Role of BRAF in Thyroid Oncogenesis

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Abstract

BRAF, a cytoplasmic serine–threonine protein kinase, plays a critical role in cell signaling as an activator within the mitogen-activated protein kinase (MAPK) pathway. The most common BRAF mutation is the V600E transversion, which causes constitutive kinase activity. This mutation has been found in a multitude of human cancers, including both papillary thyroid cancer (PTC) and papillary-derived anaplastic thyroid cancer (ATC), in which it initiates follicular cell transformation. With such a high frequency of BRAF mutations in PTC (44%) and PTC-derived ATC (24%), research in BRAF V600E detection for diagnostic purposes has shown high sensitivity and specificity for tumor cell presence. BRAF V600E in PTC has also provided valuable prognostic information, as its presence has been correlated with more aggressive and iodine-resistant phenotypes. Such findings have initiated research in targeting oncogenic BRAF in cancer therapeutics. Although multiple phase II clinical trials in patients with iodine-refractory metastatic PTC have shown significant efficacy for sorafenib, a first-generation BRAF inhibitor, the mechanism by which it mediates its effect remains unclear because of multiple additional kinase targets of sorafenib. Additionally, preclinical and clinical studies investigating combination therapy with agents such as selective (PLX 4032) and potent (BAY 73-4506 and ARQ 736) small-molecule BRAF inhibitors and MAP/extracellular signal-regulated kinase (ERK) kinase inhibitors (AZD6244) hold great promise in the treatment of BRAF V600E cancers and may eventually play a powerful role in changing the clinical course of PTC and ATC.

Background

RAF, a cytoplasmic serine–threonine protein kinase, is a member of the RAS–RAF–MEK–ERK cell-signaling pathway [also known as the MAP kinase (MAPK) pathway], and it plays an essential role in mediating cellular differentiation, proliferation, senescence, and survival in response to extracellular cues. Physiologic activation of this pathway typically occurs through a variety of plasma membrane receptors that activate Ras, a membrane-bound small G protein. Activated Ras recruits Raf to the plasma membrane for activation. Subsequently, Raf phosphorylates and activates MAP–ERK kinase (MEK), which phosphorylates and activates extracellular signal-regulated kinase (ERK). Phosphorylated Erk has more than 150 downstream targets, both nuclear and cytosolic (1). After nuclear translocation, Erk can directly phosphorylate multiple transcription factors, including c-Myc, c-Jun, Ets, and c-Fos (2). These transcription factors, in turn, have been shown to regulate cell cycle, growth, and survival (Fig. 1). Erk also phosphorylates many cytosolic proteins, including cell-cycle proteins such as retinoblastoma, apoptotic proteins such as Bad, MCL-1, and caspase 9, and cytoskeletal proteins such as paxillin, calnexin, and vinexin. The overall effects can be very divergent, and clearly are dependent on specific cell types.

The regulation of this pathway is complex because multiple isoforms of every pathway protein exist, each encoded by different genes and having both overlapping and distinct functions. There are 3 RAF isoforms: ARAF, BRAF, and CRAF (also known as Raf-1). The BRAF gene, which is found on chromosome 7, is the strongest MAPK pathway activator (3, 4) and the most frequently mutated human oncogene in the kinase superfamily (5). Additional pathway complexity arises from its lack of linearity, as BRAF can form a heterodimer with CRAF, resulting in downstream MEK–ERK signaling (6, 7), which can also occur even when one of the heterodimers is inactive. Additionally, kinase suppressor of Ras (KSR), which functions primarily as a scaffold, colocalizing Raf, Mek, and Erk, is able to trigger BRAF activation through side-to-side heterodimerization (8, 9). Thus, the intricacy of the MAPK pathway and the regulation of BRAF within it create a variety of opportunities whereby a mutation could result in aberrant BRAF signaling.
Oncogenic Mutations in BRAF

