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

Jingtai Zhi, MD 1\*, Peitao Zhang, 2,\*, Mengran Tian1, Xianhui Ruan, MD, PhD1, Shicheng Guo, PhD3,4, Weiyu Zhang, MD5, Wei Zhang, MD1, Xiangqian Zheng, MD, PhD1‡, Li Zhao, PhD2,1‡, Ming Gao, MD, PhD1‡.

1. Department of Thyroid and Neck Tumor, Tianjin Medical University Cancer Institute and Hospital, National Clinical Research Center for Cancer, Key Laboratory of Cancer Prevention and Therapy, Tianjin’s Clinical Research Center for Cancer, Tianjin 300060, People’s Republic of China
2. Department of Biochemistry and Molecular Biology, School of Basic Medical Sciences, Tianjin Medical University, Tianjin, 300070, People’s Republic of China
3. Center for Precision Medicine Research, Marshfield Clinic Research Institute, Marshfield, WI, United States, 54449
4. Department of Medical Genetics, School of Medicine and Public Health, University of Wisconsin-Madison, Madison, WI, USA
5. State Key Laboratory of Medicinal Chemical Biology, Nankai University, Tianjin 300350, People’s Republic of China, 2College of Pharmacy, Nankai University, Tianjin 300350, People’s Republic of China

\*These authors contributed equally to this work.

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

‡Corresponding author

Xiangqian Zheng, MD, PhD

Department of Thyroid and Neck Tumor, Tianjin Medical University Cancer Institute and Hospital, National Clinical Research Center for Cancer, Key Laboratory of Cancer Prevention and Therapy, Tianjin’s Clinical Research Center for Cancer, Huanhuxi Road, Ti-Yuan-Bei, Hexi District，Tianjin, 300060，P.R. China

Tel: 86-13820881516

1. mail: xiangqian\_zheng@163.com

Li Zhao, MD, PhD

Department of Biochemistry and Molecular Biology, School of Basic Medical Sciences, Tianjin Medical University, Qixiangtai Road, Heping District，Tianjin, 300070，P.R. China

Tel: 86-13043282068

E-mail: shzhaoli@tijmu.edu.cn

Ming Gao, MD, PhD

Department of Thyroid and Neck Tumor, Tianjin Medical University Cancer Institute and Hospital, National Clinical Research Center for Cancer, Key Laboratory of Cancer Prevention and Therapy, Tianjin’s Clinical Research Center for Cancer

Huanhuxi Road, Ti-Yuan-Bei, Hexi District，Tianjin, 300060，P.R. China

Tel: 86-23340123

E-mail: [headandneck2008@126.com](mailto:headandneck2008@126.com)

# **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" \o "Cronin, 2018 #3),[2](#_ENREF_2" \o "Lim, 2017 #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" \o "Siegel, 2019 #6)]. These patients normally go through multimodal therapy including surgery, radioactive iodine therapy, external beam radiation, watchful waiting, and experimental trials [[4](#_ENREF_4" \o "Cooper, 2009 #9)]. In spite of these advancements, 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" \o "Pontius, 2017 #10),[6](#_ENREF_6" \o "Perrier, 2018 #12)]. 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. 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 [[7](#_ENREF_7" \o "Liotti, 2019 #13),[8](#_ENREF_8" \o "Angell, 2014 #15)].

