHCII in different cell types within tumor tissues and found that most MHCII signals were from cancer cells (numerous true papillae, ground glass nuclei, and big size compared with APCs). Among the cohort of PTC samples, 87 cases of PTC showed positive staining for tsMHCII (**Fig. 1G**). Sample analysis by positive ratio and staining intensity revealed a lower proportion of positively-stained MHCII PTC samples with concurrent BRAFV600E expression compared to BRAF wildtype samples (34 BRAFV600E samples out of total of 93 BRAFV600E samples were positive versus 53 positive wild type BRAF samples out of a total of 92 wildtype BRAF samples (**Fig. 1H**). Therefore, tsMHCII expression was decreased in BRAFV600E PTCs, which may indicate an association between BRAFV600E-modulated signaling pathways and tsMHCII expression as a mechanism for immune escape.

## Constitutively activated BRAF-MAPK signaling blocked tsMHCII expression.

Transcription of MHC II genes are under control in part by a speciﬁc non-DNA-binding protein, the class II transactivator (CIITA) ([28](#_ENREF_28)). In humans, MHCII mainly consists of three antigens: HLA-DR, HLA-DP, and HLA-DQ. To corroborate the iTRAQ and IHC findings with tsMHCII gene expression, we evaluated the mRNA levels of CIITA and tsMHCII (HLA-DQA1, HLA-DRA, and HLA-DPA1) in various cell lines. CIITA transcript showed a strong correlation with those of HLA-DQA1, HLA-DRA, and HLA-DPA1, which were usually lower in PTC cell lines harboring BRAFV600E mutation than in wildtype cell lines (**Fig. 2A**). To verify whether the constitutive activation of BRAF-MAPK signaling is the direct cause of tsMHCII decline, BRAF and MEK inhibitors were applied to PTC cell lines BCPAP and K1 with BRAFV600E mutation; tsMHCII levels were detected by flow cytometry. Treatment of cells for 72 h with the BRAF kinase inhibitor PLX4032 or the MEK inhibitor U0126 led to upregulation of tsMHCII at protein level (**Fig. 2B-C**). To confirm the BRAF-MAPK pathway is involved in tsMHCII regulation, we overexpressed BRAFV600E in TPC1 cells with wildtype BRAF using the doxycycline-induced overexpression system. Upon doxycycline treatment, tsMHCII protein expression was significantly decreased in response to ectopic BRAFV600E signal (p<0.001, **Fig. 2D-E**). Transcript levels of CIITA, HLA-DQA1, HLA-DRA, and HLA-DPA1 showed consistent change in response to BRAF and MEK inhibition (**Fig. S2A-2B**). These results suggest that constative activation of BRAF-MAPK pathway through BRAFV600E induces down-regulation of tsMHCII expression in PTC cells through transcriptional regulation.

## BRAFV600E regulates tsMHCII via TGF-β1-SMAD3 pathway

To further understand which signaling pathways were involved in the regulation and control of tsMHCII, we treated BCPAP and K1 cells with chemical inhibitors for different classical pathways (DAPT (25 µM) for NOTCH, IWR1 (10 µM) for Wnt, SB431542 (10 µM) for TGF-β1, and cyclopamine (20 µM) for Hedgehog pathway) for 48 h. Compared to other pathway inhibitors, blockage of TGF-β1 pathway with SB431542 resulted in a significant increase of CIITA and tsMHCII gene expresion in both cell lines, suggesting the potential regulatory function of the TGF-β1 pathway for MHCII regulation and immune evasion in PTC (**Fig. 3A**). We analyzed gene expression data from the Tumor Cell Genome Atlas (TCGA) database, and found that CIITA tended to have low mRNA expression in PTC tumors with high TGF-β1 levels. Analysis of the association between CIITA and MCHII transcript levels by secreted TGF-β1 level as a function of BV600E expression in our samples revealed a similar negative correlation between the two elements (p<0.001, **Fig. 3B**) and suggest a role for TGF-β1 signaling involvement in MHCII regulation. Time course analysis of CIITA and MHCII transcript levels and TGF-β1 protein expression produced by BCPAP and K1 cells over time confirm that accumulation of TGF-β1 in culture leads to a corresponding decrease in CIITA and MHCII transcript levels (**Fig. S3A-B**), which corresponds to previous findings ([29](#_ENREF_29)). External stimulation of BCPAP and K1 cells with 10 ng/mL TGF-β1 decreased mRNA levels of CIITA and MHCII in BCPAP and K1 cells and significantly decreased protein expression of MHCII in K1 cells (**Fig. 3C and Fig. S3D**). BRAF inhibition of BCPAP and K1 cells produced a corresponding decrease in secreted TGF-β1 protein levels, which indicates that TGF-β1 signaling is influenced by BRAFV600E activity in PTC cells (**Fig. S3C**). Moreover, TGF-β1 treatment abrogated BRAF kinase inhibitor induced tsMHCII elevation, which was confirmed by mRNA expression of CIITA, HLA-DQA1, HLA-DRA, and HLA-DPA1 (**Fig. 3D** and **Fig. S3E**). Overall, activation of BFAF-MAPK pathway by BRAFV600E mutation in PTC cells leads to down-regulation of CIITA-directed tsMHCII probably through elevating TGF-β1 signaling.