The first activating mutations in BRAF were described in 2002 and clustered in the kinase domain (10). The most common BRAF mutation is the T1799A transversion resulting in a glutamic acid for valine (V600E) adjacent to an activating phosphorylation site at Ser599. The Catalogue of Somatic Mutations in Cancer (COSMIC) database currently reports on its website that the V600E mutation represents more than 95% of all BRAF mutations of the 78,000 unique samples reported (11). In its wild-type conformation, residues G597 to V601 form a hydrophobic interaction with residues G465 to V472 in the ATP-binding side (P-loop), keeping it inactivated. The BRAFV600E mutation disrupts the hydrophobic interaction, enabling the BRAF kinase to fold into a catalytically active formation, resulting in an almost 500-fold increase in kinase activity (12). Presently, more than 40 BRAF mutations have been reported, with a majority located in the kinase domain and P-loop, resulting in a direct increase in MEK phosphorylation. Paradoxically, several mutations have reduced in vitro kinase activity toward MEK, but they possess enough activity to transphosphorylate and activate CRAF though differences in heterodimerization (6, 12). The BRAFV600E variant is termed “kinase-dead” as the BRAF is catalytically inactive, yet it has been found in multiple cancers. This kinase-dead BRAF or wild-type BRAF that has been chemically inhibited has been shown to bind to CRAF and potentiate oncogenic Ras mutations, thereby further stimulating the MAPK-signaling cascade and resulting in increased tumor growth (13). This aberrant growth highlights a particular danger in targeting BRAF activity in a tumor with a Ras mutation. Finally, a rare genetic alteration in the BRAF gene has also been identified, in which the long arm of chromosome 7 becomes paracentrically inverted, leading to recombinant AKAP9-BRAF oncogene formation (14). This rearrangement results in loss of the BRAF autoinhibitory domains and, thus, constitutive kinase activation.

Thyroid Cancer and BRAF Mutations

The thyroid gland is composed of 2 hormone-producing cell types: follicular cells, which incorporate iodine to produce thyroid hormone, and parafollicular cells (or C-cells), which are much less prevalent and produce calcitonin, a hormone that regulates calcium. There are 4 types of thyroid cancer. Papillary thyroid cancer (PTC) is the most prevalent, accounting for more than 80% all of thyroid cancer cases, and arises from follicular cells. Follicular thyroid cancer (FTC) is also derived from follicular cells and, similar to PTC, is treated primarily with surgery and radioactive iodine ablation. Medullary thyroid cancer (MTC) is derived from the parafollicular cell and is treated by surgery. Finally, anaplastic thyroid cancer (ATC), which
probably arises from PTC or FTC, is the most aggressive thyroid cancer, with an average survival of less than 6 months. Very few effective therapies are available except surgery and radioactive iodine for all thyroid cancers.

BRAF mutations have been discovered in a variety of human cancers, including malignant melanoma, colorectal cancer, ovarian cancer, lung cancer, and thyroid cancer (4, 10, 15, 16). BRAF mutations were initially reported in thyroid cancer in 2003 with a frequency ranging from 26% to 44% (17, 18). Mutations have only been reported in 2 types of thyroid cancer, namely PTC and ATC (19). BRAF mutations have not been identified in FTC, MTC, benign thyroid adenomas, or hyperplasia (20). From 29 studies reporting on BRAF mutations in more than 2,000 examined thyroid cancers, the average frequency of mutations in PTC is 44% and in ATC is 24% (20). Besides BRAF mutations, 2 other well-described mutations activate the MAPK-signaling pathway in PTC: RET/PTC rearrangements and activating Ras mutations. RET/PTC rearrangements are generally mutually exclusive in PTC and occur in approximately 70% to 80% of cases, indicating the importance of the MAPK-signaling pathway in this particular tumor (21).