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 [[9](#_ENREF_9" \o "Xing, 2007 #16),[10](#_ENREF_10" \o "Ruan, 2019 #56)]. In murine models of thyroid carcinoma, tumors harboring BRAFV600E displayed a high M2-like TAM infiltration. 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 [[11-13](#_ENREF_11" \o "Angell, 2014 #17)]. 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 [[14](#_ENREF_14" \o "Frederick, 2013 #24),[15](#_ENREF_15" \o "Donia, 2012 #25)]. 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 [[16](#_ENREF_16" \o "Axelrod, 2019 #27)]. 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 [[17](#_ENREF_17" \o "Kambayashi, 2014 #29)]. 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 [[18](#_ENREF_18" \o "Ostrand-Rosenberg, 1990 #40),[19](#_ENREF_19" \o "Mortara, 2006 #41)]. In lung and colon cancer, depletion of DCs or macrophages had no effect on the ability of mice to reject tsMHCII expressing tumor cells [[20](#_ENREF_20" \o "Bou Nasser Eddine, 2017 #39)]. 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 [[21](#_ENREF_21" \o "Constantinidou, 2019 #45),[22](#_ENREF_22" \o "Franklin, 2017 #46)]. Other possible strategies to increase tumor immunogenicity are stimulating the expression of MHC molecules and/or 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. 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) [[23](#_ENREF_23" \o "Sartoris, 1998 #31)]. 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 wild-type 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 (**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 decrease 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 MCHII 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 MCHII transcript levels (**Fig. S3A-B**), which corresponds to previous findings [[24](#_ENREF_24" \o "Azouzi, 2017 #49)]. of BCPAP and K1 cells with10 ng/mL sBCPAP and cells significantly decreased cells  **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 and 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 and 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**). Clinical and pathological data are summarized in **Table S4**.

To validate the IHC findings, we analyzed transcript levels of tsMHCII in various carcinomas using the TIMER web tool (cistrome.shinyapps.io/timer/) and found that HLA-DQA1, DRA, and DPA1 mRNA levels were highly expressed in PTC (**Fig. S5**). 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 [[25](#_ENREF_25" \o "Johnson, 2016 #36),[26](#_ENREF_26" \o "Roemer, 2018 #38)]. 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 byCD107 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. S6**). 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, alter the expression of immune checkpoint molecules like PD-L1, CTL4, and HLA-G, and halt the production and expression of MHCI though the detailed mechanism for MHCI loss is still unknown [8,[11-13](#_ENREF_11" \o "Angell, 2014 #17)]. 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 [[28](#_ENREF_28" \o "Arnold, 2002 #30),29]. Similarly, in patients with thyroiditis, MHCII-positive thyrocytes presented viral peptide antigens to T-cells [[30](#_ENREF_30" \o "Londei, 1984 #50)]. 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. [[31](#_ENREF_31" \o "Jo, 2008 #52)].

In both rodent and human PTC cells, optimal TGF-β1 signaling requires constitutive activation of the MAPK pathway [[24](#_ENREF_24" \o "Azouzi, 2017 #49),[32](#_ENREF_32" \o "Nicolussi, 2003 #51)]. 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 [[33](#_ENREF_33" \o "Dong, 2001 #53)]. 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 [[34](#_ENREF_34" \o "Batlle, 2019 #54),[35](#_ENREF_35" \o "Mariathasan, 2018 #55)]. Our findings suggest an autocrine effect of TGF-β1 signaling on tumor cells to alter the patterning of cell surface antigens and sensitize PTC cells to PD-1 treatment. 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 [[16](#_ENREF_16" \o "Axelrod, 2019 #27)]. 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 [[21](#_ENREF_21" \o "Constantinidou, 2019 #45),[22](#_ENREF_22" \o "Franklin, 2017 #46),[36](#_ENREF_36" \o "Wargo, 2014 #48)]. 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 [[37](#_ENREF_37" \o "Gunda, 2018 #42)]. 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 [[38](#_ENREF_38" \o "Li, 2019 #57)]. 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. In all clinical specimens, BRAF mutation status was verified by Sanger sequencing. Thirty fresh specimens from cases and controls were collected in 2017 and washed three times with pre-cooled phosphate buffered saline (PBS) for iTRAQ analysis. The remaining 185 cases were obtained from 2013 to 2014 and used for IHC analysis.

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

iTRAQ analysis was performed as previously described. Briefly, 30 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 wildtype BRAF were randomly divided into three groups. Five samples were pooled in each group and labeled with 115, 116, and 117 labels. The remaining samples were divided and pooled in the same way but with 118, 119, and 121 labels.