SinceSMAD3 phosphorylation, interaction with SMAD4, and nuclear translocation of the SMAD2/4 complex are the classic downstream molecular events of TGF-β1 signaling, we evaluated the ability of PTC cells to induce SMAD activation. As expected, treatment of PTC cells with TGF-β1 efficiently induced phosphorylation of SMAD3 and is responsive to SMAD3 inhibition, which indicates that p-SMAD3 is a reliable read-out of TGF-β1 signaling (**Fig. S4A-B**). Correlation analysis of SMAD3 and BRAF transcript levels from the TCGA database stratified by BV600E mutation status demonstrated a positive relationship between SMAD3 expression and BRAF signaling (**Fig. 3E**). This association was confirmed at the protein level with IHC staining of PTC samples for p-SMAD3 expression where PTC samples with BV600E mutation had a greater proportion of positively stained samples than wildtype BRAF samples (**Fig. 3F**). Further analysis of the inter-relationships between the TGF-β1/SMAD and BRAF-MAPK signaling axes with BRAF and MEK inhibitors revealed that downstream SMAD signaling is influenced by BRAF-MAPK inhibition suggesting the TGF-β1/SMAD and BRAF-MAPK signaling pathways are connected in PTC (**Fig. S4C-D**). Furthermore, BRAFV600E overexpression leads to an increased population of p-SMAD3 (**Fig. 3I**). Thus, BRAFV600E in PTC increases the expression of TGF-β1 levels, which signals through a SMAD3-dependent pathway in an autocrine manner. To confirm that p-SMAD3 was responsible for the downstream reduction of CIITA and tsMHCII genes, we performed another correlation analysis of SMAD3 and CIITA gene expression levels stratified by BRAF mutation status from information in the TCGA database Similar to active TGF-β1 levels, high SMAD3 expression also correlated with low CIITA expression (**Fig. 3G**). Application of p-SMAD3 inhibitor SIS3 resulted in upregulated mRNA levels of CIITA and tsMHCII as well as protein expression of tsMHCII in both cell lines (**Fig. 3H** and **Fig. S4B and E**). Importantly, treatment with p-SMAD3 inhibitor SIS3 counteracted the suppressive effects of BRAFV600E DOX-induced MHCII mRNA and protein expression (**Fig. 3J** and **Fig. S4F**). Taken together, these results show that the autocrine TGF-β1-SMAD3 pathway mediates downregulation of tsMHCII through CIITA in BRAFV600E PTC cells.

## BRAF inhibitor restores tsMHCII expression and potentiates immune elimination of PTC cells

Various immune escape mechanisms may exist in PTC with BRAF mutation. To understand the pathological significance of tsMHCII suppression via BRAFV600E, we investigated the interactions between tumor cells and peripheral blood leukocytes (PBL) with a series of *in vitro* cytotoxicity assays targeting various signaling molecules involved in tsMCHII regulation. Pre-treatment with BRAF inhibitor PLX4032 significantly enhanced the elimination effect of PBL on BCPAP and K1 cells while suppression of CIITA expression via siRNA restored the elimination response; knockdown of CIITA expression with corresponding BRAF inhibition did not rescue the elimination response indicating that BRAF mutation and CIITA regulate immune cell activity through a common pathway (**Fig. 4A-C**). As a major target of CIITA regulation, changes in tsMHCII expression could modulate the immune response of T-cells. To examine this possibility further, we investigated the interaction between tumor cells with CD4+ T-cells and used protein expression levels of INF-γ as an indicator of T-cell activation, as normal PTC cells induce secretion of INF-γ by T-cells in co-culture. Pre-treatment of PTC cells with BRAF inhibitor significantly enhanced the production of INF-γ while co-treatment of PTC with CIITA siRNA and BRAF inhibitor prevented INF-γ secretion (**Fig. 4D**). In a BRAFV600E overexpression system, DOX-induction of BRAFV600E in TPC1 cells suppressed the elimination effect of PBL and decreased PTC cell recognition by CD4+ T-cells (**Fig. 4E-F**). These results suggest that BRAF-MAPK activation and downstream tsMHCII repression in PTC may assist in immune escape, and treatment of PTC with BRAF inhibitor may restore immune recognition.

## Clinical tsMHCII expression correlated with superior PTC prognosis

Since the distribution and diversity of immune cell types within the tumor environment is closely related to tumor growth and progression, we further evaluated the clinical significance of differing levels of tsMHCII expression on PTC prognosis. IHC staining of clinical specimens revealed a greater degree of CD4+T-cell inﬁltration in samples with high tsMHCII expression compared to PTC samples with low tsMHCII expression, which is consistent with the immune cell homing function of tsMHCII (p=0.019, **Fig. 4G** and **Table S1**). Correspondingly, PTCs with high tsMHCII expression were associated with lower T stage, N stage, TNM stage, and recurrent rate (P=0.001, P=0.003, P=0.003 and P<0.001, respectively, **Table S1**) compared to samples with low tsMHCII expression. Kaplan-Meier analyses of patient samples by IHC staining indicated that patients with positive staining of tsMHCII had longer DSS (disease-specific survival) and OS (overall survival) than those with negative staining (**Fig. 4H**). After adjusting for age, gender, TNM stage, multifocality, BRAF mutation status, and CD4+ T-cell infiltration, Cox regression analysis showed that tsMHCII expression (positive versus negative) correlated with DSS (p=0.038, hazard ratio (HR) =0.205, 95% confidence interval (CI): 0.046 – 0.917) and OS (p=0.024, HR=0.240, 95% CI: 0.070–0.827) (**Table S2** and **Table S3**). Clinical and pathological data are summarized in **Table S4**.

