BRAF Mutation in Papillary Thyroid Carcinoma: Pathogenic Role and Clinical Implications

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Papillary thyroid cancer (PTC) is the most common endocrine malignancy, accounting for 85–90% of all thyroid cancers. Genetic alternations involving the mitogen-activated protein kinase (MAPK) pathway are frequently demonstrated in PTC, such as RET/PTC, RAS, and B-type Raf kinase (BRAF) mutations. Over 90% of BRAF mutations are T1799A, resulting in a $BRAF^{V600E}$ mutation. $BRAF^{V600E}$ is present in ~50% of PTC and also found in aggressive histologic variants and PTC-derived anaplastic thyroid cancer, but is rare in follicular variants, and not found in follicular thyroid cancer. The tumorigenic role of $BRAF^{V600E}$ in the development of PTC was documented in thyroid-targeted $BRAF^{V600E}$ transgenic mice, and rat thyroid cells overexpressed with $BRAF^{V600E}$ suggested that $BRAF^{V600E}$ is an initiator of tumorigenesis and is required for tumor progression in PTC. Most clinical studies have demonstrated an association of $BRAF^{V600E}$ mutation with aggressive clinicopathologic characteristics and high tumor recurrence, although the results are controversial. The association is also observed in patients with papillary thyroid microcarcinomas and low-risk PTC. As a highly specific and unique mutation in PTC, testing for $BRAF^{V600E}$ in fine-needle aspiration specimens has been shown to refine the diagnostic accuracy of PTC in indeterminate cytology. Preoperative $BRAF^{V600E}$ analysis in low-risk patients may provide important value for prognostication, and these patients might benefit from receiving more intensive management and frequent follow-up. BRAF-targeted therapies have been developed to treat various human cancers including advanced thyroid cancers. Preclinical results are encouraging, but the anticancer effects of clinical trials are disappointing. Studies of multi-kinase inhibitors and/or combination with other regimens are underway in the treatment of advanced thyroid cancers. In this article, we review the pathogenesis of PTC, and the clinical implications of $BRAF^{V600E}$ mutation in the diagnosis, prognosis and potential targeted therapeutic strategies for thyroid cancers. [*J Chin Med Assoc* 2010;73(3):113–128]

Key Words: BRAF mutation, fine-needle aspiration cytology, papillary thyroid cancer

Introduction

Thyroid cancer is the most common endocrine malignancy and accounts for ~1% of all cancers. In the United States, it was estimated that 37,200 men and women (10,000 men, 27,200 women) would be diagnosed with thyroid cancer in 2009.[1] The incidence of thyroid cancer has increased ~50% since 1973, and it is the most rapidly increasing cancer among women and the 2nd most among men. Follicular cell-derived thyroid cancers are classified into papillary thyroid cancer (PTC), follicular thyroid cancer (FTC), and anaplastic thyroid cancer (ATC). PTC is the most common type and accounts for 85–90% of all thyroid malignancies.[2,3] Differentiated thyroid cancers (DTC) including PTC and FTC exhibit evidence of follicular epithelial cell differentiation such as iodine uptake and organification, and are usually treated successfully by primary surgical excision, radioiodine therapy, and levothyroxine suppression. The overall 10-year relative survival rate of DTC is over 90%.[1,4] However, up to 35% of patients suffered from disease recurrence during a 40-year follow-up, and over 1,600 people in the United States and 35,000 worldwide die of thyroid cancer each year. The death rate for thyroid cancer in the United States is ~0.5 per 100,000 each year.[1,5,6] Thyroid cancer with undifferentiated/aggressive histologic variants, or loss of iodine uptake due to subsequent dedifferentiation, are often inoperable and exhibit poor response to radioiodine therapy, leading

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to a high recurrence rate and unfavorable prognosis. Traditional chemotherapy has low response rates, and long-term efficacy is unsatisfactory.[7,8] There is currently no effective treatment for these patients.

Conventional treatment strategies for DTC are based on various staging systems (such as the TNM system), which are designed according to a patient's clinicopathological characteristics, allowing patients at low risk to undergo less intensive therapy and less frequent follow-up than patients at high risk.[9,10] Unfortunately, 15% of tumor recurrence after a median of 11 years' follow-up, and over 10% of cancer deaths were initially classified as low risk.[11,12] Therefore, more accurate risk stratification and effective treatment for advanced thyroid cancer are important to reduce tumor recurrence and mortality.

Recently, significant progress has been made in the understanding of the B-type Raf kinase (BRAF) mutation and the mitogen-activated protein kinase (MAPK) pathway in the tumorigenesis of human cancers.[13,14] Thyroid cancers, particularly PTCs, are frequently found to have genetic alterations. A thymidine-to-adenosine transversion at exon 15 nucleotide 1799 (T1799A) of the BRAF gene, resulting in the replacement of valine with glutamic acid at position 600 ($BRAF^{V600E}$), is the most prevalent mutation in PTC.[14] As a result, BRAF mutation has recently been the subject of intensive study to investigate its tumorigenic role and its clinical implications.[15–17] In this article, we review the mechanism of $BRAF^{V600E}$ mutation and MAPK signal transduction pathway in the pathogenesis of PTC, the clinical implications of $BRAF^{V600E}$ mutation in preoperative diagnosis and prognostic stratification, and recent advances in the use of the $BRAF^{V600E}$ mutation as a potential target of therapeutic strategies for thyroid cancers.

MAPK Signal Transduction Pathway

The MAPK pathway is an intracellular signal transduction pathway that is required for maintaining cellular activities such as cell growth, proliferation, differentiation, and apoptosis responsive to cell surface receptor tyrosine kinase (RTK) stimulation.[18,19] This pathway relays the extracellular signals from various growth factors, hormones and cytokines to the nucleus through the activation of signal cascades. As shown in Figure 1, the binding of the ligands to their surface RTKs lead to the dimerization of receptors and tyrosine residue autophosphorylation. The activated receptors, through adaptor proteins, activate RAS kinase. Then, RAS kinase activates the phosphorylation of Raf kinases, which in turn activate the dual-specificity protein kinases: MAP kinase kinases (MAPKK; also known as MAP/extracellular signal-regulated kinase, MEK) 1 and 2. MEK1/2 phosphorylate and activate extracellular signal-regulated kinases (ERK) 1 and 2. ERK1/2 regulate various transcription factors leading to gene expression.