## Immunohistochemical staining

Tissue sections from 185 PTC cases were stained with antitibodies to human MHCII, pSMAD3, and human CD4 (all antibodies from 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**

## Western blot analysis and ELISA

Western blot was performed as described previously using primary antibodies to pSMAD3 (epitope location: serine 423, host species: X, Abcam), SMAD3 (epitope location: X, host species: X, Abcam),MHCII (epitope location: X, host species: X, Abcam), and GAPDH (epitope location: X, host species: X, CST). 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.). ELISA was preformed to measure secreted levels of TGF-β1 and INF-γ according to the manufacturer’s protocol (Promega).

## Plasmid and cell transfection

Plasmids packaged for lentivirus transfection were purchased from Syngen And constructed with XXX sequence of BRAFV600E with X and X restriction enzymes. BRAFV600E sequence incorporation in the plasmin was confirmed by Sanger sequencing. Small interfering RNAs (siRNA) of CIITA were obtained from Qiagen. Transfections were performed using Lipofectamine™ 3000 (Invitrogen) following the manufacturer’s instructions.

## Real-time PCR

Real-time PCR assays were performed as previously described. Primers are listed in 5′→3′ orientation as follows:

|  |  |  |
| --- | --- | --- |
|  | FORWARD | REVERSE |
| CIITA | GAAGAAGCTGCTCCGAGGTTGC | GCTCTGTCTTGGTGCTCTGTCATC |
| HLA-DPA1 | GACCGTCTGGCATCTGGAGGAG | GAGTGTGGTTGGAACGCTGGATC |
| HLA-DQA1 | GTGGACCTGGAGAGGAAGGAGAC | CATTGGTAGCAGCGGTAGAGTTGTAG |
| HLA-DRA | GCCGAGTTCTATCTGAATCCTGACC | AAGCCGCCAGACCGTCTCC |

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

Flow cytometry was performed on a BD FACS Canto II cytometer (BD Biosciences) using the following antibodies from BD: MHCII, CD45, CD3, CD4, CD8, and CD107 on PBLs stimulated with cytokines and chemokines following the manufacturer’s instructions. PBL were isolated by differential density gradient centrifugation from human subjects and divided into two groups: one group treated with anti-CD3/CD28 stimulation (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, and recombinant human IL-2 (20 ng/ml). After five days, both groups 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 collected for staining with NIR. The samples were analyzed FlowJo (Ashland, OR).

## 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 cultured as previously described. After five days, control and experimental groups were co-cultured with PTC cell lines at a 5:1 ratio with BCPAP and K1 cells (2×105 cells/well) in a 24-well plate for 24 h. Culture supernatants were collected for downstream ELISA analysis.

## 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.

## Statistical Analysis

Statistical analysis was performed using SPSS (IBM Corporation, Armonk, NY) and GraphPad Prism 6.0 software (La Jolla, CA, USC). 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.

# **References**

1. Cronin KA, Lake AJ, Scott S. Annual Report to the Nation on the Status of Cancer, part I: National cancer statistics. 2018;124(13):2785-2800.doi:10.1002/cncr.31551

2. Lim H, Devesa SS, Sosa JA, Check D, Kitahara CM. Trends in Thyroid Cancer Incidence and Mortality in the United States, 1974-2013. *Jama.* 2017;317(13):1338-1348.doi:10.1001/jama.2017.2719

3. Siegel RL, Miller KD. Cancer statistics, 2019. 2019;69(1):7-34.doi:10.3322/caac.21551

4. Cooper DS, Doherty GM, Haugen BR, Kloos RT, Lee SL, Mandel SJ.et al. Revised American Thyroid Association management guidelines for patients with thyroid nodules and differentiated thyroid cancer. *Thyroid : official journal of the American Thyroid Association.* 2009;19(11):1167-1214.doi:10.1089/thy.2009.0110

5. Pontius LN, Oyekunle TO, Thomas SM, Stang MT, Scheri RP, Roman SA.et al. Projecting Survival in Papillary Thyroid Cancer: A Comparison of the Seventh and Eighth Editions of the American Joint Commission on Cancer/Union for International Cancer Control Staging Systems in Two Contemporary National Patient Cohorts. *Thyroid : official journal of the American Thyroid Association.* 2017;27(11):1408-1416.doi:10.1089/thy.2017.0306