In order to evaluate the effect to the cancer prognosis to the genes involved in this study in a pan-cancer style, we downloaded 11,093 gene expression quantification data derived from RNA-seq data from the TCGA database (<https://portal.gdc.cancer.gov/repository>) on February 24, 2019. We found high expression of all HLA genes including HLA-DRA (HR=0.93, P=1.1x10-6), HLA-DPA1 (HR=0.94, P=1.83x10-4), HLA-DQA1 (HR=0.94, P=4.2x10-5) while TGFB1 showed risk effect (HR=1.1, P=1.6x10-5) in a pan-cancer style which is consistent with our current result (**Figure S5**). In addition, we found CIITA showed significant protective roles for the overall survival (**Figure S5**). Using the TIMER web tool (cistrome.shinyapps.io/timer/), we analyzed transcript levels of tsMHCII in various carcinomas and found that HLA-DQA1, DRA, and DPA1 mRNA levels were highly expressed in PTC (**Fig. S6**). Transcript analysis of tsMHCII genes in our cohort indicated that high expression of tsMHCII correlated with B-cell, CD4+ T-cell, and CD8+ T-cell infiltration (**Fig. 4I**). Additional Kaplan-Meier analysis revealed that higher HLA-DQA1, DRA, and DPA1 mRNA levels were associated with a better prognosis in patients with PTC (**Fig. 4J**). After adjusting for age and gender, Cox regression analysis showed that CIITA (positive versus negative) significantly correlated with OS (HR=0.504, 95% CI: 0.27-0.938, *P*=0.031, **Table S3**). These findings suggest that tsMHCII expression may serve as a prognostic biomarker for good survival of patients with PTC.

## BRAF inhibitor up-regulated tsMHCII and enhances the sensitivity of PTC cells to PD-1 antibody treatment.

TsMHCII expression is associated with improved survival in patients with melanoma and classic Hodgkin lymphoma treated with anti-PD-1/anti-PD-L1 ([30](#_ENREF_30), [31](#_ENREF_31)). However, whether BRAF inhibitor treatment alters the homing and elimination functions of immune cells through up-regulation of tsMHCII expression following anti-PD-1 treatment remains unclear. We thus performed *in vitro* cytotoxicity assays in the presence and absence of BRAF inhibitor, PD-1, and combination BRAF inhibitor and PD-1 treatment and noticed that a combination treatment of BRAF inhibitor and Nivolunab enhanced the elimination effect of PBL on PTC cells (**Fig. 5A**). Furthermore, combination treatment also activated CD4+ T cells more efficiently and resulted in higher INF-γ secretion than either treatment alone (**Fig. 5B**). The enhanced homing, elimination, CD4+ T-cell activation, and INF-γ secretion effects in response to combination BRAF PD-1 treatment were attenuated in response to cells with siRNA-mediated knockdown of CIITA suggesting that combination BRAF inhibitor and PD-1 blockade treatment modulates immune cell function through increasing tsMHCII expression in PTC cells.

To confirm our results in an *in vivo* model, we further tested the antitumor activity of combined PLX4032 and Nivolunab treatment in a transgenic mouse model of spontaneous thyroid cancer (**Fig. 5C**). Mice treated with PLX4032 or anti-PD-1 antibody treatment alone had reduced tumor volume to about 70-80% of the initial volume though the combination regimen of PLX4032 and anti-PD-1 antibody produced a greater reduction in tumor volume and weight than either therapy alone (**Fig. 5D-E**). Flow cytometry analysis of immune cell composition within tumor tissues indicated that combined therapy largely increased CD4+ and CD8+ T-cell infiltration and elevated the percentage of activated T-cells as determined by CD107 staining (**Fig. 5F**). IHC analysis of tumor tissues from mice treated with PLX4032, anti-PD-1 antibody, or combination treatment revealed a consistent up-regulation of tsMHCII expression that was greatest in the combination regimen (**Fig. 5G**). Hematoxylin and eosin staining of liver and kidney tissues showed that combination therapy did not impair liver or kidney function in these animals (**Fig. S7**). Collectively, these data indicate that a combination regimen of BRAF inhibitor and Nivolunab increases the elimination effect of PTC cells by the immune system via up-regulation of tsMHCII. A schematic summarizing the regulation of tsMHCII involved in BRAFV600E related PTC is presented in **Fig. 6**.

# **Discussion**

Thyroid carcinomas frequently use several mechanisms to escape immune destruction, including recruitment of M2-like TAM cells, myeloid-derived suppressor cells (MDSCs), and Treg cells, expression of immune checkpoint molecules like PD-L1, CTL4, and HLA-G, and production and expression of MHCI though the detailed mechanism for MHCI loss is still unknown 8,([16-18](#_ENREF_16)). Our results indicate that BRAFV600E associated tsMHCII repression may also mediate immune escape in advanced PTC through MCHII. Compared with MHCI, MHCII has the capability to present greater repertoire of antigens to immune cells, and Veatch et al. noted that patients with melanoma can have BRAFV600E peptides incorporated into MCHII receptors onto the tumor cell surface for immune cell presentation ([32](#_ENREF_32)),29. Similarly, in patients with thyroiditis, MHCII-positive thyrocytes presented viral peptide antigens to T-cells ([33](#_ENREF_33)). Our data supports the findings of Jo et al. that PTC tissues frequently express tsMHCII antigen, and the level of tsMHCII positively correlates with superior prognosis though whether tsMHCII is used more frequently for antigen presentation by PTC cells and induces a stronger immune response than MHCI-mediated presentation requires further investigation ([34](#_ENREF_34)).

