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The role of BRAF in the pathogenesis of thyroid carcinoma

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1. ABSTRACT

BRAF is a cytoplasmic serine-threonine protein kinasethat plays a critical role in the MAPK signaling pathway. BRAF is the only member of the RAF family activated by mutation in human cancers. A single amino acid substitution (V600E) accounts for a multitude of human cancers, which causes constitutive kinase activity. In papillary thyroid cancer (PTC) and papillary-derived anaplastic thyroid cancer (ATC), the $BRAF^{V600E}$ mutation promotes follicular cell transformation. The $BRAF^{V600E}$ mutation could provide valuable prognostic information for thyroid cancer, because this mutation has been correlated with more aggressive and iodine-resistant phenotypes. Evidence has also shown that the detection of the $BRAF^{V600E}$ mutation in cancer is crucial in order to identify novel avenues for thyroid cancer treatment. Based on the BRAF kinase structure, novel drugs can potentially be designed to target oncogenic BRAF in cancer therapeutics. This review will focus on the recent progress in understandingthe functions of BRAF, the role of the BRAF mutations in thyroid carcinoma, and the correlation between BRAF mutations and cancer microenvironment.

2. INTRODUCTION

Thyroid carcinoma is the most common endocrine cancer and accounts for ~1% of all cancers. The thyroid gland is composed of two hormone-producing cell types: follicular cells and parafollicular cells.Follicular cells incorporate iodine to produce thyroid hormone, while parafollicular cells are much less prevalent and produce calcitoninto regulate calcium levels. Follicular cell-derived thyroid carcinomas are usuallyclassified into four types of cancers: papillary thyroid cancer, follicular thyroid cancer (FTC), anaplastic thyroid cancer, and medullar carcinoma.

The incidence of thyroid cancer is rapidlyincreasingthroughout the world, especially among women.PTC is the most common type and accounts for approximately 80% of all thyroid malignancies (1). Differentiated thyroid cancer, including PTC, is usually curable and has an excellent prognosisafterstandard surgical treatment, radioiodine ablation therapy, and levothyroxine suppression. Furthermore, thyroid cancer treatment has been aided through the use of thyroid ultrasound and fine-needle aspiration.Thyroid ultrasonography, which is a noninvasive imaging technique, is becoming increasingly important method used to evaluate thyroid nodules. Recently, a new technique named virtual touch tissue quantification of acoustic radiation force impulse, has a higher diagnostic performance than conventional elasticity imaging (2-5). However, many patients still succumb to thyroid carcinoma. Thyroid cancers can present undifferentiated variants and a loss function of iodine uptake, causing resistance to radioiodine therapy. These patients thus have a high recurrence rate and poor prognosis, and there are no effective therapeutic options.

Thyroid carcinomas carry several highly prevalent genetic alterations involving the mitogen-activated protein kinase (MAPK) pathway, some of which are exclusive tothis type of cancer. These oncogenic alterations include: RAS mutations (6, 7), RET/PTC rearrangements (8, 9), B-type RAF kinase (BRAF)

mutationsand PAX8-peroxisome proliferator-activated receptor γ (PPARγ) rearrangements (10, 11). In PTC, RET/PTC rearrangements,BRAF point mutations, and RAS mutations are found in over 70% of papillary carcinomas and rarely overlap in the same cancer (12).

The BRAF gene is highly mutated in tumor cells; over 40 different mutations have been identified. The $BRAF^{V600E}$ mutation is the most common and accounts for more than 90% of all mutations found in the BRAF gene (13). Moreover, this mutation is frequently present in thyroid cancer (14-19). Intriguingly, the $BRAF^{V600E}$ mutation in thyroid cancer occurs in approximately 50% of PTC and PTC-derived anaplastic thyroid carcinoma cases, but rarely occursin follicular thyroid carcinoma or other types of thyroid tumors (20).

PTC in patients harboring the $BRAF^{V600E}$ mutation appears to display a more aggressive clinical behavior but little is known about the role of this mutation in tumor adhesion,migration,invasion and metastasisin the tumor microenvironment. The extracellular matrix(ECM) microenvironment not onlyserves as a structural scaffold for malignant cells, but alsoinfluences cell behavior and affects viability and proliferation. $BRAF^{V600E}$, on cell surface receptors and ECM, appears to trigger different pathological and biological effects in a cell context-dependent manner.