6. Perrier ND, Brierley JD, Tuttle RM. Differentiated and anaplastic thyroid carcinoma: Major changes in the American Joint Committee on Cancer eighth edition cancer staging manual. *CA: a cancer journal for clinicians.* 2018;68(1):55-63.doi:10.3322/caac.21439

7. Liotti F, Prevete N, Vecchio G, Melillo RM. Recent advances in understanding immune phenotypes of thyroid carcinomas: prognostication and emerging therapies. 2019;8.doi:10.12688/f1000research.16677.1

8. Angell TE, Lechner MG, Jang JK, LoPresti JS, Epstein AL. 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. *Clinical cancer research : an official journal of the American Association for Cancer Research.* 2014;20(23):6034-6044.doi:10.1158/1078-0432.ccr-14-0879

9. Xing M. BRAF mutation in papillary thyroid cancer: pathogenic role, molecular bases, and clinical implications. *Endocrine reviews.* 2007;28(7):742-762.doi:10.1210/er.2007-0007

10. Ruan X, Shi X, Dong Q, Yu Y, Hou X, Song X.et al. Antitumor effects of anlotinib in thyroid cancer. *Endocrine-related cancer.* 2019;26(1):153-164.doi:10.1530/erc-17-0558

11. Angell TE, Lechner MG, Jang JK, Correa AJ, LoPresti JS, Epstein AL. BRAF V600E in papillary thyroid carcinoma is associated with increased programmed death ligand 1 expression and suppressive immune cell infiltration. *Thyroid : official journal of the American Thyroid Association.* 2014;24(9):1385-1393.doi:10.1089/thy.2014.0134

12. Na KJ, Choi H. Immune landscape of papillary thyroid cancer and immunotherapeutic implications. *Endocrine-related cancer.* 2018;25(5):523-531.doi:10.1530/erc-17-0532

13. Varricchi G, Loffredo S. The Immune Landscape of Thyroid Cancer in the Context of Immune Checkpoint Inhibition. 2019;20(16).doi:10.3390/ijms20163934

14. Frederick DT, Piris A, Cogdill AP, Cooper ZA, Lezcano C, Ferrone CR.et al. BRAF inhibition is associated with enhanced melanoma antigen expression and a more favorable tumor microenvironment in patients with metastatic melanoma. *Clinical cancer research : an official journal of the American Association for Cancer Research.* 2013;19(5):1225-1231.doi:10.1158/1078-0432.ccr-12-1630

15. Donia M, Fagone P, Nicoletti F, Andersen RS, Hogdall E, Straten PT.et al. BRAF inhibition improves tumor recognition by the immune system: Potential implications for combinatorial therapies against melanoma involving adoptive T-cell transfer. *Oncoimmunology.* 2012;1(9):1476-1483.doi:10.4161/onci.21940

16. Axelrod ML, Cook RS, Johnson DB, Balko JM. Biological Consequences of MHC-II Expression by Tumor Cells in Cancer. 2019;25(8):2392-2402.doi:10.1158/1078-0432.ccr-18-3200

17. Kambayashi T, Laufer TM. Atypical MHC class II-expressing antigen-presenting cells: can anything replace a dendritic cell? *Nature reviews Immunology.* 2014;14(11):719-730.doi:10.1038/nri3754

18. Ostrand-Rosenberg S, Thakur A, Clements V. Rejection of mouse sarcoma cells after transfection of MHC class II genes. *Journal of immunology (Baltimore, Md : 1950).* 1990;144(10):4068-4071

19. Mortara L, Castellani P, Meazza R, Tosi G, De Lerma Barbaro A, Procopio FA.et al. CIITA-induced MHC class II expression in mammary adenocarcinoma leads to a Th1 polarization of the tumor microenvironment, tumor rejection, and specific antitumor memory. *Clinical cancer research : an official journal of the American Association for Cancer Research.* 2006;12(11 Pt 1):3435-3443.doi:10.1158/1078-0432.ccr-06-0165