In both rodent and human PTC cells, optimal TGF-β1 signaling requires constitutive activation of the MAPK pathway ([29](#_ENREF_29), [35](#_ENREF_35)). We confirmed that BRAF inhibitor PLX4032 blocked the activation of the TGF-β1/SMAD3 pathway and that TGF-β1 signaling positively correlates with BRAFV600E-mediated downregulation of tsMCHII gene expression in patients with PTC. Furthermore, our series of signaling blockage experiments proved that TGF-β1-SMAD3 pathway mediates the repression of tsMHCII by BRAF-MAPK and supports previous work by Dong et al. in who demonstrated that TGF-β1 signaling through SMAD3 inhibited expression of CIITA and MHCII expression by repressing the activity of CIITA type IV promoter in astrocytes ([36](#_ENREF_36)). Similarly, we also found that exogenous TGF-β1 treatment repressed CIITA expression and consequently decreased the level of tsMHCII expression on PTC cell surface while inhibitors targeting specific TGF-β1/SMAD3 pathway components produced an opposite effect. Thus, down-regulation of CIITA/MHCII by TGF-β1 in PTC cells is likely due to a decrease in CIITA promoter activity induced by SMAD3 at the transcriptional regulation level.

As a multi-functional morphogen, TGF-β1 plays a role in shaping an immunosuppressive tumor microenvironment and restrain PD-L1 blockade ([37](#_ENREF_37), [38](#_ENREF_38)). Our findings suggest an autocrine effect of TGF-β1 signaling on tumor cells to alter the patterning of cell surface antigens. Whether increased TGF-β1 secretion induced by BRAFV600E induces a signaling response in other cell types or exerts a different function is worthy of additional study.

CIITA-driven MHCII expression by tumor cells has been found to prime naive CD4+ T-cells *in vivo* and efficiently vaccinate the host against parental MHCII negative tumor cells ([21](#_ENREF_21)). However, the role of tsMCHII expression in the recognition and elimination activities of CD4+ T-cells on PTC cells remains unclear. Using a combination of IHC staining information, transcript analysis, flow cytometry, and gene expression data from the TCGA database, we found that increased tsMHCII expression in PTC is correlated with increased CD4+ T-cell infiltration and better survival. *In vitro*co-culture experiments further demonstrate that increased tsMHCII expression induced by BRAF inhibition significantly activates the immune system, suggesting that downregulation of tsMHCII expression by BRAF mutation could be one of the key immune escape mechanisms for PTCs.

In melanoma and other solid malignancies such as lung, liver, and renal cell cancers, PD-1 blockade has produced remarkably durable tumor regression in patients with advanced disease. However, compared with targeted therapy, this exciting treatment has some limitations, particularly low response rate of patients ([39-43](#_ENREF_39)). The characteristic high response rate seen with targeted therapy combined with the durable response of immunotherapy provide the rationale for a multi-pronged approach with targeted therapy and immunotherapy to improve survival in patients with PTC.

Given the prevalence of BRAFV600E mutations in PTC and its association with the immune escape process, a combination regimen of BRAFV600E inhibitor with immunotherapy is of particular interest. Combinations of BRAF inhibitor and anti-PD-1/PD-L1 antibody have improved survival and tumor immunity in an immunocompetent model of orthotopic murine anaplastic thyroid cancer though the mechanism for this effect was unknown ([44](#_ENREF_44)). Our i*n vitro* assay revealed that increased tsMHCII expression by BRAF inhibition effectively induced PBL elimination of PTCs treated with Nivolunab, illustrating that combined treatment of BRAF inhibitor and Nivolunab produce a synergistic effect via upregulation of tsMHCII. *In vivo* assays revealed that the combination of PLX4032 and anti-PD-1 antibody provide a superior anti-tumor response compared to monotherapy alone, and previous work by our group indicate that tumor shrinkage is associated with increased T-cell infiltration and CD107 production ([45](#_ENREF_45)). Based on these findings, the combination of BRAF inhibitors and Nivolunab may be a promising treatment regimen for clinical application in the treatment of advanced PTC.

**Conclusion**

BRAFV600E down-regulates the expression of tsMHCII through TGF-β1/SMAD3 pathway and enhances the immune escape ability of PTC tumors. Treatment of PTC cells with BRAF inhibitor PLX4032 up-regulated tsMHCII and enhanced the recognition, homing, and elimination effects of immune cells in response to anti-PD-1 antibody treatment *in vitro* and in a transgenic mouse model of spontaneous thyroid cancer. This combination regimen of targeted small molecule inhibitor and immunotherapy may provide an effective therapeutic strategy for patients with advanced PTC.