Fine-needle aspiration is a useful approach to screen for the $BRAF^{V600E}$ mutation and refine the diagnostic accuracy of PTC. Pre-operative $BRAF^{V600E}$ analysis may provide more important prognostic value for patients. In addition, the development of BRAF-targeted therapy may provide a promising treatment for various human cancers, especially for PTC patients harboring this molecular aberration (20).

In this article, we review the mechanisms of BRAF and the MAPK signaling pathway in the pathogenesis of PTC, recent advances in the use of the $BRAF^{V600E}$ mutation as a potential target for thyroid cancertherapeutics, and the correlation between BRAF mutations and the cancer microenvironment.

3. RAF STRUCTURE

RAF is a 766amino acid protein kinase that regulates signal transduction.Similar to other protein kinases, RAF has a characteristic bilobar conformation in its tertiary structure and of three conserved regions characteristic of the Raf kinase family:conserved region 1 (CR1), a RAS-GTP-binding self-regulatory region (CR2), and a serine-rich hinge region (CR3) encompassing the kinase domain (21). Within the NH2 terminus, CR1 is a regulatory domain that auto-inhibits the C-terminal BRAF kinase domain (CR3). In CR1, residues 155-227 bind to RAS, releasing CR1 and halting kinase inhibition.Residues 234-280 contain a RAS-binding domain that targets localization to the plasma membrane. CR3 makes up the catalytic protein kinase domain that phosphorylates a consensus sequence on protein substrates. CR3 contains two important lobes, connected by a short hinge region. The smaller N-region is responsible for ATP binding, and the larger lobe binds to substrate proteins. The kinase site is in the cleft between the two lobes, while the catalytic Asp576 residue is on the C-lobe.Phosphorylation of two key residues(T599 and S602 for BRAF) on the C-lobe is necessary for RAF activation. The other motif that requires phosphorylationis the N-region which contains a SSYY motif.For activation, the first serine and last tyrosine residues of the N-region in ARAF (S298SYY) and CRAF (S338SYY) must be phosphorylated. However, the S446residue in BRAF is constitutively phosphorylated;the last tyrosine residue phosphorylation is necessary for activation, and aspartic acids can substitutefor tyrosine residues to mimic phosphorylation.BRAF has a higher constitutive kinase activity than the two other RAF family members because the phosphorylationof the S446residuein BRAFleads to a highlynegatively charged amino territory (22).

4. RAF SIGNALING PATHWAY

RAF isacentralcomponentofthehighlyconserved MAPK signaling pathway (also known asthe RAS-RAF-MEK-ERK cell signaling pathway) (Figure 1). The MAPK pathway transferssignals from the extracellular matrix to the nucleus through receptor tyrosine kinases (RTKs),and plays a critical role in mediating cellular proliferation, differentiation, apoptosis, and survival(23, 24). RAS is activated by a variety of plasma membrane signals such as cytokines, hormones, and growth factors. In its active GTP-bound state,RAS recruits wild type RAF to the plasma membrane for activation.Activated RAFthen specifically phosphorylates and activates MEK1/2, which in turn phosphorylates and activates extracellular signal-regulated kinase(ERK1/2). In thenucleus and cytosol, phosphorylated ERK has more than 150 downstream targets(25). The activated ERK1/2 translocates into the nucleus and directly phosphorylates multiple transcription factors, including c-Jun, c-Myc, Ets and c-Fos(26). These transcription factors play important roles in regulatingthe cell cycle, cell growth, and cell survival. ERK also phosphorylates many cytosolic proteins, including cell-cycle regulators such as the retinoblastoma protein and apoptotic proteins such as Bad, MCL-1, and caspase 9, and cytoskeletal proteins such as paxillin, calnexin, and vinexin.The mechanisms of the RAF family kinases are divergent and mainly dependent on the specific cell types involved. The multiple steps in the MAPK signaling pathway enable signal amplification and modulation by different protein kinases and scaffolding proteins(27).