BRAFV600E has been shown to initiate thyroid follicular cell transformation both in culture and in transgenic mice (22). In transgenic mice, conditional endogenous expression of BRAFV600E in melanocytes and lung alveolar epithelial cells generally results in initial increased proliferation, frequently followed by senescence, as opposed to endogenous expression in thyrocytes, where fully penetrant PTC is seen by 5 weeks (23). The knock-in of BRAFV600E in mice thyrocytes, under the control of the thyroid peroxidase promoter, results in classic appearing PTC with frequent local invasion, and its short latency seems dependent on the presence of thyrotropin receptor signaling. BRAFV600E has been found in microcarcinomas, further supporting the idea that this mutation may be an inciting factor in the oncogenic transformation.

The prevalence of BRAF mutations within PTC has a subtype-specific pattern, such that BRAF mutations are most common in tall-cell PTC, slightly less common in conventional PTC, and rarely found in follicular-variant PTC (24, 25). This preferential cell-specific distribution may partially explain the wide range in BRAF mutation prevalence reported in PTC, as many reports may not stratify by subtype during data analysis (20). There also seems to be a reciprocal age association between BRAF mutation and RET/PTC rearrangements in PTC, such that increasing age is a predisposing factor to sporadic BRAF mutations (20, 26). Conversely, RET/PTC rearrangements are more prevalent in childhood PTC, as well as in all age groups with radiation-induced PTC.

Clinical–Translational Advances

BRAF mutation analysis for diagnosis

With such a high prevalence of BRAF mutations in PTC and PTC-derived ATC, a great deal of interest has been expressed in the use of BRAFV600E detection for diagnostic purposes. Fine-needle aspiration (FNA) with cytologic analysis is widely used as the initial step for evaluating thyroid nodules. BRAFV600E detection in FNA specimens has been evaluated in multiple studies (27, 28). Using a colorimetric assay to detect the BRAFV600E mutation from a FNA sample, Xing and colleagues showed 100% sensitivity and specificity at a prevalence similar to that of the BRAFV600E PTC patient population (44%), with no false-positive findings; however, negative BRAFV600E results could not discriminate between the presence or absence of PTC, especially in specimens with unclear cytology (27).

Because 20% of FNAs yield ambiguous or indeterminate results, surgical intervention is often the next step, despite the fact that approximately 80% of this population does not have thyroid cancer upon further histologic analysis (29). Most indeterminate FNAs are due to the inability of cytology to distinguish a benign follicular adenoma from a follicular carcinoma. Because BRAF mutations are not found in follicular carcinomas, mutational analysis is generally not helpful for this group of thyroid nodules (28). In a recent study of 110 indeterminate FNAs with definitive surgical pathology, 3 had BRAF mutations (29 cancers; ref. 30). A review of several studies of BRAF mutations in FNA samples totaled 7 BRAF mutations in 100 samples (28).

Detection of BRAF mutations in serum DNA is also of interest, as cancerous cells can sometimes break off a tumor and circulate peripherally in the blood. In previous lines of research, detection sensitivity was insufficient; however, with the use of a mutant allele-specific PCR amplification technique, plasma DNA detection of BRAF mutations in patients with colon cancer was completed with 100% sensitivity (31). Another study was able to detect 1 heterozygous BRAF mutation in a population of more than 20,000 cells using real-time PCR, and when this technique was applied to blood samples from PTC patients, a BRAF mutation was detected in 20% of patients (32). The benefit of these detection methods is the potential to eliminate the need to perform FNA in patients with a positive BRAF mutation; however, additional research investigating specificity and sensitivity will need to be completed before these methods can be put into practice clinically.