RAS kinase belongs to a family of small G-proteins (KRAS, HRAS, NRAS) located on the inner surface of cell membranes and function as a GTPase, switching between active GTP-bound form and inactive GDP-bound form. Cycling between GDP/GTP is regulated by adaptors (e.g. growth-factor-receptor bound-2; GRB2) and guanine nucleotide exchange factors (e.g. son of sevenless; SOS). These proteins facilitate the RAS active GTP-bound form formation, and RAS GTPase catalyzes GTP hydrolysis, resulting in return to its inactive GDP-bound form.[20–22] Raf kinase was the first identified and most characterized downstream cytosolic effector of RAS.[23,24] It belongs to a family of serine/threonine kinases (A-Raf, B-Raf, and C-Raf or Raf-1), and all isoforms share 3 common conserved regions—CR1 (RAS-binding domain and cysteine-rich domain), CR2 (N-terminal regulatory domain), and CR3 (C-terminal kinase catalytic domain)—as well as several regulatory phosphorylation sites (Figure 2).[25,26]

The activation of Raf is a complex process taking place at the membrane, where Raf undergoes multisite phosphorylation and protein interactions before being rendered active.[17,27–29] The binding of RAS to RAS-binding domain (RBD) of Raf is regulated by dimeric adaptors such as 14-3-3 proteins that are bound to the phosphorylated proteins. BRAF contains conserved phosphorylation sites at S365 and S729 that are phosphorylated in the inactive state. Dimeric proteins 14-3-3 bind to these phosphorylated sites, creating a conformation that interferes with the binding of RAS to RBD.[29] The activation of BRAF is initiated with the recruitment of the inactive BRAF to the inner membrane, where the N-terminal 14-3-3 binding site is dephosphorylated to dissociate 14-3-3 proteins, and followed by the phosphorylation of T599 and S602 at the activation segment (Figures 1 and 2).[26] Inactive BRAF exhibits a characteristic bilobar structure by forming a hydrophobic interaction between residues G596-V600 of the activation loop and residues G464-V471 of the P loop (ATP binding sites), resulting in a conformation that the catalytic residues cannot bind to ATP. It is postulated that phosphorylation of T599 disrupts the hydrophobic interaction between these 2 loops, resulting in the binding of ATP and the activation of BRAF.[17] The basic mechanisms in the activation of the 3 Raf isoforms are similar, except that A-Raf and

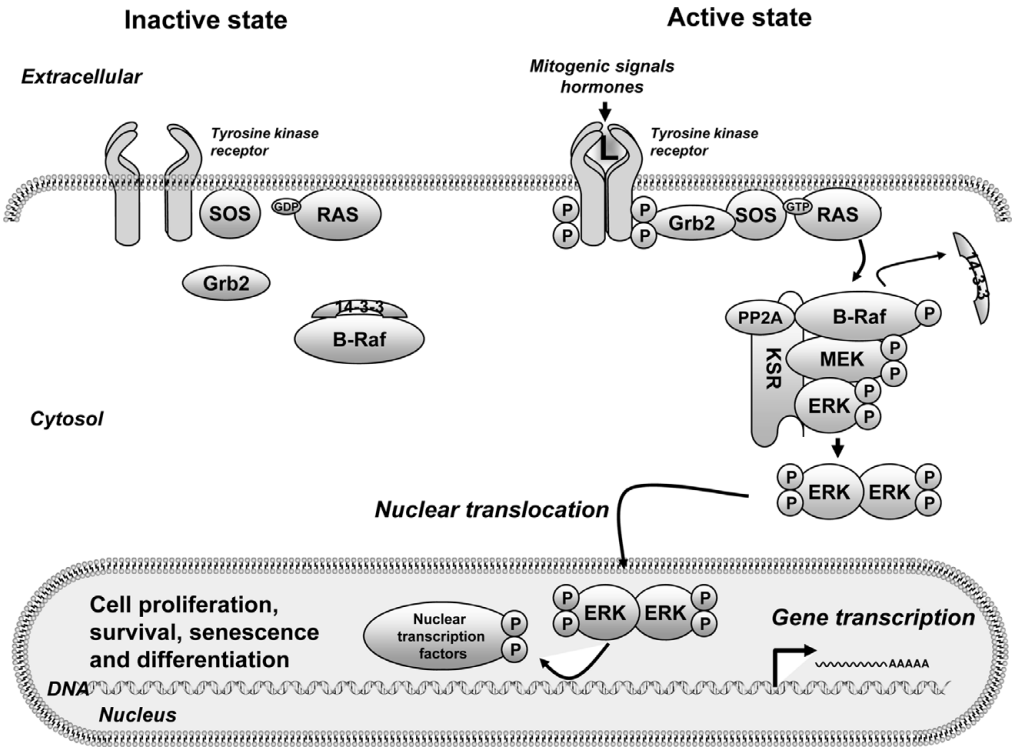

Figure 1. MAPK signal transduction pathway. In normal cells, ligands' (L) binding of the extracellular domain of their membrane tyrosine kinase receptors (RTK) triggers dimerization of the receptor → autophosphorylation of tyrosine residues in the intracellular TK domain → activation of adaptor proteins/guanine nucleotide exchange factors (e.g. Grb2 and SOS) → inactive GDP-bound form of RAS → active GTP-bound form → BRAF recruitment to the membrane → BRAF phosphorylation → MEK phosphorylation → ERK phosphorylation → nuclear translocation → ERK-induced phosphorylation of nuclear transcription factors → gene expression → proliferation, survival, senescence and differentiation. Raf–MEK–ERK kinase cascade is scaffolded by kinase suppressor of Ras (KSR). Protein phosphatase 2A (PP2A) is involved in the dephosphorylation of inhibitory sites of Raf kinases during their activation process.

C-Raf require additional kinases (e.g. SRC) and more phosphorylation steps at the N-terminal side of CR3 (N-region). Negative charge within the N-region is essential for Raf kinase activation. BRAF is constitutively phosphorylated at S446, and a regulatory tyrosine residue is occupied by an aspartic acid at D449, such that its constant negative charge will act like the phosphorylation at this site.[30] As a result, BRAF is activation-ready and only requires the RAS-mediated membrane recruitment of BRAF.

MEK1/2 are the physiological downstream effectors of BRAF. BRAF has the highest basal kinase activity and is the strongest Raf activator of downstream MEK.[31,32] Activated BRAF induces phosphorylation at 2 serine residues, S217 and S221, within the activation segment of MEK. Downstream of MEK are ERK1/2, which are activated by phosphorylation at the T202 and Y204 residues of ERK.[32] Phosphorylation of ERK activates substrates located in the nucleus and cytoplasm. The majority of ERK substrates are nuclear proteins, and the nuclear translocation of ERK phosphorylates various transcription factors, which in turn regulate gene expression.[33–35] Meticulous regulation of ERK is crucial to maintain biological homeostasis responsive to various extracellular signals. For example, hyperactivation of the ERK pathway can induce cell cycle arrest and senecense.[36,37] In contrast, aberrant activation of the pathway may induce tumor transformation (Figure 3).[13,17] The kinetics and amplitude of ERK signaling induced by different ligands can regulate biological programs differentially, such as proliferation, differentiation or apoptosis. The regulation of cellular responses is a complicated mechanism that may involve various substrates at different levels of the cascade, such as scaffold proteins and feedback inhibitors (Figure 4).[38] Apparently, tumors prefer ERK activities' programming for proliferation and survival.