20. Bou Nasser Eddine F, Forlani G, Lombardo L, Tedeschi A, Tosi G, Accolla RS. CIITA-driven MHC class II expressing tumor cells can efficiently prime naive CD4(+) TH cells in vivo and vaccinate the host against parental MHC-II-negative tumor cells. *Oncoimmunology.* 2017;6(1):e1261777.doi:10.1080/2162402x.2016.1261777

21. Constantinidou A, Alifieris C, Trafalis DT. Targeting Programmed Cell Death -1 (PD-1) and Ligand (PD-L1): A new era in cancer active immunotherapy. *Pharmacology & therapeutics.* 2019;194:84-106.doi:10.1016/j.pharmthera.2018.09.008

22. Franklin C, Livingstone E, Roesch A, Schilling B, Schadendorf D. Immunotherapy in melanoma: Recent advances and future directions. *European journal of surgical oncology : the journal of the European Society of Surgical Oncology and the British Association of Surgical Oncology.* 2017;43(3):604-611.doi:10.1016/j.ejso.2016.07.145

23. Sartoris S, Valle MT, Barbaro AL, Tosi G, Cestari T, D'Agostino A .et al. HLA class II expression in uninducible hepatocarcinoma cells after transfection of AIR-1 gene product CIITA: acquisition of antigen processing and presentation capacity. *Journal of immunology (Baltimore, Md : 1950).* 1998;161(2):814-820

24. Azouzi N, Cailloux J, Cazarin JM, Knauf JA, Cracchiolo J, Al Ghuzlan A.et al. NADPH Oxidase NOX4 Is a Critical Mediator of BRAF(V600E)-Induced Downregulation of the Sodium/Iodide Symporter in Papillary Thyroid Carcinomas. *Antioxidants & redox signaling.* 2017;26(15):864-877.doi:10.1089/ars.2015.6616

25. Johnson DB, Estrada MV, Salgado R, Sanchez V, Doxie DB, Opalenik SR.et al. Melanoma-specific MHC-II expression represents a tumour-autonomous phenotype and predicts response to anti-PD-1/PD-L1 therapy. 2016;7:10582.doi:10.1038/ncomms10582

26. Roemer MGM, Redd RA, Cader FZ, Pak CJ, Abdelrahman S, Ouyang J.et al. Major Histocompatibility Complex Class II and Programmed Death Ligand 1 Expression Predict Outcome After Programmed Death 1 Blockade in Classic Hodgkin Lymphoma. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology.* 2018;36(10):942-950.doi:10.1200/jco.2017.77.3994

27. Knauf JA, Ma X, Smith EP, Zhang L, Mitsutake N, Liao XH.et al. Targeted expression of BRAFV600E in thyroid cells of transgenic mice results in papillary thyroid cancers that undergo dedifferentiation. *Cancer research.* 2005;65(10):4238-4245.doi:10.1158/0008-5472.can-05-0047

28. Arnold PY, La Gruta NL, Miller T, Vignali KM, Adams PS, Woodland DL.et al. The majority of immunogenic epitopes generate CD4+ T cells that are dependent on MHC class II-bound peptide-flanking residues. *Journal of immunology (Baltimore, Md : 1950).* 2002;169(2):739-749.doi:10.4049/jimmunol.169.2.739

29. Veatch JR, Lee SM, Fitzgibbon M, Chow IT, Jesernig B, Schmitt T.et al. Tumor-infiltrating BRAFV600E-specific CD4+ T cells correlated with complete clinical response in melanoma. *The Journal of clinical investigation.* 2018;128(4):1563-1568.doi:10.1172/jci98689

30. Londei M, Lamb JR, Bottazzo GF, Feldmann M. Epithelial cells expressing aberrant MHC class II determinants can present antigen to cloned human T cells. *Nature.* 1984;312(5995):639-641.doi:10.1038/312639a0

31. Jo YS, Lee JC, Li S, Choi YS, Bai YS, Kim YJ.et al. Significance of the expression of major histocompatibility complex class II antigen, HLA-DR and -DQ, with recurrence of papillary thyroid cancer. *International journal of cancer.* 2008;122(4):785-790.doi:10.1002/ijc.23167

