Title: Role of BRAF in Thyroid Oncogenesis

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Running Title: BRAF and thyroid cancer

Keywords: BRAF, Thyroid Cancer, Oncogene, Tyrosine Kinase, Targeted therapy

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Disclosure of Potential Conflicts of Interest: The authors have declared that no conflict of interest exists.

Word Count: 2963

Abstract: 216

Figures: 1
BRAF, a cytoplasmic serine/threonine protein kinase, plays a critical role in cell signaling as an activator within the 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 (PTC) and papillary derived-anaplastic thyroid carcinoma (ATC), where it initiates follicular cell transformation. With such a high frequency of BRAF mutations in PTC (44%) and PTC-derived ATC (24%), research in BRAF\textsuperscript{V600E} detection for diagnostic purposes for has shown high sensitivity and specificity for tumor cell presence. BRAF\textsuperscript{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 demonstrated significant efficacy for sorafenib, a first generation BRAF inhibitor, the mechanism by which it mediates its effect remains unclear due to multiple additional kinase targets of sorafenib. Additionally, pre-clinical 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/ERK kinase inhibitors (AZD6244) hold great promise in the treatment of BRAF\textsuperscript{V600E} cancers and may eventually play a powerful role in changing clinical course of PTC and ATC.
Background

Raf, a cytoplasmic serine/threonine protein kinase, is a member of the RAS-RAF-MEK-ERK (mitogen activated protein kinase – MAPK) cell signaling pathway and 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 which activate Ras, a membrane-bound small G protein. Activated Ras, recruits Raf to the plasma membrane for activation. Subsequently, Raf phosphorylates and activates Mek, which phosphorylates and activates Erk. Phosphorylated Erk has over 150 downstream targets, both nuclear and cytosolic (1). After nuclear translocation, Erk can directly phosphorylate multiple transcription factors including c-Myc, c-June, Ets, and c-Fos (2). These transcription factors in turn have been shown to regulate cell cycle, growth, and survival (figure 1). Erk also phophorylates many cytosolic proteins including cell cycle proteins such as Rb, 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 dependant on specific cell types.

The regulation of this pathway is complex as multiple isoforms of every pathway protein exists, each encoded by different genes and having both overlapping and distinct functions. There are three 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 is 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, co-localizing 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, creates 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 their website that the V600E mutation represents >95% of all BRAF mutations of the 78,000 unique samples reported (11). In its wild-type conformation, residues G597 – V601 form a hydrophobic interaction with residues G465-V472 in the ATP binding side (P-loop) keeping it in an inactive form. 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 over 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 towards MEK, but possess enough activity to transphosphorylate and activate CRAF though differences in heterodimerization (6, 12). The BRAFD594V 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 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 whereby 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 comprised of two 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 which regulates calcium. There are four types of thyroid cancer. Papillary thyroid cancer (PTC) is the most prevalent, accounting for over 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 six months. There are very few effective therapies for all types of thyroid cancers except surgery and radioactive iodine.