Genetic Alterations in PTC

Aberrant activation of the MAPK pathway is frequently found in human cancers (Figure 3). The consistent finding of RAS and BRAF mutations in similar cancer

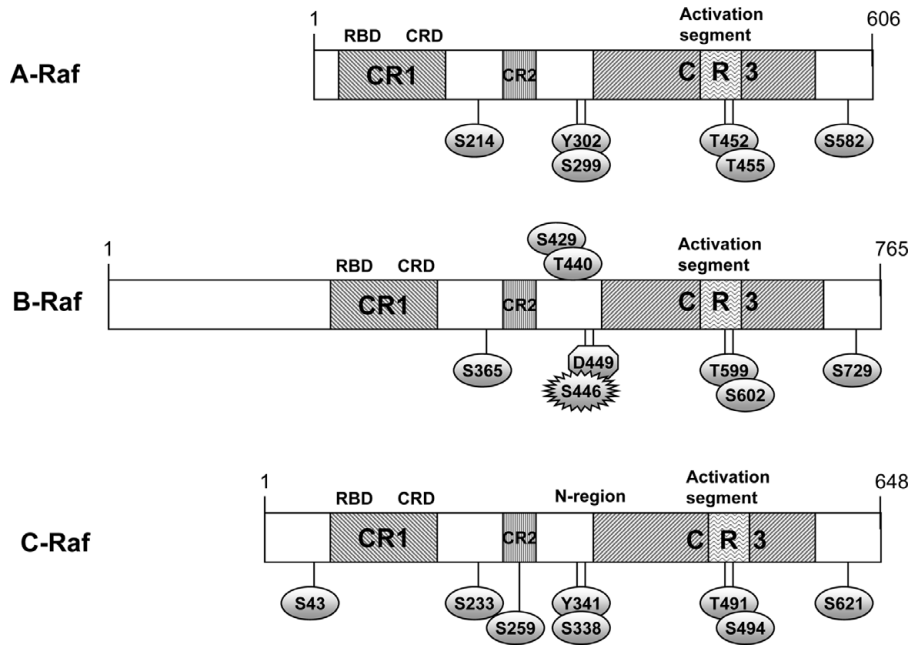

Figure 2. Structure of the Raf proteins. The Raf isoforms, A-Raf, B-Raf and C-Raf, share 3 conserved regions: CR1, CR2 and CR3. The amino acids shown refer to known phosphorylation sites. CR1 contains the RAS-binding domain (RBD) and the cysteine-rich domain (CRD), which are both required for membrane recruitment. CR2 and C-terminal contain the 14-3-3 binding sites. CR3 contains the catalytic domain (including the activation segment). The negative-charge regulatory region (N-region) contains residue C-Raf (Y341), which is conserved in A-Raf (Y302) but is replaced by aspartic acid at D449 in BRAF. S338 of C-Raf is conserved in all RAF proteins (S299 in A-Raf and S446 in BRAF), but it is constitutively phosphorylated in BRAF (star shape). The catalytic domain contains the 2 activation-segment phosphorylation sites C-Raf (T491 and S494), which are conserved in A-Raf (T452 and T455) and BRAF (T599 and S602).

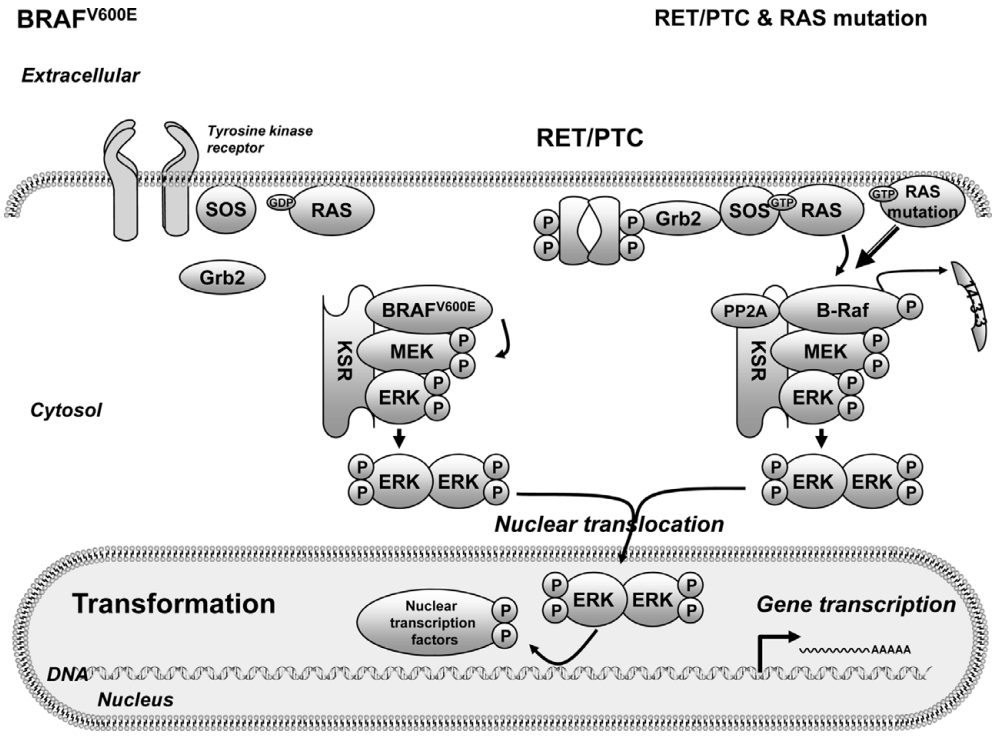

Figure 3. Activation of MAPK signaling pathway by RAS, RET/PTC and $BRAF^{V600E}$ mutations. The mechanism is similar to the physiological condition described in Figure 1, except that the signal is generated through RAS, RET/PTC and $BRAF^{V600E}$ mutations. The activation of the MAPK pathway becomes constitutive to induce cell transformation.