The regulation of this MAPK pathway is complicated because multiple isoforms of every pathway

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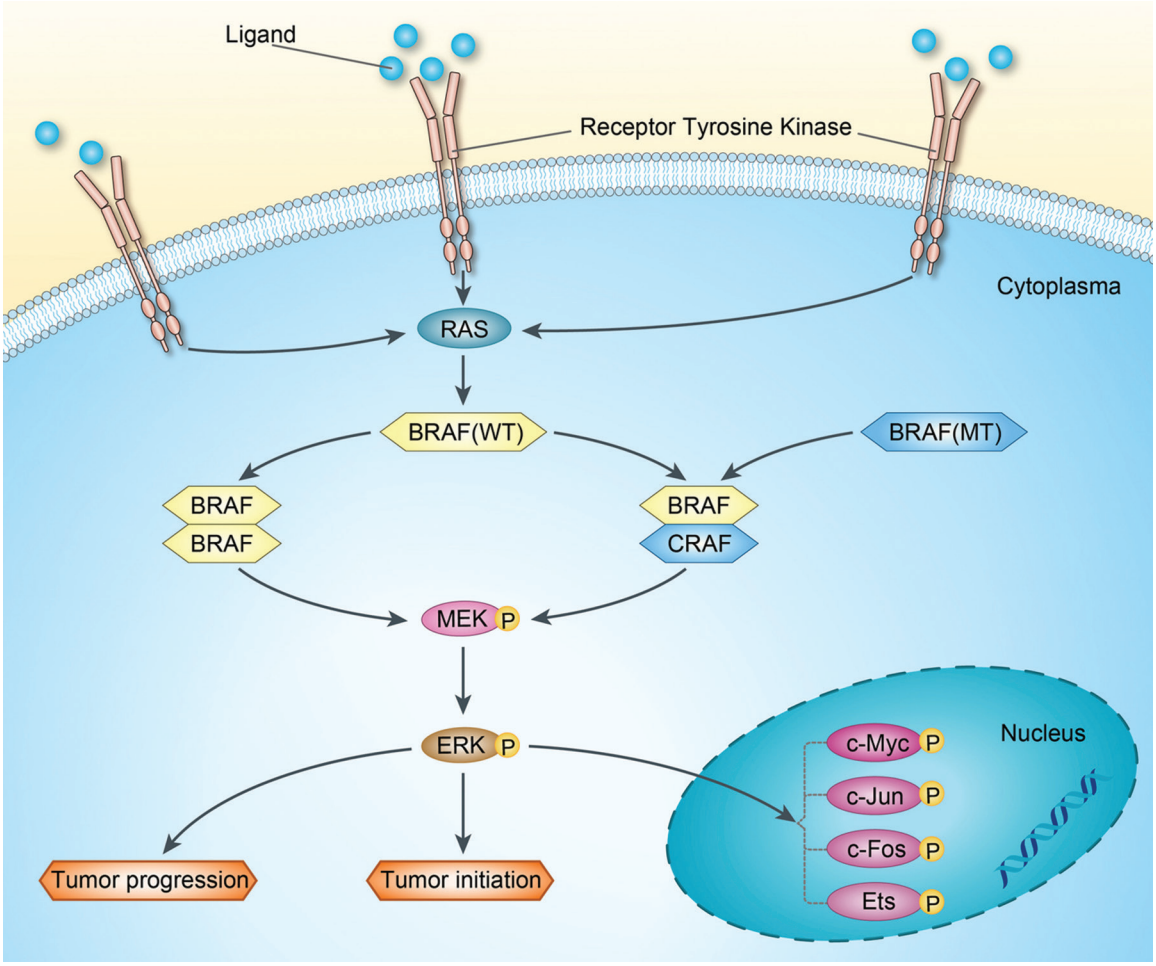

Figure 1. The MAPK signaling pathway. This pathway is initiated by a receptor tyrosine kinase locating on the cell membrane, which subsequently activated RAS facilitating dimerization of BRAF. MEK phosphorylated by activated BRAFphosphorylates ERK, resulting in the variety of cellular effects.

protein exist, each encoded by different genes with overlapping yet distinct functions. RAF kinases,cellular homologues of V-Raf oncogenes, were first acquired by retrovirus (28-30). Mammals have three RAF members: ARAF, BRAF, and CRAF (31) that share the three conserved regions (CR1, CR2, and CR3). Among the RAF isoforms, BRAF is significantly different from ARAF and CRAF significantly for fewer regulatory steps for activation (27). BRAF is expressed in many human cells, including thyroid follicular cells. The BRAF gene, found on chromosome 7q24, encodes a cytoplasmic serine-threonine protein kinase, has a higher level of activitythan the other RAF isoforms, and is the major activator of MEK1/2 (32-34).