32. Nicolussi A, D'Inzeo S, Santulli M, Colletta G, Coppa A. TGF-beta control of rat thyroid follicular cells differentiation. *Molecular and cellular endocrinology.* 2003;207(1-2):1-11.doi:10.1016/s0303-7207(03)00238-7

33. Dong Y, Tang L, Letterio JJ, Benveniste EN. The Smad3 protein is involved in TGF-beta inhibition of class II transactivator and class II MHC expression. *Journal of immunology (Baltimore, Md : 1950).* 2001;167(1):311-319.doi:10.4049/jimmunol.167.1.311

34. Batlle E, Massague J. Transforming Growth Factor-beta Signaling in Immunity and Cancer. *Immunity.* 2019;50(4):924-940.doi:10.1016/j.immuni.2019.03.024

35. Mariathasan S, Turley SJ, Nickles D, Castiglioni A, Yuen K, Wang Y.et al.TGFbeta attenuates tumour response to PD-L1 blockade by contributing to exclusion of T cells. *Nature.* 2018;554(7693):544-548.doi:10.1038/nature25501

36. Wargo JA, Cooper ZA, Flaherty KT. Universes collide: combining immunotherapy with targeted therapy for cancer. *Cancer discovery.* 2014;4(12):1377-1386.doi:10.1158/2159-8290.cd-14-0477

37. Gunda V, Gigliotti B, Ndishabandi D, Ashry T, McCarthy M, Zhou Z.et al. Combinations of BRAF inhibitor and anti-PD-1/PD-L1 antibody improve survival and tumour immunity in an immunocompetent model of orthotopic murine anaplastic thyroid cancer. 2018;119(10):1223-1232.doi:10.1038/s41416-018-0296-2

38. Li H, Li CW, Li X, Ding Q, Guo L, Liu S.et al. MET Inhibitors Promote Liver Tumor Evasion of the Immune Response by Stabilizing PDL1. *Gastroenterology.* 2019;156(6):1849-1861.e1813.doi:10.1053/j.gastro.2019.01.252

**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 (TT). 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. C) Protein expression of tsMHCII in BCPAP and K1 cells with (U0126) and without (Ctrl) treatment with MEK inhibitor U0126 for 72 h. 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.

**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. D) Flow-cytometric analysestsMHCII in BCPAP and K1 cells after treatment by PLX4032 with or without with TGF-β1. 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. 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.

**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.** 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. 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. 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. E) *In vitro* cytotoxicity assays of PBL against TPC1 cells overexpressing BRAFV600E with corresponding response in F) INF-γ produced by CD4+ T-cells. 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 CD4+ T-cell, CD8+ T-cell, and B-cell infiltration and J) survival in patients with thyroid carcinoma.

**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 after pre-treatment with PLX4032, anti-PD-1 antibody (Nivolunab, 20 µg/ml), or combination PLX4032 and Nivolunab for X hours . B) INF-γ produced by CD4+ T-cells were analyzed after co-culture with BCPAP or K1 cells that have been treated with PLX4032, Nivolunab, or combination treatment of PLX4032 with Nivolunab. 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. 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.

**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.

**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.

**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.

**Figure S5. 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 S6. Representative hematoxylin and eosin staining in mouse liver (left) and kidney (right) specimens.**

**Table S1. Correlation between MHCII expression and clinicopathological factors in PTC patients**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Variable | Low expression | | High expression | | X2 | *P* |
| Age |  |  |  |  |  |  |
| <55 | 36 | | 33 | | 0.028 | 0.867 |
| ≥55 | 62 | | 54 | |  |  |
| Sex |  |  |  |  |  |  |
| Female | 74 | | 58 | | 1.763 | 0.184 |
| Male | 24 | | 29 | |  |  |
| CD4 T cell infiltration | | | | |  |  |
| Present | 17 | | 28 | | 5.511 | 0.019 |
| Absent | 81 | | 59 | |  |  |
| BRAF V600E mutation | | | |  |  |  |
| Present | 59 | | 34 | | 8.226 | 0.004 |
| Absent | 39 | | 53 | |  |  |
| Multifocality | | |  |  |  |  |
| Present | 30 | | 28 | | 0.053 | 0.818 |
| Absent | 68 | | 59 | |  |  |
| Tumour stage | |  |  |  |  |  |
| T1/2 | 82 | | 85 | | 10.325 | 0.001 |
| T3/4 | 16 | | 2 | |  |  |
| Lymph node metastasis | | | | |  |  |
| N0 | 37 | | 52 | | 8.947 | 0.003 |
| N1a/b | 51 | | 35 | |  |  |
| AJCC stage | | |  |  |  |  |
| I+II | 86 | | 86 | | 8.684 | 0.003 |
| III+IV | 12 | | 1 | |  |  |
| Recurrence | | |  |  |  |  |
| Absent | 70 | | 85 | | 23.414 | <0.001 |
| Present | 28 | | 2 | |  |  |