# **Materials and Methods**

## PTC specimen preparation

Tumor tissue specimens were obtained from patients and PBL from healthy donors at Tianjin Medical University Cancer Institute and Hospital. Informed consent was obtained from each donor, and the study was approved by the Ethics Committee of Tianjin Medical University Cancer Institute and Hospital. Exclusion criteria were (1) patients had other malignancy, (2) patients with a history of previous operation for PTC (3) no lymph node was resected, (4) distant metastasis, (5) age less than 18 year s and (6) patients with a history of thyroiditis. In all clinical specimens, BRAF mutation status was verified by Sanger sequencing. Thirty fresh tumor specimens were collected from August 2017 to December 2017 and washed three times with pre-cooled phosphate buffered saline (PBS) for iTRAQ analysis. The remaining tumor tissue of 185 cases was obtained from 2013 to 2014 and used for IHC analysis. Clinicopathologic data of involved cases was listed in **Table S4-S5.**

## iTRAQ labeling and two-dimensional LC-MS/MS analysis.

iTRAQ analysis was performed as previously described ([46](#_ENREF_46)). Briefly, 30 tumor tissues (15 expressing wildtype BRAF and 15 expressing BRAFV600E) were used for proteomic analysis. Samples were divided into six groups according to BRAF mutation status while fifteen samples expressing BRAFV600E were randomly divided into three groups. The remaining samples were divided in the same way.

## Immunohistochemical staining

Tissue sections from 185 PTC cases were stained with antitibodies to HLA DR + DP + DQ (human MHCII, host species: Rabbit, #ab7856, Abcam),human pSMAD3 (phospho S423 + S425, host species: Rabbit, #ab52903, Abcam), and human CD4 (host species: Rabbit, #ab183685, Abcam), mouse MHCII (host species: Rat, #ab25333, Abcam) for IHC. Stained slides were examined independently by two pathologists blinded to the clinical and pathological information of the cases. The MHCII staining score was assessed as follows: negative/ little staining (0-30% of cancer cells), weak/focal staining (30-50% of cancer cells), positive/moderate staining (50-70% of cancer cells), or strong staining (70-100% of cancer cells).

**Cell signaling analysis**

## Cell lines and cell culture

Nthy-ori-3-1, BCPAP, K1, KTC-1, 8305C, TPC-1 and TT were all purchased from American Type Culture Collection. All cell lines were identified by short tandem repeat (STR) analysis. All three cell lines were cultured in RPMI 1640 (Gibco) supplemented with 10% fetal bovine serum, penicillin/streptomycin (5000 units/mL; Gibco) and l-glutamine (2 mM; Gibco). The passage number of the cells used for the experiments was about 20–30. All the cell lines tested for mycoplasma contamination. PLX4032 (selleck), U0126 (selleck), doxycycline (selleck), DAPT (selleck), IWR1 (selleck), SB431542 (selleck), Cyclopamine (selleck), SIS3 (selleck), Nivolunab (selleck), recombinant human TGF-β (peprotech) were used in this study.

## Western blot analysis

Western blot was performed as described previously using primary antibodies to pSMAD3 (phospho S423+S425, host species: Rabbit, #ab52903, Abcam), SMAD3 (host species: Rabbit, #ab40854, Abcam), HLA DR + DP + DQ (MHCII, host species: Rabbit, #ab7856, Abcam), and GAPDH (host species: Rabbit, #ab181602, Abcam) ([12](#_ENREF_12)). The proteins were visualized by enhanced chemiluminescence (Amersham) using secondary antibodies conjugated to horseradish peroxidase specifically, goat anti-rabbit IgG (Southern Biotech) and goat anti-mouse IgG (Santa Cruz Biotechnology, Inc.). Western blot analysis were performed

in duplicate or triplicate.

## Plasmid and cell transfection

Lentivirus containing pHS-AVC-LW521 plasmid were purchased from syngentech. Transfection result was confirmed by westernblot. Small interfering RNAs (siRNA) of CIITA were obtained from Qiagen. Transfections were performed using Lipofectamine™ 3000 (Invitrogen) following the manufacturer’s instructions. Cells were seeded in 6-well plates at a density of 50000 cells/well. For transfection,cells were incubated with cultured in RPMI 1640 with 10% fetal bovine serum and lentivirus vectors for 48h. After that, cells were transferred to RPMI 1640 with 10% fetal bovine serum and puromycin (4µM) for further screening.

## Real-time PCR

Real-time PCR assays were performed as previously described ([47](#_ENREF_47)). Each sample was carried out in triplicate. Primers are listed in **Supplementary table 6**.

## Flow-cytometric analyses and *in vitro* cytotoxicity assays

Flow cytometry was performed on a BD FACS Canto II cytometer (BD Biosciences).The PTC cell lines were stained with HLA-DR,DP, DQ (PE, BD, clone Tu39, cat.562008). Isotype immunoglobulin G was used as a negative control.PBL were isolated by differential density gradient centrifugation from human subjects and divided into two groups: one group treated with anti-CD3/CD28 stimulation (50 µl/ml) (Invitrogen, Grand Island, NY) and recombinant human IL-2 (20 ng/ml) (PeproTech, Rocky Hill, NJ), and the other treated with Nivolunab, anti-CD3/CD28 stimulation (50 µl/ml), and recombinant human IL-2 (20 ng/ml). PBL were pre-treated with or without anti-PD-1 antibody (Nivolunab, 20 µg/ml) for 5 days. BCPAP and K1 cells were pre-treated with or without PLX4032 (10 µM) for 72 h. After pre-treatment, both PBL and PTC cells (including BCPAP cells, K1 cells and TPC1 cells overexpressing BRAFV600E) were co-cultured at a 5:1 ratio with CFSE-labeled PTC cells (2×105 cells/well) in a 24-well plate for 6 h. All cells were then collected for staining with Zombie NIRTM dye (#423105). The samples were analyzed FlowJo 10 (Ashland, OR). Dead cells were gated based on CFSE-label via flow cytometry analysis. Each experiment was performed in triplicate.