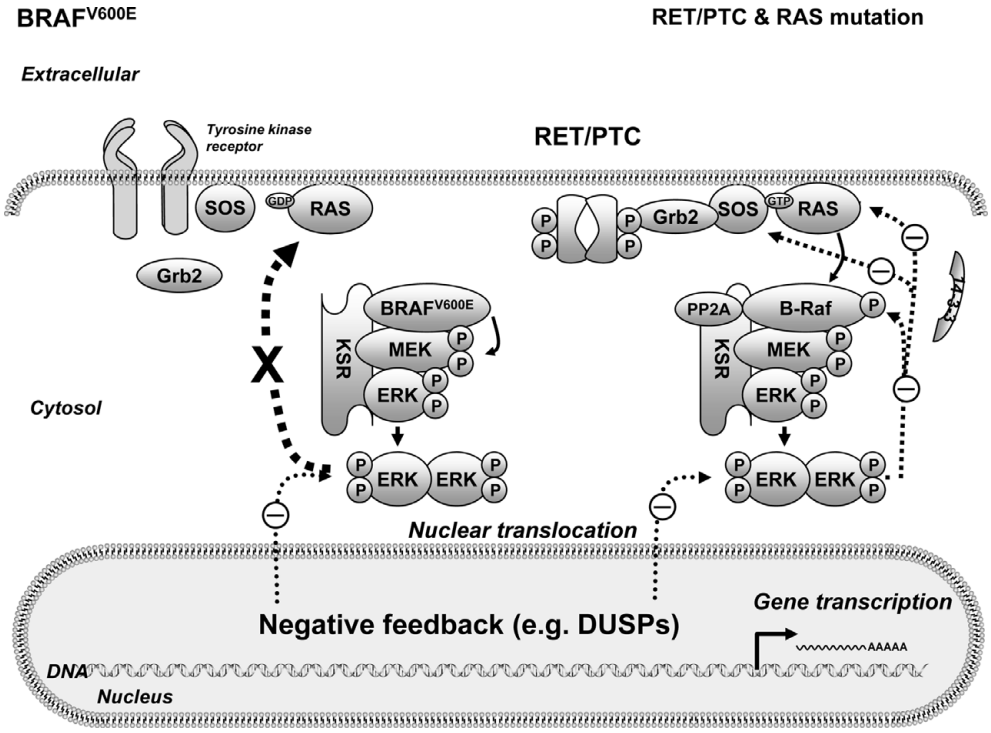

Figure 4. Proposed model of feedback inhibition in tumor cells with RET/PTC or RTKs and with BRAF^{V600E}. RET/PTC or RTKs activate the tumor cell feedback mechanism and inhibit the MAPK pathway at multiple levels. Feedback mechanism or mediators such as dual-specificity phosphatases (DUSPs) downregulate both RAF/MEK activation and ERK phosphorylation in RTK cells. BRAF^{V600E} is constitutively active and insusceptible to negative feedback.

types, and the mutation rarely involving more than 1 component of the pathway (mutually exclusive), suggest that the constitutive activation of these mutants in the pathway might be the pathogenesis of tumor formation. They also imply that a single gene mutation in the pathway is sufficient to induce cell transformation.[13,38–40] About 70% of patients with PTC are found to have genetic alterations related to the MAPK pathway, such as RET/PTC rearrangement, and RAS and BRAF mutations, indicating that the MAPK signaling pathway plays an important role in the pathogenesis of PTC.[40,41]

RET protooncogene is a tyrosine kinase receptor that is highly expressed in parafollicular C cells, but its expression is low in thyroid follicular cells. The RET gene can be activated in follicular cells by chromosomal rearrangements, linking the promoter and N-terminal domains of unrelated genes to the tyrosine kinase domain of the RET gene. The aberrant production of different chimeric forms of the receptor is known as RET/PTC. More than 11 types of RET/PTCs have been reported, and RET/PTC1 and RET/PTC3 are the most common rearrangements seen in PTCs, probably because RET/PTC1 and RET/PTC3 are intrachromosomal (chromosome 10q) paracentric inversions by fusion of the 3' portion of RET to the 5' portion of the H4 (D10S170) and NCOA4 (ELE1) genes, respectively.[42,43] The other RET/PTCs are rearranged by interchromosomal translocations. As shown in Figure 3, RET/PTCs constitutively activate the RAS/BRAF/MAPK pathway,[44–46] and the transformation of thyroid cells could be induced by overexpression of either RET/PTC1 or RET/PTC3 in cultured thyroid follicular cells and transgenic mice. Silencing the BRAF in RET/PTC-transformed follicular cells reversed the tumorigenic effect of RET/PTC, confirming that signaling along the BRAF-MAPK pathway is required for its tumorigenesis.[43,44,47–49] The prevalence of RET/PTC rearrangements in PTC is varied in different geographic regions and account for ~20% of PTC, which is particularly common in young patients and individuals with history of previous radiation exposure.[50]

RAS proteins are plasma membrane GTPases switching between active GTP-bound form and inactive GDP-bound form to activate downstream effector pathways.[20–22] Point mutations of RAS, either with increased affinity for GTP (at codons 12 and 13) or with decreased autocatalytic GTPase function (at codon 61), will withhold RAS at the active GTP-bound condition leading to the constitutive activation of the

MAPK pathway (Figure 3). Mutations of all family members of RAS genes have been reported in different types of thyroid follicular cell-derived tumors, particularly in follicular adenomas and carcinomas. However, it rarely occurs in PTCs (~10%) and is almost solely found in follicular variants.[51–53]

BRAF is highly expressed in hematopoietic cells, neurons, testis and thyroid follicular cells.[54,55] Contrary to the extremely rare mutation found in A-Raf and C-Raf, BRAF is the most common Raf mutation and is the second most common somatic mutation in all human cancers. The BRAF mutation is frequently detected in malignant melanomas, and in colon, ovarian and thyroid carcinomas.[13,14,17] Although >45 BRAF mutations have been identified in human cancers, about 90% of BRAF mutations are T → A transversion in exon 15 at nucleotide 1799 (T1799A) leading to a valine → glutamic acid replacement at position 600 ($BRAF^{V600E}$). Except for the rare BRAF mutations (K601E, AKAP9-BRAF, V600E+K601del, V599ins, V600D+FGLAT601-605ins) reported in thyroid cancer, over 90% of BRAF mutation in PTCs are $BRAF^{V600E}$.[13,56–60] The prevalence of $BRAF^{V600E}$ in PTCs varies from 29% to 83%, and is ~60% in classic PTC, ~77% in tall-cell variant, and ~25% in PTC-derived ATC, but it is rare (0–12%) in follicular-variant PTC and is not found in FTC.[15] An *in vitro* study showed that the replacement of a negative-charge residue glutamate at V600 adjacent to T599 induces an effect similar to phosphorylation at T599 and S602, which disrupts the hydrophobic interaction between the P loop and the activation loop.[17] The kinase activity of $BRAF^{V600E}$ is 460-fold higher than the wild-type BRAF, and this active conformation can constitutively activate its downstream effectors to transform normal cells or induce cancer proliferation without the need of RAS for activation.[13,17]

$BRAF^{V600E}$ mutation is frequently detected in papillary thyroid microcarcinomas (PTMC), suggesting that the mutation is an early event during PTC development.[61–65] The tumorigenic role of $BRAF^{V600E}$ in the development of PTC was documented in thyroid-targeted $BRAF^{V600E}$ transgenic mice, and tumors developed from these transgenic mice were found to progressively transform into poorly differentiated cancers with aggressive characteristics.[66] *In vitro*, $BRAF^{V600E}$-overexpressed rat thyroid cells grown on Matrigel™ showed an increase in migration of thyroid cells. It has also been reported to be associated with the upregulation of metalloproteinases (MMPs), particularly matrix MMP3, MMP9 and MMP13 genes, which are related to tumor invasion.[45,67] The proliferation of $BRAF^{V600E}$-harbored or transfected cell lines could be inhibited by MAPK pathway inhibitors or siRNA specific BRAF knockdown.[68,69] The above observations suggest that $BRAF^{V600E}$ is an initiator of tumorigenesis through the MAPK pathway, and is required for the progression of PTC.[70] Both RET/PTC and $BRAF^{V600E}$ mutations can constitutively activate MAPK pathways, resulting in follicular cell transformation. However, several studies have shown that RET/PTC-expressed rat thyroid cells induce a weaker tumorigenic effect than $BRAF^{V600E}$ mutation.[71] Conditional expression of $BRAF^{V600E}$ in thyroid cells markedly increased the Matrigel™ invasion of the transformed thyroid cells, which is more invasive than RET/PTC expressed cells.[67] Furthermore, microarray studies have also shown that human PTCs harbor $BRAF^{V600E}$ and RAS mutation, and RET/PTCs exhibit different gene expression profiles, suggesting that different mutants may affect the pathway outcomes differently. Among them, $BRAF^{V600E}$ mutation is the most potent activator in the stimulation of MAPK pathway output.[72]