BRAF mutation analysis for prognosis

More than 30 studies have examined the correlation of BRAFV600E mutation with a variety of clinicopathologic PTC characteristics (33, 34). Most of these studies, including a meta-analysis, have shown a correlation with at least 1 poor prognostic factor, such as extrathyroidal extension, lymph node metastases, advanced stage, greater predilection to develop iodine-131 resistance, and recurrent disease (35–38). Conversely, several studies have not shown any correlation, including 2 that analyzed a relatively large
Targeting BRAF in thyroid cancer for treatment

With the findings that BRAF mutations in PTC, ATC, and other human tumors tend to have more aggressive phenotypes and often become resistant to traditional therapies, developing a therapeutic agent that can selectively target oncogenic BRAF kinase may be of great clinical utility. Multikinase inhibitors, which act on multiple components of the MAPK pathway, have shown great promise in the treatment of malignancies harboring a BRAF mutation. Sorafenib (BAY 43-9006) is one such therapeutic agent that targets BRAF, CRAF, VEGF receptors 1 to 3, platelet-derived growth factor (PDGF) receptor, and RET kinases to inhibit tumor proliferation and angiogenesis (47, 48). Using RNA interference (siRNA) to knock down BRAF in human ATC cell lines, preclinical studies showed the importance of BRAF for intracellular MAPK signaling and proliferation, as tumor growth was significantly inhibited (49, 50). These findings suggested that BRAF could be an effective target for thyroid cancer treatment. The effect of sorafenib was similar to that of siRNA BRAF knockdown, inhibiting BRAF\textsubscript{V600E}-mediated intracellular signaling in vitro in xenograft models and in thyroid carcinoma cells, yet having minimal effects on normal thyrocytes (49).

Our group reported results of a National Cancer Institute–sponsored investigator-initiated phase II clinical trial in patients with iodine-refractory metastatic PTC (51, 52). This study was the first phase II trial of a multikinase inhibitor showing significant clinical and biologic activity of sorafenib in patients with iodine-refractory metastatic PTC. Based on the Response Evaluation Criteria in Solid Tumors (RECIST), sorafenib showed a 15% (6 of 41 patients) partial response rate in patients with metastatic PTC. A median progression-free survival of 15 months (51). Significant and sustained decreases in the serum tumor marker thyroglobulin were also observed. Fourteen (64%) of 22 patient tumor samples had a BRAF\textsubscript{V600E} mutation, whereas 3 (14%) had a BRAF\textsubscript{K601E} mutation. Of 9 PTC patients who had tumor samples from multiple sites, 8 showed concordant BRAF mutation status. Because of the high frequency (78%) of BRAF mutations in the study population, statistical comparison of objective response with BRAF mutation status was not possible. Paired tumor biopsies were collected in a subset of patients and showed a significant reduction in phosphorlated VEGFR and phosphorlated ERK and an increase in VEGF expression after 8 weeks of sorafenib compared with baseline. Tumor perfusion was also decreased when assessed with serial dynamic contrast-enhanced MRI (DCE-MRI). In 10 of 14 assessable PTC patients, the 8- or 16-week on therapy DCE-MRI scans revealed a median decrease of 46% (range, 27%–92%) in exchange rate (Kep, exchange rate constant) in the index lesions compared with baseline. Of note, no objective response occurred in any of the 4 patients who did not show a change in Kep. Results of correlative studies in this trial reveal significant antiangiogenic activity of sorafenib in addition to inhibiting the BRAF pathway. Another single-institution phase II clinical trial of sorafenib in metastatic, iodine-refractory thyroid carcinoma (N = 30) yielded similar results, with a 23% partial response rate and 18-month median progression-free survival (53). Differences in patient population may underlie the variation in outcomes observed between the 2 trials. Sorafenib failed to restore iodine avidity in patients with iodine-refractory PTC when tested in a phase II clinical trial (54). Taken together, these phase II clinical trials of sorafenib show its efficacy in the treatment of iodine-refractory metastatic PTC. A phase II study of sorafenib in ATC patients and an international multicenter phase III trial of sorafenib versus placebo in patients with iodine-refractory thyroid cancer (NCT00984282) are ongoing.