## Measurement of CD4+ T-cell activation

CD4+ T-cells were purified from PBL with an EasySep™ Human T-cell Enrichment kit (STEMCELL Technologies Inc., Vancouver, BC) and divided into two groups: one group treated with anti-CD3/CD28 stimulation (50 µl/ml) (Invitrogen, Grand Island, NY) and recombinant human IL-2 (20 ng/ml) (PeproTech, Rocky Hill, NJ), and the other treated with Nivolunab, anti-CD3/CD28 stimulation (50 µl/ml), and recombinant human IL-2 (20 ng/ml) ([10](#_ENREF_10)). CD4+ T-cells were pre-treated with or without anti-PD-1 antibody (Nivolunab, 20 µg/ml) for 5 days. BCPAP and K1 cells were pre-treated with or without PLX4032 (10 µM) for 72 h. After pre-treatment, PBL and PTC (including BCPAP cells, K1 cells and TPC1 cells overexpressing BRAFV600E) cells were co-cultured at a 5:1 ratio (2×105 cells/well) in a 24-well plate for 24 h. Culture supernatants were collected for downstream ELISA analysis. ELISA was preformed to measure secreted levels of TGF-β1 and INF-γ according to the manufacturer’s protocol (Promega). Each experiment was performed in triplicate.

## Animal studies

Animal experiments were approved by the Tianjin Medical University Cancer Institute and Hospital Ethics Committee and handled according to IACUC protocol. Cre recombinase was overexpressed in TPO-Cre mice under the control of human thyroid peroxidase (TPO) gene promoter. Activated BrafFV600E was expressed at physiological levels in BrafCA mice. Endogenous Braf alleles were replaced by BrafV600E by crossing BrafCA mice with TPO-Cre mice to produce a set of double transgene positive off-spring that develop PTC around the age of 4-5 weeks 27. In our conditions, the mice spontaneously developed PTC at 6-12 weeks of age. Six-week-old TPO-Cre BrafCA mice were randomly assigned to four groups according to weight. For antibody-based drug intervention, PD-1 antibody at 200 µg (RMP1-14; Bio X Cell, West Lebanon, NH) or rat immunoglobulin G (control; Bio X Cell) were injected intraperitoneally every three days. For drug-based intervention, mice received daily oral doses of PLX4032 10 mg/kg body weight or PBS.The tumor homogenate was stained for immunophenotyping with following antibodies : CD45 (PE-Cyanine7, BD, clone 30-F11, cat.552848), CD3 (FITC, BD, clone 17A2, cat.555274), CD4 (APC-Cyanine7, BD, clone GK1.5, cat. [561830](https://www.bdbiosciences.com/cn/applications/research/t-cell-immunology/th-1-cells/surface-markers/mouse/apc-cy7-rat-anti-mouse-cd4-gk15/p/561830) ), CD8a (PE, BD, clone 53-6.7, cat. 561095), CD107a (BV421, BD, clone 1D4B, cat.564347). Immue cells were gated based on the expression of CD45. The samples were analyzed FlowJo 10 (Ashland, OR).

# **Pan-cancer Survival Analysis to Involved Genes with the Cancer Genome Atlas (TCGA) Dataset**

11,093 gene expression quantification data was derived from RNA-seq data from the TCGA database (<https://portal.gdc.cancer.gov/repository>) on February 24, 2019. The RNA-seq data covered 32 cancer types include thyroid cancer. Nine cancer types were excluded due to low sample size for control samples (N<=1). Log2-transformed fragments per kilobase of transcript per million mapped reads upper quartile (FPKM-UQ) derived from HTSeq ([48](#_ENREF_48)) was applied for differential gene expression analysis. Bayesian generalized linear model (bayesglm) from ARM package (v1.10-1) was applied for differential gene expression analysis. Metafor package (v2.1-0) was applied for meta-analysis across the 23 cancer types. Cox proportional hazards regression model was applied for survival analysis to the TCGA dataset for overall survival times (R survival package v0.9), and results presented as hazard ratios (HR). Meta-analyses to HR were then conducted under fix-effect model to evaluate the effect of the gene expression to overall survival time in which HR<1 indicates high expression shows protective effects to overall survival time.

## Statistical Analysis

TIMER was a web interface for visualization and dynamic analysis for molecular characterization of tumor-immune interactions ([49](#_ENREF_49)). Statistical analysis was performed using SPSS (IBM Corporation, Armonk, NY) and GraphPad Prism 6.0 software (La Jolla, CA, USC). For statistical comparisons, the twotailed Student t test was used for two groups. X2 test was performed in comparisons of categorical variables. Kaplan-Meier and Cox regression were performed in IHC analysis to investigate the relationship between the IHC markers and patients’ prognosis. P < 0.05 was considered statistically signiﬁcant.

## Genes Involved in the study

HLA-DQA1, HLA-DRA, HLA-DPA1, CIITA, and BRAF

# **Conflict of interest**

The authors declare no conflict of interest.

# **Acknowledgements**

The authors gratefully acknowledge all the patients who donated the samples for the study and Emily Andreae, PhD, for reviewing and editing their manuscript.

# **Funding**

This work was partially supported by grants from National Natural Science Foundation of China (Grant Nos. 81872169, 81872235), Tianjin key research and development program science and technology support key projects (Grant No. 17YFZCSY00690), and Tianjin Research Innovation Project for Postgraduate Students.