**Table S2. Hazard ratios of disease specific survival and overall survivalaccording to the xpression of tsMHCII in patients with PTC**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Variable | Disease-speciﬁc Survival | | Overall Survival | |
|  | P-value | HR（95% CI） | P-value | HR（95% CI） |
| Female | 0.608 | 1.311 (0.466-3.690) | 0.392 | 1.501 (0.592-3.807) |
| Stage III+IV | <0.001 | 15.305 (4.578-51.166) | <0.001 | 18.201 (6.381-51.917) |
| Multifocality | 0.820 | 0.885 (0.307-2.549) | 0.613 | 0.783 (0.302-2.025) |
| BRAF mutation | 0.012 | 4.612 (1.405-15.141) | 0.001 | 5.524 (1.942-15.719) |
| MHC II expression | 0.038 | 0.205 (0.046-0.917) | 0.024 | 0.240 (0.070-0.827) |
| CD4 T-cell infiltration | 0.872 | 0.903 (0.258-3.154) | 0.904 | 0.935 (0.315-2.774) |
| Abbreviations: CI=Confidence Interval; HR=Hazard Ratio | | | | |

**Table S3. Hazard Ratios of overall survival according to mRNA levels of CIITA using TIMER webtool**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  |  | 95% CI | |  |
|  | HR | Lower | Uper | *P* |
| CIITA | 0.504 | 0.27 | 0.938 | 0.031 |
| Purity | 14.540 | 1.286 | >100 | 0.031 |
| Stage I | Ref. | - | - | - |
| Stage II | 6.304 | 0.738 | 53.849 | 0.093 |
| Stage III | 11.326 | 1.883 | 68.117 | 0.008 |
| Stage IV | 41.385 | 5.495 | >100 | <0.001 |
| mate | 1.109 | 0.312 | 3.930 | 0.874 |
| B-cell | 0.006 | <0.001 | >100 | 0.312 |
| CD8 T-cell | <0.001 | <0.001 | 0.500 | 0.041 |
| CD4 T-cell | 0.021 | <0.001 | >100 | 0.046 |
| Marcrophage | <0.001 | <0.001 | >100 | 0.211 |
| Neutrophage | 0.050 | <0.001 | >100 | 0.983 |
| Dendritic | >100 | >100 | >100 | 0.007 |
| Abbreviations: CI=Confidence Interval; HR=Hazard Ratio | | | | |

**Table S4. Clinicopathological characteristics of patients with PTC**

|  |  |
| --- | --- |
| Patient Characteristics | N=185 |
| Age |  |
| ≥55 | 116 (62.7%) |
| Sex |  |
| Female | 132 (71.4%) |
| Tumour stage |  |
| T3/4 | 18 (9.7%) |
| Lymph node metastasis |  |
| N1a/b | 96 (51.9%) |
| Multifocality |  |
| Present | 58 (31.4%) |
| AJCC stage |  |
| III+IV | 13 (7.0%) |
| BRAF mutation |  |
| Present | 93 (50.3%) |
| CD4 T cell infiltration |  |
| Present | 45 (24.3%) |
| Recurrence |  |
| Present | 30 (16.2%) |
| Overall mortality | 28 (15.1%) |
| Disease-specific mortality | 21 (11.4%) |
| Follow-up years, median (range) | 68 (20-72) |