Interestingly, the high kinase activity of $BRAF^{V600E}$ mutation seems to be not effectively translated to ERK activity; it only increased ERK activity 2- to 4.6-fold, suggesting the existence of regulatory mechanisms in the controlling of the signal output.[17,27] Several possible feedback mechanisms have been reported to inhibit the ERK pathway output.[73–76] ERK stimulates gene expression of feedback regulators (e.g. dual-specificity phosphatases; Sprouty) to inhibit RAS activating proteins (e.g. SOS), and also ERK's own activities (Figure 4).[73,74] Phosphorylated-ERK has been demonstrated to phosphorylate Raf directly to induce hyperphosphorylation of Raf, leading to conformational changes that might interfere with the binding of Raf to RAS, MEK or scaffold proteins.[77] The physiological role of these feedback loops is unclear, and it may aim to prevent cell cycle arrest and senescence due to hyperactivation of the ERK pathway. The constitutive activation of the MAPK signal pathway by RAS and $BRAF^{V600E}$ mutations was also found to induce feedback downregulation similar to the physiological condition.[78] Conceivably, these oncoproteins might take steps to minimize the effects of feedback inhibition by either insensitivity of the mutant protein to normal negative feedback or directly affecting the mediators of the feedback mechanism. Recent investigation demonstrated that feedback inhibition of Raf/MEK signaling was found to downregulate ERK output in RTK cells, but not in $BRAF^{V600E}$ cells (Figure 4). The evasion of the feedback mechanism in $BRAF^{V600E}$ cells was evidenced by the increase in transcriptional output and MEK/ERK-dependent transformation. This phenomenon may partly explain

the stronger tumorigenic effect observed in the $BRAF^{V600E}$-expressed cells than in the RET/PTC-expressed cells.[78]

Tumorigenesis is a complex process involving multiple signaling networks instead of a single linear unidirectional cascade of the MAPK pathway. The high prevalence of $BRAF^{V600E}$ mutation in tall-cell variant and PTC-derived ATC suggests that $BRAF^{V600E}$ mutations may play a role in the progression of PTC to more aggressive thyroid carcinomas.[15] Recent studies have shown that induction of $BRAF^{V600E}$ expression in rat thyroid cells facilitated the acquisition of secondary genetic events through induction of genomic instability, but not in RET/PTC-expressed cells.[79] Genetic alternations in the PI3K/Akt pathway and PTEN have also been found in thyroid cancers such as ATC and metastatic tumors from radioactive iodine-refractory (RAIR) PTC, particularly in the later stages of cancer progression.[80] Aberrant activation of the PI3K/Akt pathway is often coexistent with $BRAF^{V600E}$ in ATC and RAIR tumors, suggesting that the genetic instability induced by the primary $BRAF^{V600E}$ mutation in PTC may facilitate the secondary genetic alternations involving the PI3K/Akt pathway. The secondary mutation might lead to the progression of DTC to the more aggressive thyroid cancer, and dedifferentiation of the cancer cells.[81] This hypothesis is supported by observations that tumors in thyroid-targeted $BRAF^{V600E}$ transgenic mice progressed to more aggressive phenotype, and $BRAF^{V600E}$ mutation is associated with advanced patient age and not frequently detected in childhood PTCs.[15,66,82] Further investigations of the feedback and alternation pathways are important in understanding the mechanisms involved in the tumorigenesis of PTC, which might provide valuable information regarding clinical implications.

Clinical Implications Associated With $BRAF^{V600E}$ Mutation in PTC

Recently, $BRAF^{V600E}$ has taken center stage due to the findings that it may be associated with tumorigenesis and aggressiveness. Many studies have been done to investigate the clinicopathologic characteristics, and the potential utility of $BRAF^{V600E}$ mutation on the diagnostic, prognostic and therapeutic aspects of PTC. Unlike the highly consistent results obtained from *in vitro* studies, the current clinical data show controversial results regarding $BRAF^{V600E}$ mutation as a genetic prognostic marker of PTC.

Environmental and various predisposing factors have been reported to increase the risk of thyroid cancer, such as radiation exposure, dietary iodine, genetics and life style.[83] Radiation-associated PTCs are usually associated with RET/PTC and to a lesser extent with NTRK1, but not with $BRAF^{V600E}$ mutation.[84–86] Ciampi et al[57] reported that AKAP9-BRAF fusion was more commonly found in radiation-induced PTC than sporadic PTC. AKAP9-BRAF is caused by paracentric inversion of chromosome 7q, resulting in an in-frame fusion between exons 1–8 of the AKAP9 gene and exons 9–10 of BRAF.[87] This implies that radiation-induced PTCs are likely caused by the chromosome-paracentric inversion linked to constitutive activators, while sporadic PTCs are predominantly activated by point mutations on the effector kinases of the MAPK pathway. In Italy, a higher incidence of thyroid cancer and $BRAF^{V600E}$-PTC were found in people residing in Eastern Sicily, including the volcanic area of Etna (45.9%), than people in Western Sicily (22.7%). Iodine deficiency was not found to be the cause of the difference, and the unidentified carcinogens were suspected to be the volcanic soil, water or atmosphere.[88] A large Chinese epidemiological study of 1,032 conventional PTCs demonstrated that cities with high iodine content had significantly higher incidence of $BRAF^{V600E}$ mutation (69%) than cities with normal iodine content (53%). The results suggest that high iodine intake is a risk factor for $BRAF^{V600E}$ mutation and may therefore be a risk factor for PTC development.[89]