Mutations in BRAF,the most frequently mutated human oncogene in the kinase superfamily,can be found in a variety of human cancers (35).These mutations enable constitutive BRAF kinase activation. Interestingly, a couple of mutations reduce BRAF kinase activity and thus MRK phosphorylation, but they can form a heterodimer with CRAF, resulting in downstream MEK-ERK signalingin response to mitogenic signals (36, 37). Additionally, the kinase suppressor of RAS (KSR), which primarily functions as a scaffold, co-localizing RAF, MEK, and ERK, is able to trigger BRAF activation through side-to-side heterodimerization (38,39). Thus, the intricacy of the MAPK pathway and BRAF regulation creates a variety of opportunities whereby a mutation could result in aberrant BRAF signaling.Recent evidence has shown that oncogenic BRAF mutations function by bypassing the requirement for BRAF dimerization or weakening the interactionsof BRAF with MEK1. This may indicate a regulatory role for BRAF dependent on its interaction with MEK instead of RAF kinase activity (40).

5. BRAF MUTATION IN THYROID CANCERS

Constitutive activation of the MAPK signaling pathway is universal to numerous cancers.Approximately 15% of human cancers have activating RAS mutations (41).BRAF mutations have been discovered in a variety

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of human cancer cell lines, including those derived from thyroid cancers, colorectal cancers, lung cancers,ovarian cancersandmalignant melanomas (42-45).

BRAF mutations have only been reported in two types of thyroid cancer, PTC and ATC (46). From 29 studies reporting on BRAF mutations in more than 2000 examined thyroid cancers, the average frequency of mutations in PTC and ATC is 44% and 24% respectively (20).Compared to other RAF isoforms, BRAF plays a central role in regulating thyroid-specific protein expression and proliferative capacity (47).

In 2002, the first activating mutations in BRAF were reported and clustered in the kinase domain (43). Since then, over 40 BRAF mutations have been reported. Most mutations are in the kinase domain and ATP-binding site (P-loop), resulting in increased MEK phosphorylation. The most common BRAF-activating mutation,accounting for approximately 90% of cancer-associated mutations, is the T1799A substitutionlocated on exon15 resulting in a V600E amino acid substitution.

With the crystal structuredetermination of both inactive and active BRAF, a mechanism hasbeen proposed to explain the relevance of the $BRAF^{V600E}$ mutation to oncogenic and constitutive activation of BRAF kinase. In its natural conformation, residues T599 to S602 form a hydrophobic interaction with a residuein the P-loop, keeping this BRAF kinase inactivated. When the $BRAF^{V600E}$ mutation is present,two activating phosphorylation events occur on residues T599 and S602adjacent to the V600 residue.The negatively charged phosphates destabilize the inactive conformation, disrupting hydrophobic interactions, and resulting in folding into an active conformation. The level of kinase activity can be elevated almost 500-fold by a BRAF V600E substitution (41).At the same time, favorable salt bridge interactions can form with either residue K507 or the highly conserved R575 residue from the catalytic loop, which in turn stabilizes the active conformation of the kinase (43, 48-50).Approximately 45% of PTCs in adults have mutations that result in a V600E substitution in BRAF and consequent constitutive activation, making BRAF mutations the most common defined genetic abnormality in thyroid cancers (20). *In vitro*, $BRAF^{V600E}$ shows high kinase activity and a high level of transformation in fibroblasts and melanocytes (41,43,51-53).

Besides the $BRAF^{V600}$ Emutation, rare thyroid tumors have been described with BRAF kinase activating mutations at residues 599 and 601.Nearly seventy percentages of BRAF mutations elevate kinase activity and signal to ERK *in vivo*; whereas $BRAF^{G465E}$, $BRAF^{G465V}$, and $BRAF^{G595R}$ can impair kinase activity towards MEK *in vitro*, but activate C-RAF and endogenous ERK*in vivo* (41). In addition, there are BRAF-inactivating mutations. The $BRAF^{D594V}$ variant, found in a variety of cancers, is “kinase-dead”and devoid of catalytic activity. In addition, $BRAF^{D594E}$ reportedlybindsto CRAF and cooperates with the RAS mutation, stimulating the MAPK signaling cascade and increasing tumor growth (54). Another BRAF-activating mutation detected in thyroid tumors is the $BRAF^{K601E}$ mutation, which has been observed in two benign thyroid adenomas (18,55) and three follicular-variant PTCs(56). Finally, the long arm of chromosome 7 has been found inverted, resulting in recombinant AKAP9-BRAF oncogene formationin radiation-induced papillary thyroid carcinomas, whereas BRAF mutations were almost not detected in this fusion (57). This rearrangement inhibits the function of BRAF auto-inhibitory domains, resulting in constitutive kinase activation. Overall, the direct oncogenic activation of BRAF is an extremely common event in PTC tumorigenesis.