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**Figure Legends**

**Fig 1. TsMHCII is decreased in PTCs with BRAFV600E mutation.** A) Flow chart of iTRAQ-labeling LC-MS/MS analyses. B) A Volcano plot highlighting differentially expressed proteins (DEPs). 4,722 peptides were identified with greater than 1.2-fold change in BRAFV600E relative to BRAF wildtype samples and plotted as red (down-regulation) or green (up-regulation), respectively. C) Proteins with significant expression changes were plotted in the heatmap. D) KEGG analysis of total identified DEPs. Proteins of the antigen processing and presentation related pathways had the greatest degree of up-regulation while proteins involved in metabolic pathways had the greatest degree of down-regulation between BRAFV600E and wildtype BRAF samples. E) Heatmap of MHCI and MHCII proteins identified by iTRAQ. F) Representative immunohistochemical staining of tsMHCII in PTC specimens. G) Percentage of samples with varying expression levels of tsMHCII in 185 PTC samples. H) Stacked histogram of tsMHCII staining levels in PTC samples. PTC samples with BRAFV600E mutation showed a signiﬁcantly lower rate of tsMHCII expression than BRAF wildtype tumors.

**Fig 2. Constitutive activation of BRAF-MAPK pathway down-regulated tsMHCII expression in PTC.** A) Real-time PCR detection of CIITA and tsMHCII genes (HLA-DQA1, HLA-DRA, and HLA-DPA1) expression in PTC cell lines normalized to that in an immortalized thyroid cell line Nthy-ori-3-1. (B) Flow-cytometric analyses of tsMHCII in BCPAP and K1 cells with (PLX4032) and without (Ctrl) treatment with BRAF inhibitor PLX4032 (10 µM) for 72 h. The data were presented as mean ± SD. C) Protein expression of tsMHCII in BCPAP and K1 cells with (U0126) and without (Ctrl) treatment with MEK inhibitor U0126 for 72 h. The data were presented as mean ± SD. D) Western blot analysis of BRAF expression in doxycycline (DOX)-inducible BRAFV600E over-expressing TPC1 cells. E) Flow-cytometric analysis of tsMHCII in BRAFV600E over-expressing TPC1 cells compared to control cells. The data were presented as mean ± SD. \*, P <0.05; \*\*, P <0.01;\*\*\*, P <0.001.

**Fig 3. BRAFV600E downregulated tsMHCII expression through TGF-β1/SMAD3 pathway.** A) Real-time PCR detection of CIITA and tsMHCII mRNAs in BCPAP and K1 cells after treatment with inhibitors for various signaling pathways (DAPT at 25 µM for NOTCH, IWR1 at 10 µM for Wnt, SB431542 at 10 µM for TGF-β1, and Cyclopamine at 20 µM for Hedgehog pathway) or additional medium (Ctrl) for 48 h. Results presented as a heatmap. SB431542 inhibition significantly decreased transcript levels of MCHII genes in BCPAP and K1 PTC cell lines. B) Correlation analysis of TGF-β1 and CIITA gene expression levels in all PTCs or in PTCs with BRAFV600E mutation according to expression data in the TCGA database. C) Flow-cytometric analyses of tsMHCII in BCPAP and K1 cells after treatment with exogenous TGF-β1 (10 ng/ml) for 72 h. The data were presented as mean ± SD. D) Flow-cytometric analysestsMHCII in BCPAP and K1 cells after treatment by PLX4032 with or without with TGF-β1. The data were presented as mean ± SD. E) Correlation analysis of SMAD3 expression and BRAF mutation status according to information in the TCGA database. F) Representative IHC staining of phosphorylated SMAD3 in PTC specimens. Tumors with BRAFV600E mutation showed a signiﬁcantly higher rate of p-SMAD3 expression than BRAF wildtype tumors. G) Correlation analysis between SMAD3 and CIITA in all PTCs or in PTCs with BRAFV600E mutation according to information in the TCGA database. H) Flow-cytometric analyses of tsMHCII in BCPAP and K1 cells after treatment with SMAD3 inhibitor SIS3 (10 µM) for 72 h. The data were presented as mean ± SD. I) Western blot analysis in doxycycline-inducible BRAFV600E over-expressing TPC1 cells. J) Flow-cytometric analyses oftsMHCII in BRAFV600E over-expressing TPC1 cells treated with SIS3. The data were presented as mean ± SD. The data were presented as mean ± SD. \*, P <0.05; \*\*, P <0.01;\*\*\*, P <0.001.

**Fig 4. TsMHCII increased the elimination effect of immune cells in vitro, and increased expression of tsMHCII correlated with good prognosis in patients with PTC.** A) In vitro cytotoxicity assays of peripheral blood leukocytes (PBL) to BCPAP and K1 cells after pre-treatment with PLX4032 or combined with siCIITA transfection. The data were presented as mean ± SD. B). Western blot analysis of tsMHCII in BCPAP and K1 cells after transfection with siCIITA. C) Real-time PCR analysis of CIITA in BCPAP and K1 cells after transfection with siCIITA. The data were presented as mean ± SD. D) Extracellular INF-γ produced by CD4+ T-cells in co-culture with BCPAP or K1 cells pre-treated with PLX4032 or combined with siCIITA transfection. The data were presented as mean ± SD. E) *In vitro* cytotoxicity assays of PBL against TPC1 cells overexpressing BRAFV600E with corresponding response in F) INF-γ produced by CD4+ T-cells. The data were presented as mean ± SD. G) Representative immunohistochemical staining of tsMHCII and CD4 in PTC specimens. The expression of tsMHCII was remarkably associated with high CD4 T-cell infiltration. H) Kaplan-Meier analyses of patients with PTC according to staining status of tsMHCII. Association analysis of tsMHCII expression by immune cell type with the TIMER web tool analysis revealed I) a positive association between tsMHCII and survival in patients with thyroid carcinoma. The data were presented as mean ± SD. \*, P <0.05; \*\*, P <0.01;\*\*\*, P <0.001.