Over 30 studies on the relationship between $BRAF^{V600E}$ and the clinicopathological characteristics in PTC have been reported worldwide. The majority of them suggested that $BRAF^{V600E}$ mutation was associated with advanced disease stages and aggressive phenotype, while others did not find this association; the results remain controversial to date. Several studies reported a significant association of $BRAF^{V600E}$ mutation with high-risk clinicopathological characteristics such as older age,[65,90–96] male sex,[97,98] tumor size,[88,98–101] and aggressive subtype.[63,65,92,94,102–104] Many studies found that extrathyroidal invasion, lymph node metastasis, and advanced stages III/VI are the 3 most common risk factors consistently associated with $BRAF^{V600E}$ mutation.[63,65,87,88,90–96,98–100,102,105,106] Oler et al[60] and Vasko et al[107] observed that $BRAF^{V600E}$ mutation in lymph node metastasis was occasionally not found in their primary lesion, suggesting that tumor cells that acquire the mutation *de novo* are probably prompted to metastasis. Rodolico et al[95] further demonstrated that metastatic lymph nodes harboring the $BRAF^{V600E}$ mutation were larger in size and had a higher prevalence of extracapsular invasion than those without the mutation. However, other studies on paired primary and lymph node metastatic lesions did

not find discordant mutation in most of the lesion pairs, indicating that the acquisition of $BRAF^{V600E}$ mutations are not a requirement in the progression from localized to metastatic PTC.[92,105,108] In a large Italian cohort study, Lupi et al[63] found that $BRAF^{V600E}$ mutation was associated with the absence of tumor capsule, particularly in follicular- and micro-PTC variants, but not in conventional variant. Two meta-analyses also reported an association of $BRAF^{V600E}$ mutation with extrathyroidal invasion, aggressive histotype and advanced disease stages, but not with age, sex, or tumor size, and the association of $BRAF^{V600E}$ mutation with lymph node metastasis is not a uniform finding.[15,109] In fact, a recent large Chinese cohort study investigating the association of iodine intake with $BRAF^{V600E}$ mutation in different cities demonstrated that overall results of extrathyroidal invasion, lymph node metastasis and advanced disease stages were significantly associated with $BRAF^{V600E}$ mutation, but the association with extrathyroidal invasion and lymph node metastasis were not seen in all cities when analyzed city by city.[89]

Clinicopathological characteristics and staging systems are designed to predict tumor recurrence and disease prognosis.[9] Three studies, including a multicenter study of 219 patients, an American study of 245 conventional PTC cases, and an Italian study of 102 patients, demonstrated that $BRAF^{V600E}$ mutation was associated with aggressive clinicopathological features, and was also an independent predictor of tumor recurrence after a median of 15 months, 6 years, and 15 years of follow-up, respectively.[92,100,106] Kim et al[98] reported that $BRAF^{V600E}$ mutation was associated with tumor recurrence in 203 conventional PTC follow-ups for a median of 7.3 years, but was not an independent predictor after adjustment for clinicopathological prognostic factors. More importantly, the association was also observed in patients with low disease stages I/II, and conventional PTC.[92,98,106] Another finding from a study of 54 recurrent PTCs showed that 77.8% of tumors were found to harbor $BRAF^{V600E}$ mutation and 9.3% had both BRAF and RET/PTC mutations.[110] The results give further support to the premise that secondary mutations may cause tumors to progress to a more aggressive status. In contrast to the studies above, in a recent large Japanese cohort study of 631 patients with PTC who were followed-up for a median of 83 months, neither clinicopathological characteristics nor tumor recurrence was associated with $BRAF^{V600E}$ mutation.[111] A number of studies from various ethnic groups and geographic regions also did not find any association of $BRAF^{V600E}$ mutation with the aggressive clinicopathological features.[62,64,112–119] The conflicting results of these studies might be due to variations in the study populations in terms of size, age distribution, histological variants, genetic factors, environmental factors, disease stages at the time of initial diagnosis, and the methods or criteria used.

Some studies demonstrated that $BRAF^{V600E}$-mutated PTC is associated with high recurrence rate, and a decrease in radioiodine uptake in the recurrent tumor.[102,106] These observations were supported by a recent study of patients with RAIR-differentiated PTC, in which 62% of patients were found to be $BRAF^{V600E}$-positive, and 54% were 18F-fluorodeoxyglucose positron emission tomography (18-FDG-PET)-positive. Interestingly, all of the 18-FDG-PET-positive patients were found to be $BRAF^{V600E}$-positive.[80] In the process of thyroid hormone synthesis, inorganic iodine is actively transported into the thyroid cells via a basal membrane protein—sodium iodide symporter (NIS). Iodide is, in turn, transported into the follicle via an apical protein—pendrin, where iodide is oxidized by thyroid peroxidase (TPO) and incorporated into thyroglobulin in the synthesis of thyroid hormone. This process is regulated by thyroid-stimulating hormone (TSH) through the binding of the membranous TSH receptor (TSHR). Recent immunohistochemistry study showed that tumor tissues with $BRAF^{V600E}$ mutation had lower NIS expression, and failure of NIS targeted to the membrane when compared with PTC without the mutation.[102] $BRAF^{V600E}$ mutation was also found to be associated with a decrease in gene expression of TSHR, TPO, NIS, thyroglobulin and pendrin in primary or recurrent tumors.[72,99,102,113,120,121] Instead of iodide-metabolizing gene silencing, glucose transporter-1 (GLUT-1) expression was found to be increased in PTC, which was significantly higher in tumors with $BRAF^{V600E}$ mutation than wild-type.[120] These data support the biological basis for the clinical use of 18-FDG-PET to detect recurrent/metastatic lesions in patients with RAIR PTC. Recently, Romei et al[119] reported that there was neither association of $BRAF^{V600E}$ mutation with clinicopathological characteristics nor with GLUT-1/3 expression in PTC, but there was consistently lower expressions of NIS and TPO in $BRAF^{V600E}$-mutated PTC; the lower expressions of NIS and TPO were not seen in PTC with RET/PTC rearrangement. A recent Brazilian study also reported that decreased NIS gene expression was found in conventional PTC and PTMC harboring $BRAF^{V600E}$ mutation.[99] *In vitro*, conditional $BRAF^{V600E}$ expression in rat thyroid cell lines suppressed iodide-metabolizing genes. Inhibition of MAPK pathway or silencing BRAF using inhibitors or siRNA restored the expression of the iodide-metabolizing genes.[79,102,122]

The results indicate that the MAPK pathway plays an important role in the regulation of iodide-metabolizing genes, and several studies further demonstrated that the MAPK pathway promotes expression of DNA methyltransferase, which silences these genes through promoter methylation.[122–124]

The current treatment strategies for PTC allow patients at low risk to undergo less intensive postoperative adjunctive therapy and less frequent follow-up than patients at high risk, whereas patients in advanced stages, with aggressive histotype, or at high risk are managed by more aggressive therapy such as total/near total thyroidectomy, lymph node dissection, adjuvant radioiodine ablation and thyroid hormone suppression therapy.[10,125,126] For patients at high risk, the preoperative diagnosis of $BRAF^{V600E}$ mutation seems to have little additional value in the current treatment and follow-up protocols. However, the majority of patients with DTC are classified as low risk, and many of them suffer from tumor recurrence in later years. Testing for $BRAF^{V600E}$ mutation might be helpful in tailoring the therapeutic strategies for these patients, in case $BRAF^{V600E}$ mutation is proven to be a marker of poor prognosis.