The $BRAF^{V600E}$mutation is usually found in papillary thyroid microcarcinomas, indicating that this mutation is an early event during PTC development. Evidence indicates that $BRAF^{V600E}$ has a capacity to induce thyroid follicular cell dedifferentiation both *in vitro* and in transgenic mice (58). In transgenic mice, conditional endogenous expression of $BRAF^{V600E}$ transforms tumors into poorly differentiated cancers with aggressive characteristics (58). The $BRAF^{V600E}$ mutation may thus be an initiating factor in oncogenic transformation.In addition, $BRAF^{V600E}$ over-expressed in rat thyroid cells *in vitro* shows an increase in migration and the upregulation of matrix metallo-proteases (MMPs), particularly MMP3 and MMP9,in tumor invasion (59, 60). The proliferation of cells with the $BRAF^{V600E}$mutation can be inhibited using MAPK pathway inhibitors or BRAF knockdown (61, 62). These data further indicatethat $BRAF^{V600E}$ is an oncogene in thyroid carcinoma.

6. MICROENVIRONMENT AND BRAF MUTATION IN THYROID CARCINOMA

The tumor microenvironment is a dynamic system orchestrated by blood vessels, malignant cells, fibroblasts, signaling molecules, and the ECM.Decades of investigations have found that tumorigenesis is strongly affected by nonmalignant cellsin the tumor microenvironment (63). Tumors cells can influence the microenvironment by releasing extracellular signals, promoting tumor angiogenesis, and inducing peripheral immune tolerance, whileimmune cells in the microenvironment can affect the growth and evolution of cancerous cells (64, 65).Tumor epithelial cells and microenvironment stromal cells interact with each other through cell adhesion molecules (e.g. integrins, CD44),cytokines, and non-cellular ECM components (e.g. thrombospondin-1, fibronectin) (65).

The ECM is a fundamental component of the cell microenvironment, and has been substantially expanded

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during the evolution of vertebrates. It provides more than mechanical support, and is a locus for cell adhesion, with potential roles in basement membranes and tumors. All epithelial cells associate with the ECM; therefore, radical alterations in the composition and organization of the ECM in human cancers could affect cell survival, proliferation, adhesion, migration, and other properties of both tumor and stromal cells.

So far, some data have shed light on how the $BRAF^{V600E}$ oncogene affects the tumor environment in thyroid cancer, including interactions between neoplastic thyroid follicular cells and ECM components.The $BRAF^{V600E}$ mutation in cancer up-regulates Skp-2 and NF-κB signaling, and deregulates downstream targets including tumor suppressor genes (TIMP-3) and micro-RNAs (66). In PTC, $BRAF^{V600E}$ correlates with VEGF (vascular endothelial growth factor) protein expression perhaps via the $BRAF^{V600E}$ modulation of hypoxia-inducible factors (66). In addition, the activation of $BRAF^{V600E}$ in a normal rat thyroid causes the expression of genes such as matrix metallo-proteases (60). Traditionally, these enzymes may promote tumor invasion by breaking down various non-cellular components of the ECM. PTC harboring $BRAF^{V600E}$ shows a more aggressive clinico-pathological behavior and a significant increase in MMP-2 and MMP-9 protein levels, thus suggesting that these proteins may play a critical role in PTC progression (67).

7. INHIBITORS TARGETING BRAF

Thyroid cancers and other human tumors with BRAF mutations tend to have more aggressive phenotypes and often become resistant to traditional therapies.In addition, as mentioned above, the constitutive activation of $BRAF^{V600}$ mutation and the MAPK pathway are primarily responsible for tumorigenesis and tumor progression in PTC (13, 41, 43). Pharmacologic targeting $BRAF^{V600E}$ may give great promise to the patients with PTC harboring a BRAF mutation. Using inhibitors that target the BRAF kinase or its downstream effectors is the logical therapeutic approach to inhibit tumor growth and progression.Different trials have evaluated the anticancer effects of BRAF inhibitors, and the preclinical results are encouraging. Structure-based drug design can further develop novel small molecules inhibitors of $BRAF^{V600E}$ that can be inserted into the ATP-binding site and trap oncogenic $BRAF^{V600E}$ in an inactive conformation (68, 69). These chemical compounds could inhibit $BRAF^{V600E}$ kinase activity through blocking the ERK1/2 phosphorylation and G1-phase cell cycle arrest (68, 69).