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 two types of thyroid cancer, namely papillary thyroid cancer and anaplastic thyroid cancer (19). BRAF mutations have not been identified in follicular thyroid cancer, medullary thyroid cancer, or benign thyroid adenomas or hyperplasia (20). From 29 studies of over 2,000 examined thyroid cancers reporting BRAF mutations, the average frequency of mutations in PTC is 44% and in ATC is 24% (20). Besides BRAF mutations, two other well described mutations activate the MAPK signaling pathway in PTC: RET/PTC rearrangements and activating Ras mutations. RET/PTC rearrangements are a somatic chromosomal fusion where the 3’ terminal activation sequence of RET (a receptor tyrosine kinase that activates Ras) is placed under the expressional control of another gene, leading to ligand independent activation of RET, and subsequently Ras. BRAF, RET/PTC, and Ras mutations are generally mutually exclusive in PTC and occur in approximately 70-80% of cases, indicating the importance of the MAPK signaling pathway in this particular tumor (21).
BRAF\(^{V600E}\) has been shown to initiate thyroid follicular cell transformation both in culture and transgenic mice (22). In transgenic mice, conditional endogenous expression of BRAF\(^{V600E}\) 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 five weeks (23). The knock-in of BRAF\(^{V600E}\) in mice thyrocytes under the control of the thyroid peroxidase (TPO) promoter results in classic appearing PTC with frequent local invasion, and its short latency appears dependent on the presence of thyrotropin receptor signaling. BRAF\(^{V600E}\) 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 appears 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, there is great interest in the utility of the BRAF\(^{V600E}\) detection for diagnostic purposes. Fine needle aspiration (FNA) with cytological analysis is widely used as the initial step for evaluating thyroid nodules. BRAF\(^{V600E}\) detection in FNA specimens has been evaluated in multiple studies (27, 28). Using a colorimetric assay to detect
the BRAF<sub>V600E</sub> mutation from a FNA sample, Xing et al demonstrated 100% sensitivity and specificity at a prevalence similar to that of the BRAF<sub>V600E</sub> PTC patient population (44%) with no false positive findings; however, negative BRAF<sub>V600E</sub> results could not discriminate between the presence or absence of PTC, especially in specimen with unclear cytology (27). As 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 histological analysis (29). Most indeterminate FNA’s are from the inability of cytology to distinguish a benign follicular adenoma from a follicular carcinoma. Since 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 FNA’s with definitive surgical pathology, there were 3 with a BRAF mutations (29 cancers) (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 of a tumor and circulate peripherally in the blood. In previous lines of research, detection sensitivity was insufficient; however, using 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 one heterozygous BRAF mutation in a population of over 20,000 cells using real time PCR, and when applying this technique to blood samples from PTC patients, a BRAF mutation was detected in 20% of patients (32). The benefit of these detection methods is that they could potentially eliminate the need to perform FNA in patients with a positive BRAF mutation; however additional research investigating specificity and sensitivity will need to be performed before these methods can be put into practice clinically.

**BRAF Mutation Analysis for Prognosis**
Over 30 studies have examined the correlation of BRAF<sup>V600E</sup> mutation with a variety of clinicopathologic characteristics of PTC (33, 34). Most of these, including a meta-analysis, have shown a correlation with at least one poor prognostic factor such as extra-thyroidal extension, lymph node metastases, advanced stage, greater predilection to develop iodine-131 resistance, and recurrent disease (35-38). Conversely, several studies have not demonstrated any correlation, including two that analyzed a relatively large number of patients (631 and 260) (39-41). Only one study has demonstrated a correlation with distant metastases (18, 42, 43). Papillary thyroid microcarcinomas (PMTC) are tumors less than 1 cm in diameter and are generally indolent, but have also been found to have BRAF<sup>V600E</sup> mutations that are associated with extra-thyroidal extension and lymph node metastasis (44, 45). An increased frequency of BRAF mutation, up to 85%, has been reported in recurrent PTC tumors (42, 43). Additionally, in the setting of advanced PTC, BRAF mutations were also noted to be at an increased frequency (62%) of recurrent/metastatic tumors from iodine refractory PTC patients (46). In this study, frequency of BRAF mutation varied based on type of PTC: 100% (12 of 12) of tall cell variant of PTC, 85% (6 of 7) of well differentiated PTC, 47% (15 of 32) of poorly differentiated PTC had BRAF mutations. Among patients with metastases from multiple sites, 8 of 8 patients showed between-sample concordance for BRAF mutations. Despite these conflicting studies, a BRAF mutation is likely an independent marker for a more aggressive PTC, and its presence may aid in the management of PTC throughout its clinical course.

*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, vascular endothelial growth factor (VEGF) receptors 1-3, platelet derived growth factor (PDGF) receptor, and RET kinases to inhibit tumor proliferation and angiogenesis (47, 48). Using
RNA interference (siRNA) to knockdown BRAF in human ATC cell lines, preclinical studies demonstrated 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$^{V600E}$-mediated intracellular signaling \textit{in vitro}, in xenograft models, and in thyroid carcinoma cells, yet having minimal effects on normal thyrocytes (49).