The incidence of PTMC (tumor size < 1 cm in diameter) has dramatically increased since the introduction of high-resolution ultrasound-guided fine-needle aspiration (FNA) biopsy for patients with nodular thyroid disease. The prevalence of PTMC has increased approximately 2- to 4-fold in various countries during the last 2 decades, and the rapid rise of PTC in recent years is mainly due to the increased rate of diagnosis of PTMC.[127–130] PTMC accounts for approximately a quarter of thyroid cancers.[131] It is generally considered to be a low-risk cancer, and most cases are classified as stages I/II. Recommended treatment for these small low-risk tumors, in the absence of known risk factors or lymph node metastasis, is lobectomy with or without isthmectomy.[10,125] However, multifocality, extrathyroidal extension, and lymph node metastasis are often reported in PTMC, with incidences of 7.1–56.8%, 2–62.1%, and 0–64%, respectively.[131] Several studies have compared the clinical and histologic characteristics between PTC and incidental or nonincidental PTMC, and found that the prevalence of multifocality, extrathyroidal extension and lymph node metastasis are similar in PTMC and PTC. The aggressive phenotypes are more frequently found in nonincidental PTMC and PTMC with size larger than 5 mm and 8 mm, respectively.[132–135] $BRAF^{V600E}$ mutation is also the most common genetic alteration in PTMC, accounting for 17–65.6%, with incidence lower than that of PTC in general.[61–65,88,136] Recently, several studies have investigated the relationship between clinicopathological characteristics and $BRAF^{V600E}$-mutated PTMC. Park et al[136] reported a surprisingly high prevalence of extrathyroidal invasion (52.2%) and lymph node metastasis (32.9%) in PTMC; the frequency of $BRAF^{V600E}$ mutation and the recurrence rate of PTMC were similar to those of PTC. Lupi et al[63] and Frasca et al[88] observed that $BRAF^{V600E}$-PTMC was associated with extrathyroidal extension and advanced disease stages. Their findings are consistent with the recent report from Lee et al[137] that more $BRAF^{V600E}$ mutation was detected in PTMC with advanced disease stages, extrathyroidal extension, and nodal metastasis than in those without these aggressive clinicopathological characteristics. Ugolini et al[61] reported an association of $BRAF^{V600E}$ mutation with the lack of tumor capsule in PTMC. Rodolico et al[95] further found that $BRAF^{V600E}$ mutation was associated with lymph node metastases, a wider diameter of the largest metastatic area, a higher number of involved lymph nodes, and a higher percentage of metastatic lesions with extracapsular extension in PTMC. Together, the clinicopathological characteristics of $BRAF^{V600E}$-PTMC seem to be no different from those of its larger counterpart, and $BRAF^{V600E}$-PTMCs exhibit signs of greater aggressiveness and higher recurrence rate than wild-type. Most patients with PTMC are classified as stages I/II. According to current treatment strategies, these patients might receive "inadequate" treatment and less frequent follow-up. Thus, some investigators have suggested evaluating $BRAF^{V600E}$ mutation in these patients preoperatively, and treating patients with positive $BRAF^{V600E}$ mutation more aggressively.

Use of BRAF Mutation for Preoperative Diagnosis

Preoperative evaluation of nodular goiter is based on FNA cytology to select patients for surgical treatment or medical follow-up.[138] As $BRAF^{V600E}$ mutation and RET/PTC rearrangements are exclusively found in PTC, examining these markers in DNA specimens obtained from FNA can make a diagnosis in most PTCs. Salvatore et al[139] detected 38% and 18% of $BRAF^{V600E}$ and RET/PTC in FNA samples, respectively. The identification of $BRAF^{V600E}$ mutation and RET/PTC refined the diagnosis of PTC in 5 of 15 samples that were considered either indeterminate or insufficient at cytology. Using FNA $BRAF^{V600E}$ analysis, Cohen et al[140] confirmed the $BRAF^{V600E}$ mutation in 72% of carcinomas within the malignant group, and established the diagnosis of PTC in 16% of the

indeterminate group. No $BRAF^{V600E}$ mutation was detected in the benign group. Two recent studies from Marchetti et al[141] and Zatelli et al[142] demonstrated that combining traditional cytology and molecular analysis of $BRAF^{V600E}$ mutation on FNA specimens improved the diagnostic accuracy of PTCs from 62.3% to 82.2% and from 77.3% to 86.7%, respectively. However, $BRAF^{V600E}$ mutation is only positive in ~50% of PTCs, and negative results cannot exclude malignancy. Therefore, the sensitivity of $BRAF^{V600E}$ mutation analysis for PTC diagnosis is limited, although the specificity is high. Most of the indeterminate specimens are follicular neoplasm and follicular variant of PTC, which are rarely found to harbor $BRAF^{V600E}$ mutation. Inadequate FNA may lead to insufficient tumor DNA recovery from the nucleic acid preparations, which might lead to false-negative results. In fact, traditional FNA cytology by expert pathologists can provide reliable information on PTCs with an overall accuracy >90%.[138] Therefore, the value of routine $BRAF^{V600E}$ mutation analysis for PTC diagnosis in FNA is marginal. It is more reasonable to reserve the test for patients with indeterminate/inadequate FNA cytology, which may improve the diagnostic yield in these patients.

A recent study reported that RAS and PAX8/PPAR gene analysis in addition to $BRAF^{V600E}$ and RET/PTC analysis in FNA specimens enhanced the diagnostic accuracy of FNA cytology, particularly in indeterminate cytology. In the study, 97% of nodules with positive mutations were ultimately found to be malignant, suggesting that additional RAS and PAX8/PPAR analysis improved the diagnostic accuracy of indeterminate cytology which are predominantly follicular neoplasm and follicular variant of PTC.[143] Xing et al[144] investigated the utility of $BRAF^{V600E}$ mutation analysis of FNA specimens for preoperative risk stratification in PTC. Their results showed a significant association of $BRAF^{V600E}$ mutation with poor clinicopathological outcomes, and $BRAF^{V600E}$ mutation predicted extrathyroidal extension, thyroid capsular invasion, and lymph node metastasis. More importantly, 36% of PTCs with $BRAF^{V600E}$ mutation were found to have tumor persistence/recurrence, compared with 12% of PTCs without $BRAF^{V600E}$ mutation, after a median of 3 years' follow-up (odds ratio, 4.16). The positive and negative predictive values for the test to predict tumor persistence/recurrence were 36% and 88% for all PTCs, and 34% and 92% for conventional PTCs, respectively.[144] Preoperative FNA for $BRAF^{V600E}$ diagnosis seems to be helpful for tailoring the treatment strategies for PTC with low grade and for PTMC. However, further investigations in large randomized, controlled, prospective trials are necessary to confirm the role of $BRAF^{V600E}$ mutation as a clinically useful prognostic marker.