PLX4032, a small molecule specific BRAF inhibitor, shows a potential inhibition of cell growth, migration,and aggression of $BRAF^{V600E}$ human ATC cell lines (70).PLX4032 induce partial or even complete tumor regression in the patients of melanoma with the $BRAF^{V600E}$. In thyroid cancer cell lines harboring wild type BRAF, PLX4032 showed an approximately 50-fold higher IC50 value than $BRAF^{V600E}$ cell lines, indicating that PLX4032 has a selective growth inhibitory effect on $BRAF^{V600E}$-mutated thyroid carcinoma (70).

BAY43-9006 (sorafenib) is the most studied multi-kinase therapeutic agent for targeting BRAF, CRAF, VEGF receptors 1 to 3, RET kinases to inhibit tumor angiogenesis and proliferation in thyroid carcinoma cells and xenograft models (62, 71, 72). However, this effect seems to be caused by blocking angiogenesis via the VEGFR (vascular endothelial growth factor receptor) signaling pathway rather than by selectively inhibiting BRAF (73). A couple of clinical trials on sorafenib monotherapy for the treatment of various malignancies, including iodine-refractory thyroid cancer, have recently been completed. Although Phase I trials showed sorafenib was a well-tolerated agent, however Phase II trials showed little effects in advanced melanoma patients when sorafenib was used as a single-agent therapy (74). Recently, a Phase II trial of sorafenib (longer than 16weeks)in 30 patients with metastatic iodine-resistant thyroid carcinomawere treated by sorafenib showed an overall clinical benefit of 77% and 70% with thyroglobulin reduction (75). 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 15 months, and a significant reduction in the levels of phosphorylated VEGFR and phosphorylated ERK and VEGRF expression in tumor biopsies (76).

To date, there is no convinced evidence to show that the substrates of sorafenib are targeting to BRAF. Sorafenib is a multi-kinase inhibitor that may also target other kinase pathways besides VEGFR to inhibit tumorigenesis and angiogenesis. The mechanism of sorafenib mediating therapeutic effects in PTC remains to be illuminated. Indeed, antitumor effects were also observed with other kinase inhibitors such as axitinib, sunitinib, pazopanib and motesanib that are not shown to inhibit BRAF, but targeting VEGFRs and PDGFRs (platelet-derived growth factor receptor) (77, 78). In addition to BRAF mutations, other factors may affect the tumor response to sorafenib;therefore, the cocktail of various kinase inhibitors provides a potential therapeutic strategy in the future.

8. CONCLUSION

Theoncogenic $BRAF^{V600E}$mutation was first identified in malignant melanoma by Davies in 2002(43). BRAF plays a critical role in the MAPK signaling pathway andin the past twelve years, BRAF mutations have been indentified in a variety of humanmalignancies. Furthermore, there has been the development of prognostic methods for thyroid cancers based on screening for BRAF mutations, and the development of structure-based drug design

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targeting the BRAF kinase.The correlation between BRAF mutations and the tumor microenvironment remains elusive. The $BRAF^{V600E}$ mutation dysregulates the expression of ECM non-cellular components and changes the microenvironment in PTC. Similar to the tumor ECM microenvironment, $BRAF^{V600E}$also appears to impact both the migration and invasion of thyroid carcinoma cells. The identification of these new downstream targets induced by $BRAF^{V600E}$ may provide a complementary avenue to the therapeutic treatment for thyroid cancer, such as drug design targeting novel substrates. In the future, a further understanding of the mechanisms of $BRAF^{V600E}$ in thyroid cancer might offer additional therapeutic opportunities.

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Abbreviations: ARAF, A-type RAF kinase; ATC, anaplastic thyroid cancer; BRAF, B-type RAF kinase; CR1, conserved region 1; CR2, conserved region 2; CR3, conserved region 3; CRAF, C-type RAF kinase; ECM, extracellular matrix; ERK, extracellular signal-regulated kinase; FTC, follicular thyroid cancer; KSR, kinase suppressor of RAS;

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MAPK, mitogen-activated protein kinase; MCL-1, myeloid cell leukemia-1; MMPs, matrix metalloproteases; PDGFR, platelet-derived growth factor receptor; PPARγ, PAX8-peroxisome proliferator-activated receptor γ; PTC, papillary thyroid cancer; RTK, receptor tyrosine kinase; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor

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