**Fig 5. PLX4032 combined with anti-PD-1 treatment offers durable therapeutic response.** A) *In vitro* cytotoxicity assays of PBL to BCPAP and K1 cells. PBL were pre-treated with or without anti-PD-1 antibody (Nivolunab, 20 µg/ml) for 5 days. BCPAP and K1 cells were pre-treated with or without PLX4032 (10 µM) for 72 h. After pre-treatment, PBL and PTC cells were co-cultured at a 5:1 ratio (2×105 cells/well) in a 24-well plate for 6 h. The data were presented as mean ± SD. B) INF-γ produced by CD4+ T-cells were analyzed after co-culture with BCPAP or K1 cells. The data were presented as mean ± SD. C) Therapy protocol for spontaneous thyroid cancer generated in transgenic mice. D) Representative images of dissected mouse thyroid tumors after different therapies. E) Tumor weight of each group. The data were presented as mean ± SD. F) Tumor immune profiles after single and combined therapy regimens in transgenic mice depicted by heatmap of fold-changes compared to vehicle+α-IgG treatment (control). G) Representative hematoxylin and eosin staining and IHC staining of tsMHCII expression in mouse tumor specimens. The data were presented as mean ± SD. \*, P <0.05; \*\*, P <0.01;\*\*\*, P <0.001

**Fig 6. Schematic model of oncogenic BRAF-dependent regulation of tsMHCII.** Our data show that BRAFV600E downregulates tsMHCII expression via the TGF-β1/SMAD3 pathway in PTCs. BRAF inhibitor treatment up-regulates tsMHCII level to increase tumor cell elimination by the immune system and enhance the response to anti-PD-1 antibody treatment.

**Supplementary Information**

**Supplemental Figure Legends**

**Figure S1. Tandem mass spectrum and corresponding iTRAQ reporter ions.** A).Tandem mass spectrum identifying a peptide from HLA-DPA1. B). iTRAQ reporter ions for the HLA-DPA1 peptide.

**Figure S2. TsMHCII expression in PTC was downregulated by BRAFV600E at the transcriptional level.** A) Real-time PCR detection of CIITA and tsMHCII in BCPAP and K1 cells after treatment by PLX4032 for 72 h; abbreviations used: Ctrl=untreated cells, PLX4032=BRAF inhibitor treated cells. B) Transcript levels of CIITA and tsMHCII in BCPAP and K1 cells after treatment by U0126 72 h; abbreviations used: Ctrl=untreated cells, U0126=MEK inhibitor treated cells. The data were presented as mean ± SD. \*, P <0.05; \*\*, P <0.01;\*\*\*, P <0.001

**Figure S3. BRAFV600E-upregulated TGF-β1 altered tsMHCII expression.** A) Time course expression of CIITA and tsMHCII in BCPAP and K1 by real-time PCR. B) Quantitative detection of TGF-β1 in the extracellular medium of BCPAP and K1 cells over time via ELISA. C) ELISA quantification of secreted TGF-β1 after treatment with PLX4032; abbreviations used: Ctrl=untreated cells, PLX4032=BRAF inhibitor treatment. D) Transcript levels of CIITA and tsMHCII in BCPAP and K1 cells after treatment with TGF-β1 (10 ng/ml) for 72 h. E) Transcript levels oftsMHCII in BCPAP and K1 cells after treatment with PLX4032 alone or combined with TGF-β1. The data were presented as mean ± SD. \*, P <0.05; \*\*, P <0.01;\*\*\*, P <0.001

**Figure S4. TGF-β1 mediates downregulation of tsMHCII in BRAFV600E PTC cells via SMAD3.** Western blot analysis of p-SMAD3 and SMAD3 expression in BCPAP and K1 cells treated for 48 h with or without C) PLX4032, D) U0126, A) TGF-β1, or B) SIS3, respectively. E) Real-time PCR detection of CIITA and tsMHCII transcripts in BCPAP and K1 cells after treatment by 10 µM SIS3 for 48 h. F) Transcript level of CIITA and tsMHCII in BRAFV600E transfected TPC1 cells after treatment by DOX or combination DOX and SIS3 for 48 hr. Untreated cell controls are labeled as “Ctrl” in the figure. The data were presented as mean ± SD. \*, P <0.05; \*\*, P <0.01;\*\*\*, P <0.001

**Figure S5. Pan-cancer Survival Analysis to Involved Genes with The Cancer Genome Atlas (TCGA) Dataset.**

**Figure S6. TIMER web tool analysis of tsMCHII components in various cancers.** Violin plots depict the association between mRNA expression of A) HLA-DQA1, B) HLA-DRA, and C) HLA-DPA1 in various tumor types. Red boxes highlight the association between tsMCHII genes and thyroid carcinomas.

**Figure S7. Representative hematoxylin and eosin staining in mouse liver (left) and kidney (right) specimens.**