Use of BRAF Mutation for Therapeutic Decisions

As mentioned above, the constitutive activation of $BRAF^{V600E}$ mutation in the MAPK pathway seems to be the cause of tumorigenesis and progression in PTC.[13,14,17] Using inhibitors that target BRAF kinase or its downstream effectors is the logical therapeutic approach to inhibit tumor growth and progression of PTCs. Different trials have evaluated the anticancer effects of BRAF inhibitors, and the preclinical results are encouraging. Nonselective BRAF inhibitors AAL-881 and LBT-613 are isoquinolone compounds that have been shown to inhibit cell cycle progression from S-phase to G2-M phase and G0-G1 arrest, resulting in growth reduction and apoptosis in several thyroid cancer cell lines and in xenograft tumors.[68] Specific knockdown of $BRAF^{V600E}$ by siRNA inhibited the growth of ATC cell lines, the growth and transformation of $BRAF^{V600E}$-mutated PTC cells, and proliferation and tumorigenesis in xenograft tumors.[69,70] PLX4032, a small-molecule-specific BRAF inhibitor, arrested the cell growth of ATC cells and NPA human thyroid cancer cell lines harboring $BRAF^{V600E}$ mutation.[145] Prolonged treatment of ATC cells with PLX4032 induced the re-expression of NIS. In thyroid cancer cell lines bearing the RET/PTC1 and wild-type BRAF, PLX4032 showed an approximately 50-fold higher IC50 value than $BRAF^{V600E}$ cell lines, indicating that PLX4032 has selective growth inhibitory effect on $BRAF^{V600E}$-mutated thyroid cancer cells.[145] U0126 is a MEK inhibitor that has been reported to inhibit the growth of the thyroid cell-expressed $BRAF^{V600E}$ and restore the expression of iodide-metabolizing genes.[122] AZD6244 is a potent MEK1/2 inhibitor that has been demonstrated to inhibit ERK phosphorylation in thyroid cancer cell lines regardless of the status of $BRAF^{V600E}$ mutation, and the dose required to inhibit the cell growth in 4 $BRAF^{V600E}$ mutant cell lines is lower than that for the 2 wild-type cell lines. AZD6244 has also been shown to inhibit the growth of xenograft tumors derived from $BRAF^{V600E}$ mutant ATC cell lines.[146] Another small-molecule potent MEK1/2-selective inhibitor, CI-1040 (PD-184352), inhibited cancer cell proliferation and tumor xenografts derived from various cancer cells harboring $BRAF^{V600E}$ or RAS mutations.[147] CI-1040 inhibited the growth and induced re-expression of

some thyroid genes in thyroid cancer cell lines with $BRAF^{V600E}$ mutation, but not in cells with RET/PTC or wild-type alleles.[148] The results suggest that the inhibition effects of CI-1040 on tumor cell proliferation are BRAF or RAS mutation-selective. CI-1040 is the first MEK inhibitor to enter clinical trials to evaluate its performance in the treatment of lung, colon, breast and pancreatic cancers. However, no significant clinical anticancer effect was observed.[149,150]

BAY43-9006 (sorafenib), is the most studied multi-kinase inhibitor for targeting BRAF and angiogenesis-related RTK. It has been shown to inhibit the proliferation of ATC lines and tumor xenografts. However, the effect seems to be caused by blocking angiogenesis via VEGFR signaling rather than by inhibiting BRAF selectively.[151] Several clinical trials that studied sorafenib monotherapy for the treatment of various malignancies including iodine-resistant thyroid cancer have been completed recently. Although phase I trials showed encouraging results that sorafenib was a well tolerated agent, phase II trials showed little or no antitumor effects in advanced melanoma patients when sorafenib was used as a single-agent therapy.[152] Recently, a longer than 16-week phase II trial of sorafenib in 30 patients with metastatic iodine-refractory thyroid carcinoma showed an overall clinical benefit of 77%, 70% with thyroglobulin reduction, and a median 79-week progression-free survival.[153] Another phase II trial of sorafenib in patients with metastatic thyroid cancer also showed a similar antitumor activity, with a median progression-free survival of ~64 weeks, and a reduction in the levels of VEGFR phosphorylation, ERK phosphorylation, and VEGF expression in tumor biopsies.[154] To date, there is no evidence to show that the antitumor effects of sorafenib are through the inhibition of BRAF. Sorafenib is a multi-kinase inhibitor that may also target other kinase pathways such as VEGFR to inhibit tumorigenesis. Indeed, antitumor effects were also observed with other kinase inhibitors such as axitinib and motesanib that inhibit VEGFRs and PDGFRs.[155,156] Factors other than BRAF mutation may affect tumor response to sorafenib, and the combination of different kinase inhibitors and/or chemotherapy may be a potential therapeutic strategy in the future.

Conclusions and Perspectives

The discovery that $BRAF^{V600E}$ is the most common mutation in PTC, and molecular studies demonstrating its tumorigenic role in PTC, suggest that BRAF mutation is the initiator of PTC. The notion of the inherited strong kinase potency of $BRAF^{V600E}$ with the ability to induce genetic instability, silence iodide-metabolizing genes, and evade the feedback mechanisms which may promote the progression and aggressiveness of PTCs is comprehensible. Indeed, the majority of clinical studies that support the association of $BRAF^{V600E}$ with aggressive clinicopathological characteristics and higher tumor recurrence make $BRAF^{V600E}$ mutation a potential diagnostic and prognostic marker, although quite a number of studies did not find any association. Using $BRAF^{V600E}$ mutation in FNA specimens as a diagnostic marker to improve the diagnostic accuracy of PTC in indeterminate and inadequate cytology is feasible. However, the high frequency of follicular neoplasm and follicular-variant PTCs in indeterminate samples rarely being $BRAF^{V600E}$-positive, and the low tumor DNA yield in inadequate specimens, limit the clinical use of the test. Multiple-genotype analysis, such as for RAS and PAX8-PPAR, improves the diagnostic accuracy in indeterminate cytology but may cause false-positive results because RAS mutation is also positive in follicular adenomas. It seems that future discovery of more specific markers is the ultimate solution for this issue.

PTC is a relatively benign cancer when compared with other malignancies, and the majority of patients can be cured after the initial treatment with an optimistic outcome. Preoperative $BRAF^{V600E}$ analysis might have value for predicting which patients with low-grade disease classified by the current staging systems will eventually have an aggressive clinical course. It would be beneficial for these patients (including $BRAF^{V600E}$-mutated PTMC) to receive more intensive primary treatment, higher dose of radio-iodide ablation, and frequent follow-up to reduce the risk of tumor metastasis and recurrence later in the course of tumor progression. It might also be clinically indicated to use 18-FDG-PET to detect early recurrence of RAIR PTC. It is noteworthy that there is currently no effective therapy for tumors that are inoperable or lose iodine avidity. However, current available data regarding the negative clinical implications of $BRAF^{V600E}$ mutation in low-risk PTC are inconclusive. Further investigations in large randomized, controlled, prospective trials are necessary to confirm the prognostic role of $BRAF^{V600E}$ mutation before it is applied in routine clinical practice.

To date, the understanding of *in vitro* molecular pathways involved in thyroid carcinogenesis supports the rationale to develop BRAF-targeted therapies for PTC. Although the preclinical results are encouraging, the anticancer effects of clinical trials for specific BRAF-targeted therapies are unsatisfactory. Tumorigenesis is a complex process that involves other signal pathways

to effect tumor aggressiveness and progression. As new targets are disclosed, future research might use selective target inhibitors or multi-kinase inhibitors alone or in combination with other regimens (e.g. cytotoxic drugs, radioiodine therapy after re-expression of thyroid-specific genes) for the treatment of advanced thyroid cancers, with the hope that these approaches can provide satisfactory results.

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