Despite its effectiveness in the treatment of thyroid cancer, sorafenib has a range of side effects that must be considered prior to the initiation of therapy. The most common adverse events reported include diarrhea, hypertension, fatigue, and hand–foot syndrome (51, 53). Other serious, yet rare events include bowel perforation, thromboembolism, and bleeding. Additionally, keratoacanthomas of the skin have been reported in a minority of patients, and they seem to be related to class effects of BRAF inhibitors. As keratoacanthoma is a low-grade squamous cell carcinoma variant that has the potential to become invasive or metastatic, the papules are generally treated surgically.

The mechanism by which sorafenib mediates its therapeutic effect in PTC remains unclear, although it is likely to be related to multiple target inhibition, including BRAF, RET, VEGF, and PDGF. It seems that angiogenesis is a major mechanism that is targeted, because several multikinase inhibitors (such as sunitinib, axitinib, motesanib, and pazopanib) that are not shown to inhibit BRAF, but target VEGF and PDGF, are also effective in patients with PTC.
Future directions

With the discovery of BRAF-targeted therapy producing exciting but modest clinical benefit, improving efficacy of such therapy in thyroid cancer is the obvious next step. To this end, attempts are being made to design combination therapies that target pathways responsible for resistance to BRAF as well as to improve specificity and potency of BRAF inhibitors. A phase I trial examined the effect of both sorafenib and tipifarnib, a farnesyltransferase inhibitor, in 50 patients with a variety of advanced malignancies, including ATC and PTC (55). All 4 patients with PTC showed significant regression lasting over 18 months, despite these patients having disease progression prior to study entry. Another study investigated the effectiveness of sorafenib and AZD6244, a MEK kinase inhibitor, in the treatment of human gastric cancer–derived xenografts (56). In vitro and in vivo, this combination therapy showed a decrease in both tumor growth and angiogenesis and an increase in apoptosis, responses that were an amplification of those seen with sorafenib alone.

Several new drugs that are either potent BRAF inhibitors (i.e., BAY 73-4506 and ARQ 736) or selectively target BRAFV600E mutation (i.e., PLX4032 and GSK2118436) are being tested in various phases of clinical trials. PLX4032 showed a preferential inhibition of cell proliferation, migration, and invasion of BRAFV600E human ATC cell lines (57). Furthermore, PLX4032 decreased tumor growth and aggressiveness in an animal model using human ATC cell lines. Although phase II clinical trials are still under development using this drug, data are available in a few patients with thyroid cancers who were treated on a phase I clinical trial.

In this trial, 2 of 3 patients with PTC had stable disease lasting 11 to 13 months, whereas 1 patient had either partial or complete response lasting 8 months (56). Of note, 32 patients with melanoma with the BRAFV600E mutation were enrolled in this phase I study. Partial or complete response was noted in 81% of patients, with an estimated median progression-free survival of more than 7 months. Recent studies have unveiled pathways of acquired resistance to BRAF-targeted therapy in melanoma (58, 59).

Overexpression of MAP kinase kinase kinase 8 (MAP3K8 or COT), CRAF, or PDGF-β, as well as mutations in NRAS, result in secondary resistance to BRAF inhibitors. Interestingly, secondary mutations in BRAFV600E are not found in this setting.

Conclusions

BRAF mutations were first identified in malignant melanoma by Davies and colleagues in 2002 while screening genes encoding the components of the MAPK pathway (10). In the past 9 years, remarkable progress has been made in the BRAF field, including the identification of oncogenic BRAF in a wide range of human malignancies including PTC, development of diagnostic techniques, and prognostic criteria for thyroid cancers based on BRAF mutations, and completion of phase II clinical trials in thyroid cancer for therapeutics targeting aberrant BRAF signaling. This research has already begun to change the clinical course of iodine-refractory PTC, for which the previous therapeutic options were limited to supportive care. In the future, further exploration of the mechanisms of primary and secondary resistance for BRAF-targeted therapies in thyroid cancer and preclinical studies identifying types of effective combination therapies and optimum dose and sequence of combination therapies will be critical.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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