Our group reported results of NCI-sponsored investigator-initiated phase II trial in patients with iodine refractory metastatic PTC (51, 52). This was the first phase II trial of a multikinase inhibitor demonstrating significant clinical and biologic activity of sorafenib in patients with iodine refractory metastatic PTC. According to response evaluation criteria in solid tumors (RECIST), sorafenib demonstrated a 15% (6 of 41 patients) partial response (PR) in patients with metastatic PTC. A median progression-free survival (PFS) in this single arm study was 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$^{V600E}$ mutation while three (14%) had a BRAF$^{K601E}$ mutation. Eight of nine PTC patients who had tumor samples from multiple sites showed concordant BRAF mutation status. Because of the high frequency (78%) of BRAF mutations in the study population, statistical comparison of objective response and BRAF mutation status was not possible. Paired tumor biopsies were performed in a subset of patients, which showed a significant reduction in pVEGFR and pERK, and an increase in VEGF expression after eight weeks of sorafenib as compared to baseline. Tumor perfusion was also decreased when assessed with serial dynamic-contrast enhanced magnetic resonance imaging (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% to 92%) in exchange rate (Kep, exchange rate constant) in the index lesions compared to baseline. Of note, no objective response occurred in any of the four patients who did not reveal change in Kep. Results of correlative studies in this trial reveal significant anti-angiogenic activity of sorafenib in addition to inhibiting 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% PR and 18 months median PFS (53). Differences in patient population may underlie the variation in outcomes observed between the two 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 demonstrate 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 are ongoing.

Despite its effectiveness in the treatment of thyroid cancer, sorafenib has a range of side effects that must be taken into consideration 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 (KA) of the skin have been reported in a minority of patients and seem to be related to class effects of BRAF inhibitors. As KA 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 though it is likely to be related to multiple target inhibition including BRAF, RET, VEGF and PDGF. It appears that angiogenesis is a major mechanism that is targeted, since several multikinase inhibitors (such as sunitinib, axitinib, motesanib, 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 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 variety of advanced malignancies including ATC and PTC (55). All four 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 demonstrated a decrease in both tumor growth and angiogenesis, and an increase in apoptosis, responses that were an amplification of that seen with sorafenib alone.

Several new drugs that are either potent BRAF inhibitors (ie: BAY 73-4506, ARQ 736) or selectively target BRAF<sup>V600E</sup> mutation (ie: PLX4032, GSK2118436) are being tested in various phases of clinical trials. PLX4032 showed a preferential inhibition of cell proliferation, migration, and invasion of BRAF<sup>V600E</sup> human ATC cell lines (57). Furthermore, PLX4032 decreased tumor growth and aggressiveness in an animal model using human ATC cell lines. While phase II clinical trials are still under development using this drug, data are available in few patients with thyroid cancers that were treated on a phase I clinical trial. In this trial, two of three patients with PTC had stable disease lasting 11-13 months while one patient had either partial or complete response lasting 8 months (reference 56). Of note, 32 patients with melanoma with the BRAF<sup>V600E</sup> mutation were enrolled on this phase I study. Partial or complete response was noted in 81% of patients with an estimated median PFS of >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-b as well as mutations in NRAS result in secondary resistance to BRAF inhibitors. Interestingly, secondary mutations in BRAF<sup>V600E</sup> are not found in this setting.
Conclusion

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 nine years, remarkable progress has been made in the BRAF field, including the identification of oncogenic BRAF in a wide range of human malignancies, such as PTC, development of diagnostic techniques and prognostic criteria for thyroid cancers based on BRAF mutations, and conduction of phase II clinical trials in thyroid cancer for therapeutics targeting aberrant BRAF signaling. Already this research has 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 mechanisms of primary and secondary resistance for BRAF-targeted therapies in thyroid cancer, pre-clinical studies identifying types of effective combination therapies, as well as optimum dose and sequence of combination therapies are critical.

Disclosure of Potential Conflicts of Interest: The authors have declared that no conflict of interest exists.


Figure 1.

The MAPK signaling pathway is typically initiated through activation of a receptor tyrosine kinase. This activates Ras which facilitates homo- or hetero dimerization of wild type BRAF. Activated BRAF phosphorylates MEK (which is bound to KSR) which phosphorylates ERK which results in multiple cellular effects such as proliferation and survival. Mutant BRAF can dimerize and activate MEK without Ras activation.
Receptor tyrosine kinase

- RAS → BRAF
- Mutant BRAF
- BRAF
- BRAF
- BRAF → CRAF
- MEK → KSR
- ERK

Cell cycle: Rb
Apoptosis: Bad, MCL-1, Caspase 9
Cytoskeletal: Paxillin, Calnexin, Vinexin

Nucleus: c-Myc, c-Jun, Ets, c-Fos

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CCR Molecular Pathways
Clinical Cancer Research

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Clin Cancer Res  Published OnlineFirst September 7, 2011.

Updated version  Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-11-1155

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